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To the Graduate Council:

I am submitting herewith a thesis written by Julius Barth entitled "Evaluation of Various Forages In Vitro Using a Modified Artificial Rumen Technique." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Husbandry.

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EVALUATION OF VARIOUS FORAGES *IN VITRO* USING A MODIFIED ARTIFICIAL RUMEN TECHNIQUE

A THESIS

Submitted to
The Graduate Council
of
The University of Tennessee
in
Partial Fulfillment of the Requirements
for the degree of
Master of Science

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by

Julius Barth

August 1958

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Julius Barth

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TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	REVIEW OF LITERATURE	6
	Differences in Digestibility Due to Grass Species and Variety	7
	The Effect of Nitrogen Fertilization and Protein Content of Forages and Roughages on Their Digestion by Rumen Bacteria	7
	The Effect of Stage of Growth, Maturity, and Lignification of a Forage on its Digestibility by Rumen Microorganisms	10
	The Influence of Minerals, and the Mineral Content of Forages, on their Digestion by Rumen Microorganisms	16
	The Effect of Prussic Acid on Rumen Bacteria	20
III.	EXPERIMENTAL	23
	Development of Technique	23
	Experimental Procedure	27
	Source of Forage Material	27
	Preparation of Forages	29
	Preparation of the Digestion Flasks Prior to the Processing of the Rumen Microorganisms	29
	Collection, Processing, and Washing of the Rumen Microorganisms	31

CHAPTER PAGE

Cellulose Determination 34
Description of Experiments 36
Experimental Variability Trial 36
Variety and Nitration Comparisons of Forages Cut at the Hay Level 38
Stages of Maturity Comparisons of the Non Nitrated Forages 38
Stages of Maturity Comparisons of the Nitrated Forages 40
Comparisons of the Effect of the Ash Quantity and Quality of Forages as a Source of Minerals Required by Rumen Microorganisms 40
The Effect of Hydrocyanic Acid on the Activity of Rumen Microorganisms 42
The Effect of Parahydroxybenzeldahyde on the Activity of Rumen Microorganisms 42
Statistical Analysis 43
IV. RESULTS AND DISCUSSION 44
Variety and Nitration Comparisons of Forages Cut at the Pasture Stage 44
Variety and Nitration Comparisons of Forages Cut at the Hay Stage 49
Stage of Maturity Comparisons of the Non Nitrated Forages 52

CHAPTER PAGE

Stage of Maturity Comparisons of the Nitrated Forages 54

Comparisons of the Effect of the Ash Quantity and Quality of Forages as a Source of Minerals Required by Rumen Microorganisms 56

The Effect of Hydrocyanic Acid on the Activity of Rumen Microorganisms 68

The Effect of Parahydroxybenzaldehyde on the Activity of Rumen Microorganisms 69

V. GENERAL DISCUSSION 73

VI. SUMMARY 75

LITERATURE CITED 79

LIST OF TABLES

TABLE PAGE

I. Cellulose Content of the Forages Tested 45

II. Experimental Variability Trial of the Digestion of Kentucky Bluegrass In Vitro 46

III. Variety and Nitration Comparisons of Forages Cut at the Pasture Stage 48

IV. Variety and Nitration Comparisons of Forages Cut at the Hay Stage 51

V. Management Level Comparisons of the Non Nitrated Forages 53

VI. Management Level Comparisons of the Nitrated Forages . . . 55

VII. Ash Analysis of Forages Cut at Twenty Inches Down to Six Inches 58

VIII. Ash Weights of Grass Ash Supplementation to Whole Forage 59

IX. Comparisons of the Effect of the Ash Quantity and Quality of Forages on Their Utilization by Rumen Microorganisms 60

X. The Effect of Supplementation of German Millet Grass With Kentucky Bluegrass Ash and German Millet Ash 61

XI. Milligrams of Purified Cellulose Digested per Milligram of Grass Ash Present 62

XII. Additional Milligrams of Grass Cellulose Digested Due to Added Ash 63

TABLE PAGE

XIII. Corrected Ash Weight Values of Comparisons of the Effect of the Ash Quantity and Quality of Forages on Their Utilization by Rumen Microorganisms 65

XIV. The Effect of Supplementation of German Millet Grass With Kentucky Bluegrass Ash and German Millet Ash 66

XV. The Effect of Hydrycyanic Acid on the Digestion of Piper Sudan Grass by Rumen Bacteria *In Vitro* 70

XVI. The Effect of Parahydroxybenzaldehyde on the Digestion of Piper Sudan by Rumen Bacteria *In Vitro* 71

LIST OF FIGURES

FIGURE PAGE

1. Laboratory Apparatus Used in These Studies 39

CHAPTER I

INTRODUCTION

Roughages and forages make up the major portion of the diet of the ruminant animal. The various botanical species and varieties of the grasses and forages used for feeding ruminants vary not only in their adaption to climatic, soil, and topographical conditions but also in their nutritive value to the ruminant animal. In some cases a species or variety of a forage may thrive well in an area but prove to have a low nutritive value.

Experimental work is in progress in the testing, developing, breeding, and feeding of forages to discover plants of high nutritive value which are adaptable to Tennessee conditions.

Animal nutritionists work with the agronomists in the testing of pasture species and varieties with both the nutritive value to livestock and the adaptability and yield of the crop under Tennessee conditions as their final criteria of acceptability. New pasture species and hybrids are continually being developed and tested here, and varieties from other state and federal experimental stations are being brought in and tested for possible recommendation for introduction into the state of Tennessee.

There are many new varieties and species of forages and only those which show possibilities are actually tested for environmental adaptibility. Because of time, expense, and limited facilities, animal grazing trials in turn are run only on a few of the above. However, they are necessary for the final evaluation of a new forage species.

Work was begun to modify the existing artificial rumen techniques and a method was developed that may be used to evaluate the nutritive value of the various forages in vitro. This method is intended only as a screening devise to eliminate those species of grasses of lower nutritive value and is not recommended to replace actual grazing trials with live animals. However, by eliminating those grasses of lower nutritive value, the time, animals, land, and expense involved in grazing trials may be used to better advantage with those grasses which show greater promise.

This method was used in the present studies to show differences in digestibility of forages by rumen microorganisms due to stage of growth or plant maturity and fertilization. There are possibilities that this method may be used to find ways of improving the varieties which are poorer from the nutritive point of view, but which are excellent from the agronomic point of view, by supplementing them with the various minerals and other nutrients which are very critical to rumen microorganisms. This work would, of course, have to be followed up by live animal digestion or grazing trials.

Rumen microorganisms play an integral role in the digestion of forages. They are responsible for the breakdown of cellulose to volatile fatty acids and the synthesis of essential amino acids and water soluble vitamins.

The stomach of the ox is a very voluminous organ that has a capacity of 20 to 40 gallons depending upon the size of the animal. Four distinct compartments are present which are known as the rumen, reticulum, omasum, and abomasum. According to Schalk and Amadon (1928), the first of the

three are proventriculi or dilations of the esophagus and their mucosa does not secrete any digestive fluids. The abomasum is the true stomach and it is here that the mechanically prepared food first comes into contact with the digestive secretions. Digestive glands are present throughout the entire extent of the abomasal mucosa. The pyloric extremity of the abomasum connects with the first portion of the small intestine.

It can be seen from this very brief description of the rumen anatomy that the cellulolytic bacteria form the first and most important link in the chain of digestive processes in the ruminant animal. Although the nutritive and dietary requirements for the host animal are not necessarily the same as those of the rumen bacteria, the nutritive requirements for the rumen bacteria must be provided first before the animal can receive any benefit from ingested feed.

Results of feeding trials will not necessarily follow those of the artificial rumen due to the fact that the host animal has dietary requirements which are not needed by the rumen bacteria such as the fat soluble vitamins and calcium. On the other hand, the host animal is dependant upon the rumen bacteria for the synthesis of the B vitamins and essential amino acids. The bacteria can also synthesize amino acids from non protein nitrogen found in plant materials and urea.

It must be pointed out that the quantitative requirements of the various nutrients for bacterial growth might not meet the requirements for growth, maintenance, lactation, and reproduction of the host animal. Some of the nutrients are required only in comparitively small amounts to support bacterial growth. However, if these minimum requirements of the

bacteria are not met the animal will do poorly.

Results of feeding trials will usually follow those of the artificial technique but not always. The living animal is dependent upon the rumen bacteria for the utilization of the major portion of its diet. According to Morrison (1949), no enzymes produced by the digestive tract are able to digest cellulose and pentosans, which form the cell walls of plants and make up a large part of all roughages. However, these substances are attacked by bacteria in the rumen.

Morrison (1949) states that the ability of livestock to use the fiber and pentosans in their feed depends upon bacterial digestion. This is of great importance in the nutrition of cattle and sheep and is the fundamental reason why they can live chiefly on roughage. Not only are the fiberous cell walls utilized for food, but also this digestion sets free the nutrients contained inside the cells. Thus, these materials can be more easily acted on by the digestive juices in the true stomach and in the intestines.

To summarize, the nutrition, growth, and activity of the rumen bacteria can be and frequently are a limiting factor in the overall nutrition of ruminant animals. Not only is this of great importance from the standpoint of the breakdown of roughages, but also from the standpoint that the bacteria themselves are later digested by the host animal providing a source of amino acids.

The fact that rumen bacteria are essential for the digestion of forages makes the artificial rumen an acceptable screening devise for forage evaluation. If a roughage is lacking in the proper nutrients to

support the growth of rumen bacteria, or if it is not digestible on account of its stage of growth (stemminess, coarseness, or lignification) it will be of very little value to the ruminant animal.

The major objective of this investigation was to develop an artificial rumen technique which would be sensitive enough to detect major differences in the nutritional value of various species and varieties of forages *in vitro*. Two different varieties of each of two different species, a total of four different varieties, of summer annual grasses were made available by the Department of Agronomy of the University of Tennessee for these studies.

The specific objectives in this investigation were as follows:

1. To study variations in *in vitro* digestibility due to species and variety within species.
2. To determine the effect of nitrogen fertilization of these forages upon their utilization by rumen microorganisms.
3. To determine the effect of stage of maturity of these forages upon their utilization by rumen microorganisms.
4. To determine the effect of variations in the quantity and quality of the ash of these forages on the digestion of cellulose by rumen microorganisms.

Early in the experiment it appeared that the prussic acid content of grasses might possibly have a toxic effect upon rumen microorganisms. Therefore, some investigative work was conducted to determine the effect of prussic acid upon cellulose digestion *in vitro*.

CHAPTER II

REVIEW OF LITERATURE

Development of the Technique

Most of the present artificial rumen techniques involve the use of the whole unmodified rumen juice, or a washed suspension of rumen microorganisms with a basal media containing the known nutrients required by rumen microorganisms added.

Hamilton (1955) reported that ash formed a surprisingly large proportion of the total dry matter of the rumen contents - as much as three times the percentage found in the ration. The mineral levels in the rumen ingesta varied from 12 per cent to 20 per cent of the dry matter.

The natural rumen juice also contains protein, feed debris, protozoa, and metabolic products. These and the ash could easily supply nutrients to the bacteria which could possibly be lacking in the grass variety under test, and mask any nutrient deficiencies of that grass.

Doetsch (1955), as reported by Hungate, stated that certain factors in the washed suspension technique differ from those of the rumen. Among the important ones are: exclusion of the protozoa, absence of the normal fiberous substrates and the bacteria which cling to them. On the other hand, fewer variables are involved and the nature of the substrates and the amount of growth can be controlled. For comparative studies of the integrated activities of rumen bacteria and for identification of particular reactions, the washed suspension technique is a valuable tool.

Differences in Digestibility Due to Grass Species and Variety

There is practically no literature available concerning the evaluation of forages using the artificial rumen. The literature available concerning grazing trials is comparatively small. No literature is available concerning grazing trials with the varieties of grasses used in this investigation. They are Sweet Sudan, Piper Sudan, German millet, and Georgia hybrid No. 1 pearl millet.

Studies have been made comparing other varieties of the Sudans and millets as summer pastures but there is a possibility of having as much or more difference between varieties as between species. However, work was done by Underwood et al. (1955) at the University of Georgia comparing Tift Sudan and Starr millet as temporary pastures for milking cows. The average daily 4 per cent fat corrected milk was 22.2 and 21.8 pounds for the cows grazing Sudan and millet, respectively. Average fat tests were 3.86 per cent on both pastures. The cows on the Sudan gained 1.1 pounds per day which was significantly more than the 0.7 pounds of the cows grazing millet. The Sudan was slightly more digestible than the millet.

The Effect of Nitrogen Fertilization and Protein Content of Forages and Roughages on their Digestion by Rumen Bacteria

Burroughs et al. (1949) stated that protein fed to cattle and other ruminants presumably serves two separate physiological functions.

