1972

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Gerald Byron Gill

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ALTERATIONS OF THE IN VITRO DIGESTIBILITY OF
DEER BROWSE AS AFFECTED BY SYSTEMATIC
MIXING OF PAIRED SPECIES

By
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B.S., Colorado State University, 1963

Presented in partial fulfillment of the requirements
for the degree of
Masters of Science in Wildlife Biology
UNIVERSITY OF MONTANA
1972

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Chairman, Board of Examiners

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Dean, Graduate School

Sept. 27, 1972
Experimentation was conducted to determine if mixing forages improved the digestibility of individual species. Mixtures of winter browse species were used since winter forage is generally low in nutrient levels, especially deciduous species; and that period is the most crucial season in terms of energy demands on the animal.

In vitro digestion for evaluating forage quality was selected in preference to feeding live animals and measuring digestion in vivo. With the "all glass" or "closed" system used in this study, rumen microbial fermentation is simulated, but the removal of digestion products and other rumen processes are not.

Five browse species were collected from two deer winter ranges near Missoula, Montana: Douglas fir (Pseudotsuga menziezii), big sagebrush (Artemisia tridentata), bitterbrush (Purshia tridentata), evergreen ceanothus (Ceanothus velutinus), and serviceberry (Amelanchier alnifolia). After the samples were dried and ground, three browse pairs were formed from the five species. The species of each pair were then combined in various proportions to produce seven substrates.

Rumen fluid inocula collected from wild, free-ranging mule deer (Odocoileus hemionus hemionus) were used to inoculate the Douglas fir-sagebrush and sagebrush-bitterbrush substrates. A white-tailed deer (Odocoileus virginianus ochrourus) was the inoculum source for the ceanothus-serviceberry series.

Volatile fatty acid (VFA) production and dry matter loss (DML) were selected as the criteria to evaluate substrate digestibility. Based on VFA production, the data indicate that mixing species does improve the digestibility or microbial utilization of the substrate with the species used in this investigation. Douglas fir-sagebrush substrates produced consistently higher VFA levels than the sagebrush-bitterbrush substrates, although Douglas fir and sagebrush are considered less palatable than bitterbrush.

Another interesting phenomenon was suggested by the VFA patterns of certain substrates. It appears, with those substrates, the amount of digestible substrate material was reduced to a level at which the VFA's became a more readily available energy source for the microorganisms, and VFA utilization exceeded production.
ACKNOWLEDGEMENTS

To individually thank everyone who contributed to this project would require as much space as the thesis narrative. Instead, it is my hope that interpretation and presentation of the results have been accomplished well enough to evoke a feeling of pride from all who contributed to the project. However, some individuals and groups deserve special recognition.

I am indebted to my committee chairmen: Dr. James A. Bailey, now at Colorado State University, Fort Collins, Colorado, who assisted me during the 1968-69 school year; and Dr. W. L. Pengelly who returned from sabbatical leave for the 1969-70 school year. My special gratitude also to my committee members: Drs. Bart O'Gara and Lee Eddleman, University of Montana; Dr. L. Jack Lyon, U.S. Forest Service, Forestry Sciences Laboratory; and Mr. Thomas W. Mussehl, Chief of Game Research, Montana Fish and Game Department. Though not a member of the committee, my thanks to Dr. R. Ream for periodic advice on my study and for substituting on my examination board.

The U.S. Forest Service, Forestry Sciences Laboratory provided laboratory facilities and equipment. The Montana Fish and Game Department provided financial support and ran chemical analyses of the browse samples.
Drs. Wayne Van Meter of the UM Chemistry Department and Robert Irving of the Botany Department assisted me in attempting to run VFA analyses of the test samples by gas chromatography.

Dr. J. G. Nagy and Mr. C. Schwartz, Colorado State University at Fort Collins, ultimately ran the volatile fatty acid analyses, and Schwartz assisted me with interpretation of the fatty acid chromatograms.

P. R. Hildebrand and S. C. Shaffer, fellow UM graduate students, assisted me with deer collections and laboratory procedures.

Last, but by no means least, I thank my family, who forfeited many evening family hours and weekend outings. I am especially appreciative of the many hours my wife, Kathy, spent typing and retyping this paper.
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Chapter 1

INTRODUCTION

Through the process of natural selection, ruminants have been endowed with a specialized digestive system. Complex in both function and structure, the ruminant stomach has four interconnected compartments: the reticulum, rumen, omasum and abomasum. The rumen, largest of the four, contains a dynamic microbiota of bacteria and protozoa. Hungate (1966:82 and 123) tabulated 24 species of bacteria and 32 protozoa commonly associated with domestic ruminants. Nagy and Tengerdy (1968:442) identified bacteria in the rumens of wild mule deer (Odocoileus hemionus) similar to those described in domestic ruminants. Comparable findings in elk (Cervus candensis) rumens were reported by McBee et al. (1969:183). Ruminants and their microbes exhibit a symbiotic relationship; furthermore, difficulties in obtaining and maintaining pure cultures of rumen microorganisms suggest some symbiosis at the microbial level (Annison and Lewis, 1959:16).

The rumen microbes are able to use certain low value elements of the host's diet and synthesize products of value to both organisms. Significant in this respect is the evidence that both protein and nonprotein nitrogen can be
converted to amino acids and ammonia which are utilized by
the host animal and the microbes (Annison and Lewis,
1959:92 and 93; Hungate, 1966:300-306; Hatfield, 1970:44-
46). Microbial decomposition of dietary carbohydrates, and
to some extent amino acids, produces volatile fatty acids
(VFAs) which are a primary energy source for domestic
ruminants. However, microbial fatty acid production is not
to a degree entirely without sacrifice since the process results in a
10-20% loss of usable food energy (Picton, 1970).

Even with the benefit of microbial digestion, rumin­
ants require certain nutritional elements in their diet to
maintain the necessary microbial populations. Though summer
forage does not appear to be nutritionally limiting, the
quality of winter foods may be deficient to the degree of
providing a submaintenance diet especially in terms of
digestible energy (Dietz, 1965:277). Winter foods of domes­
tic livestock can be supplemented to offset nutritional
deficiencies. Wild ruminants generally manage to sustain
themselves through winter periods on natural forage if
populations are reasonably balanced with the food supply of
the range. It has been suggested that mixtures of species
in the diet compensate their individual limitations and
improve the utilization and maintenance value of each species
(Dietz et al., 1962:84; Nagy et al., 1969). In addition,
studies have shown that deer are selective in their feeding,
largely choosing the most nutritious plants and plant parts
The scope of this investigation was to evaluate the qualities of forage mixtures by digesting various combinations of paired browse species in vitro. Samples of browse species commonly used by mule deer and white-tailed deer (Odocoileus virginianus) were collected from their respective winter ranges near Missoula, Montana. Winter forage was selected to test mixture responses of plants that were assumed to vary in their nutrient levels and palatability. Specific assumptions were: (1) there would be little difference in the digestibility of Douglas fir (Pseudotsuga menziesii) and sagebrush (Artemisia tridentata) due to the low palatability of both species (Longhurst et al., 1968); (2) bitterbrush (Purshia tridentata) would be more digestible, though comparatively lower in nutrient levels, than sagebrush; and (3) the digestibility of serviceberry (Amelanchier alnifolia) would exceed that of evergreen ceanothus (Ceanothus velutinus). The expected response was that equal mixtures of the paired browse species would exhibit higher digestibility than the individuals.
Current techniques have enabled researchers to broaden the scope of their investigations in big game nutrition. Until the early 1960s, the primary interest in big game nutrition was in determining what the animals ate. One investigational technique used was feeding site evaluations. Biologists would follow game trails and record forage utilization, or they would observe animals feeding and evaluate utilization after the animals had moved to another site. This technique still finds application today though perhaps not as extensively as in the past. Development of rumen analysis techniques added depth to food habit data by revealing relative proportions of items in the diet as well as what was eaten. Recent experimentation in feeding observations has involved the use of captive deer trained to leash (Healy, 1971). The animals are allowed free choice of food items while monitors record plants and plant parts eaten, selection behavior, and forage preference. Brown (1961:67) used semi-tame deer, unleashed but approachable, in his feeding observations to determine forage preference. He compares his findings with rumen analysis data and defines certain limitations of both types of food habits investigational techniques.
Food habit studies have contributed volumes of data on what animals eat. However, depending on the scope of a project, knowledge of what is eaten may provide only a substantial base for further research. Research efforts are now being directed into other aspects of big game nutrition. Current knowledge of the specific dietary requirements of wild ruminants and metabolism of their food is limited. Most of the current concepts in big game nutrition, other than food habits, are extrapolations from knowledge gained with domestic ruminants.

Although forage quality studies provide little in terms of understanding how an animal uses its food, they do indicate the potential of forages as food sources. Some studies, such as those by Ullrey et al. (1964, 1967, 1968, 1971), have been conducted on the nutritional quality of plant species used by deer; however, evaluations of mixed diets are all but nonexistent.

EVALUATION OF FORAGE QUALITY

Dietz (1965:277) suggests that the most common nutritional deficiencies of deer are protein, phosphorus, and either available energy or digestible energy or both. Further, he lists three factors affecting the nutritional quality of a forage: "... (1) the levels of important nutrients contained in the portion eaten, (2) the ability of game animals to digest these nutrients, and (3) the
efficiency of the digested nutrients in meeting the physiological demands of the body. . . ." Nutrient levels vary within the structural components of plants and with plant phenology. Four species of deciduous shrubs (gambel oak, *Quercus gambeli*; serviceberry, *Amelanchier alnifolia*; bitterbrush, *Purshia tridentata*; and mountain mahogany, *Cercocarpus montanus*) analyzed by Dietz (1958) had the highest percentage of important nutrients in the leaves. Harshbarger and McGinnes (1971:672) found that fallen sourwood (*Oxydendrum arboreum*) leaves retained higher levels of total nitrogen and nitrogen-free extract and lower crude fiber levels through the fall and winter than previously reported for eastern browse twigs. Aldous found higher nutritive value in the tips of bitterbrush than in larger twigs (cited by Snyder, 1961:4). As phenology advances, deciduous plants generally decline in their percentages of certain nutrients, such as protein and phosphorus, while exhibiting relative increases in others, such as crude fiber, ether extract, and nitrogen-free extract (Frederiksen, 1961:8; Dietz, 1965:279). During winter dormancy, deciduous plants may provide only maintenance or even submaintenance rations depending on the relative proportions of their nutrient compositions and their digestibility. Supplementing the reduced winter value of deciduous plants, evergreen and semi-evergreen vegetation retain comparatively high nutrient levels during the winter (Dietz *et al.*, 1962:82; Dietz,
although several species may be relatively low in palatability.

