A STUDY OF THE EFFECT OF RATE OF AMMONIUM NITRATE FERTILIZATION, CHEMICAL COMPONENTS, AND TIME OF CUTTING ON THE *IN VITRO* RUMEN DIGESTION OF BERMUDAGRASS FORAGE

by

JAMES WILLIAM HOGAN

Bachelor of Science

Upper Iowa University

Fayette, Iowa

1961

Submitted to the faculty of the Graduate School
of the Oklahoma State University in partial
fulfillment of the requirements
for the degree of
MASTER OF SCIENCE
August, 1963

A STUDY OF THE EFFECT OF RATE OF AMMONIUM NITRATE FERTILIZATION, CHEMICAL COMPONENTS, AND TIME OF CUTTING ON THE IN VITRO RUMEN DIGESTION OF BERMUDAGRASS FORAGE

Thesis Approved:

James E. Webster
Thesis Adviser

Donald C. Abbott

Dean of the Graduate School

ACKNOWLEDGEMENT

The author wishes to express his sincere appreciation to Dr. James E. Webster of the Biochemistry Department for his guidance throughout this investigation. Thanks are also due to Dr. Willis D. Gallup and Dr. George R. Waller for their aid, to W.C. Elder of the Agronomy Department for growing and collecting the samples of bermudagrass forage, to Dr. Allen D. Tillman of the Animal Husbandry Department for aid in obtaining samples of rumen contents, and to the Biochemistry Department for the provision of laboratory facilities and financial aid.

TABLE OF CONTENTS

TABLES

FIGURES

FIGURES (CONTINUED)

Page

INTRODUCTION

The importance of the rumen in the digestion of forages by the ruminant animal has been recognized since the latter part of the 18th century. For many years the accepted method of determining the nutritive value of forages was through the use of approximate chemical analyses and *in vivo* digestion trials. In recent years, *in vitro* or artificial rumen techniques have been used considerably in gathering data on the nutritive value of many forages. Although early attempts to correlate the results of *in vitro* and *in vivo* digestion trials were not totally successful, recent reports have shown that good correlation is possible.

This investigation was initiated in an attempt to relate the rate of NH_4NO_3 fertilization, and time of cutting of bermudagrass forage to selected chemical components and *in vitro* digestibility, and to determine what, if any, chemical component of bermudagrass forage could be related to dry matter digestibility and hence, nutritive value.

LITERATURE REVIEW

Barnett and Reid (2), in their review on reactions in the rumen, have divided the various methods of study of rumen digestion *in vitro* into four categories which are as follows:

a. Simple incubation of rumen liquor with various substrates.

b. Impermeable artificial rumens, usually constructed of glass vessels, about which more will be said later.

c. Semi-permeable sacs which are used in the hope of simulating the part which diffusion plays in the natural rumen.

d. Artificial rumens of either the impermeable or semi-permeable type which are inoculated with washed suspensions of rumen microorganisms.

Since all the experiments to be considered were conducted with the impermeable type of artificial rumen, it is the only category which will receive further attention.

The impermeable artificial rumen is a system which attempts to approximate many of the environmental conditions found in the rumen of a living animal. In order to accomplish this, certain factors must be held constant. The temperature of the reaction vessel(s) is usually held between 38° and 40° C. through the use of a temperature-controlled water bath. The anaerobic condition which is found in the natural rumen is simulated by constantly passing CO_2 and/or N_2 through the tightly sealed reaction vessel(s). A $NaHCO_3$ buffer system is used to

assume the role of saliva as a buffer. This buffer system is initially adjusted to a pH of from 6.7 to 7.0, and is held relatively constant through intermittent additions of Na_2CO_3. Trace minerals and a nutrient salt solution necessary for the growth and reproduction of rumen microorganisms are added to the test solution. Rumen liquor samples containing microorganisms natural to the rumen are usually obtained from animals which have an indwelling rumen fistula, or from the rumen of freshly slaughtered animals. The rumen liquor obtained from such animals is subjected to various chemical and/or physical treatments and is then used to inoculate the test solution in the reaction vessel(s). The material which is to be digested is then placed in the reaction vessel(s), and degradation of the substrate is allowed to proceed for a predetermined period of time. At the end of this time, digestion is halted, and the amount of digestion which has occurred is calculated.

There are a wide variety of methods which may be used to measure the amount of digestion occuring in the artificial rumen. While the most common methods are based on the disappearance of one or more chemical components of the substrate, other methods are based on the end products produced by the digestion. Probably the most common method used currently to determine the amount of digestion which has taken place is to measure the disappearance of cellulose. This procedure has been used successfully by Quicke and Bentley (25), Reid et al. (27), Donefer et al. (12), and Kamstra et al. (17, 18), to name only a few. Other common methods currently in use are dry matter disappearance (3, 6, 7, 27, 31), and organic matter disappearance (15), as well as disappearance of other chemical components.

A wide range of techniques has been used in the preparation of

the rumen liquor used to inoculate the test solution. Some methods are very simple and require a minimum of equipment. Walker (34) merely strained the rumen liquor through cheesecloth and obtained good correlation between in vivo and in vitro dry matter digestibility. Quicke et al. (26) reported a procedure which involved extracting pressed rumen contents (pulp) with a phosphate buffer, centrifuging the buffer extract, and resuspending the sediment. Yet a third method was used by Van Dyne (33), in which the rumen contents were extracted with a phosphate buffer, and this extract used as the inoculum.

The effect of the source of the rumen liquor has been studied by many investigators. It has been reported by LeFevre and Kamstra (19) that rumen fluid obtained from sheep and cattle may be used interchangeably if they are fed a similar ration. Quicke et al. (26), in an in vitro fermentation experiment with nine dried forages, showed that feeding different types of forages to a steer used as a rumen liquor source had no significant effect on digestibility of forage cellulose. Barnett (1), reported that rumen liquor obtained from freshly slaughtered animals fed on diets of turnips, grass, silage, and oats, could be used indiscriminately without noticeable effect on results. However, Quicke et al. (26) have reported that rumen liquor obtained from alfalfa-fed animals has a significant effect on in vitro digestion.

While there is considerable variation in the methods used in in vitro fermentation experiments, the results compare very well with those obtained through the use of in vivo procedures (4, 5, 16, 27, 34). Because of the advantages of in vitro procedures over in vivo procedures (29) in the evaluation of forages, they have been and will continue to be used extensively in determining the nutritive value of forage crops.

Numerous investigators (8, 9, 17, 25, 27) have studied the chemical composition and maturity of forages in attempts to relate these properties to *in vitro* and *in vivo* digestion trial results. The decrease in digestibility of forages with increasing maturity has been shown to be associated with an increase in lignin content (13, 18, 30, 31), and perhaps with the seasonal decrease in protein content of the forages (2). While lignin is not considered a carbohydrate, it is thought to have a close physical and chemical relationship with the cellulose and other carbohydrate fractions of the plant. Meyer and Lofgreen (21) have shown that crude fiber digestibility may be correlated with lignin content, and Sullivan (31) has shown that there is a correlation between acid-insoluble lignin and digestible dry matter. Quicke and Bentley (25) have shown that with maturing forages, a slight increase in lignin content may cause a considerable decrease in cellulose digestibility. Differences in *in vitro* digestibility, at all stages of growth, were shown to be more closely related to acid-insoluble than to total lignin, suggesting a change in the nature of the lignin fraction through-out the growing period. Patton and Gieseker (23) reported that increased lignin content in forages caused decreased weight gains in cattle, and suggested the following theories to explain this result:

a. The lignin may incrust the other digestible constituents, putting them "in a nutshell which the animal can't crack."

b. Lignin may combine chemically with other constituents, forming unavailable compounds.

c. Digestion may be retarded through local inhibition of digestive enzymes due to the toxic action of phenolic groups resulting from the partial decomposition of lignin.

