# THE NATURE OF ANTIMETABOLIC FACTORS AFFECTING NUTRITIVE VALUE OF DIPLOID ALFALFA *MEDICAGO FALCATA L.*

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THESIS



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THE NATURE OF ANTIMETABOLIC FACTORS
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ABSTRACT

THE NATURE OF ANTIMETABOLIC FACTORS AFFECTING NUTRITIVE VALUE OF DIPLOID ALFALFA MEDICAGO FALCATA L.

by John A. Schillinger, Jr.

Six-day specific growth response tests of weanling voles, Microtus pennsylvanicus, in vitro rumen determinations of dry matter digestibility, and the development of chlorosis in alfalfa shoots associated with plant extracts were utilized to determine differences in nutritive value between individual diploid alfalfa Medicago falcata plants. A water-soluble antimetabolite(s) was found in alfalfa plants of low nutritive value which affected the cellulolytic capacity of rumen microorganisms, retarded growth of weanling voles, and produced chlorotic symptoms in excised alfalfa shoots.

The antimetabolite adversely affected the rumen bacteria population by significantly reducing the numbers of Gram-negative cocci in the rumen inoculum. This resulted in a pronounced reduction in the amounts of short-chained volatile fatty acids produced in fermentation media containing forage from plants of low nutritive value.

The antimetabolic effect of the plants of low nutritive value was nullified by additions of glycine, aspartic acid, and glutamine in the in vitro dry matter digestibility tests. These amino acids were also successful antidotes in the tests of vole growth response and alfalfa shoot bioassay. Partial recovery from the antimetabolic effect in dry matter digestibility studies was obtained when coenzymes DPN and DPNH were used as antidotes.

Results of these studies suggest adenine synthesis as the site of action of the antimetabolite.

The data from the $F_2$ population of a cross between two plants differing in nutritive value indicated that the antimetabolite(s) was elaborated under the control of a complex genetic system. The dry matter digestibility of the $F_1$ plants was intermediate to the parents. In the distribution of dry matter digestibility for the $F_2$ population, transgressive segregation from the parental types was observed.

Differences in dry matter digestibility between $F_2$ plants were as high as 15%. These differences are of sufficient magnitude to produce significantly different responses in animal performances and suggest successful alfalfa improvement for nutritive value through breeding programs.

# THE NATURE OF ANTIMETABOLIC FACTORS AFFECTING NUTRITIVE VALUE OF DIPLOID ALFALFA *MEDICAGO FALCATA* L.

By

John A. Schillinger, Jr.

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# INTRODUCTION

Alfalfa is considered the most important forage legume in the United States on the basis of its comparatively superior nutritive value and yield. It has been improved with regard to disease and insect resistance, persistency of stand, dry matter yields, and the quality improved by various managerial practices.

Emphasis in agronomic research has been placed on the total tonnage of alfalfa harvested annually. As a result, little attention has been given to assaying alfalfa for usable nutrients or chemical components affecting efficient utilization of forage by livestock, particularly ruminants.

Forage breeders often have selected and utilized in new alfalfa varieties, lines which possess antibiosis to a spectrum of insects and diseases. These lines are generally not evaluated for feeding value prior to varietal release. Since certain metabolic pathways are common to both higher and lower forms of life, it is possible that the accumulated levels of antibiotic factors can be detrimental to the nutritive value.

Alfalfa is known to contain both stimulatory and suppressive growth factors for poultry, laboratory animals, and microorganisms. However, the identification and mode of inheritance of these factors has not been determined. This information should be formulated to aid in further improvement of nutritive value.

The purpose of this study was (1) to determine the nature of anti-metabolic factors in 2n *M. falcata* which cause differential responses in nutritive value (2) to study the inheritance of these factors.

## REVIEW OF LITERATURE

### Inheritance of Specific Substances in Alfalfa

Studies of the inheritance of substances, such as antimetabolites, which affect the nutritive value of plants are rare. The main reason may be the lack of identity of these factors, but equally important is the lack of effective screening techniques or bioassays for individual plants.

Except for preliminary studies of the inheritance of carotene and the amino acids methionine and cysteine, little progress has been reported to date. Ham and Tysdal (1946) showed that alfalfa strains had inherent high and low levels of carotene and related these to resistance to potato leafhoppers. Selfed progenies of alfalfa clones differing in methionine levels were found to have methionine levels similar to those of the parents (Tisdale et al. 1950; Singleton et al. 1952).

Complications in the interpretation of genetic studies of alfalfa have often plagued plant geneticists. In 4n Medicago sativa, analyses of inheritance patterns of mutant genes affecting leaf markings were complicated by variation in expressivity and penetrance (Stanford 1959; Whittington and Burrage 1963). Implications arising from tetrasomic inheritance may prevent accurate interpretation of inheritance studies (Stanford 1951). Therefore, diploid alfalfa is more adapted to definitive genetic studies.

In a comprehensive study of flower color inheritance in diploid alfalfa, Cooper and Elliott (1964, 1965) proposed a genetic hypothesis based on identification and segregation of flower pigment. They found

flower pigment production to be under the control of several different genetic systems. Xanthophyll ester production was controlled by two genes, $Y_{x1}$ and $Y_{x2}$, of equal and additive effects; anthocyanin pigments were under the control of a single dominant gene, P; quercetin pigment inheritance appeared complex; and production of two kaempferol glycosides was controlled by different dominant genes, $K_2$ and $K_3$.

## Forage Quality and Its Evaluation

The primary purpose of forage in diets of ruminant animals is to provide energy. Indexes of forage quality, such as Federal hay grades, legume or protein content, method of curing, and the data of feed-value tables, are now considered inadequate and the quality of a forage is better defined by the rate at which it is consumed and its energy value per unit of weight (Reid *et al.* 1959). Baumgardt and Smith (1962) have also defined forage quality as "a high concentration of available energy in an appetizing form." The digestible energy content of a forage, in turn, can be accurately described by the dry matter digestibility of that forage (Moir 1961; McCullough 1959; Swift 1957).