Over-used ranges further intensify the nutritional deficiencies of big game diets. Dietz (1965:280) suggests that deer on overbrowsed winter ranges may consume such low-quality forage that malnutrition results. Concentrations of animals on big game winter ranges may reduce or completely eliminate preferred plants and encourage invasion of less palatable species. Among plants of low palatability (though not necessarily low nutrient value) are several species that contain bactericidal compounds. It has been postulated that these compounds were evolved as defense mechanisms; a genetic selection to protect the plant from being over-grazed (Longhurst et al., 1968).

Big sagebrush and Douglas fir are two examples of species that contain bactericidal compounds. Nagy et al. (1964:788-789) demonstrated that increasing amounts of the essential oils from sagebrush inhibited in vitro cellulose digestion. Oxygenated monoterpenes found in mature needles of Douglas fir are especially inhibitory on rumen microbes (Longhurst et al., 1968). Longhurst et al. (op. cit.) have concluded that, generally, highly aromatic plants are the most inhibitory to rumen function. Yet, tolerance levels for inhibitory species can be increased, if the species are consumed as part of a mixed diet (Dietz et al., 1962:83;
Various techniques have been used to evaluate forage quality. The proximate analysis is a combination of tests used to measure the crude protein, crude fat (ether extract), ash, nitrogen-free extract and crude fiber of plants. While limited to measuring broad categories, this method offers a means of identifying potential nutrient sources (Dietz, 1965: 275; Harshbarger and McGinnes, 1971:672).

Digestion trials provide another, and certainly more authentic means of testing the value of forages for ruminants. With captive animals, investigators are technically able to control the diet and monitor some of the metabolic processes. Nevertheless, providing and maintaining digestion trial facilities and caring for captive animals, especially inherently wild species, require considerable financial investment. In addition, extrapolating results to equivalent processes in free-ranging animals may not be reliable.

In vitro digestions have been extensively used since the late 1950s by researchers in animal science (Johnson, 1963:792); only recently has the technique been applied to nutrition of wildlife. In vitro rumen fermentation is basically a process of digesting substrates with rumen microorganisms outside of the rumen. Several advantages and disadvantages of using this technique have been identified (Burroughs et al., 1950; Van Dyne, 1963; Warner, 1964;
Pearson, 1970). In vitro research offers the advantages of speed in conducting experiments, controlled conditions, reduced expense, simplicity and portability of equipment, ability to test a large number of samples at one time, and repeatability. Johnson (1966:856) stressed the major advantage of in vitro research as the ability to study rumen microbial activity divorced from the host animal. Some of the disadvantages are: results may not be comparable to in vivo determinations; it is difficult to approximate chemical constituents similar to saliva, control the end products, maintain microorganisms comparable to those in the rumen, and maintain normal physiological conditions throughout long incubation periods; and there is a lack of standardization in techniques and procedures among laboratories.

Hungate (1966:438) emphasizes that in vitro investigations "... are useful for obtaining clues to possible differences in feeds, but the final measure of a feed is its effect on the ruminant." In the final analysis, combinations of techniques will improve the reliability of conclusions derived from forage quality evaluations.

IN VITRO FERMENTATION

Pearson (1970:85) describes in vitro rumen fermentation as a system that "... includes fermentation of a substrate with rumen microorganisms in a buffered nutrient
medium under controlled conditions of anaerobiosis, temperature, and pH." Historically, investigations which influenced the development of in vitro techniques can be traced back to the 1800s. Johnson (1963) presents a good historical review of in vitro research and also cites several sources that review earlier works on rumen microbial activity. Hungate (1966:437) credits Waentig and Gierisch in 1919 as the first to evaluate forage digestibility in vitro. Tilley and Terry (1963) described a new two-stage technique that utilizes a 24-hour microbial fermentation of the substrate concluded by an acid-pepsin digestion for 48 hours. It has been suggested that this method be used as a standard technique for all in vitro research (Pearson, 1970:88).

Reliability, consistency, and comparability of in vitro results are influenced by a variety of factors. Since rumen microbes are the basis for any in vitro study, factors affecting them will definitely influence the results. They appear to be sensitive to high temperatures and deviations in pH below or above the range 6.7 to 7.0 (Johnson, 1966:860).

Hershberger et al. (1959) identified the primary requirements of rumen microorganisms as: anaerobic environment, minerals, and a source of ammonia nitrogen, cellulose, and soluble carbohydrates. Other factors that alter in vitro results include: type of system used, substrate amount and size, source and preparation of inoculum, amount of inoculum
and buffering medium, and fermentation time.

Generally, two types of in vitro systems have been utilized—an open or "continuous flow" system and a closed or "all glass" system. Johnson (1966:857) summarizes the chief advantages and disadvantages of both systems. Continuous flow systems duplicate more closely the actual processes occurring within the rumen but are complex and limited in the quantity of samples that can be tested in one experiment. Closed systems are simple in design and can be used to test a large number of samples per experiment. Nevertheless, they are limited to studying only a few of the many rumen microbial processes. Also, with a closed system the protozoa usually are eliminated from the microbial populations.

The amount and size of substrates vary among laboratories. Hungate (1966:437) cautions that substrate quantity should be small enough to prevent accumulations of fermentation products that would be unfavorable to microbial activity—"This quantity will depend on the buffering capacity of the medium and the digestibility of the substrate." Some investigators suggest that particle size of the substrate should not be as fine as obtained by ball milling nor as large as 2.5 millimeters (Pearson, 1970:85). It was assumed that the fine grinding disrupted the cell walls of the plant structure and the large particles were not completely digested.
As with other facets of in vitro research, the source and preparation of rumen inoculum have not been standardized. Among investigators there exists a division of thought regarding source of the inoculum. Several support the concept that source animals should be on the same diet as the test substrate (Johnson, 1963:795; Harris et al., 1967:44; Pearson, 1970:86). However, dietary control is seldom possible with free-ranging, wild ruminants, and their natural diets commonly consist of a variety of plants. Bowden and Church (1962), after reviewing several in vitro studies, concluded: "... it seems advisable for most accurate estimates to use inoculum from an animal which is on a diet of nutritional ingredients as close as possible to that being digested." Bruggeman et al. (1968:203) recommend that "... the vegetative stage of the substrate should be synchronized with the respective seasonal stage of the rumen microbial population ...," which would be most applicable in evaluating natural forage with inocula from wild ruminants.

Several methods of preparing rumen inocula are available for in vitro research. The method used will depend upon the objectives of the investigation. For example, to study the nutrient requirements of rumen microorganisms, the microbial cells must be separated from the rumen liquor since the fluid itself contains adequate amounts of various nutrients (Johnson, 1963:794). Strained whole
Rumen fluid appears best suited for forage evaluation studies since it contains both the microorganisms and many of their needed growth factors (Hungate, 1966:437). Some of the inocula preparations that have been used include: strained whole rumen fluid, phosphate buffer extract, centrifuged cell suspension, washed cell suspension, and resuspended ruminal microorganisms. Johnson (1966:861-862) describes the first four inocula and discusses some of their limitations. Quicke et al. (1959) describe the methods of preparing phosphate buffer extract and resuspended ruminal microorganism inocula. The preparation and advantages and disadvantages of washed cell suspensions are discussed in some detail by Annison and Lewis (1959:52). Pearson (1970:86) experimented with fresh and frozen inocula. Digestibilities were lower with frozen inocula suggesting that freezing causes the microorganisms to die or become inactive.

Only limited sources of information were found recommending the amounts of inoculum and buffering medium to be used. It might be assumed that a large quantity of inoculum would insure a viable inoculation by introducing greater numbers of microorganisms. However, the amount of inocula must be balanced with sufficient buffer to prevent acid accumulations that will inhibit microbial activity. Marquardt and Asplund (1964), using different volumes of washed and rewashed inocula in a basal medium, found a linear relationship between increased cellulose digestion
and volume of inoculum. A curvilinear relationship between volume of inoculum and cellulose digestion accompanied low levels of the basal medium, suggesting an equilibration level between volumes of inoculum and basal medium beyond which acid accumulations became inhibitive. Barnes (1966) concluded from an informal meeting of in vitro researchers in 1959 that the most common volumes of inoculum and buffering medium were 20 milliliters of strained whole rumen fluid to 30 milliliters of buffer. Other researchers have used 50 milliliters of liquid medium mixed in a 1:4 ratio of rumen fluid to buffer (Tilley and Terry, 1963; Pearson, 1970:89).

Most buffering media in use today are based on an "artificial saliva" proposed by McDougall (1948) from his analyses of sheep saliva. They function, basically, as a neutralizer to maintain a fairly constant pH of 6.7 to 7.0 and may also serve as sources of nutrients for the microbial populations (Marston, 1948; Johnson, 1966:857-858).