More recently, Dehority and Johnson (11) presented evidence obtained through *in vitro* digestion trials, which tends to verify the theory that deposition of lignin as an "encrusting substance" rather than total concentration of lignin is responsible for decreased digestibility of forages.

The effect of hemicellulose on digestibility has received slight attention. However, Kamstra et al. (17), McBee (20), and Salisbury et al. (28) have shown that hemicellulose neither inhibits nor stimulates cellulose digestion.

EXPERIMENTAL METHODS

Preparation of Bermudagrass Forage Samples: A genetically pure strain of bermudagrass[1] was grown on five series of plots, each series of plots consisting of four replications. Each of the five series of plots received NH_4NO_3 fertilizer four times per year in total amounts varying from 0 to 1,400 pounds per acre. The Agronomy Department advised that samples were collected at approximately twenty-one-day intervals in an attempt to simulate the conditions present on normally grazed bermudagrass pasture. Samples were collected over a three year period starting in 1960. The first samples were usually collected during the last week of May, the last samples during the last week of September, with a total of seven cuttings being collected each season.

The samples were dried in a forced draft oven at 105° C. and ground in a Wiley mill to pass through a 40-mesh screen before determining their chemical composition and in vitro dry matter digestibility.

Isolation of Holocellulose: The method used to isolate holocellulose is similar to that of Wise, Murphy, and D'Addieco (37). Ely and Moore (14) reported that this method gave 99 per cent recovery of theoretical holocellulose in ten forages tested.

Initially, four 25-g. samples of bermudagrass forage that had been collected from four replicate plots were combined to form a 100-g. sample. Each of the four 25-g. samples had been collected on the same date from

[1]Midland Variety bermudagrass was used in all experiments.

plots which had received the same amount of NH_4NO_3 fertilizer. This 100-g. sample was placed in a 90 x 200 mm. double thimble[1] and was extracted for a minimum of fifteen hours with an ethanol-benzene mixture composed of 1 part ethanol to 2.5 parts benzene by volume. After extraction, the residue was dried, weighed, and thoroughly mixed. Then, 250 ml. of a 0.5 per cent ammonium oxalate solution was added to a fourth of this residue which was refluxed at 85° C. for four hours. After refluxing, the extract was filtered and washed with hot water. A 600-ml. Buchner type funnel with a coarse fritted disk was used for all filtering procedures. A second 250-ml. portion of 0.5 per cent ammonium oxalate was then added to the residue and it was refluxed at 85° C. for fifteen hours. The residue was washed and filtered again. This residue was placed in a 1.5-liter beaker with 625 ml. of water, and 2 ml. of glacial acetic acid and 7.5 g. of reagent grade $NaClO_2$ were added in the order indicated. This mixture was placed in a temperature-controlled water bath at 85° C. and stirred occasionally. After fifteen minutes, thirty minutes, and forty-five minutes, glacial acetic acid and $NaClO_2$ were added in the amounts indicated above. At the end of one hour, the beaker was cooled in ice water to 10° C. The mixture was filtered, washed six times with ice water, air dried for fifteen hours, and finally dried under an infra-red lamp for approximately four hours.

In Vitro Rumen Digestion: The in vitro method used was a modified version of the technique developed by Walker (34).

Preparation of Inoculum: The rumen fluid used as inoculum was obtained from a steer fitted with a rumen fistula. The steer was maintained on mature, weathered, prairie hay and cottonseed meal, and no

[1]Double extraction thimbles were obtained from W. and R. Balston Ltd., England.

attempt was made to feed a diet consisting solely of the forage being tested in the in vitro rumen apparatus. A sample of rumen contents was withdrawn from the steer, strained through six layers of cheesecloth, and placed in a vacuum bottle preheated to 38° C. for transportation to the laboratory. In the laboratory, the sample was again strained through six layers of cheesecloth and stirred slowly using a magnetic stirrer to prevent sedimentation and to insure that a homogenous sample could be obtained for use as the inoculum.

Preparation of Nutrient Solution: A slightly modified procedure similar to that of Walker (34) was followed in preparing the nutrient solution.

a. A solution of minerals similar to the solution originally outlined by Warner (35) was prepared and made to a volume of 2 liters. It contained the following:

$(NH_4)_2SO_4$	13.20 g.
$MgSO_4 \cdot 7H_2O$	12.30 g.
$CaCl_2 \cdot 2H_2O$	7.38 g.
$K_2HPO_4 \cdot 3H_2O$	68.50 g.
$CoCl_2 \cdot 6H_2O$	0.04 g.
$CuSO_4 \cdot 5H_2O$	0.04 g.
$FeSO_4 \cdot 7H_2O$	0.20 g.
$MnSO_4 \cdot H_2O$	0.06 g.
$ZnSO_4 \cdot 7H_2O$	0.08 g.
MoO_3	0.02 g.
Glacial acetic acid	12.00 ml.
H_3PO_4, 88%, syrupy	16.50 ml.

b. Two hundred fifty ml. of a 1.0 M H_3PO_4 solution was prepared.

c. An aqueous solution containing the sodium salts listed below was prepared with the final volume being brought to 1 liter.

NaCl	58.50 g.
Na_2HPO_4	21.30 g.
$NaC_2H_3O_2$	82.00 g.

d. An aqueous solution consisting of the volatile fatty acids listed below was prepared with the final volume being 10 ml.

Propionic acid	2.30 ml.
Isobutyric acid	0.90 ml.
Isovaleric acid	0.80 ml.
Valeric acid	0.80 ml.

e. Two hundred fifty ml. of a 1.0 M $NaHCO_3$ solution was prepared.

To prepare the nutrient solution for use in the in vitro rumen, 300 ml. of aqueous mineral solution, 60 ml. of sodium salts solution, 6.0 ml. of volatile fatty acids solution, 6.0 ml. of 1.0 M H_3PO_4 solution, and 2,508 ml. of water were mixed thoroughly. Immediately before use 120 ml. of a 1.0 M $NaHCO_3$ solution was added. The total volume of this nutrient solution was 3 liters.

In Vitro Rumen Apparatus: The in vitro rumen apparatus (see figure 1) consisted of a series of twelve wide mouth bottles, each of approximately 500-ml. capacity. The bottles were tightly stoppered with rubber stoppers through which passed two glass tubes. The inlet tube reached to the bottom of the bottle and was drawn out at the end to form a jet. The outlet tube was short and extended approximately

Figure 1. *In Vitro* Rumen Apparatus

1 cm. below the stopper. The bottles were held in position in a temperature-controlled water bath by a frame constructed of aluminum alloy rods and copper wire. Each bottle was connected in series by a length of 5/16th inch rubber tubing, and the tubing was of such length that it formed an inverted U between adjacent bottles and acted as a condenser to prevent any liquid being carried over from one bottle to another. The water bath was maintained at 38° C. and a stream of CO_2 was bubbled continuously through the bottles. Each of the twelve bottles contained 250 ml. of the nutrient solution, 20 ml. of strained rumen fluid, and 1 g. of substrate (with the exception of three blanks which contained no substrate). Digestion in vitro was continued for seventy-two hours.