One function pertains to the growth and development of microorganisms in the digestive tract in fostering roughage digestion and the synthesis of B-vitamins and amino acids. The other function relates to the growth of the ruminants body proper.

Enlow and Coleman (1929) reported that the protein content of pasture grass in a grazing condition can be increased and maintained at a somewhat higher level than ordinary by frequent light applications of a nitrogen fertilizer.

Experiments were begun by Moon (1954) in 1948 in Scotland to examine the influence of late applications of nitrogenous fertilizers on the composition and nutritive value of seed-hays and meadow hays of low protein content. Digestibility measurements with sheep showed that the fertilizer treatment had little effect on the digestibility of the non-protein fractions. Average digestibility percentages showed that the fertilizer treatment, besides increasing the percentage of protein in the hay, also increased the digestibility of the protein.

In tests run by Poulton et al. (1957) at the New Jersey Experiment Station, orchardgrass hay was grown with 100, 200, and 400 pounds of nitrogen per acre. The crude protein content of the hays was 13.89 per cent, 17.52 per cent, and 18.02 per cent, respectively. Alfalfa hay grown with conventional fertilizer applications contained 19.19 per cent crude protein.

The digestion coefficients of the crude protein for the hays were 67.4 per cent, 73.8 per cent, and 73.8 per cent for the 100, 200, and 400 pounds nitrogen per acre, respectively. The digestion coefficient for the alfalfa was 78.3 per cent. No significant variation in the digestibility

of the energy, ether extract, and nitrogen free extract components was observed. Four yearling wether sheep were used in the digestion trials.

Markley et al. (1957) used four eighteen-month-old heifers in a total collection digestion trial. A nitrogen balance was determined for each day. The crude protein and digestion coefficient of the second cutting bromegrass hays were: 25 pounds nitrogen per acre, 7.46 per cent and 61.77 per cent; 125 pounds nitrogen per acre, 12.45 per cent and 66.55 per cent; 225 pounds nitrogen per acre, 17.16 per cent and 74.33 per cent, respectively. Ether extract followed a trend similar to protein while the per cent fiber and nitrogen free extract decreased with increasing nitrogen fertilization. The digestible energy, protein, ether extract, and fiber increased while that of nitrogen free extract decreased with increasing nitrogen fertilization of bromegrass.

Crampton and Jackson (1944) noted in their experiments that there was a high negative correlation between crude protein and digestibility of the herbage. They stated that this is strong evidence toward refuting the claim that treatment to pastures resulting in increased protein correspondingly increases the nutritive value of such pastures when their lowest level of protein was much above the needs of the steers or sheep.

Walker and Hepburn (1955) studied the relationship existing between the gross digestible energy content and the chemical composition of twenty four hays with nine Cheviot wether lambs over a period of eight months. They stated that the crude protein content of the hay was of no value in predicting the digestibility of energy and was related only to the digestibility of crude protein.

Results of studies by Burroughs *et al.* (1950) indicate that protein *per se* exerts a favorable influence upon roughage digestion when roughages are fed to cattle in rations containing starch. Burroughs stated that it would appear that the minimal protein at times must exceed 11 per cent.

Burroughs further stated that the importance of protein *per se* to roughage digestion when a roughage is fed alone or in the absence of a starchy feed seems small indeed. Results in his study indicated that as little as 4 or 5 per cent protein in a roughage is ample for good digestion. Apparently the protein requirements for efficient roughage digestion decreases as starch or starchy grains are reduced in the ration. This does not mean that this small amount of protein in a roughage is ample in meeting the body needs of cattle since requirements for digestion and body needs are distinct.

The Effect of Stage of Growth, Maturity, and Lignification of a Forage on its Digestibility by Rumen Microorganisms

J. N. Bird (1943) states that the stage of growth which a plant has reached at the time of cutting is one of the most important considerations in connection with the harvesting of forage crops. Both the increased production of stem and resulting changes in proportion of stem to leaf as a plant matures have a marked influence upon the yield and feeding value of the crop obtained. The rapid increase of the stem during the period immediately preceeding bloom and the withering and loss of lower leaves in the advanced stage of maturity account for marked changes not only in yield but also in the chemical composition, palatibility, and digesti-

bility of a grass plant in the later stages of growth. Leaf tissue is known to be richer in protein, calcium, and carotene and lower in fiber than stem tissue.

Stage of cutting studies were made at McGill University by Bird (1943) from 1935 to 1940. The cutting stages used were: (1) short grass, (2) long grass, (3) begin to head, (4) begin to bloom, (5) end of bloom, and (6) seed formed. The species used were bromegrass, timothy, red top, and Kentucky bluegrass. All species showed a marked decline in percentage of crude protein during the first three stages, which is to be expected as a result of a period of rapid stem elongation. When the four species are compared with respect to crude protein content, it is noted that the difference between them are small in comparison with differences between stages of cutting between species.

The carotene content also declined with successively later stages of cutting. Differences between species were small when compared with differences within species, especially in the earlier stages. At the more mature stages Kentucky bluegrass maintained a relatively higher and Timothy a relatively lower level of carotene than the other two species.

Studies were begun in May, 1939 at the Panhandle Agriculture Experiment Station by McMillen et al. (1943) regarding (1) chemical composition, (2) grazing value, and (3) vegetative changes of herbage in a typical upland plains pasture. Blue gramma, buffalograss, and Russian thistles were the grasses used.

Young Hereford steers or heifers were turned into the pasture in the spring as soon as the grass was ready for grazing. Sheep were put in

the pasture when more complete utilization of weeds, particularly Russian thistles, was desirable.

The moisture, protein, and phosphorus content of all three herbages dropped except in the case of phosphorus in Russian thistles. Calcium was quite variable throughout the season. The carotene also decreased with maturity.

The summary of the performance of livestock on the experimental pasture for four grazing seasons revealed that yearling Herefords grazing the pasture for an average of 129 days for four seasons made an average daily gain of 1.01 pounds and produced a yearly average of 29.3 pounds of beef per acre. The average daily gain for June was 1.47 pounds as compared to 0.53 pounds in August. When the herbage contained 13 per cent to 25 per cent crude protein (dry basis) and 10 to 37 milligrams of carotene per 100 grams in June, gains were high. As the protein dropped to 6 per cent to 8 per cent and carotene to 3 to 6 milligrams in August, average livestock gains were correspondingly low. These figures indicated that gains are related to chemical composition.

Studies in Eastern Canada by Crampton and Jackson (1944) during the years 1941, 1942, and 1943 showed similar trends in digestibility of dry matter of pasture by steers and sheep due to seasonal variations. From early spring to mid-summer there was a marked decline in digestibility and this coincides with the maturing of the plants. After about July 1, the digestibility either continued to fall, or rose to spring levels depending on climatic conditions. Rate of gain of grazing cattle also followed roughly these trends in digestibility of the dry matter of the

forage. There appeared to be a characteristic steady decline in digestibility of pasture herbage dry matter from the values of the order of 75 per cent for early spring grass to 60 per cent some six weeks later.

Crampton and Jackson (1944) further stated that the digestibility of mixed pasture herbage by steers and by sheep appeared to follow quite closely the leaf/stem ratio existing at the time. A wide ratio meant high digestibility which decreased as the material increased in the proportion of stems.

A study was made at Macdonald College by Drapala et al. (1947) during 1944 and 1945 with Dollard red clover to show the effects of plant maturity on lignification and dry matter digestibility. The studies indicated that lignification proceeds regularly with advancing maturity of the plant and that regions around the vascular bundles are primarily involved in the process. During the years 1944 the lignin content increased from 7.3 per cent on May 26 to 12.2 per cent on July 10. During 1945 the lignin content increased from 7.6 per cent on June 1 to 15.2 per cent on July 12. Actually, the digestibility by sheep of the dry matter of the entire plant declined from 64 per cent to 58 per cent for forage corresponding to a June 1 and June 8 cutting to a June 22 and June 30 cutting, respectively.

The results of grazing trials conducted at Kentucky by Forbes and Garrigus (1950) with both steers and wethers showed relationships between organic matter digestibility and the protein, crude fiber, and lignin contents of the forages.

The studies revealed that the relationships between organic matter digestibility and lignin content exhibited the greatest degree of correlation,

the greatest regression coefficient and the highest statistical significance. Forbes and Garrigus stated that the best single measure for purposes of predicting organic matter digestibility of a pasture forage, is the lignin content of the forage. This is an entirely logical and expected finding in view of the non digestibility of the lignin and the fact that occurring in plant cell walls, it physically inhibits the digestion of nutrients enclosed within the cells. The fact that the protein content indicated the next best measure is probably more related to the high degree of negative correlation between lignin and protein contents than to any specific effect of the protein itself upon the total digestibility within the limits encountered in the experiments. The results obtained for two species of animals, sheep and steers, were not statistically significant.

Walker and Hepburn (1955) found that crude fiber influenced the digestibility of all nutrients except ether extract. They concluded that a close relationship existed between the grass digestible energy contents of the hays and the structural components, as represented by crude fiber, lignin, and cellulose.

Sullivan (1955) stated that lignin has been considered important from two points of view; it is practically indigestible, and it influences the digestibility of the other substances. The digestion coefficients of lignin were found to be measurable and variable with a high of 12.4 per cent in one sample. The lignin of the feces was found to be attacked more readily by strong acids and weak alkalis than the lignin isolated from the grass, an indication that the lignin was modified in its passage through

the sheep.

The digestion coefficients of the true and natural cellulose ranged from fifty six to eighty nine, a range as great as that of crude fiber. Lignin percentage was significantly correlated with digestibility of all the insoluble carbohydrates and total dry matter.

Stallcup et al. (1956) reported that there was a highly significant negative correlation between the lignin content of the hay and the per cent removal of lignin, ash, and protein from the rumen in 12 hours.

Patton and Giesker (1950) (cited by Steppler, 1951) claim that lignin acts as a physical barrier to the microflora and fauna of the rumen, impeding the digestion of the cytoplasm it occludes. Clayson (1943) (cited by Steppler, 1951) believes that lignin is not easily attacked by enzymes. Norman (1939) (cited by Steppler, 1951) also agrees that presence of lignin in the cell wall prevents the bacterial degradation of cellulose, largely through mechanical action. Studies by Watson and Horton (1936) (cited by Steppler, 1951) suggest that protein and other food reserves were protected to some degree by the presence of lignin.

Kamstra (1958) reported that a negative relationship existed between lignin content and the in vitro cellulose digestion in the whole plant materials. Kamstra pointed out that it appears that lignin is one of the main single components of the plant which tends to decrease cellulose digestion by microorganisms. As reported by Kamstra, Baker and Harriss (1947) and Howie and Baker (1952) demonstrated the manner of attack by microflora upon cellulose. They made photomicrographs showing bacterial attack at points of fracture or breaks in the fiber. Thus, it is not

unreasonable to suggest that lignin, because of its close association with cellulose, could substantially inhibit microbial attack on cellulose particles.

The Influence of Minerals, and the Mineral Content of Forages, on their Digestion by Rumen Microorganisms

Minerals are probably the most critical of the nutrients required by rumen microorganisms for their growth, metabolism, and proper function.

Results of earlier work done by Woodman and Evans (1930) does not agree with the results of more recent experiments regarding the effect of minerals of forages on digestion. Digestion trials with sheep in England in 1929 by Woodman and Evans (1930) showed that mineral deficient hay was as digestable as meadow hay which had been made from herbage in as fiberous a stage of growth as that of the mineral-deficient herbage. They stated that the comparison, therefore, does not support the contention that such herbage is characterized by low digestibility on account of deficiency of mineral matter. As with normal cultivated herbage, the digestibility of the mineral-deficient herbage is a function of maturity, being influenced by the degree of lignification of the fiber, and not by the amount and character of its inorganic constituents.

Woodman and Evans concluded that malnutrition on pasturage of subnormal mineral content is due directly to the failure of the diet to supply the necessary inorganic materials for constructional purposes and for maintaining the normal balance of minerals in the blood and tissues. It is not, even in part, to be ascribed to any indirect effect, such as

is embodied in the suggestion that the mineral deficiency leads to undernutrition of the animal by causing a depression of its appetite and its capacity to digest the organic constituents of the herbage.

Klosterman et al. (1953) (cited by Tillman et al, 1954) showed that alfalfa meal, alfalfa ash, and a trace mineral mixture containing iron, manganese, copper, zinc, and cobalt improved for steers a fattening ration containing a poor quality timothy hay. In a later study, Bently and Moxon (1952) (cited by Tillman et al., 1954) reported that the poor quality timothy hay was low in cobalt and that either alfalfa ash or a cobalt containing salt mixture corrected this deficiency.

Digestibility trials were conducted at the Oklahoma Agricultural Experiment Station by Tillman et al. (1954) to study the effect of supplementing a ration containing low quality prairie hay with a complete mineral mixture or alfalfa ash. Neither alfalfa ash nor a complete mineral mixture was found to improve the apparent digestibility of the ration or any of its proximate components.