Workers in animal nutrition have found that the digestibility of a forage can be accurately determined by artificial rumen techniques (Baumgardt *et al.* 1962, 1964; Donefer *et al.* 1960; Johnson *et al.* 1962; Lefevre and Kamstra 1960; and Simkins 1963). They have obtained significant positive correlations between artificial rumen data and *in vivo* digestibility.

Modifications in techniques of *in vitro* determinations of digestibility have been successfully employed. An improved rumen inoculum for maximum degradation of cellulose and less variation between experiments

was prepared by suspending the extracted ingesta of the rumen in a phosphate buffer (Johnson et al. 1958). The combination of fermentation first with rumen fluid and then with acid pepsin has become widely accepted as a more precise measure of the digestibility and nutritive value of a forage (Tilley and Terry 1963). A pure culture fermentation technique has been developed for the nutritive evaluation of forages (Ifkovitz 1964). The bacterium utilized was Bacteroides succinogenes, a Gram-negative coccus capable of efficiently utilizing forage cellulose as a substrate.

Research on the functions of rumen microflora has made possible a better understanding of ruminant metabolism and the utilization of forages. Bacteria characterized as Gram-negative micrococci have been identified as those with the primary cellulolytic activity of the rumen (Dehority et al. 1960; El-Shazly et al. 1961). The Gram-negative rod types of bacteria were reported to have a minor role in cellulose degradation. Bryant (1963) has cultured and identified 22 bacteria species which are found in the rumen and has established their morphological shape, Gram reaction, motility, energy sources, and major fermentation products.

Other laboratory procedures for the evaluation of forage quality have been proposed. Chemical methods based upon the solubility of the cellulose of forages in cupriethylene diamine and 1.0 N $H_2SO_4$ were well correlated with relative intake of the forage by cattle as well as dry matter digestibility and energy digestibility (Dehority and Johnson 1963, 1964). Van Soest (1964) has reviewed the new chemical procedures for evaluating forage quality and concluded that the acid-detergent digestion

of cellulose and lignin of forages provided an accurate indicator of forage quality. The cellulose and lignin content of a forage was closely correlated with consumption and digestibility of forages fed to cattle.

## Bioassays of the Nutritive Value of Forages

Various bioassays of nutritive value of forages have been proposed. Elliott (1963) suggested the use of a six-day specific growth response test of weanling meadow voles, Microtus pennsylvanicus, as a bioassay. The rates of weight gain in rabbits were closely correlated with weight gains of steers and sheep fed the same forage (Crampton et al. 1940; Richards et al. 1962). Crickets have also been used as a bioassay of the nutritive value of forages (Pfander et al. 1964). They obtained accurate measures of completeness of forage diets by evaluating 30-day growth rates, percent survivors reaching adulthood, and adult weight. The results observed in cricket feeding tests were significantly correlated with sheep feeding trials. The presence of toxic factors in tall fescue was bioassayed by measuring changes in the temperature of a cow's tail (Jacobson et al. 1962). After administration of extracts, differences between room temperature and tail temperature ranged from 8.6 to 21.7°C for toxic vs non-toxic tall fescue extracts.

The use of laboratory methods of rumen fermentations for evaluation of breeding lines of forages has recently been initiated (Ross and Kamstra 1964; Tilley and Terry 1963). The preliminary results of these studies are encouraging and suggest that in vitro dry matter digestibility will provide an efficient screening procedure for nutritive value of forage breeding materials.

Factors in Alfalfa with Biological Activity

Coumestrol is an estrogen which has been isolated in pure form from alfalfa (Bickoff et al. 1957). In a recent study, it was found that infection of alfalfa foliage with either of two leaf-spot pathogens Pseudopeziza medicaginis or Leptosphaerulina briosiana promoted coumestrol accumulation (Loper and Hansen 1964). Stilbestrol, another estrogen, has been cited as the component of alfalfa forage which is responsible for improving quality and rate of gain of fattening cattle (Bickoff 1958). Anti-estrogenic factors, as well as estrogenic factors have been extracted from the same alfalfa sample (Adler 1962; Biely and Kitts 1964).

Because of its foaming properties, saponin has been suggested as an important factor in producing bloat (Glover 1963; Pressey et al. 1961). Saponin was reported to occur in alfalfa in amounts ranging from 0.2 to 1.8 percent of the dry matter. From saponin of alfalfa, a respiratory inhibitor of rat diaphragm muscle was isolated and found to be involved with bloat in ruminants (Jackson and Shaw 1959). Fractions of saponins with the greatest activity of respiratory inhibition were found after chromatographing saponins on ion-exchange and carbon columns. A foam stabilizing protein was extracted from alfalfa leaves and purified by agar gel filtration (McArthur et al. 1964).

The second growth of forage legumes was found to contain a factor which caused profuse salivation and cessation of feeding in cattle (Byers and Broquist 1960). Hot-water extracts of these forages also contained the salivation factor, which was suggested to be an alkaloid.

Researchers in poultry nutrition have attempted to identify factors in alfalfa which affect growth of chicks. Two contrasting factors, one

which inhibits chick growth and another which stimulates growth, have been reported. Chick growth stimulation was reported when either dehydrated or sun-cured alfalfa was added to a purified diet containing all known growth factors (Binger et al. 1961; Hansen et al. 1953; Kohler and Graham 1951, 1952).