Digestion Procedure: A standard, which was a sample of dried and ground bermudagrass forage, was included in each experiment. One gram of this "standard forage" was placed in bottle 8 and this forage was used as an internal check in each experiment. In acceptable experiments its digestibility ranged between 57 and 65 per cent. As a further check, bottles 2 and 12 contained 1 g. each of a purified cellulose sample.[1] Bottles 1, 5, and 9 contained no substrate but had the normal volume of nutrient solution and strained rumen fluid. These bottles provided a value for the quantity of dry matter introduced in the form of rumen fluid. The samples to be tested were added to bottles 3, 4, 6, 7, 10, and 11. Bottles 3 and 7, 4 and 10, and 6 and 11 always contained duplicate samples. The bottles were connected in a series, and an anaerobic environment was maintained throughout the digestion period. The pH of the solution was checked initially and was adjusted to 6.7

[1] Solka Floc BW-200, obtained from Brown Comp., Boston, Mass., is 99.5 per cent pure cellulose when dry.

with Na_2CO_3 (106 g./liter). The pH was checked after digestion periods of twenty-four and forty-eight hours, and the samples were corrected to pH 6.7 if they had varied. Alteration of pH was seldom more than 0.2 pH unit per twenty-four hour period. At the end of this digestion period the bottles were disconnected, stirred thoroughly, and the contents were transferred to 250-ml. glass centrifuge bottles. At this point the total volume of each *in vitro* digestion bottle was approximately 300 ml., making two transfers of 150 ml. each necessary. The samples were centrifuged at 2,000 r.p.m. for twenty minutes in a size 2 International Centrifuge using a no. 267 head. After each 150-ml. sample had been centrifuged, the supernatant was decanted, and the sediments from both fractions were combined. The sediment was washed twice with water, centrifuged at 2,000 r.p.m. for ten minutes after each washing, and the supernatants were discarded. The sediment was then transferred to a tared platinum crucible, dried overnight at 105° C. and weighed. The weight of sediment in bottles 1, 5, and 9 was averaged and then subtracted from the weight of sediment for each *in vitro* digestion bottle. The figure obtained by these calculations was termed "undigested dry matter." The "undigested dry matter" for each duplicate sample set was then averaged, multiplied by 100, and subtracted from 100 to obtain per cent *in vitro* dry matter digestibility for each bermudagrass forage or holocellulose sample in question.

Lignin Analysis: The procedure used here combines the salient features of three different methods previously outlined by Thacker (32), Phillips and Smith (24), and Davis (10).

Preparation of Reagents:

a. A 1 per cent pepsin solution was prepared by adding 1 g.

of powdered pepsin to 100 ml. of 0.1 N HCl immediately before using.

b. A 0.25 per cent Na_2CO_3 solution was prepared by adding 1 g. of Na_2CO_3 to 400 ml. of water.

c. A 1.0 N H_2SO_4 solution was prepared by placing 55.6 ml. of concentrated H_2SO_4 in 2 liters of solution.

d. A 72 per cent (by weight) H_2SO_4 solution was prepared by adding 405 ml. of concentrated H_2SO_4 to 254 ml. of water.

Analytical Procedure: A 0.5 g. sample of the forage was extracted with 50 ml. of an ethanol-benzene mixture (1 part ethanol - 2 parts benzene) for a minimum of twenty-four hours using soil extracting flasks and air condensers. The ethanol-benzene mixture was removed by washing the flask and sample with ethanol followed by water. The sample was then placed in a 250-ml. soil flask, and 40 ml. of a 1 per cent pepsin solution in 0.1 N HCl was added. This mixture was allowed to incubate for twenty-four hours and then the pepsin solution was removed and the sample washed with hot water. At this point, 100 ml. of a 0.25 per cent Na_2CO_3 solution was added and the mixture was incubated twenty-four hours at 37° to 40° C. The sample was then filtered and washed with hot water. After transferring the sample to a 600-ml. Berzelius beaker, 100 ml. of 1.0 N H_2SO_4 was added and the solution was boiled for one hour using a reflux condenser. After boiling, the sample was filtered and washed with ethanol and ether. The ether was then removed to dry the sample. At this point the sample was placed in a dry Brezelius beaker and 15 ml. of cold 72 per cent H_2SO_4 was added. The sample was placed in an ice water bath at 18° C. and stirred frequently for two hours. At the end of two hours, the acid was diluted to approximately 1.0 N by

the addition of 350 ml. of water. This mixture was boiled gently under a reflux condenser for one hour, filtered through gooch crucibles, and washed with hot water, ethanol, and ether. The ether was removed by heating, and the sample was dried in an oven at 105° C. and weighed. As the final step, the sample was ashed at 600° C., cooled in a desiccator and weighed. The loss in weight which occurred through ashing was considered as the amount of lignin originally present in the sample.

Cellulose and Hemicellulose Analysis: The method used was essentially that of Patton (22).

Duplicate 1 g. samples of holocellulose which had been dried, and ground in a Wiley mill to pass a 40-mesh screen, were placed in 6-inch Pyrex centrifuge tubes. Then, 15 ml. of 80 per cent acetic acid and 1.5 ml. of concentrated HNO_3 were added. Small flask covers were filled with water and placed in the necks of the centrifuge tubes to act as reflux condensers. At this point the tubes were placed on a sand bath and boiled gently for twenty minutes. The centrifuge tubes were then cooled in a water bath and the condensers were removed. Approximately 20 ml. of ethanol was added and the mixture was stirred. The tubes were centrifuged until the sample had sedimented and the supernatant was discarded. The sample was washed, in the centrifuge tube, twice with ethanol, and once each with hot benzene, hot ethanol, and ether. The samples were dried under an infra-red lamp until no ether odor remained and then placed in an oven at 105° C. for one hour. After cooling in a desiccator, the tubes were weighed and ashed at 550° C. overnight. Then the tubes were cooled in a desiccator and reweighed. The loss in weight on ashing was recorded as the amount of cellulose. Since holocellulose is defined as cellulose plus hemicellulose, the weight of the original

holocellulose sample minus the weight of the cellulose was considered to be equal to the amount of hemicellulose present.

RESULTS AND DISCUSSION

Reproducibility of In Vitro Digestion Method: Tables I and II are sample results, picked at random, which show the reproducibility of this method. Table I indicates that the dry matter digestibility of duplicate bermudagrass forage samples varied only slightly. However, as Table II indicates, the digestibility of duplicate holocellulose samples varied quite a bit. This was probably due to sample variation, and was not caused by the in vitro digestion method. Figure 2 shows that relatively good correlation may be obtained between separate experiments.

Chemical Composition of Bermudagrass Forage as Affected by NH_4NO_3 Fertilization:

Holocellulose: As shown in Table III, the rate at which NH_4NO_3 fertilizer was applied appears to have had an effect on the amount of holocellulose formed in bermudagrass forage. When the total amount of fertilizer applied was increased from 0 through 1,400 pounds per acre, the holocellulose content of the forage samples was decreased. The greatest decrease in holocellulose was shown to occur in the samples collected in late June or early July. In samples which had received no NH_4NO_3 fertilizer, the holocellulose fraction comprised an average of 59.80 per cent of the total sample, while in samples which received 1,400 pounds per acre of fertilizer the holocellulose fraction amounted to an average of only 48.40 per cent of the total sample.

TABLE I

DIGESTIBILITY IN VITRO OF DRY MATTER IN BERMUDAGRASS FORAGE SAMPLES

Sample	Bottle No.	Weight of Sediment	Undigested Dry Matter	Digested Dry Matter
		g.	g.	%
Blank	1	0.1252	-	-
Blank	5	0.1275	-	-
Blank	9	0.1302	-	-
H-400,[a] 1 g.	3	0.4462	0.3186	68.00
H-400, 1 g.	7	0.4491	0.3215	
I-400,[b] 1 g.	4	0.4642	0.3366	66.00
I-400, 1 g.	10	0.4709	0.3433	
J-400,[c] 1 g.	6	0.5158	0.3882	61.22
J-400, 1 g.	11	0.5149	0.3873	
Solka Floc, 1 g.	2	0.2153	0.0877	91.04
Solka Floc, 1 g.	12	0.2192	0.0916	
Standard Forage, 1 g.	8	0.5096	0.3820	61.80

[a] H-400 - A sample of bermudagrass forage collected from four replicate plots on May 25, 1961.

[b] I-400 - A sample of bermudagrass forage collected from four replicate plots on June 11, 1961.

[c] J-400 - A sample of bermudagrass forage collected from four replicate plots on June 30, 1961.