Results obtained in studies by Burroughs et al. (1950) showed that alfalfa hay contains a nutrient(s) in addition to protein which in some way protects it from being digested to a lesser extent when fed in the presence of starch. This nutrient(s) appears to be contained in sufficient amounts in alfalfa so that when a poor quality roughage is fed such as corn cobs in conjunction with alfalfa, the poor quality roughage is also benefited.

Burroughs stated that the nature of the unknown nutrient(s) would appear to be partially or totally soluble in water and in part or totally associated with the inorganic constituents contained in alfalfa hay. The

evidence presented in the study can best be explained on the basis that the unknown material under consideration in alfalfa hay is an essential nutrient(s) for rumen microorganisms involved in roughage digestion. This view is further strengthened by in vitro roughage digestion studies (unpublished data) with rumen microorganisms involving roughage digestion. The addition of alfalfa ash and certain complex mineral mixtures definitely enhances the ability of rumen microorganisms to digest cellulose as well as roughage dry matter under controlled laboratory conditions.

Burroughs further states, that the importance of these findings to livestock production in addition to their academic interest would seem to lie in the possibility that poor quality roughages may need special mineral supplementation. Also the probability exists that quality of roughages as it applies to livestock feeding may be dependent to a large extent upon the mineral make-up of the roughage in supplying nutrients for rumen microorganisms involved directly in roughage digestion or utilization.

In digestion trials with Hereford steers, the digestion of corn cobs was improved progressively with four respective additions of alfalfa hay. A water extract of dehydrated alfalfa meal or the ash of the alfalfa meal fed at the rate equivalent to four pounds meal daily per steer improved the digestion of corn cobs materially.

Swift et al. (1951) reported that in digestion and calorimetric experiments with sheep fed a ration containing about 40.4 per cent corn cobs, the apparent digestibility of the crude fiber was significantly increased by the addition of the alfalfa ash (from 43.0 per cent to 53.8

per cent). The Increase in crude fiber digestibility was accompanied by an increase in methane production which, however was not large; the net effect of the alfalfa ash being to increase the amount of feed energy available to the animal. Swift states that the effect of the ash was to increase fermentation in the rumen, making available to the animal, after paying for the overhead (methane), distinctly more feed energy.

Chappel et al. (1955) reported that the addition of common nutrient mineral elements to a ration fed to lambs, in which corn cobs was the main roughage was without effect. However, the addition of either the ash of alfalfa or a synthetic alfalfa ash (a mixture of purified salts simulating the composition of alfalfa ash) resulted in highly significant increases in the digestion of organic matter and crude fiber fractions of the ration.

Burroughs et al. (1950) using the artificial rumen technique compared four poor quality roughages with four good quality dehydrated forages. He reported, the cellulose in the good quality roughages was digested efficiently without supplementation, digestion being as good as that occurring when the addition of complex minerals and autoclaved manure extract were included. Conversely, the cellulose in the poor quality roughages failed to be digested efficiently without supplementation. The addition of complex minerals and manure extract along with available nitrogen greatly increased cellulose digestion in these poor quality roughages. The poor quality roughages were: corn stover, wheat straw, corn cobs, and mature timothy-bluegrass hay. The good quality roughages were: dehydrated-clover hay, rye hay, and alfalfa hay.

Hamilton et al. (1955) reported that the mineral levels in the rumen ingesta varied from 12 per cent to 20 per cent of the dry matter.

Mineral levels were high on poor quality rations and vise versa. Sources of minerals other than saliva were considered.

The Effect of Prussic Acid on Rumen Bacteria

Morrison (1956) stated that both Tift Sudan and Sweet Sudan are apt to be higher than common Sudan in prussic acid which is toxic. He also reported that Piper, a new variety developed by the Wisconsin station has much less prussic acid than other Sudan grasses. Early research by Willaman and West (1916) at Minnesota showed that varietal difference is probably of more weight in determining the amount of hydrocyanic acid in sorghum than is the condition of growth.

Avery et al. (1903) (cited by Vinall, 1921) reported that the glucosides of prussic acid are harmless and become dangerous only when they are hydrolyzed and liberate prussic acid. This cyanogenetic glucoside in sorghums is called dhurrin and is said by Dunstan and Henry (1902) (cited by Vinall, 1921) to have the empirical formula $C_{14}H_{17}O_7N$, and on hydrolysis with hot dilute hydrochloric acid or the enzyme, emulsin, yielded one molecule each of prussic acid, parahydroxybenzaldehyde, and dextrose.

The theory of Dunstan and Henry (1902), as reported by Vinall (1921), was that sorghum poisoning is caused by dhurrin and emulsin coming together in the early processes of digestion when the enzyme by the addition of water to the glucoside breaks the latter down and liberates the prussic acid.

Boyd et al. (1939) stated that the various species of animals react differently when fed plants containing cyanophoric glucosides. These

differences are caused by different anatomical structures and different detoxifying abilities of various animals. Cattle and sheep are ruminants and both are known to be subject to poisoning by cyanophoric glucosides. The paunch or rumen of these animals is neither strongly acid nor alkaline in reaction, contains a large flora of microorganisms, and considerable quantities of the enzyme emulsin. An excellent medium is thus provided for the hydrolysis of the glucoside with the liberation of the toxic agent-hydrocyanic acid. Horses and hogs, being non ruminants, have only one stomach which is strongly acid in reaction due to the presence of HCl. This HCl reacts with the liberated HCN to form much less toxic substances which are formic acid and ammonium chloride.

Manges (1935) stated that the cyanide acts upon the protoplasm and suspends the activity of all forms of living matter.

Menaul and Dowell (1920) reported that the quantity of hydrocyanic acid is greatest in the young plant and decreases rapidly as the plant matures.

There is definite experimental evidence that nitration of the plants has an effect on their hydrocyanic acid content. As early as 1903, Maxwell, as reported by Willaman and West (1915), believed that the amount of the glucoside is dependent on the character of the soils - soils rich in nitrogen produce plants richer in the glucoside. Brunnich (1905), as reported by Willaman and West, in experiments with sodium nitrate in Queensland found that fertilized plants contained slightly more hydrocyanic acid than those unfertilized, and concluded that heavy nitrogenous soils and favorable climatic conditions increase the amount of the acid.

Willaman and West (1915) concluded that when sorghum is grown on poor, infertile soil, added nitrogen may slightly increase the amount of hydrocyanic acid in the plant. With a fertile soil and abundant nitrogen this effect may not be produced.

Comparatively recent work was reported by Boyd et al. (1939). The results of the effect of heavy applications of nitrogen fertilizer in pot tests on the cyanide content of Sudan grass at different stages of growth showed conclusively that Sudan grass in both the fertilized and unfertilized pots was highest in cyanide content in the early stages of growth. The Sudan grass which received the nitrogen fertilizer was consistently higher in cyanide than the unfertilized grass, but was below the toxic limit after it had reached a height of one foot.

CHAPTER III

EXPERIMENTAL

Development of Technique

This procedure was developed on the principle that, theoretically, a forage should be able to supply the entire nutrient requirements of rumen microorganisms without any additional supplementation of minerals or protein. This implies that the existing artificial rumen techniques using the whole rumen juice with or without the addition of a mineral and urea nutrient solution could not be used. Washed, mineral free rumen microorganisms were necessary.

A method for the study of cellulose digestion by washed suspensions of rumen microorganisms was developed by Cheng, Hall, and Burroughs (1955). This method, however, involves the use of pure wood cellulose as the bacterial substrate with the addition of a mineral and urea solution to supply the nutrient requirements of the microorganisms.

The method of Cheng et al. (1955) was revised and was used in this investigation. The major revisions were the elimination of the nutrient solution entirely, and modifications in glassware and equipment which would make this technique applicable for use with forages instead of cellulose. The washing technique was revised slightly and improved.

The first attempted digestion trials were carried out using the original technique of Cheng et al. (1955), the only exception being that ground grass was used in place of pure cellulose. It was observed that

the ground forage was quite buoyant and immediately floated to the top of the centrifuge tubes where it could not be saturated by the bacterial solution and proper digestion take place. Attempts to alleviate the condition by breaking the surface tension with small amounts of detergents and ethyl alcohol were unsuccessful. Upon agitation of the contents, considerable quantities of the forage remained on the sides of the tube which was an unsatisfactory condition. Also, it did not help saturate the grass. Allowing the forages to remain in water overnight helped only slightly.

It was decided that centrifuge tubes, which were satisfactory for use as digestion flasks with pure cellulose, could not be used as digestion flasks for ground forage and were discarded. Before further work could continue it was important that a method be found for the complete saturation of the ground forage without the use of foreign additives which would be toxic to the bacteria or change experimental results.

Grinding the forage as fine as possible in a Wiley Mill and then mixing with water in a Waring Blendor proved satisfactory. However, it was impossible to transfer the entire amount of the ground forage quantitatively from the blendor with a limited amount of wash liquid. It is important that the concentration of forage to liquid not be changed.

To avoid transfering the forage from the mixing bowl to the final digestion flask, it was decided to agitate the forage in the final digestion flask with a magnetic stirrer. Erlenmeyer flasks (125 milliliters) proved satisfactory as digestion flasks and were used.

Our original intentions were to use disappearance of total dry matter, correcting for the water soluble fractions, as a measure of digestibility.

Failure resulted because it was impossible to filter off the bacterial solution from the forage due to the proteinaceous nature of the bacterial solution. This plan was discarded and the digestibility of the forage cellulose was used as a criterion of bacterial activity. This involved a cellulose determination on the forage remaining in the flask, following the digestion period.

After the physical and mechanical difficulties in the technique were corrected, pilot runs were made in duplicate. The bacterial suspension was prepared exactly as described by Cheng et al. (1955) up to and including the final washing. At this point the final sediment was suspended in distilled water and bubbled with a stream of carbon dioxide for ten minutes. One-half of the bacterial suspension was added to an Erlenmeyer flask containing ground forage suspended in the mineral nutrient solution described by Cheng et al. (1955). The other half of the bacterial suspension was added to an Erlenmeyer flask containing the same ground forage suspended in distilled water. The first flask containing the nutrient mineral solution served as a control to check the efficiency of the second flask without the added nutrients.

These pilot runs resulted in failure, not only in the new technique in which the rumen microorganisms were suspended in distilled water but also in the technique of Cheng et al. (1955), in which the rumen microorganisms were suspended in the mineral nutrient solution. After repeated unsuccessful trials and careful checks of every step of the laboratory technique, the cause of failure was discovered. It was observed when checking the pH of the bacterial suspension following bubbling with carbon

dioxide; that the pH of the bacterial suspension was between pH 4 and pH 5. This acid condition resulted in the probable death of the microorganisms even before the digestion period had begun.

The fact was overlooked that the passage of carbon dioxide through distilled water results in the formation of carbonic acid causing a sharp decline of the pH of the solution. This did not occur in the original technique of Cheng et al. (1955) due to the fact that the nutrient mineral solution used has buffering properties. However, in these trials failure resulted in both techniques because the bacterial suspension was carbonated before it was divided, one half being added to the nutrient mineral solution.

It was decided that a buffer was necessary to keep the bacterial suspension close to pH 7. From the standpoint of bacterial physiology a phosphate buffer would have been the most satisfactory. However, the addition of phosphorus, which is a very critical mineral requirement for rumen bacteria, would be defeating the purpose of this technique.

A 1.0 per cent solution of sodium bicarbonate in distilled water was found to maintain a pH of about seven after being bubbled vigorously with carbon dioxide overnight. The rumen microorganisms were suspended in a sodium bicarbonate solution during all of the in vitro digestion trials thereafter. The original washing technique of Cheng et al. (1955) was revised to use a sodium bicarbonate solution as a washing media instead of distilled water. This allowed carbonation between washings and resulted in much less death of bacteria during the washing technique and higher digestion.

Those workers who did not wash the rumen microorganisms did not encounter these difficulties due to the buffering properties of the whole unwashed rumen liquor. Also, the addition of a mineral nutrient solution or basal media used by many workers alleviated this condition.

It is interesting to note that similar conditions exist in the rumen of the living animal and that a buffering action takes place. Cason et al. (1954) reported that a significant positive correlation was found to exist between the ash content of the rumen ingesta and the pH of the rumen. This relationship appears to be due to the buffering capacity of the ash which together with the buffering action of the saliva, tends to keep the pH of the rumen contents within rather narrow limits.

After the use of the sodium bicarbonate buffer was initiated, the in vitro digestion trials proceeded satisfactorily both in the new technique which did not involve the use of the nutrient mineral solution and the technique of Cheng et al. (1955). The practice of running a digestion trial using the nutrient mineral solution along with the digestion trial using the new technique, without the added nutrient mineral solution was continued throughout the pilot runs. It was noted that the digestion of the grass cellulose in the flasks containing the nutrient mineral solution was about 4 or 5 per cent higher than in the flasks containing only the forage and rumen microorganisms.