Fermentation time has considerable influence on the results of in vitro digestions. Johnson (1966:862) considered the differences in fermentation periods for various kinds of substrates. Among the types of digestible carbohydrates, there are wide variations in the time periods required for both initiation of fermentation and maximum rate of digestion. Simple sugars have a short lag period (time between initiation of an experiment and the beginning of fermentation) and are generally digested rapidly once
fermentation begins. Cellulose has a much longer lag period and slower digestion rate. Several investigators suggest a 24-hour fermentation period for reliable results from cellulose and dry matter digestions (Asplund et al., 1958:178; Kamstra et al., 1958; Donefer et al., 1960; Harris et al., 1967:48). Others feel that periods greater than 24 hours are needed for comparing in vitro cellulose digestion studies and for extrapolating in vivo cellulose digestion from in vitro results (Quicke et al., 1959; LeFevre and Kamstra, 1960; Troelsen and Hanel, 1966).

VOLATILE FATTY ACIDS

Volatile fatty acids are one of the primary end products from carbohydrate decomposition by rumen microbes (Hungate, 1966:245). Little dietary carbohydrate is absorbed in the alimentary tract; most is fermented to VFA and absorbed through the rumen wall (Annison and Lewis, 1959:121; Warner, 1964; Hungate, 1966:193). Through enzymatic splitting of the carbon-carbon bonds in carbohydrates, rumen bacteria utilize a source of energy for synthesizing microbial cell material from protein in the host's diet. The VFAs produced as a result of carbohydrate decomposition become energy sources for the host. It is estimated that VFA provides 63-90% of the energy requirements of cattle (Rodgers, 1958; Picton, 1970); however, insufficient data are available to quantify the utilization of fatty acids by wild ruminants. Picton (1970) suggests that cervids are less
efficient in microbial digestion than domestic ruminants and thereby derive a smaller proportion of their energy needs from VFAs. Short (1963:194) estimated that white-tailed deer derived less than 50% of their energy requirements from VFAs. However, until more information is available, it must be assumed that fatty acids are an important energy precursor in wild ruminants, though perhaps not to the same degree as in domestic livestock.

The amounts and proportions of VFAs are variable, depending on such factors as nature of the diet, time after feeding, and age of the animal (Stewart et al., 1958; Frederiksen, 1961:13; Bruggeman et al., 1968:201). The primary acids produced are acetic, propionic, and butyric in the general proportions: 65% acetic, 20% propionic, and 15% butyric (Huffman, 1951; Annison and Lewis, 1959:62; Frederiksen, 1961:12; Short, 1963:194). Annison and Lewis (1959:60) and Short et al. (1966; 1969b:382) have shown higher rumino- reticular levels of butyric and propionic acids and lower acetic acid with easily digested foods than with foods that are more difficult to digest. Reid et al. (1964) demonstrated varying proportions of acetic and propionic acids related to increasing herbage maturity. Annison and Lewis (1959:62) cite several investigations that show a direct relationship between protein content of the diet and levels of butyric acid and total VFA production. Acetic acid levels were inversely related to protein content.
Bailey (1970) cites several references which suggest that increased production of propionic acid is correlated with high levels of food and protein intake, a high rate of food fermentation in the rumen, and fine-grinding of the forage. Generally, these same factors result in low acetic acid levels. Thus, the ratio of acetic to propionic acid in vitro may be a reliable indicator of forage digestibility.

Most of the literature reviewed on in vitro research pertained to cellulose digestion. Reid et al. (1964) used VFA production in their evaluation of forage quality. A study by Gray, Pilgrim, and Weller in 1951 (cited by Asplund et al., 1958:171) indicates a relationship between the amount and nature of VFA production in vitro and forage quality. In their own work, using a variety of hay substrates, Asplund et al. (op. cit.:179) found correlations between dry matter digestibility in vivo and both VFA production and dry matter loss (DML) in vitro. Further, they found in vitro VFA production and DML were significantly correlated with crude protein levels of the substrates.

If in vivo digestibility is to be extrapolated from in vitro results, then cellulose and/or dry matter digestibility may be the most reliable criteria to use. Johnson (1963:795) advises caution in considering VFA production as the main criterion for interpreting rumen microbial fermentations of a substrate. Volatile fatty acids are produced from the fermentation of a variety of substrates rather than
being specific for one (i.e., VFA production lacks specificity). However, for general evaluations of forage digestibility, VFA is a valid criterion since definite relationships between forage digestibility and total VFA production and molar proportions of each acid have been demonstrated.
Chapter 3

METHODS AND MATERIALS

The laboratory phase of this study included the in vitro digestions and attempts to analyze the samples for volatile fatty acids. It was conducted at the U.S. Forest Service Forestry Sciences Laboratory, Missoula, Montana, where laboratory space and facilities were available.

The field phase consisted of collecting browse samples and wild deer (inocula donors) from two winter ranges located within 100 miles of Missoula. Equipment and funds for the field work were contributed by the Montana Fish and Game Department and the University of Montana.

COLLECTION AND PREPARATION OF BROWSE SAMPLES

Three pairs of deer browse were mixed in various proportions and tested for variations in digestibility. The browse pairs were:

Douglas fir and big sagebrush
Big sagebrush and bitterbrush
Evergreen ceanothus and serviceberry
Sagebrush, bitterbrush, and Douglas fir were collected in late January, 1970, from the Markham-Mineral mountains area in west-central Montana. This area, located
approximately 17 miles west of Lincoln, Montana, is a traditional mule deer winter range. Sagebrush and bitterbrush samples were current annual-growth stems clipped from several plants of each species. None of the stems were larger than a 1/8-inch diameter. Clippings from sagebrush, a semi-evergreen shrub, consisted of stems, leaves, and decadent flower parts. Samples of bitterbrush, a deciduous shrub, contained only stems and buds. Douglas fir material consisted of 3- to 5-inch sections of the tips of low-growing branches clipped from a single tree.

Serviceberry and ceanothus were collected from a clearcut site on the Flathead National Forest near Condon, Montana. This part of the Swan River Valley is a primary white-tailed deer (*O. v. ochrourus*) wintering area. Serviceberry was clipped on March 2, 1970, from plants in a timbered drainage adjacent to the clearcut site. As with the sagebrush and bitterbrush samples, serviceberry clippings were current annual growth with no stems larger than 1/8-inch diameter. Ceanothus samples were collected from plants within the clearcut and consisted of only leaves and stem ends stripped from the plants. Ceanothus plants were covered by deep snow when the serviceberry samples were collected; consequently, ceanothus samples could not be obtained until March 25.

All browse samples were dried and then ground in a Wiley mill to pass a 20-mesh screen. Prior to grinding,
sagebrush and bitterbrush were air-dried for 10 days. Douglas fir samples were also air-dried for 10 days, but the material retained enough moisture to plug the screen. Ultimately, the samples were placed in a Labline forced-air oven and dried at 34°C-26°C for 92 hours in a Percival circulated-air environment chamber. All samples were dried at approximately room temperature to preserve the volatile compounds of sagebrush and Douglas fir (Ward and Nagy, 1965: 2) and to retain, as closely as possible, the original nutrient characteristics of each species. After the samples were dried and ground, seven substrates, weighing 1 gram each, were prepared from the paired browse species (Table 1).

Twenty-four grams of each substrate from the Douglas fir-sagebrush and ceanothus-serviceberry pairs were analyzed for crude protein, crude fiber, ether extract, ash, phosphorus, and calcium (see Appendix, Exhibit A). Due to a shortage of bitterbrush material, only three substrates from the sagebrush-bitterbrush combination could be analyzed: sagebrush only (substrate 1), 50% sagebrush-50% bitterbrush (substrate 4); and bitterbrush only (substrate 7). The analyses were conducted at the Chemical Analytical Laboratory, Montana State University, Bozeman, Montana. Due to limited funds, it was not possible to repeat these analyses on the fermentation residues; consequently, the apparent digestibility of these chemical components was not determined.
Free-ranging mule deer and white-tailed deer were used as source animals for rumen inocula. Mule deer inocula were used in both sagebrush series, whereas whitetail inoculum was used in the ceanothus-serviceberry series. Mule deer were taken from the same area that the sagebrush, bitterbrush, and Douglas fir samples were collected. One animal was killed on February 26, 1970, for the Douglas fir-sagebrush series and the other on March 7, 1970, for the sagebrush-bitterbrush series. The white-tailed deer was killed April 7, 1970, in an area approximately 3 miles west and 4 miles north of the clearcut from which the ceanothus and serviceberry had been obtained. Whitetails had not yet moved onto the clearcut, presumably inhibited by snow depths at the time. However, some ceanothus and serviceberry were discovered in the area where the animal was shot. The value of this notation is clarified if reference is made to the previous discussion of source of inocula in the Literature Review.

All inocula were collected in the early morning, assuming the deer would be active and easily located, and rumen fatty acids would be at low levels. Short et al. (1969a:190), after analyzing rumino-reticular contents of 68 hunter-killed white-tailed deer, found lower VFA concentrations in rumens of animals killed between 8:00 a.m. and
Table 1. Percentages of each browse species in the experimental substrates. 1/

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Browse Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Douglas fir</td>
</tr>
<tr>
<td>1</td>
<td>100.0</td>
</tr>
<tr>
<td>2</td>
<td>87.5</td>
</tr>
<tr>
<td>3</td>
<td>75.0</td>
</tr>
<tr>
<td>4</td>
<td>50.0</td>
</tr>
<tr>
<td>5</td>
<td>25.0</td>
</tr>
<tr>
<td>6</td>
<td>12.5</td>
</tr>
<tr>
<td>7</td>
<td>0.0</td>
</tr>
</tbody>
</table>

1/ Each substrate weighed 1 gram.

12:00 noon than those killed later.