TABLE II

DIGESTIBILITY IN VITRO OF DRY MATTER

IN HOLOCELLULOSE SAMPLES

Sample	Bottle No.	Weight of Sediment	Undigested Dry Matter	Digested Dry Matter
		g.	g.	%
Blank	1	0.1340	-	-
Blank	5	0.1316	-	-
Blank	9	0.1323	-	-
HC-3,[a] 1 g.	3	0.3209	0.1883	85.23
HC-3, 1 g.	7	0.2397	0.1071	
HC-8,[b] 1 g.	4	0.2950	0.1624	87.11
HC-8, 1 g.	10	0.2280	0.0954	
HC-13,[c] 1 g.	6	0.2719	0.1393	83.02
HC-13, 1 g.	11	0.2612	0.1286	
Solka Floc, 1 g.	2	0.2172	0.0846	92.72
Solka Floc, 1 g.	12	0.1936	0.0610	
Standard Forage, 1 g.	8	0.5579	0.4253	57.47

[a] HC-3 - A holocellulose sample isolated from bermudagrass forage sample H-400.

[b] HC-8 - A holocellulose sample isolated from bermudagrass forage sample I-400.

[c] HC-13 - A holocellulose sample isolated from bermudagrass forage sample J-400.

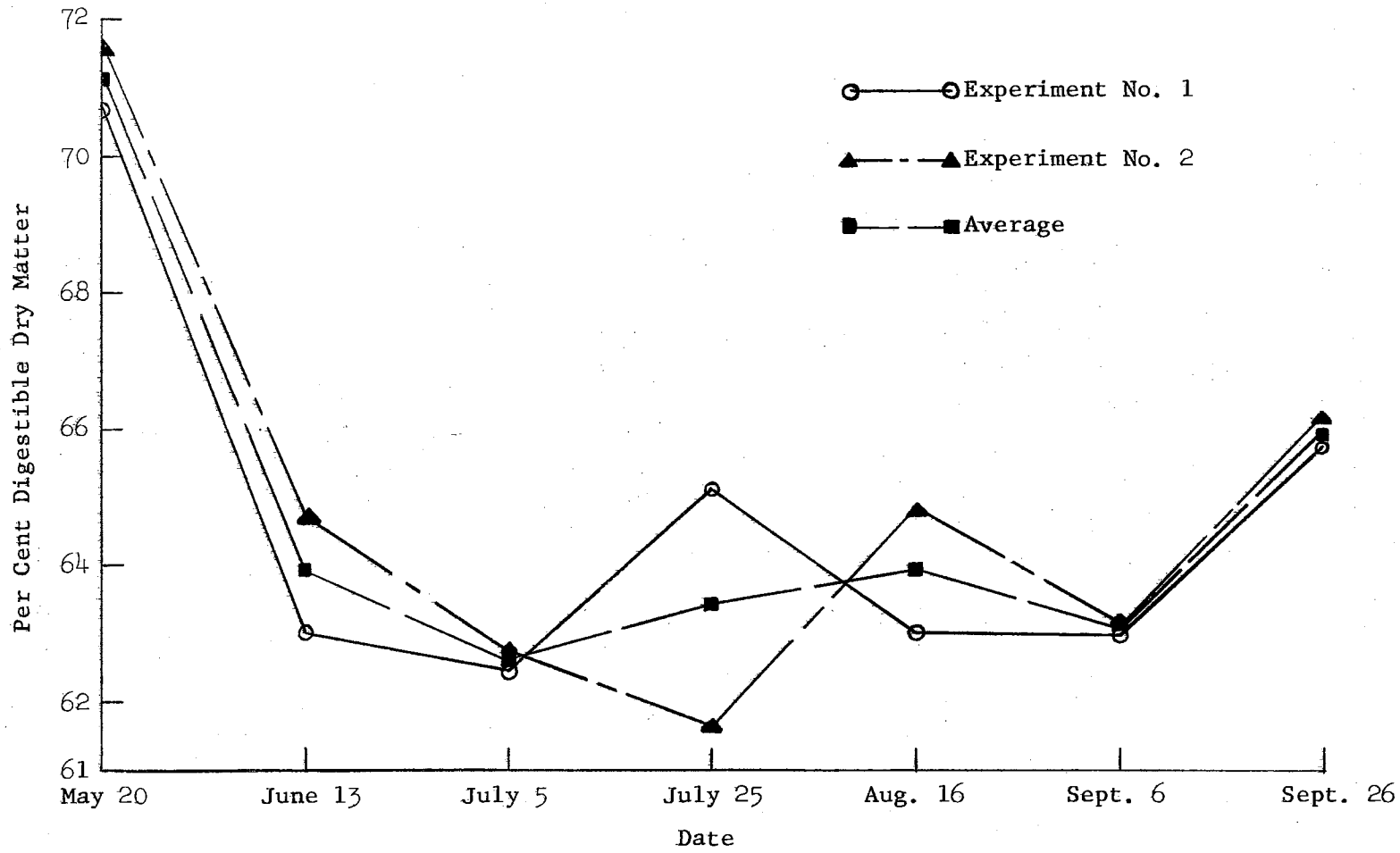

Figure 2. Percentage Digestibility In Vitro of Dry Matter in Bermudagrass Forage as Affected by Time of Cutting (1960).

Note: Experiments no. 1 and 2 were run on the same samples at different times to check reproducibility of the in vitro digestion method.

TABLE III

THE PERCENTAGE OF HOLOCELLULOSE IN BERMUDAGRASS FORAGE AS AFFECTED BY NH_4NO_3 FERTILIZATION

Sample No.[a]	0 lb./acre NH_4NO_3			1,400 lb./acre NH_4NO_3		
	1960	1961	average	1960	1961	average
	%	%	%	%	%	%
1	49.72	-	49.72	45.88	44.44	45.16
2	57.20	62.76	59.58	53.72	48.32	51.02
3	55.84	63.76	59.80	48.08	48.72	48.40
4	55.52	58.20	56.86	51.44	53.88	52.66
5	55.20	59.60	57.40	45.72	50.92	48.32
6	55.84	-	55.84	52.44	48.28	50.36
7	52.40	58.48	55.44	44.10	48.72	46.41

[a]All samples numbered from 1 through 7 were collected on the following dates: (1) May 20, 25, and 24; (2) June 13, 11, and 13; (3) July 5, June 30, and July 5; (4) July 25, 20, and 26; (5) August 16, 10, and 16; (6) September 6, August 31, and September 6, and (7) September 26, 28, and October 1 for the years 1960, 1961, and 1962 respectively.

Cellulose: The rate of fertilization seems to have had little or no effect on the amount of cellulose laid down in bermudagrass forage. As Table IV indicates, various samples from forage which was not receiving NH_4NO_3 fertilizer had a greater cellulose content than samples from forage which had received a maximum of 1,400 pounds per acre of NH_4NO_3 fertilizer. However, in other samples the reverse of the above was shown to occur, in that forage which had received 1,400 pounds per acre of fertilizer laid down a greater amount of cellulose than forage which

received no fertilizer.

TABLE IV

THE PERCENTAGE OF CELLULOSE IN BERMUDAGRASS FORAGE AS AFFECTED BY NH_4NO_3 FERTILIZATION

Sample No.[a]	0 lb./acre NH_4NO_3			1,400 lb./acre NH_4NO_3		
	1960	1961	average	1960	1961	average
	%	%	%	%	%	%
1	21.97	-	21.97	25.64	20.89	23.26
2	28.07	31.86	29.96	30.61	24.89	27.75
3	25.28	31.76	28.52	27.18	22.14	24.66
4	27.06	26.52	26.79	28.10	25.92	27.01
5	24.92	26.10	25.51	23.62	24.86	24.24
6	26.34	-	26.34	27.62	23.57	25.60
7	25.04	25.69	25.36	25.28	22.52	23.90

[a]See footnote a to Table III.