Experimental Procedure

Source of Forage Material

The forages used in this study were grown in Tennessee River bottomland under controlled experimental conditions by the Department of Agronomy

of the University of Tennessee. The varieties of grasses grown were Sweet Sudan, Piper Sudan, German millet, and Georgia hybrid No. 1 pearl millet. The two levels of nitrogen fertilization used in this investigation were: no nitrogen and 120 pounds nitrogen per acre.

The Agronomy Department made cuttings at several stages of growth but the only ones used in these studies were the twenty inches down to six inches to represent the usual pasture stage, and fifty four inches or early bloom down to four inches to represent the hay stage. Several cuttings were made at each stage of growth but only the first cutting was used in this investigation.

The grasses were planted at the rate of eight pounds per acre on June 1, 1957 at which time 350 pounds per acre of 0-20-20 were drilled in. Nitrogen treatments were top-dressed as 33.5-0-0. Two of the three replications were located on Huntington loam, the other on Huntington sandy loam. The plots consisted of three, twenty foot rows, three feet apart; the center eighteen feet of the center row was harvested as described above and was used in both this experiment and the Agronomy Department experiment. The plant material from the three replications was composited. After harvesting, the plant material was dried overnight in an electric oven, ground in a Wiley Mill and stored in paper bags.

A forage of a proven high nutritive value (Kentucky bluegrass) was included in the experiment to act as a positive control for comparison to determine the quality of the forages being tested. The only stand of pure Kentucky bluegrass available was from a lawn and the sample taken was cut with hand clippers. The sample was taken in late September when it

was in a very succulent stage of growth. The same sample of Kentucky bluegrass was used throughout the entire series of experiments.

Preparation of Forages

The ground forages, as received from the Agronomy Department, were reground six times in the Wiley mill using a number 40 mesh screen in order to get them very fine. To provide for experimental uniformity, each forage received exactly the same grinding treatment through the same screen. The forages were oven dried and kept in a dessicator until ready for use.

Preparation of the Digestion Flasks Prior to the Processing of the Rumen Microorganisms

Each forage was run in duplicate, and two trials were run on each test. The only exception to this was the grass-ash experiment where only one trial was run.

One-half gram of the ground and dried forages were weighed, the exact weight recorded, and added to numbered 125 millileter Erlenmeyer flasks. The Erlenmeyer flasks were fitted with a rubber stopper, through which passed two separate pieces of glass tubing which served as carbon dioxide lines. One length of glass tubing reached almost to, but not quite touching, the bottom of the flask. This served as the carbon dioxide inlet tube and carried carbon dioxide down into the bacterial suspension. The second piece of glass tubing was simply a short piece extending a short distance below the rubber stopper, to act as an escape tube.

In advance of going to the barn to collect the rumen juice, 25 milliliters of a 1 per cent solution of sodium bicarbonate solution in

distilled water was pipetted into each flask. Each flask was placed on a magnetic stirrer and the contents agitated rapidly for exactly five minutes. The sodium bicarbonate solution was added to all of the flasks at the same time and each flask stirred for the same period to provide for experimental uniformity. No attempt was made to wash the forage which was splashed up on the sides of the flasks back into the solution to avoid disturbing the dilution factor. This was corrected later. The stirring blade was rinsed off with a few drops of water between flasks.

The stopper was placed in each flask and they were placed in a shallow water bath set at 39.5° Centigrade. This water bath was located beneath a series of gas outlet fittings attached to a tank of carbon dioxide. A stream of carbon dioxide was started through the flasks as soon as possible, in advance of processing the bacteria.

The apparatus was set up completely in advance, and carbon dioxide passed through the digestion flasks containing the forage saturated in buffer solution for these reasons. The first, and most important, is that the sodium bicarbonate buffer solution is alkaline. By bubbling carbon dioxide through the solution, carbonic acid is formed and the pH will shift to about pH 7. If the carbon dioxide is stopped, the pH will shift back to an alkaline condition. The second reason is that once the processing of the bacteria is begun, it should be carried on continuously, without interruption. Washed rumen bacteria are far more delicate than the whole rumen juice and it is necessary that they be added to a source of nutrients, which in this case is a ground forage, as soon as possible. The third reason is that the digestion flasks and their contents need to be 39.5°

Centigrade when the bacterial suspension is added.

The apparatus was set up as follows: Two flasks were set up in series, the second flask receiving its carbon dioxide from the first. The long inlet tube on the first flask was connected to a gas outlet fitting by means of rubber tubing. A shorter second piece of rubber tubing connected the short glass escape tube on the first flask to the long glass inlet tube on the second flask. The order in which the flasks were set up will be described under each experiment.

Before going to the barn, two flasks containing 800 milliliters of 0.8 per cent sodium bicarbonate in distilled water and one flask containing 500 milliliters of the 1 per cent sodium bicarbonate buffer were placed in a deep water bath set at 39.5° Centigrade and carbon dioxide was bubbled through the solutions until used. These two 0.8 per cent buffer solutions were used to wash the rumen microorganisms.

Collection, Processing, and Washing of the Rumen Microorganisms

Rumen contents obtained from a fistulated steer, fed a predominantly orchardgrass hay diet, was strained through eight layers of cheese cloth into previously warmed thermos bottles. Actually, 2000 milliliters of whole rumen juice were required for an experiment. However, since it is difficult to accurately measure whole rumen juice due to foam and gas bubbles, about 2200 milliliters of whole rumen juice were used.

The whole rumen juice was centrifuged in an Ivan Servall angle centrifuge at 1000 r.p.m. for one minute. This step was to sediment the protozoa, feed debris, and undigested feed particles not removed when straining through the cheese cloth.

The supernatant, which contained the cellulolytic bacteria, was then poured off and strained through eight layers of cheese cloth into an Erlenmeyer flask clamped into a deep water bath set at 39.5° Centigrade. A few drops of the sediment from each centrifuge tube was added. The remaining sediment was then discarded. The supernatent was then left in the water bath to warm and a stream of carbon dioxide bubbled through it lightly for about ten minutes.

The supernatent was again centrifuged in a Sharples super centrifuge operating at its maximum speed, between 25,000 and 35,000 r.p.m. The bacterial sediment was scraped into a Waring Blendor bowl, a small amount of 0.8 per cent sodium bicarbonate buffer was added, and the packed bacteria were agitated for about twelve seconds to resuspend the bacteria. The volume of the bacterial suspension was built up to 800 milliliters with the addition of the carbonated 0.8 per cent sodium bicarbonate buffer. It is important that this buffer solution be well carbonated and the pH of the solution be checked before use.

The bacterial suspension was again warmed and carbonated for about ten or fifteen minutes while the Sharples centrifuge was reassembled. The bacterial suspension was centrifuged through the Sharples centrifuge again and resuspended in the 0.8 per cent sodium bicarbonate solution and centrifuged as before.

After the second washing a small amount of the 1 per cent of the sodium bicarbonate buffer was added to the Waring Blender bowl instead of the 0.8 per cent solution. The bacterial suspension was then built up to a volume of a little over 500 milliliters with the 1 per cent sodium

bicarbonate solution. Actually 500 milliliters were required, but a little more solution is recommended to facilitate accurate pipetting of the last aliquote. The 1 per cent sodium bicarbonate buffer must be warm and well carbonated and the pH checked before adding it to the bacterial suspension.

The bacterial suspension was returned to the water bath and bubbled briskly for about ten minutes with carbon dioxide. The pH was again checked and, if necessary, adjusted to pH 7 with a saturated solution of sodium carbonate. It was not necessary to adjust the pH during any of the trials.

Concentrating the 2000 milliliters of whole rumen juice down to 500 milliliters of a washed bacterial suspension provides a solution with twice the concentration of bacteria normally used in artificial rumen work. This was done purposely to account for the fact that 25 milliliters of the bacterial suspension was added to a digestion flask containing 25 milliliters of the sodium bicarbonate buffer. This provides a final concentration of 1000 milliliters of washed rumen bacterial suspension from 2000 milliliters of whole rumen juice which is normally used.

Twenty five milliliters of the bacterial suspension were pipetted into each digestion flask. The carbon dioxide stream was continued throughout the entire procedure. The suspension was pipetted down the side of the flask, washing all of the ground forage on the sides of the flask into the solution.

The flasks were disconnected at the water bath - one series of two flasks at a time. The bacterial suspension was pipetted into both flasks and they were returned immediately to the water bath and reconnected to the source of carbon dioxide before disconnecting the next series of two flasks.

The fermentation period was allowed to proceed for twenty four hours with an occasional gentle agitation of each flask. At the end of the fermentation period they were disconnected and processed in the same order as they were set up in an effort to correct for the time required to inocculate a total of twenty digestion flasks.

Cellulose Determination

After the fermentation period, the entire contents of the digestion flasks were carefully transferred to 75 milliliter Pyrex centrifuge tubes, washing the flasks several times with small amounts of water to remove all of the forage. The tubes were centrifuged for four minutes at a speed of about 1000 r.p.m. in an Ivan Servall angle centrifuge and the supernatent discarded.

The undigested cellulose in the forage samples was determined by using the procedure of Crampton and Maynard (1938) with slight modifications. Twenty five milliliters of 1.25 per cent sodium hydroxyde solution in distilled water was added to each tube and each tube was placed in a boiling water bath for exactly twenty minutes to digest the lignin. As soon as it was possible, the tubes were centrifuged at 1000 r.p.m. for five minutes and the sodium hydroxide poured off. The boiling should be staggered, six at a time, so that they could be centrifuged immediately after coming out of the water bath. Then 12 milliliters of glacial acetic acid followed by 2.5 milliliters of 70 per cent nitric acid was put in each tube. Immediately after the addition of the nitric acid the tubes were placed in a boiling water bath and left for exactly twenty minutes.

The entire contents of each tube was filtered through a Gooche crucible with each tube being washed with a stream of ethanol. The crucibles were then left in a vacuum oven at 95° Centigrade for at least four hours or overnight. They were cooled in a dessicator and weighed. They were ignited in a muffle furnace at 650° Centigrade, cooled in a desiccator and weighed. The undigested cellulose was determined by the loss of weight upon ignition.

In order to determine the percentage of cellulose digested it is necessary to know the original cellulose content of the forage sample. The cellulose content of the undigested grass was determined by the method just described with modifications. Due to its physical nature undigested ground grass material has a tendency to float and packs very poorly when centrifuged. However, grass will pack when alcohol is added.

A 0.5 gram oven dried sample of the forages was weighed into a 125 milliliter Erlenmeyer flask. Fifteen milliliters of distilled water plus 15 milliliters of ethanol were pipetted into each flask. Each flask was placed on the magnetic stirrer and agitated rapidly for five minutes. The contents of each flask was transferred carefully to a 75 milliliter Ivan Servall centrifuge tube and centrifuged at 1000 r.p.m. for five minutes. The supernatent was poured off slowly and carefully to avoid the loss of any of the sample material. This first step was to saturate the material so that it could be acted upon properly by the chemical reagents used.

Sodium hydroxide was added and the sample was heated exactly as described in the case of the digested forage. After removal from the

water bath, 25 milliliters of ethanol was added to each tube and the contents stirred with a glass rod. The tubes were then centrifuged at 1000 r.p.m. for five minutes and the supernatent discarded. The remainder of the procedure was carried out exactly as described for the cellulose determination of digested forages.

Description of Experiments

Experimental Variability Trial

An *in vitro* digestion trial was run using Kentucky bluegrass as the only bacterial substrate, in order to determine the variation in digestibility, if any, due to position in the water bath, position of carbon dioxide fitting, position in assembly of apparatus, and due to glassware and apparatus.

The flasks were assembled and the apparatus set up in exactly the same order as used in the forage comparisons.

Variety and Nitration Comparisons of Forages cut at the Pasture Stage

The first experiment was designed to determine differences, if any, between varieties and species, and the effect of nitrogen fertilization on the utilization of these forages by rumen microorganisms when cut at the twenty inches level down to six inches, or pasture stage.

All of the varieties of grasses under test were used in this experiment. The levels of nitrogen fertilization compared were no nitrogen and one hundred twenty pounds nitrogen per acre. All of the forages used were cut at the same level of growth, twenty inches down to six

inches. The variables in this experiment were the forage species and varieties, and the levels of nitrogen fertilization. The stage of growth was constant.

Two flasks containing Kentucky bluegrass were used as a positive control. Two flasks containing purified cellulose (Solka-Floc) were added as a negative control to show the actual stimulatory effects of the nutrients derived from the forages on the bacterial digestion of cellulose.

The samples were run in duplicate and placed in the water bath and connected, in a series of two flasks, in a definite order designed to minimize differences, if any, in position in regard to the carbon dioxide source. An example of this order follows below:

The flask which was connected directly to the carbon dioxide tank outlet fitting was referred to as being in the number one position. The flask connected to the number one flask for its carbon dioxide is referred to as the number two position.