However, the same alfalfa meal which stimulated chick growth when used at 10% of a diet inhibited growth at the 20% concentration (Heywang 1950; Mangelson et al. 1949). Factors which produce this effect were found to be concentrated in alfalfa leaves (Kodras et al. 1951). Peterson (1949) observed that the growth depressing factor was present in ethanol extracts of alfalfa meal and its action as a growth-inhibitor was counteracted by antidoting chick rations with cholesterol. Another antidote to the growth inhibitor in alfalfa was found to be Vitamin $B_{12}$ (Ayala and Johnson 1951). In a detailed study of chick growth inhibition, Wilgus and Madsen (1954) found that concentration of this factor varied considerably between samples of alfalfa meal; 20% of meals tested suppressed chick growth significantly. Deficiencies of protein, carotene, minerals, fiber, and chemical residues were eliminated as possible causes for the growth inhibition. The chick growth suppression was reported to involve the saponin content of the dehydrated alfalfa meals (Bolton 1962). The presence of an antioxidant which protects B-carotene may make it unavailable to the chicks. Bickoff et al. (1954) identified the antioxidant as ethoxyquin (6-ethoxy-2,2,4 trimethyl-1,2 dehydroquinoline). Also, a fat soluble factor of alfalfa was found to reduce the availability of tocopherol (vitamin E) to chicks by 33% (Pudelkiewiez and Matterson 1960).

Cold-water extracts of alfalfa were found to possess antibacterial activity at concentrations of less than 1:20 (Frisbey et al. 1953).

Likewise, an ether extract of alfalfa inhibited the growth of Escherichia coli (McDonald 1955).

Alfalfa ash and a warm-water extract of alfalfa increased digestibility of both dry matter and organic matter of cattle and sheep rations containing different roughages (Bentley et al. 1954; Burrough et al. 1948; Ward et al. 1957; Tillman et al. 1954).

## Amino Acids of Legumes

Changes in amino acid content of alfalfa hay within and among seasons have been associated with differences in feeding value (Smith and Agiza 1951). Decreasing percentages of amino acids, particularly methionine, with advance in maturity of alfalfa have been related to changes in nutritional value (Loper et al. 1963). In another study no positive relationship was found between amino acid content of alfalfa at various stages of maturity and bloat incidence (Meyer et al. 1965).

Raw unextracted soybean meal has been shown to possess an imbalance in amino acid content which is evidenced in bioassays of growth rates among weanling rats and chicks, and egg production of layers (Askelson and Balloun 1964; Borchers 1965; Rogler 1964). Borchers observed that either heated soybean meal or unheated soybean meal supplemented with amino acids was capable of supporting growth of weanling rats. In antidote studies using amino acids, Askelson and associates found that the combination of methionine, lysine, and glycine was essential for maximum chick growth.

The extraction and identification of amino acid analogs from leguminous plants has been reviewed by Bell (1963). From species of

*Vicia*, *Lathyrus*, and *Phaseolus*, a number of analogs were isolated. The biological effects of these analogs varied from distorted protein structure of bean seedlings to nervous disorders in higher animals.

## MATERIALS AND METHODS

Source material was obtained from five plants of diploid sickle alfalfa Medicago falcata (Russian source 22506), which had been selected on the basis of their performances in preliminary chick nutrition studies conducted by Elliott (1962).

Diallel crosses involving the above plants were made in the greenhouse during the winter of 1961-62. In the spring of 1962, parental propagules and $F_1$ seedlings were individually spaced-planted on two-foot centers in three-foot rows into a field design consisting of five completely randomized blocks. Each of the fifteen entries within a block contained eight plants.

Individual plant harvests were made on June 18, 1963 and June 13, 1964 while plants were in an early bloom stage. Each plant was cut by hand; placed in a labeled, perforated paper bag; weighed; and dried for five days in a drier in which a temperature of 100-110°F was maintained. After drying, the plants were reweighed, individually ground in both a hammer mill and a Wiley mill, screened through a 30 mesh screen, and stored at room temperature until bioassayed.

During the fall of 1963, 18 plants of the $F_1$ family of parental plants 22506-11 and 22506-13, hereafter denoted as clones 11 and 13, were moved into the greenhouse. They were induced to flower by extending the daylength to fourteen hours with fluorescent lights. The $F_1$ family was interpollinated to produce an $F_2$ generation. Attempts to self-pollinate $F_1$ plants were generally unsuccessful.

Seeds were harvested from each $F_1$ plant, bulked, scarified, and germinated on moistened blotter paper in petri dishes. The seedlings

were individually transferred to two-inch peat pots filled with a 1:2 sterilized mixture of peat and sand. Essential growth nutrients were added in water as needed. On May 23, 1964 the $F_2$ seedlings were transplanted by hand into a field nursery.

Forty-eight $F_2$ plants of the clone 11 x clone 13 cross were harvested on October 10, 1964, the crowns dug, moved to the greenhouse, and the forage harvested again on January 6, 1965. Both plant harvests were treated as described herein. Parental propagules of clones 11 and 13 and $F_1$ hybrids used to produce the $F_2$ also were moved to the greenhouse and harvested on January 6, 1965.

## Chemical Analysis

Forage protein content, ash, and ether extracts were determined by standard A.O.A.C. (1955)[1] methods. Crude fiber content of alfalfa meals was determined by Van Soest's acid-detergent method (1963). Spectrographic analysis of the elemental content of alfalfa meals was made by the Plant Analysis Laboratory, Department of Horticulture, Michigan State University.

Preparation of cold-water extracts of the parental clones and $F_2$ plants included the following steps: (1) ninety milliliters of cold distilled water was added to 10 grams of finely ground alfalfa meal; (2) the plant material was mascerated 20 minutes at low speed in a Waring blender; (3) the homogenate was filtered under low suction through No. 2 Whatman filter paper in a Buchner funnel and the filtrate was placed in

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[1] These analyses were carried out by Mr. John Grier and Dr. E. J. Benne, MSU Biochemistry Department.

refrigerator for 12 hours; (4) the filtered residue was added to 90 ml of cold distilled water in a beaker and the solution placed in the refrigerator for 12 hours; (5) the residue, water mixture was filtered into the original filtrate; (6) the extract was evaporated to a volume of 20 ml in a flask evaporator with temperature of 50°C and vacuum of 22 psi; (7) after evaporation, the extract was refiltered through a No. 4 Whatman filter paper and stored in a stoppered bottle in a 40°F refrigerator.