Hemicellulose: Hemicellulose formation in bermudagrass forage was decreased by increased amounts of NH_4NO_3 fertilizer. Table V indicates that forage which received 1,400 pounds per acre of NH_4NO_3 fertilizer formed less hemicellulose than forage which had received no NH_4NO_3 fertilizer. The greatest reduction in hemicellulose content was shown to occur in late June or early July. At that time the average percentage of hemicellulose in samples which had received no fertilizer was 31.28 per cent, while samples which had received 1,400 pounds per

acre of NH_4NO_3 fertilizer contained an average of 23.29 per cent hemicellulose.

TABLE V

THE PERCENTAGE OF HEMICELLULOSE IN BERMUDAGRASS FORAGE AS AFFECTED BY NH_4NO_3 FERTILIZATION

Sample No.[a]	0 lb./acre NH_4NO_3			1,400 lb./acre NH_4NO_3		
	1960	1961	average	1960	1961	average
	%	%	%	%	%	%
1	27.75	-	27.75	20.24	23.55	21.90
2	29.13	30.90	30.02	23.11	23.43	23.27
3	30.56	32.00	31.28	20.90	26.58	23.29
4	28.46	31.68	30.07	23.34	27.96	25.65
5	30.28	33.50	31.89	22.09	26.06	24.08
6	29.50	-	29.50	24.82	24.71	24.76
7	27.36	32.79	30.08	18.82	26.17	22.50

[a]See footnote a to Table III.

Unpublished data (36) have shown that increasing the amount of NH_4NO_3 fertilizer applied to bermudagrass forage caused an increase in the protein content of the forage. This suggests that as the protein content of bermudagrass forage increased, there was a subsequent decrease in the amount of holocellulose laid down by the forage. Since holocellulose is defined as the cellulose plus hemicellulose fractions of the forage, the decrease in holocellulose could have been caused by a

decrease in hemicellulose or cellulose, or both. However, since it was shown that the greatest decrease in holocellulose and hemicellulose occurs simultaneously, it would seem that the decreased holocellulose content can be accounted for in the hemicellulose fraction alone.

Per Cent Digestibility In Vitro of Dry Matter in Bermudagrass Forage as Affected by NH_4NO_3 Fertilization: Figure 3 and Table VI indicate that the rate at which NH_4NO_3 fertilizer was applied had negligible effect on the per cent digestibility in vitro of dry matter in bermudagrass forage. Comparison of Figure 3 with Table III shows that the holocellulose fraction of bermudagrass forage had little effect on its in vitro digestibility. Earlier reports (17, 20, and 28) have shown that hemicellulose has no effect on in vitro cellulose digestion by rumen microorganisms. When the results given in Table V are compared with Figure 3, a similar conclusion may be drawn as to the effect of hemicellulose on in vitro dry matter digestibility of bermudagrass forage.

Per Cent Digestibility In Vitro of Dry Matter in Bermudagrass Forage as Affected by Time of Cutting: Table VII and Figures 2, 4, and 5 show that there was considerable seasonal variation in the in vitro dry matter digestibility of bermudagrass forage. Samples collected in May of 1960 were 71.15 per cent digested, while similar samples collected in July were only 62.62 per cent digestible. Similar results were noted in 1961, when May samples were 68.00 per cent digestible, while samples collected on June 30 were only 61.22 per cent digestible. Samples collected during the summer of 1962 show a decrease in digestibility of similar magnitude occurring during the period from June 13 to July 5. During the period from early July to late September, digest-

TABLE VI

PERCENTAGE DIGESTIBILITY IN VITRO OF DRY MATTER IN BERMUDAGRASS FORAGE AS AFFECTED BY NH_4NO_3 FERTILIZATION

Date Collected	Rate of NH_4NO_3 Fertilization		
	0 lb./acre	400 lb./acre	1,400 lb./acre
	%	%	%
May 20, 1960	72.75	72.64	71.16
July 5, 1960	62.54	64.66	65.70
August 16, 1960	64.42	64.89	62.10
September 26, 1960	59.88	61.77	61.04

TABLE VII

PERCENTAGE DIGESTIBILITY IN VITRO OF DRY MATTER IN BERMUDAGRASS FORAGE AND HOLOCELLULOSE AS AFFECTED BY TIME OF CUTTING

Sample No.[a]	Digestibility of Dry Matter in Bermudagrass Forage			Digestibility of Holocellulose Isolated from 1961 Bermudagrass Forage
	1960[b]	1961	1962	
	%	%	%	%
1	71.15	68.00	62.02	85.23
2	63.87	65.60	67.69	87.11
3	62.62	61.22	58.26	86.60
4	63.41	62.30	62.46	-
5	63.93	61.09	63.07	89.85
6	63.12	62.43	59.88	88.92
7	66.00	61.61	63.57	88.38

[a] See footnote a to Table III.

[b] Each value for 1960 is the average result of two experiments.

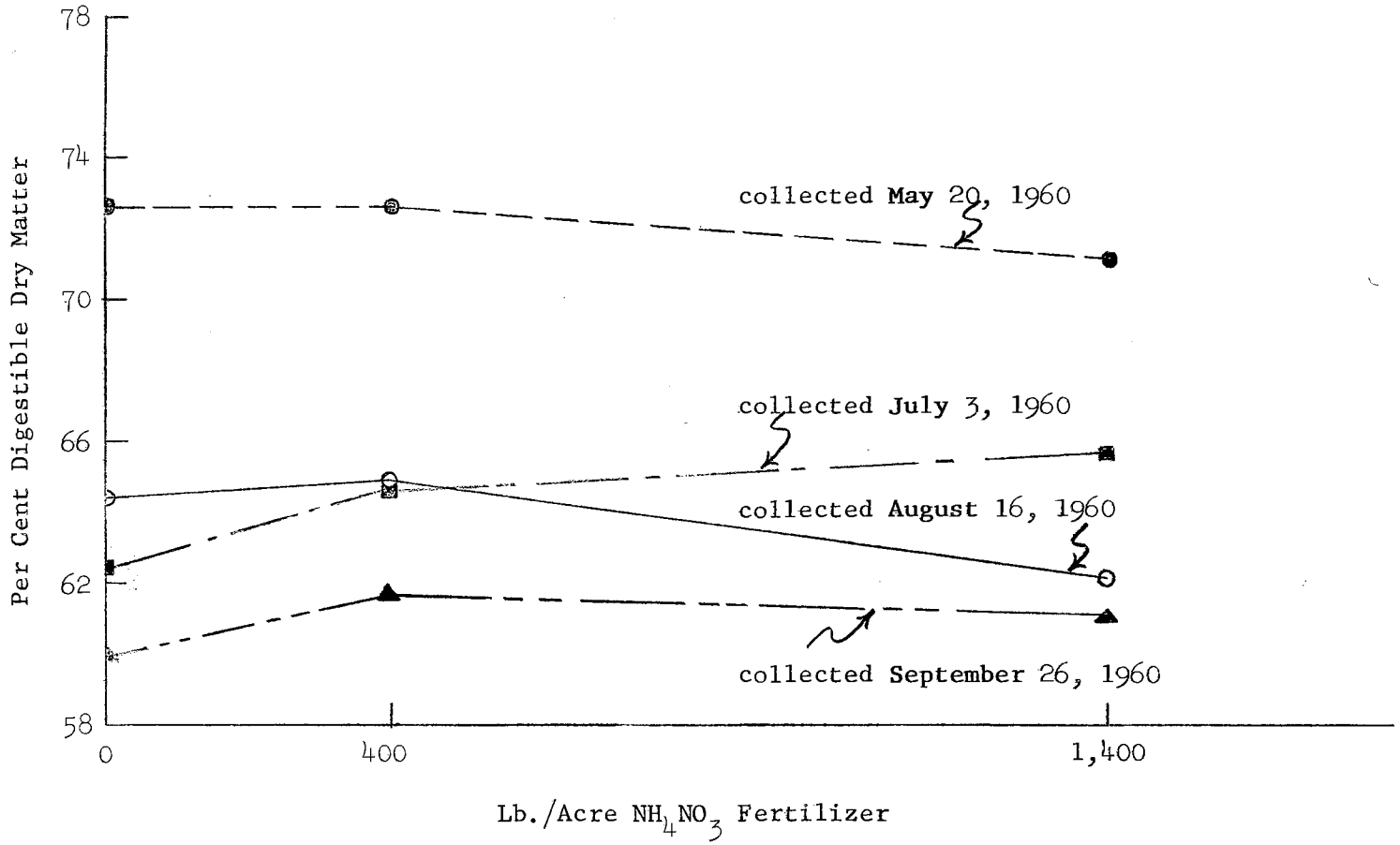

Figure 3. Percentage Digestibility In Vitro of Dry Matter in Bermudagrass Forage as Affected by NH_4NO_3 Fertilization.