Outlet fitting No. 1

Number 1 position; first duplicate of Sweet Sudan, no nitrogen, twenty inches down to six inches.

Number 2 position; first duplicate of Sweet Sudan, 120 pounds nitrogen per acre, twenty inches down to six inches.

Outlet fitting No. 2

Number 1 position; second duplicate of Sweet Sudan, 120 pounds nitrogen per acre, twenty inches down to six inches.

Number 2 position; second duplicate of Sweet Sudan, no nitrogen, twenty inches down to six inches.

This system permitted each nitrogen level to occupy a number one position and a number two position on different CO_2 trains. Two different varieties of grasses were never attached in the same series and Kentucky bluegrass and cellulose were always connected to their own duplicate. The apparatus used is shown in Figure 1.

Variety and Nitration Comparisons of Forages Cut at the Hay Level

This experiment was designed to determine differences, if any, between varieties and species and the effect of nitrogen fertilization on the utilization of these forages by rumen bacteria when cut at the fifty four inches, or early bloom stage, down to four inches.

This experiment was conducted exactly the same as the first experiment with one exception. All of the forages used were cut at the fifty four inches, or early bloom stage, down to four inches.

Stages of Maturity Comparisons of the Non Nitrated Forages

The third experiment was designed to compare the effects of plant maturity on the utilization of these forages by rumen microorganisms, when no nitrogen fertilization was given.

All of the varieties were used in this experiment. The stages of growth were fifty four inches down to four inches, and twenty inches down to six inches. All of the grasses used received no nitrogen. The order in which the apparatus was set up is very similar to that of the first and second experiments. The only difference being that the

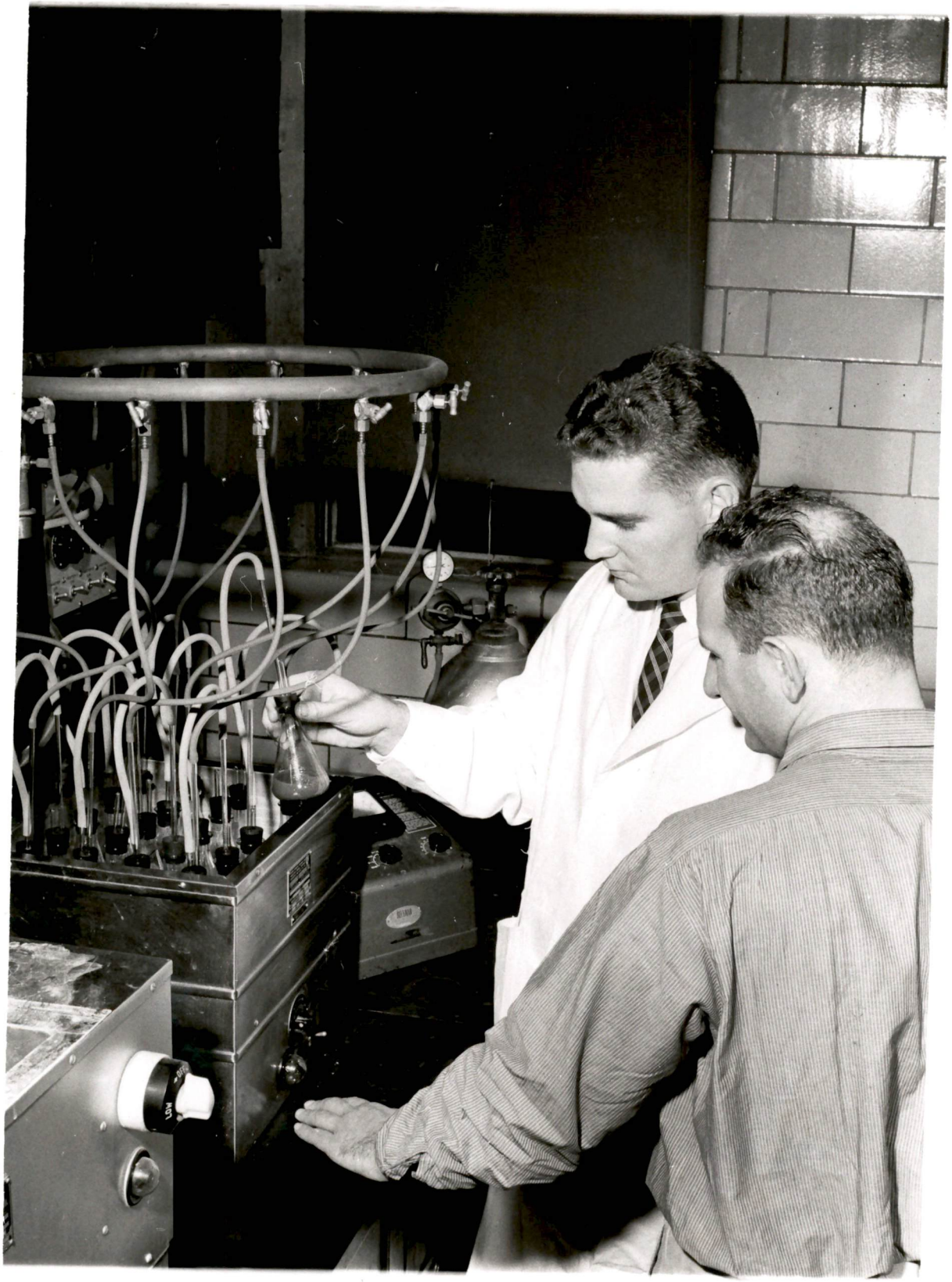


Figure 1. Laboratory apparatus used in these studies.

position was determined by the stage of growth instead of nitrogen fertilization.

Stages of Maturity Comparisons of the Nitrated Forages

This experiment was designed to compare the effects of plant maturity on the utilization of forages by rumen bacteria when 120 pounds of nitrogen fertilization was added per acre.

This experiment was conducted exactly the same as the third experiment with one exception. All of the forages used were fertilized with 120 pounds of nitrogen per acre.

Comparisons of the Effect of the Ash Quantity and Quality of Forages as a Source of Minerals Required by Rumen Microorganisms.

This experiment was designed to compare the effect of variations in the quantity and quality of the ash of these forages on the digestion of purified cellulose by rumen microorganisms. Only one trial was conducted.

In this experiment the forages were ashed and the ash used as a source of minerals. Urea was used as a source of nitrogen. Purified cellulose was used as a bacterial substrate.

A comparison was also made of the stimulatory effect of the addition of an ash supplement on the utilization of a forage by rumen microorganisms. In this case the ash of two different forages was added to flasks containing a whole forage as the bacterial substrate.

All of the varieties and species were used in this experiment. All were cut at the twenty inches down to six inches growth level and

received no nitrogen fertilization except one sample. This was a sample of Sweet Sudan which received 120 pounds of nitrogen per acre and cut at twenty inches down to six inches. Kentucky bluegrass was included and purified cellulose only was added as a control.

One-half gram of the oven dried forage was weighed into crucibles and ashed until all of the organic matter was destroyed. This ash was transferred quantitatively to the digestion flasks and 0.2 gram of purified cellulose was weighed into each flask. One gram of urea was added per liter of the 0.1 per cent sodium bicarbonate buffer used.

In the comparison of the stimulatory effects of an ash supplement on the utilization of a whole forage, 0.5 gram aliquots of non nitrated German millet forage cut at the pasture stage were weighed into six flasks. The ash from 0.5 gram of non nitrated German millet grass (pasture stage) was added to each of two flasks. Two flasks received a supplement of the ash of 0.5 gram of Kentucky bluegrass and the other two flasks received no supplement which served as a control for this portion of the experiment.

Each flask containing only purified cellulose and ash was agitated on the magnetic stirrer for two and one-half minutes to break up the ash and perhaps make it more soluble. The flasks containing the whole forage were agitated for the usual five minutes. In the assembly of the apparatus, both duplicates of a sample were connected together in the same series. In the cellulose determination the treatment with 1.25 per cent sodium hydroxide was omitted for those samples containing only pure cellulose.

The Effect of Hydrocyanic Acid on the Activity of Rumen Microorganisms

This experiment was designed to determine the effect of hydrocyanic acid, if any, on rumen microorganisms. One-half gram samples of non nitrated Piper Sudangrass (pasture stage) were weighed into ten digestion flasks. One-half gram samples of nitrated Sweet Sudan, (pasture stage) were weighed into two digestion flasks.

Immediately after inoculation with rumen microorganisms the flasks containing the Piper Sudangrass received the following treatments. In the first two flasks were pipetted 10 ppm of hydrocyanic acid; second two, 20 ppm; third two, 40 ppm; and the fifth two, 80 ppm. Two flasks containing Piper Sudan did not receive any treatment and acted as a control. The two flasks containing the nitrated Sweet Sudan did not receive any treatment.

The Effect of Parahydroxybenzeldahyde on the Activity of Rumen Microorganisms

This experiment was designed to determine the effect of parahydroxybenzaldehyde, if any, on rumen microorganisms. This experiment was conducted similar to the hydrocyanic acid experiment. The only exception being that parahydroxybenzaldehyde was used in place of hydrocyanic acid. The concentrations of the reagents used were the same.

Due to the fact that parahydroxybenzaldehyde is insoluble in water, it was first dissolved in 2 milliliters of acetone. The solution was made up to the required volume with distilled water. Additions of acetone were added to all flasks, so that all of the samples received the same amount of acetone.

Statistical Analysis

Analysis of variance techniques as described by Snedecor (1950) were used to statistically analyze the data obtained in all experiments. Least significant mean differences (L.S.D.) were calculated for each trial as well as for the average values of all trials conducted in an experiment.

CHAPTER IV

RESULTS AND DISCUSSION

The results of the *in vitro* digestion trials using the various forages as a bacterial substrate are presented in the tables given. It will be noted that in all cases the cellulose content of the forages was digested to a much larger extent than was the purified cellulose in the control flasks. This is a positive indication that nutrients essential for the growth of cellulolytic bacteria were supplied by the forages. It must be kept in mind that in the cellulose control flasks the cellulose was supplied *per se*, and there were no physical factors or barriers inhibiting digestion such as lignin and fiber, as in the case of the grasses.

In order to know the percentage of cellulose digested it was necessary to run cellulose determinations on all of the forage samples involved. The results are listed in Table I.

The results of the experimental variability trial are presented in Table II. The average digestion for the entire trial was 51.7 per cent with a range of 8.7 per cent and a standard error of 2.19 per cent. The average digestion of the flasks in the number one position was 51.48 per cent and in the number two position was 51.95 per cent, or practically the same.

Variety and Nitration Comparisons of Forages Cut at the Pasture Stage

Varieties of these grasses were compared when cut at a height of twenty inches tall down to a level of six inches above the ground. A

TABLE I

CELLULOSE CONTENT OF THE FORAGES TESTED

Forage	Percentage cellulose[a]	
	20 inches down to 6 inches	54 inches down to 4 inches
Sweet Sudan (non nitrated)	29.13	34.10
Sweet Sudan (120 pounds N per acre)	25.11	31.34
Piper Sudan (non nitrated)	30.93	37.75
Piper Sudan (120 pounds N per acre)	26.45	33.03
German millet (non nitrated)	32.77	34.47
German millet (120 pounds N per acre)	29.44	34.53
Georgia hybrid No. 1 pearl millet (non nitrated)	30.46	34.68
Georgia hybrid No. 1 pearl millet (120 pounds N per acre)	26.62	32.69
Kentucky bluegrass	23.24	

[a]Each value is an average of two replicates.

TABLE II

EXPERIMENTAL VARIABILITY TRIAL OF THE DIGESTION OF KENTUCKY BLUEGRASS IN VITRO

Flask number	Percentage cellulose digested
1	47.7
2	50.7
3	51.1
4	53.4
5	56.2
6	54.7
7	51.9
8	54.1
9	50.0
10	50.2
11	51.2
12	49.6
13	48.1
14	48.4
15	53.2
16	54.3
17	53.7
18	52.6
19	51.9
20	50.4

$\bar{X}$ = 51.7 per cent

Standard error of mean = 2.19 per cent

Number 1 position $\bar{X}$ = 51.48 per cent

Number 2 position $\bar{X}$ = 51.95 per cent

comparison was made between the non nitrated forages and the same forages receiving 120 pounds of nitrogen per acre. The results of this experiment are shown in Table III. The digestion of the grasses was much higher than in the purified cellulose control flasks indicating a highly significant increase in digestibility due to the nutrients received from the grasses.

In the case of the non nitrated forages there was no significant differences between varieties within a species. There was no significant difference between either of the non nitrated Sudans and the Kentucky bluegrass control. The digestion of the non nitrated Sudans and Kentucky bluegrass was higher than either of the non nitrated millets. The differences in digestion were highly significant.