After the rumen had been exposed and opened, a household soup ladle was used to transfer the rumen contents to a premade cheesecloth sack. The sack, constructed of 16 layers of coarse (80 grade) cheesecloth, could be closed by means of a drawstring. Early experimentation in collecting steer rumen fluid, using unmodified pieces of cheesecloth, resulted in spilling a quantity of rumen contents when attempting to filter more than a handful of material. The cheesecloth sacks increased the volume of rumen contents that could be strained at one time which accelerated transfer of the inoculum to Thermos containers and minimized
exposure of the microorganisms. The Thermos containers were modified with Bunsen valves (Johnson, 1966:859) inserted through the caps to vent accumulating fermentation gases (Fig. 1). Otherwise, if confined, the gases would have developed sufficient pressure to blow the caps off the containers and waste a large quantity of inoculum.

Figure 1. Inoculum container—a Thermos bottle modified with Bunsen valve to vent accumulating gases.

DESIGN OF IN VITRO DIGESTION SERIES

Technique

Three digestion series were conducted in this investigation, one for each browse pair. The basic components of a series consisted of seven substrates (Table 1) and a control for determining digestion of food material. Only two replications were possible with
the Douglas fir-sagebrush components due to a shortage of inoculum. These were digested for periods of 2, 6, and 12 hours with the complete series involving 48 test samples (8 basic components x 2 replications x 3 fermentation periods) and 2 replicate 0-hour controls. Components of the sagebrush-bitterbrush and ceanothus-serviceberry combinations were replicated three times with a series for each pair consisting of 72 test samples (8 basic components x 3 replications x 3 fermentation periods) and 3 replicate 0-hour controls. The 0-hour controls were to correct for original VFA and dry matter content of the inocula. Fermentation controls allowed correction for VFA production and dry matter loss from the digestion of rumen material.

Each substrate, totaling 1 gram of air-dried browse material, was placed in a 100-milliliter centrifuge tube, hereafter referred to as a digestion chamber. Forty milliliters of buffering solution (see Appendix, Exhibit B) were added to each substrate prior to inoculation, and the digestion chambers were capped with single-hole rubber stoppers and immersed in a water bath.

The microbial digestion process was initiated by adding 10 milliliters of inoculum to each digestion chamber (Fig. 2). Inadvertently, the 4DF-4Sa, 2-hour replicates were inoculated twice; and one of the all sagebrush, 2-hour replicates in the Douglas fir-sagebrush series was not inoculated. They were treated as missing observations in
the analysis of data. Control samples received the buffer and inocula only. Maximum exposure of the rumen fluid to atmospheric oxygen was 20 minutes. Pearson (1970:06) suggests that microbial activity is 40% inhibited if the inoculum is aerated for 15 minutes and completely inhibited if aeration is 30 minutes or longer. However, microscopic examination of the rumen fluid, after the digestion chambers of a series were inoculated, verified the presence of active organisms.

Figure 2. Adams aupette used to inoculate the digestion chambers.

When a fermentation period was completed, the test samples for that period were removed from the water bath, the microbial activity was terminated (Fig. 3) by adding 4 milliliters of 25% HCl solution (1 part hydrochloric acid in
4 parts distilled water). The 0-hour controls were acidified immediately after inoculation to retain initial VFA and dry matter contents. All deactivated samples were refrigerated until VFA production and dry matter loss could be determined.

Figure 3. Apparatus and technique for terminating microbial fermentation activity.

Apparatus

The in vitro apparatus constructed for this study (Fig. 4) was a slightly modified version of the "artificial rumen" described by Van Dyne (1963). The equipment components and their functions will be discussed in reference to Figures 5 and 6, using a numerical sequence. The system utilized a continuous flow of carbon dioxide (CO₂) to establish and maintain an anaerobic environment. The CO₂
Figure 4. "Artificial rumen," or *in vitro* fermentation apparatus.

Figure 5. *In vitro* apparatus showing the carbon dioxide source and flow pathway.
was released from a commercial cylinder (1), through a water trap to remove impurities (2), into the main distribution manifold (3). From the main manifold, gas flow was regulated by screw clamps (4) into bench manifolds (5). From each bench manifold, CO₂ could be dispersed into as many as 18 digestion chambers (6). The digestion chambers had pour spouts to vent accumulating CO₂ and fermentation gases. The CO₂ flow rate was regulated into each digestion chamber to minimize agitation of the inoculum microorganisms and simultaneously prevent fine substrate particles from plugging the gas jets. The jets were 8-inch lengths of 5-millimeter glass tubing tapered at the discharge end that were inserted through the rubber stoppers of the digestion chambers.

The water bath (Figs. 4 and 5) maintained a relatively
constant environmental temperature of 39°C, simulating an average of the rumen temperature (Annison and Lewis, 1959: 17). The digestion chambers were suspended in the bath by wooden supports illustrated in Figure 6.

ANALYTICAL PROCEDURES

Approximately 35 milliliters of liquid from each sample were centrifuged for 1 hour at 3,600 RPM in an International clinical centrifuge. Part of the supernatant was retained and frozen for VFA analysis; the remainder was discarded. Undigested substrates and their corresponding centrifugation precipitates were washed onto pre-weighed muslin filters and air-dried for dry matter quantification. The residue weights were calculated by simple subtraction of original filter weights from the air-dried weights of the filters plus their residues. The residues were then corrected for undigested rumen material by subtraction of the average residue weight of their respective control replicates. Computation of percent dry matter loss (DML) was derived using a ratio of the difference between the dry weight of the original substrate (x) and the dry weight of undigested substrate (y) to the dry weight of the original substrate (x-y).

Preparatory to VFA analyses, the frozen fluid samples were thawed and filtered through Millipore filters (AAWP O13 00, 13-millimeter diameter, 0.8 micron porosity).
Two-milliliter quantities of each sample were drawn into a syringe and injected through a Swinny adapter which held the filter. Each sample was filtered twice and refrozen.

Chromatography for VFA content was attempted by the author using a Varian Aerograph model 600 D gas chromatograph with a hydrogen flame detector. Hydrogen gas for the detector was supplied by a Varian Aerograph model 650 hydrogen generator. Chromatograms of the individual fatty acids were recorded on a Beckman chart recorder.

A column for the gas chromatograph was constructed by the investigator with assistance from Dr. Robert Irving of the University botany department. Packing for the column was 2.5% FFAP on 100/120 Chromosorb G A/W DMCS H.P. Standard solutions that contained known quantities of acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids were prepared as described by Nagy (personal communication; Appendix, Exhibit C). These provided a basis for determining VFA content of the unknown samples.

After the chromatograph was readied for VFA detection, injections of the browse digestion samples were begun. However, it soon became evident that the chromatograph was losing sensitivity with each injection. Chromatograms of repeated injections of a standard acid solution exhibited diminishing resolutions of the acid curves. Improved resolutions were restored after flushing the column with distilled water. This indicated a deficiency in the
column, but a replacement was not available to test the supposition. Ultimately, all samples were sent to Dr. J. C. Nagy, Colorado State University, for analyses.

A detailed outline of the laboratory procedures used in this study are appended (Appendix, Exhibit D), including suggestions for improving techniques where applicable.
RESULTS AND DISCUSSION

In Montana and most of the intermountain states, energy expenditure of wild ruminants is highest during the winter. Maintaining body temperature and other metabolic processes requires a diet that will meet these energy demands. At the same time, deciduous forage species adapt to winter conditions by entering a stage of dormancy. During their dormant period, plants develop relatively high crude fiber levels and lower levels of nutrients needed for growth and fat production. Therefore, during the winter, natural forages provide only a maintenance ration at the most.

Selective feeding and mixtures of plants in the diet apparently enable big game animals to derive the highest nutritional value from available food items. To investigate the additive effects of forage mixtures and determine whether mixtures would increase the amount of available energy, samples of deer browse collected during the winter were paired, and combinations of the pairs were digested in vitro. Volatile fatty acid (VFA) production and dry matter loss (DML) were the criteria used to evaluate microbial responses to the browse substrates. Since VFAs are apparently a valuable energy source to ruminants (see Lit. Rev.),
this section will primarily center around interpretation of the VFA data. DML will be discussed in its relationship to VFA production.

VOLATILE FATTY ACID PRODUCTION

Dietz (1971:2) states that 75% of the dry weight of plants is composed of carbohydrates. VFAs are an end product of carbohydrate decomposition; therefore, VFA production should logically reflect the digestibility of the bulk of plant dry matter.

The results of each digestion series, both VFA production and DML, are presented in Table 2. The first series was comprised of mixtures of Douglas fir and sagebrush. Both species are browsed by deer, although Longhurst et al. (1968) have them categorized as moderately palatable and unpalatable, respectively. Their chemical compositions include essential oils (fat components) that have demonstrated bactericidal properties (Nagy et al., 1964; Longhurst et al., 1968). Certain kinds of the oxygenated monoterpenes in mature needles of Douglas fir were especially inhibitory to rumen microorganisms compared to the others tested. In vitro digestions of the Douglas fir-sagebrush substrates suggest that pure Douglas fir was rapidly utilized reaching a peak VFA level after 6 hours fermentation (Fig. 7). Declining VFA levels with an additional 6 hours fermentation suggest that the digestible substrate was
Figure 7. Douglas fir - sagebrush VFA production as a function of time.  
1/ Observations missing for 4DF-4Sa substrate. Estimate for the 2-hour VFA level is an average of the 2-hour levels observed in the 6DF-2Sa and 2DF-6Sa substrates.
largely eliminated and VFAs became a more readily available energy source for the rumen organisms, or that the substrate digestion was being inhibited by low levels of the bactericidal compounds. Possibly the combined effects of both factors influenced the observed response.