Ethanol extracts were made by using 25 ml of 95% ethanol as solvent in which to homogenize 2 g of alfalfa meal for 15 minutes. The homogenate was filtered through No. 2 Whatman filter paper and washed with 80% ethanol. To one volume of filtrate, three volumes of chloroform was added, the solution shaken, allowed to separate into two layers, and the upper aqueous layer was removed and reduced to a volume of 10 ml by steam evaporation.

Comparisons of relative amino acid concentrations between $F_2$ plants of significantly different dry matter digestibilities were made by two methods. The free amino acid content of ethanol extracts and the amino acid concentration of the protein hydrolysate were compared by paper chromatographic techniques similar to those of Thompson and Morris (1959). The hydrolysates were prepared by refluxing 250 mg of alfalfa meal in 50 ml of 6N HCl for 20 hours, filtering the mixture through No. 40 Whatman filter paper, evaporating the filtrate to dryness over steam, storing the residue in a vacuum dessicator for 24 hours over dry sodium hydroxide, and finally redissolving the residue in 20 ml of distilled water.

Fifty λ of ethanol extract or hydrolysate were spotted on a 22.5 inch sheet of No. 1 Whatman chromatogram paper with a micropipette. Two

solvent systems, 77 percent ethanol and n-butanol:formic acid:water (10:3:2.5), were utilized for separation of amino acids. After chromatograms were developed and dried, they were sprayed with a ninhydrin (in 0.2 percent n-butanol) reagent and returned for 15 hours to a chromatogram drying oven set at 100°C.

Water extracts of parental clones and certain $F_2$ plants were compared by paper chromatographic procedures set forth by Elliott (1963). In this case, a solvent system of 3 parts n-butanol:1 part acetic acid: 1.5 parts water (BAW) was used to separate the components of water extracts. The chromatograms were streaked with either 750 or 1500 λ of water extract, and developed with the BAW solvent in a chromatocab for 17 hours. Inch-wide strips were cut from the edges and the center of the chromatograms and sprayed with modified Dragendorff's reagent or bromcresol green reagent (in 0.2 percent n-butanol) to identify stained areas of the chromatogram. Stained areas were cut out and eluted with 200 λ of distilled water. The eluates from the first chromatogram were rechromatographed, developed with a BAW solvent system, sprayed with the same reagents, and each stained area eluted. The eluates from the second chromatogram were stored in stoppered bottles until assayed for physiological activity.

Extensive research on individual plants could not be carried out because of the limited amount of forage available. Therefore, seven of the $F_2$ plants which were lowest in dry matter digestibility were bulked and referred to as the poor $F_2$ or low DMD $F_2$ sample. Likewise, the seven best $F_2$ plants were bulked to form a good $F_2$ composite sample.

## Bioassays

### 1 Specific Growth Response of Weanling Voles

A specific growth response test of weanling meadow voles, Microtus pennsylvanicus L. previously described by Elliott (1963) was used to screen alfalfa plants for their nutritive value. Experimental diets comprised of

| | |
|---|---|
| 75.0 g | alfalfa meal from an individual plant |
| 37.5 g | carbohydrate mix (flour 20 g, corn starch 40 g, confectioner's sugar 20 g, dextrin 10 g, and corn oil 10 g) |
| 6.0 g | vitamin fortification mix (NBC) |
| 6.0 g | mineral salt mix (NBC) |
| 15.0 g | honey |

were fed to two sib pairs of weanling voles for seven days. At least one and usually two weanling voles from each litter were fed a control diet which consisted of

| | |
|---|---|
| 114 g | alpha cel |
| 75 g | carbohydrate mix (see above) |
| 30 g | casein |
| 11 g | vitamin fortification mix (NBC) |
| 11 g | mineral salt mix (NBC) |
| 23 g | honey |
| 3 g | casein |
| 3 g | sucrose. |

Weight gains were recorded daily, but only the weight gains of the final six days were used to calculate the growth response.

The average percent weight gain of the pair and the specific growth response, Gsp, which is defined by the following equation

$$Gsp = \frac{Ge - Gc}{Gc}$$

where Ge = average percent weight gain of pair of voles on an experimental diet

Gc = average percent weight gain of pair of siblings on a control diet

were used as indicators of a plant's nutritive value.

Litters of weanling voles were obtained from a large colony originating from voles captured from the wild in 1962 and supplemented annually by the addition of several large, aggressive males. Matings within the colony were arranged to minimize inbreeding. Also, only healthy litters numbering six or more were retained after nutrition tests were completed. After the voles reached maturity, each litter was divided by sex, sibling females were mated engroup to an unrelated male, and the largest males of the litter were saved for future matings. The population cage of three or four females and one male was maintained for a seven to nine month period, during which time each female gave birth to six to ten litters.

Figure 1. A pair of weanling voles in plastic cage used during growth tests. Note the feed hopper with experimental diet.

II In Vitro Rumen Digestibility Tests

Estimates of the digestible nutrient content of individual alfalfa plants were obtained by means of 6 and 36 hour in vitro rumen fermentations. These results are presented as percent dry matter disappearance (DMD) after fermentation. Each forage sample was tested in duplicate or triplicate depending on the amount of forage available. The field-grown $F_2$

plants were tested in duplicate in two experiments, with a different rumen fluid used for each experiment. $F_2$ plants from the greenhouse were tested in triplicate in one experiment.