Nitration of the Sudans resulted in decreased digestibility. Nitration of Sweet Sudan resulted in a decrease in digestibility which was highly significant, while the decrease in the case of the Piper Sudan did not reach a significant level.

Nitration of the German millet showed an increase in digestibility, which was not statistically significant. Nitration had no effect on the digestibility of the Georgia hybrid No. 1 pearl millet. These results were in agreement with the literature concerning live animal trials which generally show that the nitration of pastures results in an increase in protein, an increase in the digestibility of the protein, but has little effect on the digestibility of the non protein fractions.

In the case of the nitrated forages there were significant differences in digestion, both, between species and between varieties within species. The inhibition of the Piper Sudan due to nitration was 6.2 per

TABLE III

VARIETY AND NITRATION COMPARISONS OF FORAGES
CUT AT THE PASTURE STAGE

Forage	Percentage cellulose digested		
	Trial I	Trial II	Average
	Per cent	Per cent	Per cent
Purified cellulose	17.00	12.50	14.75
Kentucky bluegrass	73.00	73.50	73.25
Sweet Sudan (non nitrated)	73.00	77.00	75.00
Sweet Sudan (120 pounds N per acre)	41.50	56.50	49.00
Piper Sudan (non nitrated)	70.00	74.50	72.25
Piper Sudan (120 pounds N per acre)	62.50	69.50	66.00
German millet (non nitrated)	52.50	62.00	57.25
German millet (120 pounds N per acre)	59.50	66.00	62.75
Georgia hybrid No. 1 pearl millet (non nitrated)	54.50	59.00	56.75
Georgia hybrid No. 1 pearl millet (120 pounds N per acre)	49.00	61.50	55.25
L. S. D. (5 per cent level)	4.50	5.32	8.99
L. S. D. (1 per cent level)	6.40	7.57	12.94

cent compared to 26 per cent inhibition due to nitration in the Sweet Sudan; the difference in inhibition due to nitration being highly significant. The nitrated Piper Sudan was more digestible than the nitrated Sweet Sudan. The difference was highly significant. While there was no significant difference between the non nitrated Sudans and Kentucky bluegrass, the Kentucky bluegrass was highly significantly more digestible than the nitrated Sweet Sudan.

While the nitrated German millet showed a slightly higher digestibility than the nitrated Georgia hybrid No. 1 pearl millet, the difference was not significant.

The nitrated Piper Sudan was more digestible than either of the millets. The difference was significant when compared to the Georgia hybrid No. 1 millet though not significant when compared to the German millet. The nitrated Sweet Sudan was highly significantly less digestible than the German millet. Although the nitrated Sweet Sudan was less digestible than the Georgia hybrid No. 1 pearl millet the difference was not significant. Due to the fact that it is known that nitrogen fertilization increases the prussic acid content of Sudans and sorghums, and Piper Sudan which has a normally lower prussic acid content was less affected by nitration, it was hypothesized that the prussic acid glucoside may have an inhibiting effect on rumen microorganisms. This will be discussed in further detail later.

Variety and Nitration Comparisons of Forages Cut at the Hay Stage

Varieties of these grasses were compared when cut at a height of fifty four inches tall, early bloom, down to four inches above the ground

A comparison was made between the non nitrated forages and the same forages receiving 120 pounds nitrogen per acre.

The results of this experiment are shown in Table IV. The digestion of the Kentucky bluegrass control was highly significantly higher than any of the forages tested. This indicates that the rumen microorganisms, following the washing and processing procedure, still maintained a high cellulolytic potential where the physical and chemical conditions of the forage permitted.

At this stage of maturity, there was no significant difference between any of the species or varieties tested. This is not surprising since the stage of maturity and lignification have been considered by most workers to be the most important factors in relation to organic matter digestibility of a forage.

There was a slight but not significant increase in the digestion of these forages at this stage of maturity due to nitration. These results follow the general pattern of the results obtained with live animals which tend to agree that nitration of forages has no significant effect on their digestibility.

In the first trial there was a highly significant increase due to nitration in the digestibility of all of the forages tested except the German millet. In three subsequent trials, the increase in digestibility of these forages could not be reproduced. There is no explanation for this, except that the steer did suffer from a disorder of the rumen prior to the first trial. There might possibly have been a residual effect from the therapeutic rumen stimulant that was administered by a veterinarian.

TABLE IV

VARIETY AND NITRATION COMPARISONS OF FORAGES CUT AT THE HAY STAGE

Forage	Percentage cellulose digested				
	Trial I	Trial II	Trial III	Trial IV	Average
Purified cellulose	10.50	17.00	9.50	21.50	14.62
Kentucky bluegrass	61.00	61.00	64.00	76.00	65.30
Sweet Sudan (non nitrated)	27.00	30.50	42.50	44.00	36.00
Sweet Sudan (120 pounds N per acre)	39.50	41.50	40.00	40.00	40.29
Piper Sudan (non nitrated)	29.00	33.50	42.50	40.00	35.71
Piper Sudan (120 pounds N per acre)	37.50	35.00	39.00	39.00	37.43
German millet (non nitrated)	30.00	34.00	54.50	35.50	38.50
German millet (120 pounds N per acre)	37.50	31.00	51.00	40.50	40.00
Georgia hybrid No. 1 pearl millet (non nitrated)	26.50	35.50	39.50	39.50	35.25
Georgia hybrid No. 1 pearl millet (120 pounds N per acre)	42.50	37.00	40.00	40.00	39.88
L. S. D. (5 per cent level)	5.50	7.27	7.87	5.17	7.83
L. S. D. (1 per cent level)	7.80	10.33	11.19	7.35	10.58

There was no inhibition of the digestion of the Sudans with increased nitrogen at this stage of maturity. This further supports the hypothesis that the prussic acid glucoside may be involved in the variety and nitration comparisons cut at the pasture stage.

Stage of Maturity Comparisons of the Non Nitrated Forages

Comparisons were made of the two different stages of maturity of the non nitrated forages. The digestibility of the forages when cut at fifty four inches tall, early bloom stage, down to four inches was compared with the same forages when cut at twenty inches tall down to six inches. None were nitrated.

The results of this experiment are shown in Table V. The advanced stage of maturity had a highly significant negative effect on the digestibility of all of the forages tested except German millet. In the case of the German millet there was a negative effect due to maturity, however, it did not quite reach the level of significance.

It is evident from these results that digestibility of these forages, when not nitrated, decreases with plant maturity. These results follow the general pattern of the results of live animal trials where the influence of plant maturity on digestibility was studied. These results confirm the work of Kamstra et al. (1958) who reported that a negative relationship existed between lignin content and the in vitro cellulose digestion in the whole plant materials.

TABLE V

MANAGEMENT LEVEL COMPARISONS OF THE NON NITRATED FORAGES

Forage	Percentage cellulose digested		
	Trial I	Trial II	Average
	Per cent	Per cent	Per cent
Purified cellulose	8.00	11.00	9.50
Kentucky bluegrass	70.00	71.50	70.75
Sweet Sudan (20 in. down to 6 in.)	60.00	67.50	63.75
Sweet Sudan (54 in. down to 4 in.)	31.50	34.50	33.00
Piper Sudan (20 in. down to 6 in.)	61.00	65.50	63.25
Piper Sudan (54 in. down to 4 in.)	24.00	33.00	28.50
German millet (20 in. down to 6 in.)	34.00	35.50	34.75
German millet (54 in. down to 4 in.)	27.00	32.50	29.75
Georgia hybrid No. 1 pearl millet (20 in. down to 6 in.)	45.50	49.50	45.00
Georgia hybrid No. 1 pearl millet (54 in. down to 4 in.)	23.00	32.50	27.75
L. S. D. (5 per cent level)	4.95	5.91	5.42
L. S. D. (1 per cent level)	7.04	8.40	7.80

Stage of Maturity Comparisons of the Nitrated Forages

Comparisons were made of the two different stages of maturity of the nitrated forages. The digestibility of the forages when cut at fifty four inches down to four inches was compared with the same forages when cut at twenty inches tall down to six inches. All of the forages were nitrated at the rate of 120 pounds nitrogen per acre.

The results of this experiment are shown in Table VI. In the case of the nitrated forages the results of the Sudans do not follow the pattern of the millets as affected by plant maturity. It will be noted that the mature Sweet Sudans were digested to a greater extent than the plants cut at twenty inches down to six inches. The nitration of the Sweet Sudan had a stronger inhibiting effect on its utilization by cellulolytic bacteria than did plant maturity. The fact that there was no significant difference between the varieties of the Sudans and millets when cut at fifty four inches down to six inches indicates that the nitrated Sudans were influenced by maturity to the same extent as the millets. It was pointed out in the nitration comparisons of forages cut at the hay stage that there was no inhibition due to nitration at the early bloom stage of maturity. The fact that the results obtained with the nitrated Sweet Sudan are contradictory to the results of the other forages in relation to plant maturity can best be explained by the fact that nitration did not inhibit digestion when cut at the early bloom stage of growth, but had a definite inhibiting effect when cut at twenty inches down to six inches. The inhibition due to nitration of the forage cut at

TABLE VI

MANAGEMENT LEVEL COMPARISONS OF THE NITRATED FORAGES

Forage	Percentage cellulose digested		
	Trial I	Trial II	Average
	Per cent	Per cent	Per cent
Purified cellulose	11.50	10.00	10.80
Kentucky bluegrass	69.00	71.50	70.25
Sweet Sudan (20 in. down to 6 in.)	36.00	41.50	38.75
Sweet Sudan (54 in. down to 4 in.)	52.00	42.00	47.00
Piper Sudan (20 in. down to 6 in.)	53.00	53.50	53.25
Piper Sudan (54 in. down to 4 in.)	44.00	39.50	41.75
German millet (20 in. down to 6 in.)	54.50	59.00	56.75
German millet (54 in. down to 4 in.)	41.50	48.50	45.00
Georgia hybrid No. 1 pearl millet (20 in. down to 6 in.)	48.00	57.00	52.50
Georgia hybrid No. 1 pearl millet (54 in. down to 4 in.)	42.00	42.00	42.00
L. S. D. (5 per cent level)	6.44	6.78	9.11
L. S. D. (1 per cent level)	9.16	9.64	13.10

the pasture stage had a greater effect on its utilization than did plant maturity.

The digestion of the Piper Sudan at the early bloom stage was significantly lower than that cut at twenty inches down to six inches. The effect of nitration on immature Piper Sudan was not nearly as great as that on Sweet Sudan which accounts for the fact that the effect of plant maturity was far more evident. However, in the case of the non nitrated Piper Sudan, digestion decreased almost 50 per cent due to plant maturity, while the digestion of the nitrated Piper Sudan decreased about 20 per cent due to plant maturity. This, again, was due to inhibiting effect of nitration on the immature Sudans; rather than any stimulatory effect on the digestion of the mature forage due to nitration.
The millets followed the expected pattern of a significant decrease in digestion by cellulolytic bacteria due to plant maturity, as was the case with the non nitrated millets.

Comparisons of the Effect of the Ash Quantity and Quality of Forages as a Source of Minerals Required by Rumen Microorganisms

This experiment provided an opportunity to compare the influence of both quantity and quality of these forage varieties as a source of minerals required by rumen microorganisms. To compare the effects of ash quality and make corrections in the results due to experimental variations of the ash present, it was necessary to determine the exact amount of ash added to each flask. The actual amount of the grass ashed, the

ash weight, and the average ash percentage are shown in Tables VII and VIII.

The results of this trial are shown in Tables IX and X. An examination of the results in Table IX shows considerable experimental variation between the two samples of several of the varieties tested. After a comparison of the ash weights per flask shown in Table VII and the digestion per flask it was concluded that this experimental variation was probably due, to a large part, to the difference between the weight of the ash in each of the two flasks.

The reason for this variation in ash weight per flask was due to the fact that when the grass samples were weighed for ashing, it was not considered of prime importance that each sample weigh exactly 0.5000 gram. Instead, each sample was weighed to as close to 0.5000 gram as possible, the two samples of each variety being within one-half milligram of each other. It was not felt at the time, that so minute a difference in the ash per flask would have any observable effect on the digestion of cellulose. Also, dried ground grass will pick up moisture while being weighed and, of course, there was some human error.