Including 12.5 and 25% sagebrush in mixture with Douglas fir improved the substrate digestibility over Douglas fir alone, as progressively higher VFA levels were observed with the mixtures. However, with a substrate of 87.5% Douglas fir and 12.5% sagebrush, a lag period in VFA production was observed during the 2-hour to 6-hour interval. An abrupt change in VFA production after the 6-hour period suggests adjustment of the microbial composition in favor of bacteria capable of utilizing the mixed substrate during the lag period, followed by an accelerated rate of substrate digestion up to 12 hours. When the mixture contained 25% sagebrush, a nearly linear VFA production rate after the 2-hour period indicates microbial adaptation to the combined browse species was more uniform over the 12 hours, and substrate digestion was relatively constant among the three periods. With 50% sagebrush, VFA accumulation was relatively constant up to 6 hours. Depression of the VFA production rate after 6 hours suggests a diminishing supply of readily digestible material and microbial utilization of less digestible substrate, or inhibition of microbial activity by low toxicity levels of essential oils, or a
Table 2. VFA production and DML by fermentation period for the experimental substrates. 1/

<table>
<thead>
<tr>
<th>Substrates</th>
<th>VFA Production (milliMoles/liter of acid)</th>
<th>DML (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 hours</td>
<td>6 hours</td>
</tr>
<tr>
<td>Douglas fir</td>
<td>65.55</td>
<td>121.15</td>
</tr>
<tr>
<td>7DF-1Sa</td>
<td>71.00</td>
<td>81.60</td>
</tr>
<tr>
<td>6DF-2Sa</td>
<td>69.40</td>
<td>105.95</td>
</tr>
<tr>
<td>4DF-4Sa</td>
<td>---</td>
<td>109.05</td>
</tr>
<tr>
<td>2DF-6Sa</td>
<td>77.75</td>
<td>92.15</td>
</tr>
<tr>
<td>1DF-7Sa</td>
<td>67.00</td>
<td>114.80</td>
</tr>
<tr>
<td>Sagebrush 3/</td>
<td>62.50</td>
<td>111.95</td>
</tr>
<tr>
<td>Sagebrush</td>
<td>62.20</td>
<td>84.53</td>
</tr>
<tr>
<td>7Sa-1Bit</td>
<td>56.93</td>
<td>49.33</td>
</tr>
<tr>
<td>6Sa-2Bit</td>
<td>84.77</td>
<td>63.13</td>
</tr>
<tr>
<td>4Sa-4Bit</td>
<td>72.90</td>
<td>63.67</td>
</tr>
<tr>
<td>2Sa-6Bit</td>
<td>42.27</td>
<td>61.17</td>
</tr>
<tr>
<td>1Sa-7Bit</td>
<td>14.53</td>
<td>46.70</td>
</tr>
<tr>
<td>Bitterbrush</td>
<td>18.60</td>
<td>57.43</td>
</tr>
<tr>
<td>Ceanothus</td>
<td>9.23</td>
<td>68.13</td>
</tr>
<tr>
<td>7Ce-1Se</td>
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<td>6Ce-2Se</td>
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<td>4Ce-4Se</td>
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<td>60.37</td>
</tr>
<tr>
<td>2Ce-6Se</td>
<td>27.83</td>
<td>40.37</td>
</tr>
<tr>
<td>1Ce-7Se</td>
<td>29.43</td>
<td>68.10</td>
</tr>
<tr>
<td>Serviceberry</td>
<td>11.67</td>
<td>27.23</td>
</tr>
</tbody>
</table>

1/ Mean value of replicated samples.
2/ Both replications received double inoculations.
3/ One replication was overlooked when the digestion chambers were inoculated.
combination of both factors. An obvious lag period observed between the 2-hour and 6-hour periods with 75% sagebrush in the substrate suggests a second adjustment of the microbes to a Douglas fir-sagebrush mixture. Combining 12.5% Douglas fir with 87.5% sagebrush appears to influence an almost linear rate of VFA production up to 6 hours. A depressed production rate after 6 hours indicates a diminishing supply of digestible material, or increasing toxic levels of the essential oils, or a combination of reduction in digestible material and microbial inhibition by the essential oils. Sagebrush alone produced a higher level of VFAs than did the Douglas fir substrate. Continuous production of VFAs, but at a slower rate than for Douglas fir, suggests Douglas fir was more easily digested, but sagebrush had the greater quantity of total digestible material. Obvious depression of VFA production after 6 hours may be attributed to depletion of digestible material, or increasing toxic levels of the essential oils, or a combination of both factors.

Graphing the peak VFA level of the three time periods for each substrate illustrates apparently real differences among substrates (Fig. 8). Including small amounts of sagebrush in the mixture (12.5 and 25%) increased the peak VFA levels of the substrates. With 50 and 75% sagebrush, peak VFAs appeared to be progressively depressed although the reductions were small and still remained above the peak VFA level of Douglas fir alone. The highest VFA yield was
Figure 8. Peak VFA level of three fermentation periods for Douglas fir - sagebrush substrates.
observed with a mixture of 12.5% Douglas fir and 87.5% sagebrush, and sagebrush alone had the second highest level. The data suggest that sagebrush provided a greater quantity of digestible material than Douglas fir; however, including a small amount of Douglas fir in the substrate appeared to improve utilization more than with the other mixtures.

Sagebrush-bitterbrush mixtures exhibited effects on VFA production over time that were more easily interpreted than those for Douglas fir-sagebrush (Fig. 9). Patterns of VFA production for sagebrush were somewhat conflicting between this series and the Douglas fir-sagebrush series. In the latter, sagebrush exhibited a suppressed rate of VFA production from the 6-hour to 12-hour period, while in this series the production rate was depressed between the 2-hour and 6-hour fermentation interval and accelerated after 6 hours of fermentation. These differences suggest that the Douglas fir-sagebrush inoculum contained microorganisms more adapted to sagebrush which were able to digest the sagebrush substrate more rapidly. The inoculum for sagebrush-bitterbrush substrates was from a different deer, and the data suggest its microbial populations were adjusting to the sagebrush during the 2-hour to 6-hour interval. The depressed rate of VFA production during that interval indicates a period of microbial adaptation. In addition, experimental error may have contributed to the pattern contrasts. Including a small amount of bitterbrush in the substrate (12.5%
Figure 9. Sagebrush - bitterbrush VFA production as a function of time.
in this study) appeared to influence some reduction in the amount of easily digested material as evidenced by a slightly depressed 2-hour VFA level. Increasing the proportion of bitterbrush to 25% apparently had the opposite effect as more of the easily digestible material became available. Further increases of bitterbrush in the substrate progressively depressed the 2-hour VFA levels. Including 12.5, 25, and 50% bitterbrush in the substrate influenced a suppression of substrate utilization from the 2-hour to 6-hour period. Depressed levels of VFA during this interval and a comparatively rapid rate of VFA accumulation after 6 hours suggests microbial utilization of VFAs exceeded VFA production while the composition of microbes adjusted to utilize the mixed substrate. With the 25% bitterbrush mixture VFA depletion was more rapid during the 2-hour to 6-hour interval, indicating that an even smaller quantity of digestible substrate was available than with 12.5 and 50% bitterbrush; therefore, the microorganisms appeared to concentrate more on VFAs as an energy source. When the substrate consisted of 75% bitterbrush, microbial adaptation to the mixed browse appeared to be faster than when the mixtures contained more sagebrush. Although VFA levels were depressed during the 2-hour to 6-hour interval, production exceeded utilization. With a small amount of sagebrush (12.5%) in the substrate, VFA production was almost level from 6 to 12 hours, suggesting most of the
digestible substrate had been eliminated after 6 hours. A bitterbrush, only, substrate exhibited a peak VFA level at 6 hours, suggesting that so little digestible material remained after 6 hours the microorganisms again concentrated on VFAs as the most readily available source of energy.

Peak VFA levels for the sagebrush-bitterbrush substrates centered around those that were predominantly sagebrush (Fig. 10). Maximum substrate digestibility (based on peak VFA levels) was attained with a mixture of 75% sagebrush and 25% bitterbrush. A substrate composition including 50% bitterbrush or more progressively reduced the levels of peak VFA production. Bitterbrush alone had a higher VFA peak than a mixture of 12.5% sagebrush and 87.5% bitterbrush, suggesting that a small amount of sagebrush suppressed the potential for bitterbrush digestion. However, the VFA peak for bitterbrush was considerably lower than for a mixture of 25% sagebrush and 75% bitterbrush, indicating the suppressant effect of sagebrush on bitterbrush digestion is only realized when the mixture composition is quite low in sagebrush (below 25%).

VFA production as a function of time is illustrated for ceanothus-serviceberry substrated in Fig. 11. Although the 2-hour VFA levels of this series were, on the average, much lower than those of the other two sets, increasing amounts of serviceberry, up to 50% in the substrate, improved the 2-hour VFA levels. Above 50% serviceberry appeared to
Figure 10. Peak VFA level of three fermentation periods for sagebrush - bitterbrush substrates.
Figure 11. Ceanothus - serviceberry VFA production as a function of time.
depress the 2-hour VFAs. Ceanothus exhibited a slow initial rate of digestion but a rapid VFA accumulation from 2 to 6 hours. After 6 hours, VFA production exhibited an abrupt leveling off, suggesting most of the digestible material was eliminated. With 12.5% serviceberry, the rapid digestion of substrate observed during the 2-hour to 6-hour interval with ceanothus was not illustrated. A small proportion of serviceberry seemed to reduce the amount of easily digested material but, apparently, not the total digestible material. Increasing the serviceberry content to 25% of the substrate exhibited an almost linear rate of VFA production from 2 to 12 hours, indicating a relatively constant supply of digestible material being utilized at about the same rate. An equal mixture of ceanothus and serviceberry seemed to reduce the quantity of digestible material in the substrate while increasing its digestibility. A peak VFA level at 6 hours and declining levels from 6 to 12 hours indicated most of the digestible substrate was eliminated with 6 hours fermentation, and microbial utilization of the VFAs exceeded that of the substrate after 6 hours. A mixture of 25% ceanothus and 75% serviceberry appeared to increase the amount of digestible material available to the microorganisms, but the digestion rate was depressed between the 2-hour and 6-hour periods and resumed after 6 hours. A small proportion (12.5%) of ceanothus mixed with serviceberry greatly improved the amount of digestible substrate and rate of
digestion over serviceberry alone. VFA levels peaked at 6 hours, but VFA utilization exceeded substrate utilization after 6 hours. Peak VFA accumulation for serviceberry appeared after 6 hours of fermentation. From 6 to 12 hours, the VFA level declined slightly, suggesting initial microbial utilization of accumulated VFAs in response to a diminishing supply of digestible substrate.