Rumen fluid was obtained from a fistulated Holstein non-lactating cow fed alfalfa hay alone. The fluid was collected two hours after the cow had been fed and one hour after the removal of the remainder of her feed and water.

After removing the rumen ingesta, it was strained through three layers of cheesecloth and collected in a pre-warmed thermo-insulated jug. The rumen fluid was taken immediately to the laboratory, poured into large conical flasks, and allowed to stand for one-half hour in a 39°C water bath. Suction was then used to draw off the bottom, fluid layer from the flasks. In order to displace the entrapped air, a stream of carbon dioxide was passed through the fluid.

Either a one or one-half gram (±0.0004) sample of dried ground alfalfa was weighed and placed in a 125 ml Erlenmeyer flask. To each flask, 20 ml of buffer solution was added and the flasks placed in a 39°C water bath. The buffer solution was prepared by dissolving 8.2 g of potassium phosphate, 17.4 g of dibasic sodium phosphate, 4.0 g of urea, and 7.4 g of monohydrated sodium carbonate in 2000 ml of distilled water. The pH of the buffer solution was adjusted to 6.8 by bubbling carbon dioxide through it.

To the pre-warmed mixture of forage and buffer, 24 ml of rumen fluid was added. The atmosphere of the flask was then thoroughly flushed with carbon dioxide, the flask sealed with a rubber stopper fitted with a Bunsen gas release valve, and returned to the 39°C water bath. An

estimation of residual non-filterable dry matter originating from the inoculum was obtained by taking two 24 ml rumen fluid samples. Microbial activity was stopped by adding three drops of a 20% thymol solution (dissolved in 95% ethanol).

Contents of fermentation flasks and 24 ml rumen fluid samples were filtered through fritted glass crucibles containing a layer of Solka Floc.[1] Preparation of the crucibles included adding a one-half inch layer of a 1:2 Solka Floc:water mixture, filtering off excess water, drying in an 80°C oven for 36 hours, and recording the dry weight. The fermentation residue was twice washed with distilled water.

The crucibles containing the residues were dried for 36 hours in an 80°C oven, placed in a dessicator for 30 minutes and then reweighed. Percent dry matter disappearance (DMD) or dry matter digestibility was calculated by the following procedure:

$$\text{Final Crucible Weight} - \text{Original Crucible Weight} = A$$

$$A - \text{mean inoculum weight} = B$$

$$\frac{\text{Forage Weight} - B}{\text{Forage Weight}} \times 100 = \%\ \text{DMD}$$

Amount of Cellulose Degraded by Clone 11 and Clone 13 Fermentation Media

The effect of clones 11 and 13 on cellulolytic activity of rumen inocula was determined by adding one gram of Solka Floc to their fermentation media at 0 and 6 hours after the onset of fermentation. The cellulose-forage mixture was allowed to ferment for 24 hours after the cellulose was added. Forage standards were allowed to ferment for

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[1] Product of Brown Company, Berlin, New Hampshire

both 24 and 30 hours, while cellulose standards were given 24 hours to ferment before the rumen bacteria were killed. The amount of cellulose degradation of the forage--cellulose mixture was estimated as follows:

Final Crucible Weight - Original Crucible Weight = A
A - Forage DMD of standard = B
B - Mean rumen inoculum weight = C

$$\frac{\text{Cellulose} - C}{\text{Cellulose}} \times 100 = \%\ \text{DMD of cellulose}$$

Determinations of the Effects of Alfalfa Extracts Upon Dry Matter Disappearance

Water extracts of the parental clones and water and ethanol extracts of $F_2$ plants exhibiting high or low dry matter disappearances were introduced into various fermentation media to determine their effects upon dry matter digestibility. Extracts were added in quantities of 0.5, 1.0, and 1.5 ml/g of alfalfa.

Antidote Studies

After pronounced differences in clonal dry matter disappearance were observed, a series of antidote experiments were conducted in which various metabolically active substances were added via the buffer solution to the fermentation medium. Emphasis was placed upon components of the diphosphopyridine nucleotide coenzymes DPN and DPNH (NADP and NADPH), since Elliott (1964) had reported the occurrence in alfalfa of antimetabolites which were related to these coenzyme systems. Substances tested were:

DPN (NADP)
DPNH (NADPH)
Vitamin B Mixture
Nicotinamide
Nicotinic Acid
Adenine
Amino Acids in Mixtures and Alone
Complete Vitamin Mixture.

Several concentrations of these, usually in the range of 1 to 30 mg/g of alfalfa meal, were exploited until a maximum response, if any, was found.

Niacin (nicotinic acid) was incorporated into experimental diets of clone 13 at the rate of .25 g/75 g of alfalfa at the time the diets were prepared. Likewise, additions of glycine, glutamine, and aspartic acid at the rate of 150 mg of each/75 g of alfalfa were made to clone 13 experimental diets. These diets were then bioassayed with the weanling voles as with other experimental diets.

Bacteria Counts

Gram stains and microscopic analyses were made of rumen bacterial populations exposed to various alfalfa water extracts to determine gross effects of plant extracts upon the rumen bacteria population. Bacterial samples were taken at the following times:

(1) 0 hour before addition of extracts

(2) ½ hour after addition of extracts (sources of the 4 extracts used were 2 parents, $F_2$ plants of low DMD, and $F_2$ plants of high DMD)

(3) 6 hours after ½ hour exposure to extracts (rumen fluid was centrifuged at 12,500 rpm for 10 minutes, supernatant poured off, bacteria resuspended in centrifuged untreated rumen fluid, the suspension added to 1 g alfalfa meal and 20 ml buffer solution in 39°C water bath, and allowed to ferment for six hours. Samples were obtained by straining fermentation media through three layers of cheesecloth)

Slides containing 1 drop of either 1:10, 1:50, or 1:1000 dilutions of rumen fluid were fixed onto the slide and then stained with 1% crystal violet for two minutes, fixed for 2 minutes with Lugol's iodine solution (1 iodine:2 potassium iodide), washed with a 1:1 acetone:ethanol solution, and counterstained with 2% safranin for

two minutes. The slides were examined under a 97x oil immersion lens with 10x ocular. Estimates of numbers of Gram-negative and positive rod- and cocci-shaped bacteria per grid square were made.