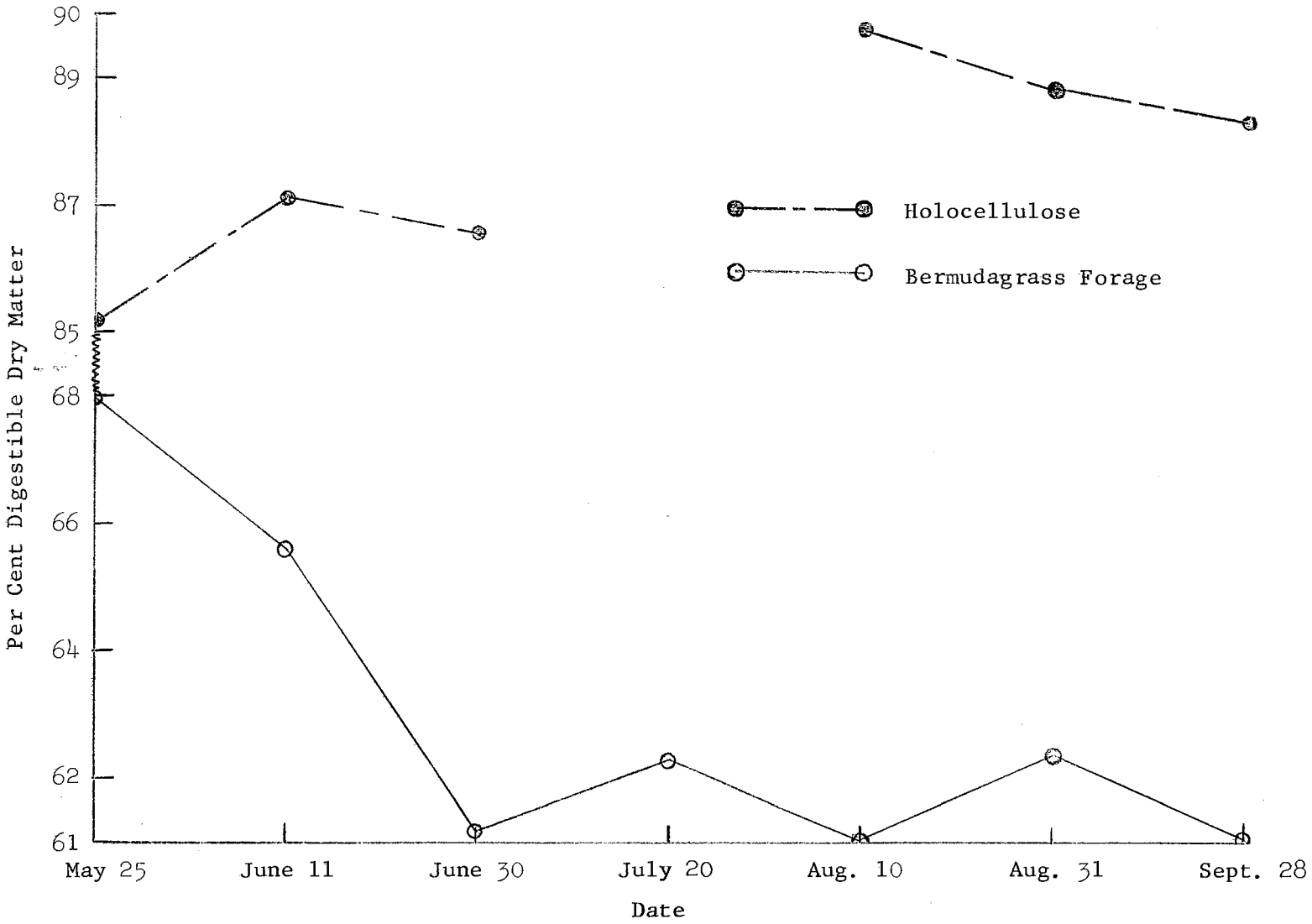

Figure 4. Percentage Digestibility In Vitro of Dry Matter in Bermudagrass Forage and Holocellulose as Affected by Time of Cutting (1961).