To correct for this experimental variation, the milligrams of cellulose digested per milligram of ash added was calculated individually for each flask as shown in Tables XI and XII. The difference between the two sample ash weights per treatment was calculated from Tables VII and VIII. This difference was multiplied by the milligrams cellulose digested per milligram of ash present shown in Tables XI and XII using the data given for the sample number having the lesser amount of ash. This product

TABLE VII

ASH ANALYSIS OF FORAGES CUT AT TWENTY INCHES DOWN TO SIX INCHES

Forage ashed	Sample weight	Ash weight	Average ash percentage
	Grams	Grams	Per cent
Kentucky bluegrass			8.997
Sample 1	0.5001	0.0443	
Sample 2	0.5003	0.0457	
Sweet Sudan (120 pounds N per acre)			10.373
Sample 1	0.5004	0.0514	
Sample 2	0.5003	0.0524	
Sweet Sudan (non nitrated)			9.469
Sample 1	0.5000	0.0465	
Sample 2	0.5001	0.0482	
Piper Sudan (non nitrated)			9.206
Sample 1	0.5000	0.0458	
Sample 2	0.5006	0.0463	
German millet (non nitrated)			10.342
Sample 1	0.5006	0.0515	
Sample 2	0.5002	0.0520	
Georgia hybrid No. 1 pearl millet (non nitrated)			14.305
Sample 1	0.5002	0.0719	
Sample 2	0.5004	0.0712	

TABLE VIII

ASH WEIGHTS OF GRASS ASH SUPPLEMENTATION TO WHOLE FORAGE

Forage ashed	Sample weight	Ash weight
	Grams	Grams
Kentucky bluegrass ash added to German millet grass (non nitrated, 20 in. down to 6 in.)		
Sample 1	0.5002	0.0456
Sample 2	0.5003	0.0439
German millet (non nitrated, 20 in. down to 6 in.) ash, added to German millet (non nitrated, 20 in. down to 6 in.) whole forage		
Sample 1	0.5004	0.0538
Sample 2	0.5004	0.0522

TABLE IX

COMPARISONS OF THE EFFECT OF THE ASH QUANTITY AND QUALITY OF FORAGES ON THEIR UTILIZATION BY RUMEN MICROORGANISMS

Source of ash[a]	Per cent cellulose per se digested		
	Sample 1	Sample 2	Average
	Per cent	Per cent	Per cent
Purified cellulose (control)	23.0	17.0	20.0
Kentucky bluegrass	91.0	92.0	91.5
Sweet Sudan (120 pounds N per acre)	83.0	88.0	85.5
Sweet Sudan (non nitrated)	70.0	80.0	75.0
Piper Sudan (non nitrated)	77.0	80.0	78.5
German millet (non nitrated)	73.0	81.0	77.0
Georgia hybrid No. 1 pearl millet (non nitrated)	86.0	88.0	87.0
L. S. D. (5 per cent level)			9.75
L. S. D. (1 per cent level)			14.46

[a]All of the forages used were cut at the pasture stage.

TABLE X

THE EFFECT OF SUPPLEMENTATION OF GERMAN MILLET GRASS WITH KENTUCKY BLUEGRASS ASH AND GERMAN MILLET ASH

Treatment[a]	Per cent cellulose digested		
	Sample 1	Sample 2	Average
German millet grass (control)	47.0	41.0	44.0
German millet ash added to German millet grass	67.0	68.0	67.5
Kentucky bluegrass ash added to German millet grass	73.0	72.0	72.5
L. S. D. (5 per cent level)			8.01
L. S. D. (1 per cent level)			14.72

[a]All of the German millet ash and grass used was non nitrated, cut at twenty inches down to six inches.

TABLE XI

MILLIGRAMS OF PURIFIED CELLULOSE DIGESTED
PER MILLIGRAM OF GRASS ASH PRESENT

Source of ash[a]	Milligrams of cellulose digested per milligram of ash		
	Sample 1	Sample 2	Average
Kentucky bluegrass	4.09	4.02	4.55
Sweet Sudan (120 pounds N per acre)	3.23	3.37	3.30
Sweet Sudan (non nitrated)	3.01	3.34	3.17
Piper Sudan (non nitrated)	3.36	3.48	3.42
German millet (non nitrated)	2.83	3.13	2.98
Georgia hybrid No. 1 pearl millet (non nitrated)	2.40	2.49	2.44
L. S. D. (5 per cent level)			.348
L. S. D. (1 per cent level)			.527

[a]All grass ashed was cut at twenty inches down to six inches.

TABLE XII

ADDITIONAL MILLIGRAMS OF GRASS CELLULOSE DIGESTED DUE TO ADDED ASH

Treatment[b]	Additional mgs. cellulose digested[a]	Additional mgs. cellulose digested per mg. of ash added
Kentucky bluegrass ash added to German millet grass		
Sample 1	47.80	1.05
Sample 2	46.80	1.07
Average		1.06
German millet ash added to German millet grass		
Sample 1	36.60	0.68
Sample 2	38.20	0.73
Average		0.71

[a]These values are based on the fact that 44 per cent of the cellulose was digested in the German millet control flasks as shown in Table X. These values represent the difference in milligrams of cellulose digested between the control and the ash supplements.

[b]All of the German millet ash and grass used was cut at twenty inches down to six inches.

was then added to the cellulose digested of the sample having the lesser amount of ash and the percentage of cellulose digested for that sample recalculated. The results of the ash weight corrected digestion values are shown in Tables XIII and XIV.

These figures represent the theoretical percentage of cellulose digested had each sample of a treatment received exactly the same amount of ash. The corrections were based on the actual digestion of the sample being adjusted. The corrections were made only between samples of a single treatment and not between treatments due to variations in the normal ash content of the forages used.

An examination of Table XIII shows that the effects of adding ash on the digestion of purified cellulose was highly significant in all cases. The highest digestion of cellulose occurred in the treatment containing the ash of Kentucky bluegrass. This was followed by the non nitrated Georgia hybrid No. 1 pearl millet and the nitrated Sweet Sudan. However, these differences were not significant. The differences in cellulose digestion between the Kentucky bluegrass and the non nitrated Sweet Sudan, the non nitrated Piper Sudan and the non nitrated German millet ash treatments were highly significant. Cellulose digestion in both the nitrated Sweet Sudan and the non nitrated Georgia hybrid No. 1 pearl millet ash treatments was significantly higher than the non nitrated sweet Sudan, non nitrated Piper Sudan, and the non nitrated German millet ash treatments. There was no significant difference in the digestion between the non nitrated Sweet Sudan, non nitrated Piper Sudan, and non nitrated German millet ash treatments.

TABLE XIII

CORRECTED ASH WEIGHT VALUES OF COMPARISONS OF THE EFFECT OF THE ASH QUANTITY AND QUALITY OF FORAGES ON THEIR UTILIZATION BY RUMEN MICROORGANISMS

Source of ash[a]	Percentage of cellulose per se digested		
	Sample 1	Sample 2	Average
Purified cellulose (control)	23.0	17.0	20.0
Kentucky bluegrass	93.0	92.0	92.5
Sweet Sudan (120 pounds N per acre)	84.0	88.0	86.0
Sweet Sudan (non nitrated)	72.0	80.0	76.0
Piper Sudan (non nitrated)	78.0	80.0	79.0
German millet (non nitrated)	74.0	81.0	79.0
Georgia hybrid No. 1 pearl millet (non nitrated)	86.0	89.0	87.5
L. S. D. (5 per cent level)			8.43
L. S. D. (1 per cent level)			12.50

[a]All of the forages used were cut at twenty inches down to six inches.

TABLE XIV

THE EFFECT OF SUPPLEMENTATION OF GERMAN MILLET GRASS WITH KENTUCKY BLUEGRASS ASH AND GERMAN MILLET ASH

(Corrected for Ash Weight)

Treatment[a]	Per cent cellulose digested		
	Sample 1	Sample 2	Average
German millet grass (control)	47.0	41.0	44.0
German millet ash added to German millet grass	67.0	68.0	67.5
Kentucky bluegrass ash added to German millet grass	73.0	72.0	72.5
L. S. D. (5 per cent level)			8.01
L. S. D. (1 per cent level)			14.72

[a]All of the German millet ash and grass used was non nitrated, cut at twenty inches down to six inches.

In this trial there was a significant increase in digestion of cellulose due to the nitration of Sweet Sudan. As shown in Table VII, nitration also increased the ash content.

A comparison of the data in Table VII with that in Table XIII shows that rumen microorganisms respond to both the quantity of ash in the forage and the quality of the ash in the forage. Georgia hybrid No. 1 pearl millet, which was second in digestion only to Kentucky bluegrass, had the highest ash content of the forages tested, 14.30 per cent. However, Kentucky bluegrass, which was the highest in cellulose digestion, had the smallest ash content of the forages tested, 9.00 per cent.

The effect of ash quality on cellulose digestion by rumen microorganisms can best be seen by examination of data in Table XI. This shows the milligrams of cellulose per se digested per milligram of ash present. Kentucky bluegrass was the highest in this respect, the difference being highly significant in all cases. This indicates that the high response of the rumen microorganisms due to the addition of Kentucky bluegrass ash was due to ash quality rather than ash quantity. Georgia hybrid No. 1 millet was lowest in this respect, the difference being highly significant in all cases. This indicates that the high response of the rumen microorganisms due to the addition of Georgia hybrid No. 1 millet ash was due to the quantity of the ash rather than quality.

There was no significant difference between the nitrated and non nitrated Sweet Sudan. This indicates that the significant increase in cellulose digestion due to the addition of the ash of the nitrated forage was due to ash quantity rather than quality.

A comparison of the values of the ash of Kentucky bluegrass and German millet as a supplement for German millet grass are shown in Table XIV. The added ash resulted in a highly significant increase in the digestion of the German millet grass cellulose in all cases. This indicates possibilities that the nutritive value of some forages can be improved by the addition of mineral supplements.

The statistical analysis of the comparison between the treatments of German millet ash and Kentucky bluegrass ash only show that the digestion was significantly higher in the samples containing the Kentucky bluegrass ash.

The additional digestion, due to the ash supplements, in terms of milligrams of grass cellulose digested per milligram of grass ash present was calculated and is shown in Table XII. As a supplement for German millet grass, the Kentucky bluegrass ash was of a higher quality than the German millet ash. The difference was highly significant.

The Effect of Hydrocyanic Acid on the Activity of Rumen Microorganisms

It was noticed early in the experimental work that the nitration of Sudans caused them to be considerable less digestible than the non nitrated Sudans. This was especially evident in the Sweet Sudan and less evident in the Piper Sudan. In some of the early preliminary work, the digestion of the nitrated Sweet Sudan was practically inhibited while the digestion of the Piper Sudan was decreased considerably as compared to the non nitrated forages.

Due to the fact that the early literature states that nitrogen fertilizer increases the prussic acid content of Sudans and sorghums and the Piper Sudan which has a lower prussic acid content was less affected, it was hypothesized that the prussic acid glucoside may have an inhibiting effect on rumen bacteria.

The results of the addition of a pure solution of hydrocyanic acid to digestion flasks containing non nitrated Piper Sudan, cut at twenty inches down to six inches, are shown in Table XV. Two samples containing the nitrated Sweet Sudan, which was usually inhibited, were used as a comparison.

The results shown in Table XV do not indicate any inhibition of rumen microorganisms, due to hydrocyanic acid, under the experimental conditions and levels of hydrocyanic acid used.

This does not imply that hydrocyanic acid has no effect on rumen bacteria, but that in these trials it could not be shown. Perhaps an effect could be shown if higher levels of hydrocyanic acid were used. Also, it must be borne in mind that hydrocyanic acid was extremely volatile and did not remain in the bacterial solutions at the concentrations added for very long. Perhaps an effect, if any, could be shown if a different type of apparatus could be adapted.

The Effect of Parahydroxybenzaldehyde on the Activity of Rumen Microorganisms

The results of this experiment are shown in Table XVI. Although mathematically, the results are significant, the inhibition shown nowhere

TABLE XV

THE EFFECT OF HYCROCYANIC ACID ON THE DIGESTION OF PIPER SUDAN GRASS BY RUMEN BACTERIA IN VITRO

Forage[a]	HCN added ppm	Per cent cellulose digested		
		Trial I	Trial II	Average
Sweet Sudan (120 pounds N per acre)	None	54.00	29.00	41.00
Piper Sudan (non nitrated)	None	75.00	64.50	69.75
Piper Sudan (non nitrated)	10	75.00	67.50	71.25
Piper Sudan (non nitrated)	20	72.50	67.00	69.75
Piper Sudan (non nitrated)	40	71.50	64.50	68.00
Piper Sudan (non nitrated)	80	70.50	63.50	67.00
L. S. D. (5 per cent level)		3.09	4.68	12.69
L. S. D. (1 per cent level)		4.67	7.09	19.21

[a]All of the forages used were cut at twenty inches down to six inches.

TABLE XVI

THE EFFECT OF PARAHYDROXYBENZALDEHYDE ON THE DIGESTION OF PIPER SUDAN BY RUMEN BACTERIA IN VITRO

Forage[a]	PHBA added ppm	Per cent cellulose digested		
		Trial I	Trial II	Average
Sweet Sudan (120 pounds N per acre)	None	58.00	55.00	56.50
Piper Sudan (non nitrated)	None	78.50	73.00	75.75
Piper Sudan (non nitrated)	10	77.50	71.50	74.50
Piper Sudan (non nitrated)	20	75.50	70.00	72.75
Piper Sudan (non nitrated)	40	76.50	71.00	73.75
Piper Sudan (non nitrated)	80	76.00	72.00	74.00
L. S. D. (5 per cent level)		2.01	4.07	2.26
L. S. D. (1 per cent level)		3.04	6.16	1.47

[a]All of the forages used were cut at twenty inches down to six inches.

reaches the magniture of that indicated for the nitrated Sweet Sudan and the results must be considered as inconclusive.