Volatile Fatty Acid Analyses

Analyses for short-chain ($C_2$ to $C_4$) volatile fatty acid (VFA) concentrations of a 25 ml filtrate sample from the fermentation flasks were made by employing an aerograph model A-600-D "Hi Fi" gas chromatogram with a hydrogen flame ionization detector coupled with a Sargent SRL recorder. The absorption column was 5 feet long, 1/8 inch in diameter, and contained 15% versamid 900 and 5% isopthatic acid on 60/80 chromosorb W. An injection port temperature of 190°C was maintained and nitrogen was used as a carrier gas.

A standard curve was made by injecting 2.0 µl of known dilutions of barium acetate, sodium propionate, and sodium butyrate solutions and measuring the peak heights. Concentration of sample VFA were calculated by injecting 2.0 l of filtrate, measuring peak heights for acetic, propionic, and butyric acids and comparing them to the standard curve. VFA concentrations were expressed as micromoles (µM) per milliliter of fermentation filtrate.

III Alfalfa Axillary Shoot Bioassay

Axillary shoots, at least 4 inches long, of two $F_2$ plants, one of high and the other of low in vitro dry matter digestibility ratings, were placed in 4 inch, aluminum foil-wrapped shell vials containing water extracts (obtained by methods already described) of low and high DMD $F_2$ plants. Extract:distilled water dilutions from 1:0 to 1:500

were exploited. To reduce evaporation and add support for the cuttings, loose cotton plugs were wrapped around the cuttings and fitted into tops of the vials. The rate and degree of translocation of test solutions were estimated by the rate of distribution of an aqueous solution of Amaranth dye of 3.5 g/l. As a control, one cutting from each plant was placed into a vial of distilled water. Stem elongation, leaf development, and chlorosis of the leaves were recorded for each cutting.

## EXPERIMENTAL RESULTS

### Screening and Inheritance Studies

Initial screening studies for nutritive value using specific growth responses of weanling voles indicated that clones of diploid alfalfa *M. falcata* differed significantly (Table 1). Average percentage weight gains for a six-day test ranged from 67.50 to 0.29 while the specific growth response, Gsp, varied from +4.38 to -0.81. From the plants tested, clones 11 and 13 were selected on the basis of their diversity in nutritive value and utilized in inheritance studies of factors affecting nutritive value.

When fed plant material from clone 13, weanling voles gained very poorly and in many instances lost weight. However, their rate of feed consumption was not noticeably different from other experimental diets, thus ruling out differences in taste preferences. One batch of experimental diet consisting of 75 g of alfalfa meal was sufficient to feed four voles seven days.

Forage from clones 11 and 13 was tested in *in vitro* rumen fermentation tests after the above differences were observed. Table 2 contains the percentage dry matter disappearance (DMD) data for these clones during 6 and 36 hour fermentation periods. These results reflected the same trend in nutritive value as the vole growth response, inasmuch as clone 13 was significantly lower in digestibility than clone 11. At the end of fermentation clone 11 fermentation media always had a lower pH than that of clone 13 (see Table 2). Data of this table were obtained from 16 entries of each clone using 4 different rumen fluid samples.

Table 1. Growth responses of weanling meadow voles to experimental diets of alfalfa clones (*M. falcata*).

| | 23506 Clone No. | | | | |
|---|---|---|---|---|---|
| | 4 | 7 | 11 | 13 | 14 |
| Average % Weight Gain | | | | | |
| A | 45.83 | 23.91 | 56.18 | 3.80 | 5.88 |
| B | 38.07 | 22.00 | 57.50 | 0.29 | 45.65 |
| Mean | 41.95 | 22.95 | 56.84 | 2.04 | 25.76 |
| Gsp | | | | | |
| A | +1.66 | -0.35 | 3.90 | -0.67 | -0.70 |
| B | +1.06 | -0.50 | 4.38 | -0.81 | +0.10 |
| Mean | +1.36 | -0.42 | +4.14 | -0.74 | -0.30 |

Table 2. Comparisons of 6 and 36 hour dry matter disappearances and end of fermentation pH readings of clones 11 and 13 grown in the field in 1963. Mean values represent 16 entries and 4 different rumen inocula.

| | Percent Dry Matter Disappearance After: | | | | | |
|---|---|---|---|---|---|---|
| | 6 Hours | | | 36 Hours | | |
| Clone No. | Mean | Range | pH | Mean | Range | pH |
| 11 | 36.82 | 35.8 to 37.2 | 6.45 | 49.38 | 47.7 to 51.8 | 6.10 |
| 13 | 31.92 | 30.8 to 33.1 | 6.60 | 46.18 | 44.6 to 47.1 | 6.40 |

Figure 2 illustrates the distribution for dry matter disappearance of the $F_2$ generation of the clone 11 x clcne 13 cross when grown in the field. The mean six-hour digestibility ratings for this population ranged from 20.43 to 33.92%. The dry matter disappearance distribution of the same $F_2$ population from the greenhouse is shown in Figure 3. The range of mean digestibility was from 24.90 to 36.44%. The standard error of the mean of duplicate entries, within an experiment using one rumen fluid sample, was 0.79 and 0.64 digestibility units for the field and greenhouse $F_2$ populations, respectively. The field and greenhouse $F_2$ results were significantly correlated, $r = +.89$.