Peak VFA levels centered around the substrates proportionately high in ceanothus (Fig. 12). Including 12.5% serviceberry in mixture with ceanothus did not appear to improve the substrate digestibility over that of ceanothus alone. However, a mixture of 75% ceanothus and 25% serviceberry yielded the highest VFA level, suggesting that ratio provided the greatest amount of digestible substrate. Equal proportions of ceanothus and serviceberry depressed the peak VFA below that of ceanothus. Further increasing the proportions of serviceberry to 75 and 87.5% progressively increased substrate VFA production to almost the same level as that for ceanothus alone. Serviceberry, alone, appeared to provide only a small amount of digestible substrate; the peak VFA level appeared at the 6-hour period and was considerably lower than the peaks of the other substrates.

Two comments are considered necessary as a postscript to this discussion of the VFA results. (1) Maximum fermentation time was considered too short for determining
Figure 12. Peak VFA level of three fermentation periods for ceanothus - serviceberry substrates.
true VFA peaks of almost half of the substrates tested. VFA production for these was increasing, after 6 hours, at relatively rapid rates and would have peaked higher. Perhaps the true peaks would have indicated that other mixtures provided greater quantities of digestible material than those cited. (2) Narrower time intervals between fermentation periods might have revealed slightly higher VFA levels between 6 and 12 hours for those substrates that exhibited 6-hour peaks.

With all the browse pairs, protein and VFA levels increased in the same direction, suggesting that with increasing levels of protein more substrate was utilized for energy to synthesize microbial cell material (Figs. 13, 14). Relationships between protein availability and carbohydrate utilization have been expressed by Hungate (1966:312) and Annison and Lewis (1959:62). However, peak VFA levels exhibited by certain mixtures of the browse pairs suggest that, in addition to increased protein, the synergistic effects of mixing also influenced a maximum utilization of the energy potential in the plant materials.

DRY MATTER LOSS

Many discrepancies were observed in the DML data which made interpretation of the results difficult. Initially, DML for the control samples did not behave as expected. Theoretically, the 2-hour fermentation controls
Figure 13. Comparison of VFA production and crude protein levels for Douglas fir - sagebrush and sagebrush - bitterbrush substrates.  
1/ Protein analyses were possible for only 3 substrates of the sagebrush - bitterbrush combinations (see Meth. and Mat.)  
Peak VFA level of substrates not analyzed for crude protein are illustrated by dashed line (- - -).
Figure 14. Comparison of VFA production and crude protein levels for ceanothus - serviceberry substrates.
should have retained about the same quantity of dry matter as the 0-hour controls, or slightly less. The 6-hour and 12-hour controls should have yielded progressively less dry matter as microbial digestion eliminated digestible substrate components. In all three time periods of the ceanothus-serviceberry series and the 2-hour and 6-hour periods of the sagebrush-bitterbrush series fermentation controls were higher in dry matter content than the 0-hour controls. In the Douglas fir-sagebrush series, each of the fermentation controls was lower in dry matter quantity than the 0-hour control; however, the 2-hour and 12-hour controls were equal in dry matter, while the 6-hour control had 0.05 grams less.

Douglas fir-sagebrush DML, graphed as a function of time, did not illustrate any distinguishable kind of consistency in patterns among substrates (Fig. 15). The only substrates that exhibited logical responses (either no DML or increasing DML over increasing fermentation time) were 6DF-2Sa, 1DF-7Sa, and sagebrush. Curves for the other substrates suggested that at various intervals between fermentation periods some of the digested dry matter was regained. With 25% sagebrush in the substrate, the curve for 6DF-2Sa indicates that after the easily digested substrate material was exhausted (DML after 2 hours fermentation) there was no more digestible dry matter—a logical theory but not very probable. The 12.5% Douglas fir and 87.5% sagebrush mixture
Figure 15. Douglas fir - sagebrush DML as a function of time. 
1/ Observations missing for 4DF-4Sa substrate. Estimate for the 2-hour DML level is an average of the 2-hour levels observed in the other substrates.
was one of the substrates exhibiting the highest dry matter loss; the same mixture produced a higher VFA level than the other substrates. Depressions of the rate of DML from 2 to 12 hours observed with this substrate suggested a diminishing quantity or quality of digestible dry matter. Sagebrush, alone, had approximately the same peak DML. However, it exhibited a higher 2-hour DML level than the mixture, but a greater depression of the rate of DML from 2 to 12 hours. Both substrates exhibited logical and realistic DML responses.

Sagebrush-bitterbrush substrates had a distinguishable DML pattern among substrates, but the responses were illogical (Fig. 16). All but the sagebrush and 6Sa-2Bit substrates produced curves suggesting that varying amounts of the digested dry matter were regained after 6 hours fermentation. Determining which substrate produced the greatest quantity of digestible dry matter was not possible with this series.

All of the ceanothus-serviceberry substrates illustrated comparatively consistent and logical DML responses which were more easily interpreted than the patterns of the other two series (Fig. 17). The rate of DML (indicated utilization of the easily digested substrate material) was comparatively rapid for the seven substrates up to 2 hours. Varying degrees of depressed digestion rate from 2 to 6 hours were observed with all the substrates. A mixture of 87.5%
Figure 16. Sagebrush - bitterbrush DML as a function of time.
Figure 17. Ceanothus - serviceberry DML as a function of time.

1/ The lowest replicate of this substrate appeared atypical and significantly lowered the average DML. A dashed line (---) shows the direction of DML when an average of the two similar replicates was used.
and 12.5% serviceberry yielded the highest DML; however, the 1Ce-7Se and serviceberry substrates exhibited DML levels very nearly as high. VFA production data indicated that substrates high in ceanothus were more digestible than those proportionately higher in serviceberry. A mixture of 75% ceanothus and 25% serviceberry was the most VFA productive substrate, while serviceberry, alone, produced the least.

In two of the three series (Douglas fir-sagebrush and sagebrush-bitterbrush) the DML data exhibited either inconsistent or illogical digestion patterns; therefore, little in the way of conclusions or comparisons with VFA could be determined. Investigators evaluating cellulose digestion in vitro normally run their experiments for 24 hours or longer to reduce variability in the data. Asplund et al. (1958:178 and 179) found a high correlation between VFA production and DML with a 24-hour fermentation, but not with data from a 12-hour period or less due to the variation in DML. However, logical conclusions about VFA patterns could be derived from graphs of the data.
Chapter 5

CONCLUSIONS

Sagebrush is the dominant vegetation on many winter ranges utilized by mule deer. Due to its bactericidal properties, deer can only digest sagebrush if it is mixed with other forage. Sagebrush retains relatively high nutrient levels during winter; in mixture with other species, it provides nutritious food. In this study, mixing up to 25% bitterbrush with sagebrush improved the digestibility of both species (indicated by increased VFA production). Extrapolating these results to habitat management of winter ranges dominated by sagebrush, and ignoring the influence of other species that would also be consumed by deer, it might be assumed that 25% bitterbrush to 75% sagebrush in the vegetative composition would allow maximum utilization of sagebrush.

In the Northern Rocky Mountains, wildfires have created winter game ranges dominated by evergreen ceanothus. While evergreen ceanothus is a good deer food by itself, this investigation indicated that its digestibility was progressively improved when it was mixed with increasing amounts of serviceberry, up to 25%. As with sagebrush and bitterbrush, 25% serviceberry in a site dominated by ceanothus would improve the food value of the ceanothus; again ignoring other plants which the deer eat.
On ranges where sagebrush and Douglas fir occur, they may be the most available forages during certain winter periods. In this study, more VFA was produced by sagebrush (indicating a greater quantity of digestible material) than by Douglas fir. With a mixture of 87.5% sagebrush and 12.5% Douglas fir, VFA production was greater than that of sagebrush. Such a ratio could easily be obtained by deer on most winter ranges where both sagebrush and Douglas fir occur.

The results of this study indicated that a closed-system in vitro technique could be used to evaluate mixed diets. However, because of differences in plant communities and the many factors affecting plant chemistry on different ranges, to be practical in management in vitro digestions should be performed on key forage species of each range. Furthermore, since deer food habits and range management practices are not limited to 2 species, investigating the digestibility of triple or quadruple mixtures would be desirable.
LITERATURE CITED


Snyder, W. A. 1961. A chemical analysis of thirteen major deer forage plants from the Guadalupe Mountains of New