The same limitations due to experimental conditions and the levels of parahydroxybenzaldehyde used that were mentioned in regard to the hydrocyanic acid work were applicable. The acetone used to make the parahydroxybenzaldehyde soluble in water was very volatile and as it escaped from solution, no doubt carried parahydroxybenzaldehyde with it.

CHAPTER V

GENERAL DISCUSSION

The experimental procedure previously described was used successfully to evaluate forage utilization by microorganisms *in vitro*. Differences, though not always significant, were found between grass species, varieties, and stages of plant maturity.

No increase in utilization of forages by rumen microorganisms, due to nitration, could be observed using this technique. However, this was in general agreement with most of the live animal studies cited in the review of literature.

Several details in the technique are due for criticism and could possibly be improved. When 50 milliliters of the bacterial suspension were added to the 125 milliliter Erlenmeyer flask, a large surface area of the liquid resulted. This was undesirable due to the fact that aeration may result by having a large portion of the liquid exposed to the atmosphere. The results of this effect were mere obvious in the number two position flask of the series because the outlet tube opens directly into the air.

This condition was overcome by bubbling the carbon dioxide through the contents of the flasks comparatively briskly. If too little carbon dioxide was used, digestion may proceed normally in the number one flask and be inhibited in the number two flask. If too much carbon dioxide was used, a small amount of the liquid in the number one flask may be carried over into the number two flask resulting in a greater experimental variation. It was found that it was preferable to supply the carbon dioxide in slight

excess than to use too little.

There are several possible methods of alleviating the situation. If a larger volume of bacterial suspension was used, the level of the liquid would rise into the narrower portion of the flask. However, this would require processing a greater volume of rumen juice which would take additional time.

Each digestion flask should be connected to an individual carbon dioxide outlet instead of in a series. This would be an ideal situation when the experimental conditions permit. In this experiment only thirteen outlets were available to supply the carbon dioxide for twenty flasks, so that the series arrangement was the only alternative.

The 125 milliliter flask could be replaced by a 50 milliliter flask and the volume of bacterial suspension reduced. This solution appears to be the most satisfactory and should be worthy of trial.

CHAPTER VI

SUMMARY

An experimental technique was developed using the artificial rumen to evaluate the utilization of various forages by rumen microorganisms *in vitro*. This technique is unique in the respect that washed rumen microorganisms were used without the addition of a nutrient mineral solution. The sole source of nutrients required by rumen microorganisms were supplied by the various grasses used in this investigation. The varieties of the grasses used were Sweet Sudan, Piper Sudan, German millet, and Georgia hybrid No. 1 pearl millet.

In all cases the digestion of the grass cellulose was much higher than when the cellulose was supplied *per se*, indicating that the forages supplied essential nutrients required by cellulolytic bacteria.

When cut at the pasture stage the non nitrated Sudans were utilized to a highly significantly greater extent than the non nitrated millets. At this stage of maturity, nitration had no significant effect upon the digestion of the millets while it had an inhibiting effect on the digestion of the Sudans. The decrease in digestion of the Sweet Sudan was highly significant while the decrease in the case of the Piper Sudan was not significant.

When cut at the hay stage there was no significant difference between any of the species or varieties tested, and nitration had no significant effect. At this stage of maturity there was no decrease in the digestion of the Sudans due to nitration.

Comparisons made between non nitrated forages cut at the pasture stage and the hay stage showed that maturity had a highly significant negative effect on the digestibility of all of the forages tested except German millet. In the case of German millet the decrease in digestion due to maturity was not quite significant.

Comparisons made between the nitrated forages cut at the pasture stage and the hay stage showed that maturity had a significant negative effect upon the digestion of the millets and Piper Sudan. The Sweet Sudan was digested to a significantly greater extent when mature than when cut at the pasture stage, because there was no inhibition due to nitration in the case of the mature plants.

A technique was developed to compare the influence of the quantity and quality of the grass ash upon the digestion of purified cellulose. One-half gram of the forage was ashed and the ash used as the sole source of minerals required by rumen microorganisms. There are indications that bacterial growth was influenced by an interaction between the total quantity of the minerals in a forage and the quality of the minerals in a forage. The balance of minerals may be an important factor in mineral quality.

Kentucky bluegrass ash supported the highest cellulose digestion with the smallest amount of ash present due to the comparatively low ash content of Kentucky bluegrass. Georgia hybrid No. 1 pearl millet ash was the next highest in cellulose digestion with the largest amount of ash present.

Both the ash of Kentucky bluegrass and the ash of German millet were supplemented to German millet grass. The ash supplements caused a

highly significant increase in grass cellulose digested in both cases. The digestion of cellulose was significantly higher in the flasks containing the Kentucky bluegrass ash than the German millet ash.

In an effort to explain the inhibiting effect of the nitration of Sudans, trials were run to determine the effect, if any, of the segments of the prussic acid glucoside on bacterial activity. Under the experimental conditions used, no inhibiting effect on bacterial activity could be produced by additions of either hydrocyanic acid or parahydroxybenzaldehyde.

LITERATURE CITED

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Bird, J. N., 1943. Stage of Cutting Studies: I. Grasses. Jour. Am. Soc. of Agron. 35:845.

Boyd, F. T., O. S. Asmodt, G. Bohstedt, and E. Truag. 1939. Sudan Grass Management for Control of Cyanide Poisoning. Jour. Am. Soc. of Agron. 30:569.

Burroughs, Wise, Paul Gerlaugh, B. H. Edgington and R. M. Bethke. 1949. Further Observations on the Effect of Protein Upon the Roughage Digestion in Cattle. Jour. Animal Sci. 8:9.

Burroughs, Wise, Paul Gerlaugh, and R. M. Bethke. 1950. The Influence of Alfalfa Hay and Fractions of Alfalfa Hay Upon the Digestion of Ground Corn Cobs. Jour. Animal Sci. 9:207.

Burroughs, Wise, L. S. Gall, Paul Gerlaugh, and R. M. Bethke. 1950. The Influence of Casein Upon Roughage Digestion in Cattle with Rumen Bacteriological Studies. Jour. Animal Sci. 9:214.

Burroughs, Wise, H. G. Headley, R. M. Bethke, and Paul Gerlaugh. 1950. Cellulose Digestion in Good and Poor Quality Roughages Using an Artificial Rumen. Jour. Animal Sci. 9:513.

Cason, James L., E. S. Ruby, and O. T. Stallcup. 1954. The Influence of the Ash Content of the Rumen Ingesta on the Hydrogen Ion Concentration in the Bovine Rumen. Jour. Nutr. 52:457.

Chappel, Charles F., Robert J. Sirny, C. K. Whitehair, and Robert MacVicar. 1955. Effect of Mineral Supplements on Digestibility of a Corncob Ration by Sheep. Jour. Animal Sci. 14:153.

Cheng, E. W., Glen Hall, and Wise Burroughs. 1955. A Method For the Study of Cellulose Digestion by Washed Suspensions of Rumen Microorganisms. Jour. Dairy Sci. 38:1225.

Crampton, E. W., and L. A. Maynard. 1938. The Relation of Cellulose and Lignin Content to the Nutritive Value of Animal Feeds. Jour. Nutr. 15:383.

Crampton, E. W., and I. R. C. Jackson. 1944. Seasonal Variation in Chemical Composition of Pasture Herbage and the Relation to its Digestibility by Steers and Sheep. (Pasture Studies-XXIV). Jour. Animal Sci. 3:333.

Drapala, W. J., L. C. Raymond, and E. W. Crampton. 1947. Pasture Studies XXVII. The Effects of Maturity of the Plant and its Lignification and Subsequent Digestibility by Animals as Indicated by Methods of Plant Histology. Sci. Agr. 27:36.

Enlow, C. R., and J. M. Coleman. 1929. Increasing the Protein Content of Pasture Grasses by Frequent Light Applications of Nitrogen. Jour. Am. Soc. of Agron. 21:845.

Forbes, R., and W. P. Garrigus. 1950. Some Relationships Between Chemical Composition, Nutritive Value, and Intake of Forages Grazed by Steers and Wethers. Jour. Animal Sci. 9:354.

Hamilton, Peter, B. E. Plummer, Jr., and H. C. Dickey. 1955. Measures of Digestion in the Ruminant. Maine Agr. Exp. Sta. Bul. 543:1.

Hungate, R. E. 1955. Symposium on Microbiology of the Rumen. Bacteriol Rev. 19:277.

Kamstra, L. D., A. L. Moxon, and O. G. Bentley. 1958. The Effect of Stage of Maturity and Lignification on the Digestion of Cellulose in Forage Plants by Rumen Microorganisms in vitro. Jour. Animal Sci. 17:199.

Manages, J. D. 1935. Cyanide Poisoning. Vet. Med. 30:347.

Markley, R. A., J. L. Cason, and B. R. Baumgardt. 1957. Digestibility of Bromegrass Hays Grown Under Various Levels of Nitrogen Fertilization. Abstracts of Papers to Be Presented at the Forty-Ninth Annual Meeting of the American Society of Animal Production. 16:1053.

McMillen, Warren N., Quentin Williams, and Wright Langhorn. 1943. Chemical Composition, Grazing Value and Vegetative Changes of Herbage in a Typical Plains Pasture. Jour. Animal Sci. 2:237.

Menaul, Paul, and C. T. Dowell. 1920. Cyanogenesis in Sudan Grass: A Modification of the Francis-Connell Method of Determining Hycrocyanic Acid. Jour. Agr. Research. 18:447.

Moon, F. E. 1954. The Composition and Nutritive Value of Hay Grown in the East of Scotland and the Influence of Late Applications of Nitrogenous Fertilizers. Jour. Agr. Sci. 44:140.

Morrison, Frank B. 1949. Feeds and Feeding. Twenty-First Edition. The Morrison Publishing Co., Ithica, New York.

Morrison, Frank B. 1956. Feeds and Feeding. Twenty-Second Edition. The Morrison Publishing Co., Ithica, New York.

Poulton, B. R., G. J. McDonald, and G. W. Vander Noot. 1957. The Effect of Nitrogen Fertilization on the Nutritive Value of Orchardgrass Hay. Jour. Animal Sci. 16:462.

Snedecor, George W. 1950. Statistical Methods. The Iowa State College Press, Ames, Iowa.

Stallcup, O. T., James L. Cason, and B. J. Walker. 1956. Influence of the Lignin Content of Hay on the Passage of Nutrients Through the Rumen. Arkansas Agr. Ext. Bul. 572:1.

Steppler, H. A. 1951. Pasture Studies XXXI. Lignification Studies With Various Grass Species. Sci. Agr. 31:1.

Sullivan, J. T. 1955. Cellulose and Lignin in Forage Grasses and Their Digestion Coefficients. Jour. Animal Sci. 14:710.

Swift, R. W., R. L. Cowan, G. P. Barron, K. H. Moddy, and E. C. Grose. 1951. The Effect of Alfalfa Ash Upon Roughage Digestion in Sheep. Jour. Animal Sci. 10:434.

Tillman, A. D., C. F. Chappel, R. J. Sirny, and Robert MacVicar. 1954. The Effect of Alfalfa Ash Upon the Digestibility of Prairie Hay by Sheep. Jour. Animal Sci. 13:417.

Underwood, L. M., W. J. Miller, H. L. Dalton, and T. H. Rogers. 1955. A Comparison of Tift Sudan and Starr Millet as a Temporary Summer Pasture for Milking Cows. Association of Southern Ag. Workers, Proceedings. 52nd Annual Convention, 1955.

Vinall, H. N. 1921. A Study of the Literature Concerning Prussic Acid in Sorghum, Sudan Grass, and Johnson Grass. Jour. Am. Soc. of Agron. 13:267.

Walker, D. m., and W. R. Hepburn. 1955. The Nutritive Value of Roughages for Sheep, the Relationship Between the Grass Digestibile Energy and the Chemical Composition of Hays. Jour Agr. Sci. 45:298.

Willaman, J. J., and R. M. West. 1915. Notes on the Hydrocyanic Acid Content of Sorghum. Jour. Agr. Research. 4:179.

Willaman, J. J., and R. M. West. 1916. Effect of Climatic Factors on the Hydrocyanic Acid Content of Sorghum. Jour. Agr. Research. 6:261.

Woodman, H. E., and R. E. Evans. 1930. Nutritive Value of Pasture, the Utilization by Sheep of Mineral Deficient Herbage. Jour. Agr. Sci. 20:587.