A similar distribution pattern was observed in both the field and greenhouse material; however, the average digestibility was increased from 29.12 to 30.07 when plants were grown under greenhouse conditions. The average digestibilities of the parents and $F_1$ plants grown under similar conditions as the $F_2$ population are also shown on the histograms. The highest frequency of $F_2$ plants was found near the parental means, especially near the clone 13 mean digestibility. Nevertheless, as is evident from the histograms, transgressive segregation from the parental types occurred. Certain $F_2$ plants were as much as 4% better than clone 11 and others were 6% poorer than clone 13 in dry matter disappearance. The $F_2$ distribution did not fit a pattern which could be explained by the segregation of a single gene; instead, it suggested that differences in dry matter digestibility were under control of several genes.

Table 3 shows that plants which gave the poorest digestibility when grown in the field were also the lowest when grown in the greenhouse. The same pattern was observed for plants of high digestibility. Also,

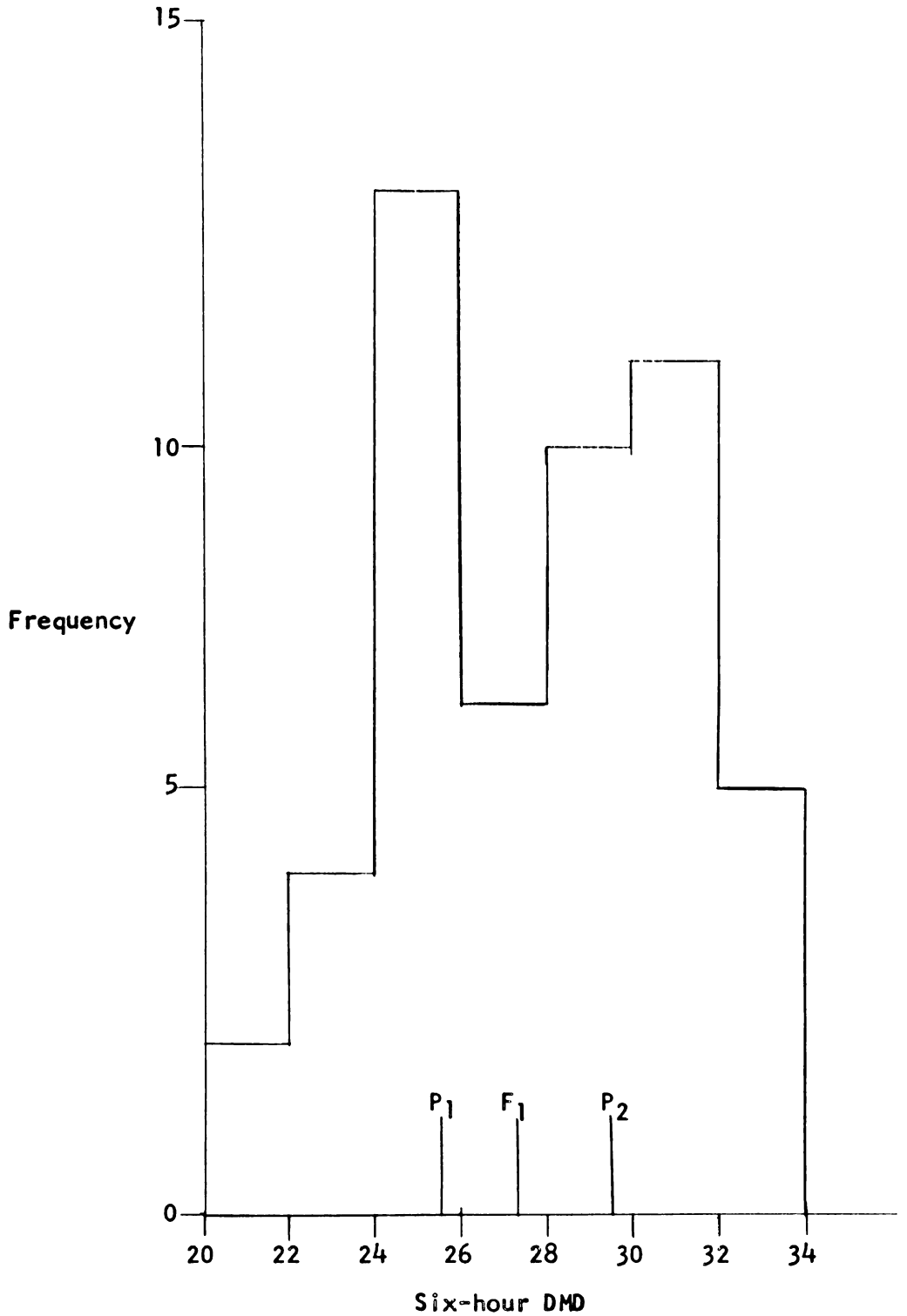



Figure 2. Distribution of six-hour dry matter disappearances of $F_2$ generation of clone 11 and 13 cross grown in field nursery and average DMD of parents, $P_1$ = clone 13 and $P_2$ = clone 11.