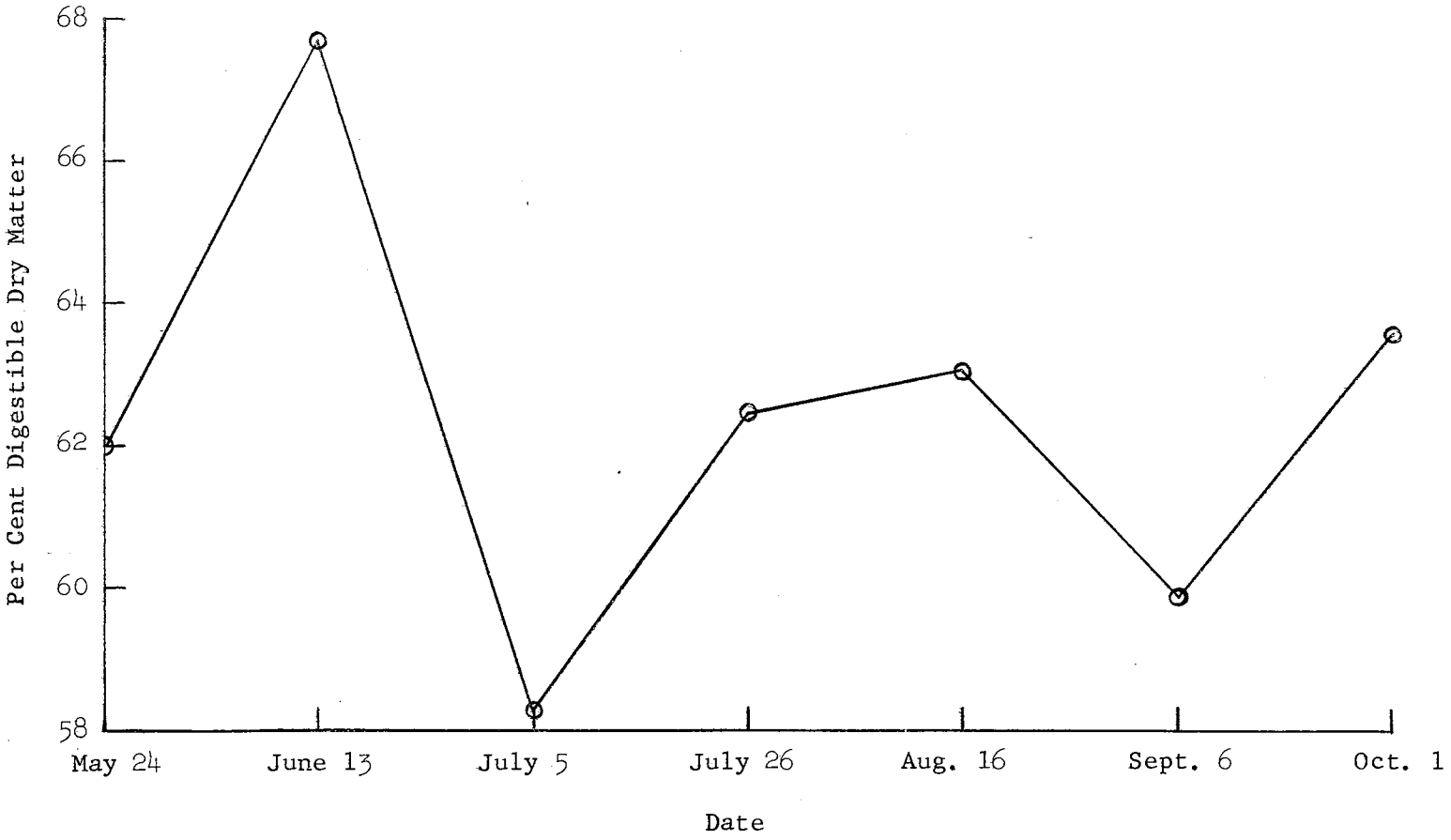

Figure 5. Percentage Digestibility In Vitro of Dry Matter in Bermudagrass Forage as Affected by Time of Cutting (1962).

ibility leveled off, with a few minor fluctations, at an average figure of approximately 62 per cent.

Per Cent Digestibility In Vitro of Dry Matter in Bermudagrass Forage as Affected by Lignin Content: Comparison of percentage lignification with in vitro digestibility data indicates that the lignin in bermudagrass forage affected its digestibility. Table VIII and Figure 6 show that, in general, as lignification increases, in vitro digestibility decreases. Analysis of these date proves that they are statistically significant at the 5 per cent level of probability. Figure 4 indicates that when bermudagrass forage was subjected to the acid chlorite treatment for removal of lignin, the digestibility was increased by as much as 30 per cent. Dehority and Johnson (11) have presented evidence showing that the manner in which lignin was laid down may affect digestibility as much as the total concentration of lignin present in the forage, and this may be true for bermudagrass forage.

TABLE VIII

PERCENTAGE DIGESTIBILITY IN VITRO OF DRY MATTER IN BERMUDAGRASS FORAGE AS AFFECTED BY LIGNIN CONTENT

Sample No.[a]	Digestibility			Lignin Content		
	1960	1961	average	1960	1961	average
	%	%	%	%	%	%
1	70.70	68.00	69.35	6.63	6.11	6.37
2	63.03	65.60	64.32	7.07	6.48	6.78
3	62.45	61.22	61.84	6.62	7.46	7.04
4	63.48	60.41	61.94	8.08	6.60	7.34
5	61.36	59.20	60.28	8.87	7.18	8.02
6	66.15	60.12	63.14	7.65	6.72	7.18
7	68.87	59.30	64.08	7.18	7.40	7.29

[a]See footnote a to Table III.

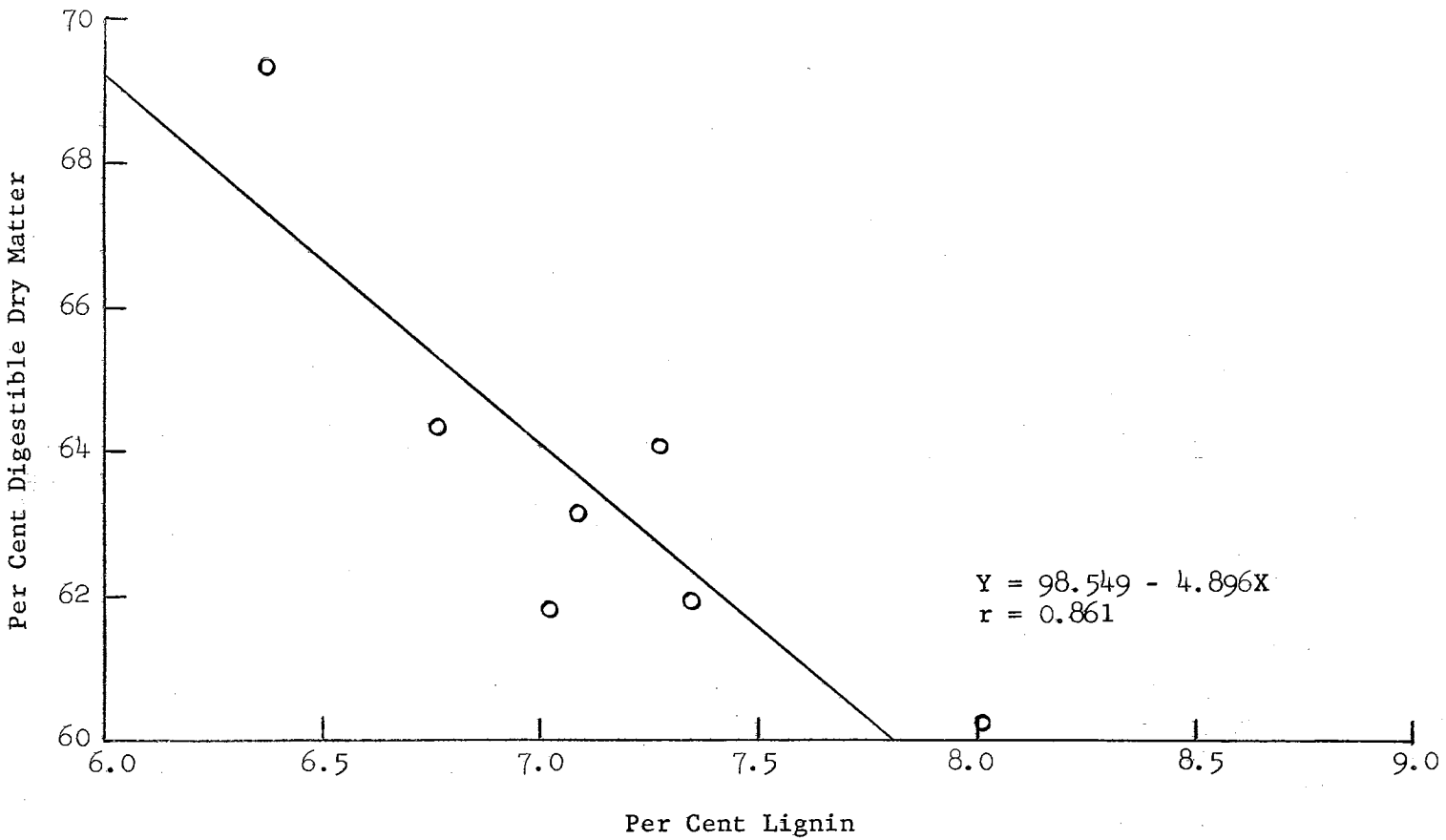

Figure 6. Percentage Digestibility In Vitro of Dry Matter in Bermudagrass Forage as Affected by Lignin Content.

SUMMARY

Samples of bermudagrass forage collected over a three year period were studied to determine the effect of rate of NH_4NO_3 fertilization on selected chemical components, and the effect of rate of fertilization, chemical composition, and time of cutting on *in vitro* dry matter digestibility.

The forage samples were analyzed for holocellulose, hemicellulose, cellulose, lignin, and *in vitro* dry matter digestibility.

The following general trends were noted:

a. Increased NH_4NO_3 fertilization caused a decrease in the percentage of holocellulose laid down by the forage.

b. The rate of NH_4NO_3 fertilization had little effect on the percentage of cellulose laid down by the forage.

c. Increased NH_4NO_3 fertilization caused a decrease in the percentage of hemicellulose laid down by the forage.

d. *In vitro* dry matter digestibility appeared to be little affected by the rate of NH_4NO_3 fertilization, and holocellulose, cellulose, and hemicellulose content of the forage.

e. Forage samples collected during May and early June were more digestible than similar samples collected during the remainder of the growing season.

f. Increased lignification caused a decrease in *in vitro* digestibility.