Exhibit A

Chemical Analyses of the Substrate Material

<table>
<thead>
<tr>
<th>Substrate</th>
<th>% Moisture</th>
<th>% Protein</th>
<th>% Ether Extract</th>
<th>% Ash</th>
<th>% Crude Fiber</th>
<th>% Phos.</th>
<th>% Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Douglas fir</td>
<td>5.5</td>
<td>5.7</td>
<td>6.8</td>
<td>4.0</td>
<td>22.0</td>
<td>.17</td>
<td>.99</td>
</tr>
<tr>
<td>7DF-1Sa</td>
<td>6.1</td>
<td>6.4</td>
<td>7.6</td>
<td>3.7</td>
<td>22.0</td>
<td>.12</td>
<td>.97</td>
</tr>
<tr>
<td>6DF-2Sa</td>
<td>6.3</td>
<td>6.4</td>
<td>8.0</td>
<td>3.5</td>
<td>22.1</td>
<td>.17</td>
<td>.87</td>
</tr>
<tr>
<td>4DF-4Sa</td>
<td>6.9</td>
<td>6.9</td>
<td>8.7</td>
<td>3.3</td>
<td>22.1</td>
<td>.18</td>
<td>.88</td>
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<tr>
<td>2DF-6Sa</td>
<td>7.8</td>
<td>7.6</td>
<td>9.4</td>
<td>3.0</td>
<td>21.7</td>
<td>.13</td>
<td>.60</td>
</tr>
<tr>
<td>1DF-7Sa</td>
<td>8.3</td>
<td>7.8</td>
<td>9.9</td>
<td>3.0</td>
<td>21.5</td>
<td>.11</td>
<td>.54</td>
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<tr>
<td>Sagebrush</td>
<td>7.4</td>
<td>8.1</td>
<td>11.1</td>
<td>3.0</td>
<td>21.1</td>
<td>.18</td>
<td>.48</td>
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<tr>
<td>Sagebrush</td>
<td>8.6</td>
<td>8.2</td>
<td>9.9</td>
<td>2.8</td>
<td>21.4</td>
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<td>.47</td>
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<tr>
<td>7Sa-1Bit</td>
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<td>---</td>
</tr>
<tr>
<td>6Sa-2Bit</td>
<td>---</td>
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<td>---</td>
<td>---</td>
<td>---</td>
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<td>---</td>
</tr>
<tr>
<td>4Sa-4Bit</td>
<td>7.5</td>
<td>7.8</td>
<td>7.1</td>
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<td>.13</td>
<td>.50</td>
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<tr>
<td>2Sa-6Bit</td>
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<td>---</td>
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<td>---</td>
</tr>
<tr>
<td>1Sa-7Bit</td>
<td>---</td>
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<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Bitterbrush</td>
<td>6.7</td>
<td>7.7</td>
<td>3.9</td>
<td>2.0</td>
<td>30.1</td>
<td>.10</td>
<td>.54</td>
</tr>
<tr>
<td>Ceanothus</td>
<td>7.5</td>
<td>10.9</td>
<td>7.5</td>
<td>3.0</td>
<td>10.9</td>
<td>.11</td>
<td>.82</td>
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<td>7Ce-1Se</td>
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<td>10.1</td>
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<td>6.2</td>
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<td>15.6</td>
<td>.11</td>
<td>.91</td>
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<tr>
<td>4Ce-4Se</td>
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<td>8.5</td>
<td>5.4</td>
<td>3.4</td>
<td>19.8</td>
<td>.10</td>
<td>1.00</td>
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<tr>
<td>2Ce-6Se</td>
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<td>6.8</td>
<td>4.9</td>
<td>3.5</td>
<td>24.3</td>
<td>.12</td>
<td>1.10</td>
</tr>
<tr>
<td>1Ce-7Se</td>
<td>6.8</td>
<td>6.1</td>
<td>4.5</td>
<td>3.6</td>
<td>26.3</td>
<td>.11</td>
<td>1.10</td>
</tr>
<tr>
<td>Serviceberry</td>
<td>6.8</td>
<td>4.3</td>
<td>3.9</td>
<td>3.8</td>
<td>28.6</td>
<td>.12</td>
<td>1.10</td>
</tr>
</tbody>
</table>
Composition of the Acid Buffering Solution Used in Each Digestion Chamber

<table>
<thead>
<tr>
<th>Component 2/</th>
<th>Amount Grams per Liter of Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO₃</td>
<td>9.800</td>
</tr>
<tr>
<td>Na₂HPO₄ (Anhydrous)</td>
<td>3.710</td>
</tr>
<tr>
<td>KCl</td>
<td>0.570</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.470</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.120</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.053</td>
</tr>
</tbody>
</table>

1/ Suggested by Nagy (pers. comm., 1969).

2/ Component parts were dissolved in distilled water.
### Procedure for Preparing Standard VFA Solutions

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Molecular weight gr./liter</th>
<th>m.Moles/liter of VFA solution (100% VFA Standard)</th>
<th>Grams of acid/liter of VFA Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic</td>
<td>60.054</td>
<td>244.56</td>
<td>14.6</td>
</tr>
<tr>
<td>Propionic</td>
<td>74.080</td>
<td>80.32</td>
<td>5.95</td>
</tr>
<tr>
<td>Isobutyric</td>
<td>88.108</td>
<td>4.28</td>
<td>0.377</td>
</tr>
<tr>
<td>Butyric</td>
<td>88.108</td>
<td>60.92</td>
<td>5.365</td>
</tr>
<tr>
<td>Isovaleric</td>
<td>102.14</td>
<td>18.38</td>
<td>1.875</td>
</tr>
<tr>
<td>Valeric</td>
<td>102.14</td>
<td>25.80</td>
<td>1.635</td>
</tr>
</tbody>
</table>

Include 1 ml. of Metaphosphoric Acid/12.5 ml. VFA solution or 80 ml./liter of solution. (I used 1 ml. of HCl solution to 12.5 ml. of a mixture of 10 ml. rumen fluid and 40 ml. buffer. However, my standards were prepared as shown below using the Metaphosphoric Acid solution described.)

To make up VFA standards:

1. Weigh out 14.6g Acetic, 5.95g Propionic, 0.377g Isobutyric, 5.365g Butyric, 1.875g Isovaleric, and 1.635g Valeric acid. This mixture should be equal to 30 ml.

2. Take 3 ml. of above mixture and add it to 97 ml. of distilled H₂O.

3. Make up a 25% Metaphosphoric Acid solution. (2.5g M.P.A. and fill with distilled H₂O to 100 ml. mark on graduated cylinder.)

4. Add 8 ml. of 25% M.P.A. solution to the VFA and H₂O solution; this is a 100% VFA standard.

5. To make up remaining % standards, take appropriate amounts of the 100% VFA standard and add appropriate amounts of distilled H₂O.

---

1/ Nagy (pers. comm., 1969).
<table>
<thead>
<tr>
<th>VFA Standard</th>
<th>Amount of 100% VFA Standard</th>
<th>Amount of Distilled H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>75%</td>
<td>7.5 ml.</td>
<td>2.5 ml.</td>
</tr>
<tr>
<td>50%</td>
<td>5.0 ml.</td>
<td>5.0 ml.</td>
</tr>
<tr>
<td>40%</td>
<td>4.0 ml.</td>
<td>6.0 ml.</td>
</tr>
<tr>
<td>30%</td>
<td>3.0 ml.</td>
<td>7.0 ml.</td>
</tr>
<tr>
<td>20%</td>
<td>2.0 ml.</td>
<td>8.0 ml.</td>
</tr>
<tr>
<td>15%</td>
<td>1.5 ml.</td>
<td>8.5 ml.</td>
</tr>
<tr>
<td>10%</td>
<td>1.0 ml.</td>
<td>9.0 ml.</td>
</tr>
<tr>
<td>7.5%</td>
<td>0.75 ml.</td>
<td>9.25 ml.</td>
</tr>
<tr>
<td>5.0%</td>
<td>0.5 ml.</td>
<td>9.5 ml.</td>
</tr>
<tr>
<td>2.5%</td>
<td>0.25 ml.</td>
<td>7.75 ml.</td>
</tr>
</tbody>
</table>
Preparation Procedures for Each Digestion Series and Data Analyses

Forage Preparation

1. Current annual growth of 1/8 inch stem diameter or smaller was hand-clipped from sagebrush, bitterbrush, and serviceberry plants. Douglas fir clippings consisted of the first 2 to 4 inches of branch tips. Ceanothus samples were leaves and stem tips stripped from several plants.

2. Sagebrush and bitterbrush samples were air-dried for 10 days. Douglas fir samples were air-dried 10 days then placed in a Labline forced-air oven and dried at 34-36°C for 18 hours. Ceanothus and serviceberry were dried at 24-26°C in a Percival circulated-air environmental chamber. For future studies of a similar nature, drying methods should probably be the same for all samples and selected to best suit the purposes of the study.

3. Each browse sample was ground through a 20-mesh screen in a Wiley mill and thoroughly mixed. Mixing the ground vegetation uniformly distributed stem, bud, and leaf material.

4. The seven 1 g. substrates were comprised of complementary quantities of 1,000, 0.875, 0.750, 0.250, and 0.125 g. of each of the two browse species being tested.

5. Each substrate was placed in a 100 ml. glass
centrifuge tube with pour spout, referred to as a digestion chamber.

Preparation of in vitro Equipment

1. Buffering solution (Exhibit A, page 70) based on that proposed by McDougall (1948) was prepared in sufficient quantity for 75 digestion chambers. Forty ml. of buffer was pipetted into each chamber.

2. Carbon dioxide flow was adjusted prior to each digestion series in tubes of distilled water. The settings were by ocular estimate and judged to be the same from all discharge jets (5 mm. glass tubing, 8 inches long, and tapered at the outlet end).

3. The prepared digestion chambers were immersed in a Blue M water bath thermostatically regulated at 39° C. The discharge jets were inserted through single-hole rubber stoppers, and the stoppers were wedged into the tops of the digestion chambers. Positioning the discharge jets above the settled substrate material prior to a series prevented their becoming plugged with fine substrate particles. Once a series was in progress, a constant flow of carbon dioxide kept the discharge openings free of particulate matter.

At this point I would like to reflect on the in vitro apparatus used in this study. The system was complex and bulky with its maze of tubes for carbon dioxide dispersion which made inoculating and handling the digestion chambers
awkward. Carbon dioxide flow into each digestion chamber required periodic adjustments; although only minor alterations were required in many cases. Due to the type of gas regulator used, the flow volume fluctuated. Alterations in gas pressure would often reduce the flow into one or more chambers to such a degree that the discharge jets would become plugged. Adjustments were required, particularly after the chambers assigned to a given fermentation period were removed. A simpler system that is commonly used functions without the need of continuous carbon dioxide flow. The digestion chambers are completely sealed with single-hole rubber stoppers. Short sections of glass tubing are inserted through the stoppers, positioned above the liquid and substrate media. A Bunsen valve (Johnson, 1966:859) is attached to the section of glass tubing outside the digestion chambers to vent CO₂ and fermentation gases without introducing atmospheric oxygen. Immediately after inoculation, each digestion chamber is flushed with carbon dioxide and capped; fermentation gases maintain an anaerobic environment throughout the experiment. However, some means of mild agitation should perhaps be incorporated into this system to keep some substrate material in suspension and expose more surface area for microbial attachment.