LITERATURE CITED

1. Barnett, A.J.G. Studies on the Digestibility of the Cellulose Fraction of Grassland Products. J. Agr. Sci., 49:467(1957).

2. Barnett, A.J.G. and Reid, R.L. Reactions in the Rumen, Edward Arnold (Publishers) Ltd., London, 1961, 137-169.

3. Baumgardt, B.R. and Hill, D.L. Factors Affecting the Dry Matter Digestion of Various Roughages in the Artificial Rumen. J. Dairy Sci., 39:943(1956).

4. Baumgardt, B.R., Cason, J.L. and Markley, R.A. Comparisons of Several Laboratory Methods as Used in Estimating the Nutritive Value of Forages. J. Animal Sci., 17:1205(1958).

5. Bowden, D.M. and Church, D.C. Correlations Between In Vitro and In Vivo Measures of Digestibility and Chemical Components of Forages. J. Dairy Sci., 45:980-985(1962).

6. Church, D.C. and Peterson, R.G. Effect of Several Variables on In Vitro Rumen Fermentation. J. Dairy Sci., 43:81-92(1960).

7. Clark, K.W. and Mott, G.O. The Dry Matter Digestion In Vitro of Forage Crops. Can. J. Plant Sci., 40:123(1960).

8. Crampton, E.W. and Forshaw, R.R. The Nutritive Values of Kentucky Blue Grass, Red Top and Brome Grass. J. Nutrition, 19:161(1940).

9. Crampton, E.W. and Forshaw, R.R. The Intra-Seasonal Changes in the Nutritive Value of Pasture Herbage. Sci. Agr., 19:701(1939).

10. Davis, R. Personal Communication.

11. Dehority, B.A. and Johnson, R.R. Effect of Ball-Milling Upon In Vitro Digestibility of Cellulose of Mature Forages. J. Animal Sci., 19:1257(1960).

12. Donefer, E., Crampton, E.W. and Lloyd, L.E. In Vitro Rumen Fermentation Data as Indicies of Effective Nutritive Value of Forages. J. Animal Sci., 18:1538(1959).

13. Drapala, W.J., Raymond, L.C. and Crampton, E.W. The Effects of Maturity of the Plant and Its Lignification and Subsequent Digest-

ibility by Animals as Indicated by Methods of Plant Histology. Sci. Agr., 27:36(1947).

14. Ely, R.E. and Moore, L.A. Yields of Holocellulose Prepared from Various Forages by Acid Chlorite Method. J. Agr. Food Chem., 2: 826-829(1954)

15. Forbes, R.M. and Garrigus, W.P. Some Effects of Forage Composition on Its Nutritive Value When Cut and Fed Green to Steers and Wethers, as Determined Conventionally and by the Lignin Ratio. J. Animal Sci., 9:531(1950).

16. Hershberger, T.V., Long, T.A., Hartsock, E.W. and Swift, R.W. Use of the Artificial Rumen Technique to Estimate the Nutritive Value of Forages. J. Animal Sci., 18:770(1959).

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

18. Kamstra, L.D., Moxon, A.L. and Bentley, O.G. Effect of Lignification in Plants on Digestion of the Plant Cellulose In Vitro. J. Animal Sci., 14:1238(1955).

19. LeFevre, C.F. and Kamstra, L.D. A Comparison of Cellulose Digestion In Vitro and In Vivo. J. Animal Sci., 19:867-872(1960).

20. McBee, R.H. Manometric Method for the Evaluation of Microbial Activity of Rumen with Application to Utilization of Cellulose and Hemicellulose. Appl. Microbiol., 1:106-110(1953).

21. Meyer, J.H. and Lofgreen, G.P. The Estimation of the Total Digestible Nutrients in Alfalfa from Its Lignin and Crude Fibre Content. J. Animal Sci., 15:543(1956).

22. Patton, A.R. Seasonal Changes in the Lignin and Cellulose Content in Some Montana Grasses. J. Animal Sci., 2:59-62(1943).

23. Patton, A.R. and Gieseker, L. Seasonal Changes in the Lignin and Cellulose Content of Some Montana Grasses. J. Animal Sci., 1:22 (1942).

24. Phillips, T.G. and Smith, T.O. The Composition of Timothy. Technical Bulletin 81, Agricultural Experiment Station, University of New Hampshire, Durham, N.H., 1943.

25. Quicke, G.V. and Bentley, O.G. Lignin and Methoxyl Groups as Related to the Decreased Digestibility of Mature Forages. Science, 18:365(1959).

26. Quicke, G.V., Bentley, O.G., Scott, H.W. and Moxon, A.L. Cellulose Digestion In Vitro as a Measure of the Digestibility of

Forage Cellulose by Ruminants. J. Animal Sci., 18:275-287(1959).

27. Reid, R.L., Shelton, D.C., Welch, J.A. and Jung, G.A. Pasture Quality as Determined by In Vitro and In Vivo Techniques. J. Animal Sci., 18:1537(1959).

28. Salsbury, R.L., Vanderkolk, A.L., Baltzer, B.V. and Luecke, R.W. The Rates of Digestion of the Cellulose of Some Plant Fractions by Rumen Microorganisms In Vitro. J. Animal Sci., 17:293(1958).

29. Shelton, D.C. Pasture and Range Research Techniques, Comstock Publishing Associates, Ithaca, New York, 1962, 57-59.

30. Sosulski, F.W. and Patterson, J.K. Correlations Between Digestibility and Chemical Constituents of Selected Grass Varieties. Agron. J., 53:145(1961).

31. Sullivan, J.T. A Rapid Method for the Determination of Acid-Insoluble Lignin in Forages and Its Relation to Digestibility. J. Animal Sci., 18:1292(1959).

32. Thacker, E.J. A Modified Lignin Procedure. J. Animal Sci., 13: 501(1954).

33. Van Dyne, G.M. Micro-Methods for Nutritive Evaluation of Range Forages. J. Range Management, 15:303-314(1962).

34. Walker, D.M. The In Vitro Digestion of Roughage Dry Matter. J. Agr. Sci., 53:192-197(1959).

35. Warner, A.C.I. Criteria for Establishing the Validity of In Vitro Studies with Rumen Microorganisms in so-called Artificial Rumen Systems. J. Gen. Microbiol., 14:733-748(1956).

36. Webster, J.E. Personal Communication.

37. Wise, L.E., Murphy, M. and D'Addieco, A.A. Chlorite Holocellulose, Its Fractionation and Bearing on Summative Wood Analysis and on Studies on the Hemicelluloses. Paper Trade J., 122:35 (1946).

VITA

James William Hogan

Canidate for the Degree of

Master of Science

Thesis: A STUDY OF THE EFFECT OF RATE OF AMMONIUM NITRATE FERTILIZATION, CHEMICAL COMPONENTS, AND TIME OF CUTTING ON THE IN VITRO RUMEN DIGESTION OF BERMUDAGRASS FORAGE

Major Field: Chemistry

Biographical:

Personal data: Born in Independence, Iowa, December 2, 1935, the son of A.C. and Agnes Hogan.

Education: Attended grade school in Masonville, Independence, New Hampton, and Fayette, Iowa; graduated from Fayette Consolidated High School in 1953; received the Bachelor of Science degree from Upper Iowa University, with majors in Chemistry and Biology, in May, 1961; completed requirements for Master of Science degree in August, 1963.

Professional experience: Research Assistant, Biochemistry Department, Oklahoma State University, 1961-63.

Professional organizations: Member of the American Chemical Society, American Association for the Advancement of Science, and the Iowa Academy of Science.