**Inoculum Collection and Preparation**

1. Cheesecloth sacks were designed to filter inocula from the rumen contents. The sacks were constructed of 16
layers of 80-grade cheesecloth, 24 inches long by 6 inches wide, folded to 12-inch lengths, and sewed on two sides. A drawstring was hemmed around the open end to close the sack during filtration.

2. Thermos containers used to transport the inocula were modified by inserting Bunsen valves through the caps to vent accumulating gases (Fig. 1, page 24).

3. Just prior to the time of inoculum collection, the water bath and carbon dioxide were turned on, the carbon dioxide flow was adjusted, and the discharge jets were lowered to approximately 1/4 inch above the bottoms of the digestion chambers.

4. Ice water was poured into the Thermos containers, assuming the chilled containers would suppress bacterial activity during transport of the inocula. It was later felt that ice water was unnecessary as the Bunsen valves seemed to adequately vent fermentation gases that would otherwise have caused increasing pressure within the sealed containers.

5. Inocula were collected from free-ranging, wild deer which had been shot by the investigator.

6. After the rumen was exposed and opened, its contents were transferred to the filter sacks using an ordinary household ladle. The ice water was poured from the Thermos containers, and rumen fluid was filtered into them by hand-squeezing the sack of rumen contents. Though
it was not done in this study, when wild, free-ranging ruminants are used as inocula donors, the filtered forage material should be preserved in a formalin solution. Analyses of the rumen contents allow a comparison of the animal's diet and the in vitro substrates, which should be similar according to some investigators.

7. After the rumen fluid was transported back to the laboratory, each digestion chamber was inoculated with 10 ml. of the fluid using an Adams aupette.

8. Replicate samples of the seven substrates and a control were digested for periods of 2, 6, and 12 hours. The data for both VFA production and DML from this study, and investigators using cellulose and dry matter digestion in other studies, suggest longer incubations than 12 hours. With VFA production as a criterion, the fermentation time should be long enough to obtain peak VFA levels for each substrate (18 to 24 hours). Intervals between the fermentation periods ought to be relatively small and consistent to detect the peaks of substrates that level off between initiation and termination of the experiment. Variability in DML could probably be reduced by using the two-stage digestion method described by Tilley and Terry (1963). This method combines a 24-hour in vitro microbial digestion with additional digestion in an acid-pepsin solution for 24 to 48 hours. During the microbial digestion stage, replicate samples could be deactivated every 2 hours for VFA analyses;
the samples for incubation periods spaced 4 hours apart (4-hour, 8-hour, 12-hour, etc.) would probably be adequate for DML analyses. Thus, considerably more digestion chambers will be required than were used in this study.

Additional experimentation is needed to evaluate the full potential for applying the *in vitro* technique to evaluating mixed forage digestibility. Increasingly complex mixtures of 3, and more, forages should be investigated to determine at what capacity of forages interaction obscures any reliable evaluation of the real effects of mixing. To do so, it would appear necessary to test the consistency of patterns for VFA production, DML, or whatever criteria are used, by repeating each experiment or digestion series at least once.

Sample Preparation

1. At the end of fermentation period, the digestion chambers for that period were removed. After the gas jets were removed from the completed chambers, carbon dioxide flow was maintained through them and discharged into the water bath. Thus, overall gas pressure was slightly affected, requiring only minor readjustments of carbon dioxide flow into the active digestion chambers.

2. Microbial activity was terminated by adding 4 ml. of 25% HCl solution (1 part concentrated HCl to 4 parts distilled water) to the digestion chambers. The HCl
solution was dispensed from a 50 ml. burette with a section of rubber tubing attached, and a squeeze clamp was used to regulate the amount of solution into each chamber (Fig. 6). All deactivated samples were refrigerated until the digestion series was completed.

3. Approximately 33 ml. of the liquid from each sample were poured into 50 ml. polyethylene centrifuge tubes and centrifuged at 3,600 rpm for 1 hr. in an International clinical centrifuge. Approximately 15 ml. of the supernatant was retained and frozen for volatile fatty acid (VFA) analysis. Precipitated material on the bottom of the centrifuge tubes was washed onto pre-weighed muslin filters that had been depressed into perforated muffin tin compartments and taped in place to prevent spillage of the residue. In future studies it might be of interest to run 4 replicates of each substrate, filtering 2 with muslin filters and 2 with a Gooch crucible or coarse fritted crucibles (suggested by Phillip Dillon, Research Chemist, Moorman Mfg. Co.; pers. comm.). Replicates of each filtration technique could be compared for variability in residue quantity. Replicates from the method of least variability may then be used to improve the validity of the investigation.

4. Undigested substrate material remaining in the digestion chambers was also washed onto the filters. After filtration, the filters with their residues were air-dried and weighed. Original filter weights were subtracted from
their respective filter-residue weights to determine the quantity of DM residue. These residues were corrected for undigested rumen material by subtraction of the average residue weight of their respective control replicates. Percent DML was computed as described in the Methods and Materials section.

Unnatural discrepancies noted between DM quantities of the 0-hr. controls and fermentation controls were discussed in the Results and Discussion. The author recommends a system that allows mild agitation of the inoculum prior to and during inoculation to minimize variances in the control DML. Phillip Dillon, Research Chemist, Moorman Mfg. Co. (pers. comm., 1972), suggests continuous flow of carbon dioxide through a batch mixture of rumen fluid and buffering medium. Such a system would prevent stratification of solid material and should minimize the DM discrepancies of control samples; anaerobiosis is also maintained with this technique.

5. All frozen VFA samples were thawed and 2 ml. quantities of each were filtered twice through a 0.8 micron millipore filter using a Swinney adapter attached to a 2 cc. syringe. The filtered samples were then refrozen.

VFA Analysis

1. Attempts were made by the investigator to quantify the following fatty acids by gas chromatography: acetic,
propionic, isobutyric, butyric, isovaleric, and valeric. A Varian Aerograph model 600 D gas chromatograph with a hydrogen flame detector was the basic instrument used. Hydrogen gas for the detector was supplied by a Varian Aerograph model 650 hydrogen generator adjusted to a flow rate of 25 ml. of gas/min. Helium was used as the carrier gas and was also set at a flow rate of 25 ml./min. The investigator constructed his own column for the chromatograph using 12.5 ft. of 1/8-in. stainless steel tubing. The column was packed with 25% FFAP on 100/120 Chromosorb G A/W DMCS H.P. and placed in the chromatograph to be conditioned for 48 hrs. at 96° C. Chromatograms were recorded on Beckman number 106770 chart paper using a Beckman 10-in. linear recorder. A Hamilton microliter syringe was used to inject 1 microliter of each sample into the gas chromatograph. However, the chromatograph appeared to lose sensitivity with each successive injection. Due to the investigator's limited experience in gas chromatography, the problem was unresolved.

2. All samples were finally sent to Dr. Julius Nagy, Colorado State University, Fort Collins, Colorado, for VFA analysis. Nagy conducted the analyses using a Varian Aerograph gas chromatograph series 1700 with a hydrogen flame detector. Flow rate of the hydrogen gas was 60 ml./min. Nitrogen was used as the carrier gas or mobile phase of the system and was adjusted to a flow rate of 40 ml./min.
Compressed air was also flushed through the system at a rate of 300 ml./min. Chromatograph settings were: (1) column oven temperature, 135° C; injection port temperature, 200° C; detector temperature, 200° C; range setting -10^-10 amps./mv. and attenuation setting -4. The column was constructed of a 10-ft. length of 1/8-in. stainless steel tubing packed with 20% TWEEN 80 plus 2% H_3PO_4 (85%) on Chromosorb W 60-80 mesh. Chromatograms were produced on a Varian Aerograph strip chart recorder model 20 with impulses transmitted in series first to a Varian Aerograph digital integrator model 477 and finally to a Victor digit-matic printer. Samples were injected at random in groups of 5. Each group of 5 samples was preceded and followed by an injection of a 15% standard solution (Exhibit D) to monitor daily and day-to-day changes in the column. Due to the functional characteristics of a gas chromatograph, some carry-over of products from the previous sample is inherent in this type of analysis. Double injections of each sample and averaging the two or using only the second chromatogram compensates for or reduces the bias in product carry-over.

3. On the chromatograms, the base width of each curve was marked, and the area of each curve was measured as a summation of all the integration units within the base width.

4. During the VFA analyses, which were begun in
early January, 1971, the initial column was replaced on 1-20. Since the second column gave higher values than the initial column, it was felt that all data should be adjusted to the higher readings rather than to an average of the values for both columns. An overall, mean area value was calculated for each acid curve on chromatograms of the 15% standard solutions injected from 1-20 to the end of the analyses. Daily means were calculated for the areas of each curve on chromatograms of the 15% standards injected on that day. Correction factors for each day that samples were injected could then be derived for the acid curves by subtracting the daily mean area values from the overall mean value. Subsequently, all of the curve areas were adjusted (perhaps weighted is a better term) using the daily correction factor that applied to each acid.

5. A table provided by Dr. J. C. Nagy (pers. comm., 1969) gave the milliMoles of each acid per liter of VFA solution for each standard solution prepared for analysis (see Exhibit C, page 72). With this information, and using adjusted curve areas of the standards, regressions of adjusted curve area on mM/liter of acid could be constructed. From these the quantity of each acid in a sample could be determined from its adjusted curve area. Total VFA content was a summation of the individual acids in the sample.