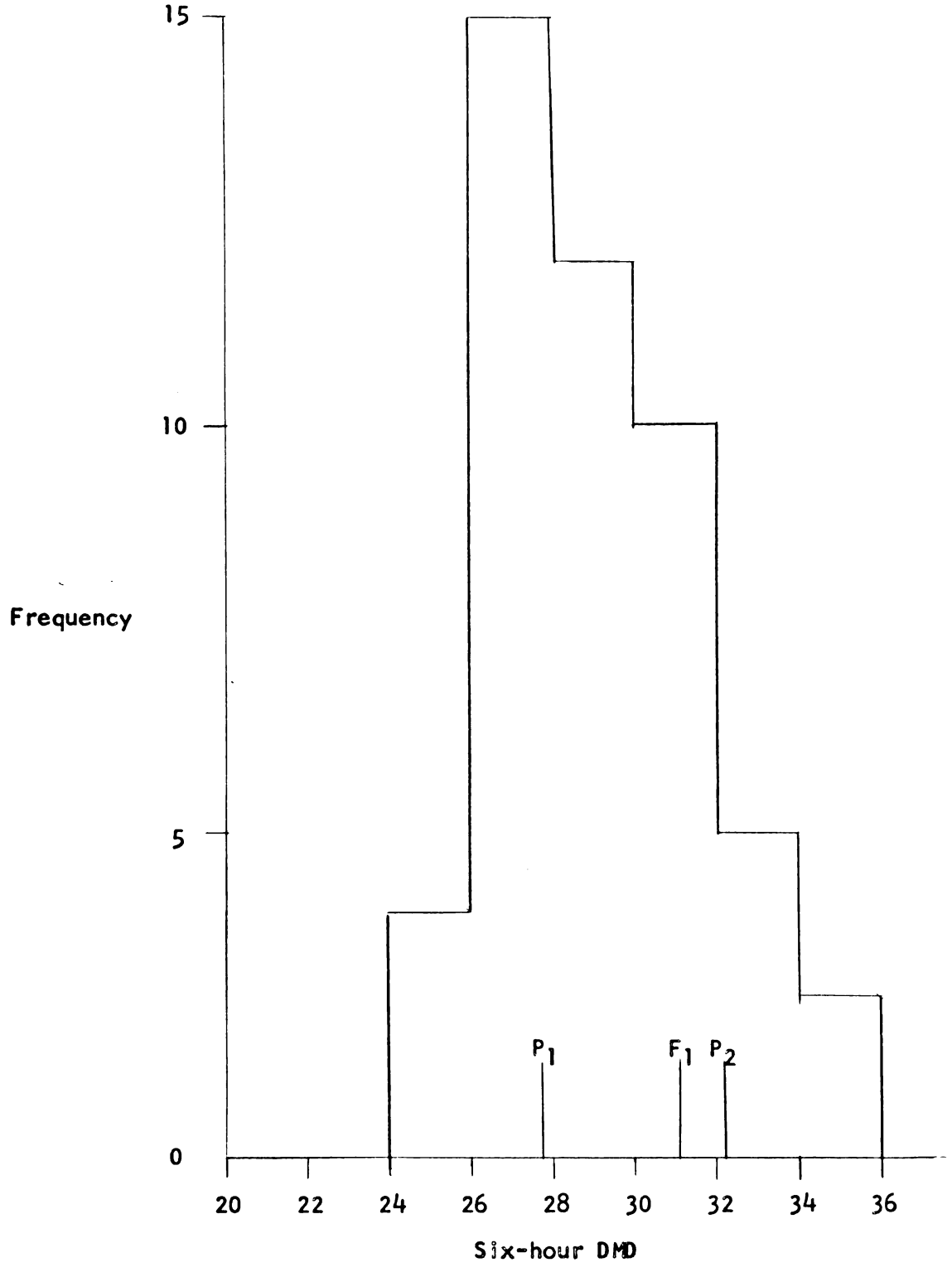



Figure 3. Distribution of six-hour dry matter disappearances of the $F_2$ generation of the clone 11 x clone 13 cross grown in the greenhouse and the average DMD of parents, $P_1$ = clone 13 and $P_2$ = clone 11.

Table 3. Comparisons of six-hour DMD of parents, 18 $F_1$ plants and 6 $F_2$ plants--3 lowest and 3 highest DMD--grown in the field (1964) and the greenhouse (1964-65).

| | Six-Hour DMD | | | |
|---|---|---|---|---|
| | Field Grown | | Greenhouse Grown | |
| Parents and $F_1$* | Mean | Range | Mean | Range |
| Clone 11 | 29.75 | 29.61 to 30.26 | 32.47 | 30.42 to 35.69 |
| Clone 13 | 26.20 | 25.57 to 26.89 | 28.32 | 26.29 to 30.51 |
| $F_1$ | 27.64 | 27.02 to 28.25 | 31.61 | 29.52 to 33.30 |
| $F_2$ Plant Number | | | | |
| 4 | 20.43 | 20.23 to 20.54 | 24.90 | 24.71 to 25.10 |
| 11 | 20.80 | 18.87 to 22.36 | 24.85 | 24.34 to 25.36 |
| 13 | 25.30** | 23.73 to 26.29** | 27.48 | 27.26 to 27.51 |
| 38 | 38.85 | 33.69 to 34.01 | 36.44 | 36.21 to 36.67 |
| 1 | 33.92 | 31.88 to 35.28 | 32.89 | 32.42 to 33.35 |
| 40 | 31.25** | 29.91 to 32.57** | 32.18 | 31.72 to 32.63 |

* Average of 13 trials (6 different rumen fluids)
** Average of 24 trials (8 different rumen fluids)

Table 3 and the histograms show that the average dry matter disappearance of the $F_1$ hybrids was always located between the digestibility of the parents. When these data are considered, complete dominance of the gene or genes which controls the presence of the antimetabolic principle is doubtful.

Antimetabolic Effect on Cellulolytic Rumen Bacteria

The effect of *in vitro* rumen fermentations of clone 13 forage upon the cellulolytic activity of rumen bacteria is indicated in Table 4. When additions of cellulose were made at the start of the fermentation period, there was very little effect of the clone 13 antimetabolite upon cellulose breakdown. However, after the population of ruminal bacteria had been exposed to clone 13 forage for six hours, cellulose degradation was markedly affected. In fact, the estimated amount of cellulose broken down was less than half of that degraded by clone 11 fermentation media. These data suggested that the clone 13 antimetabolite had an influence upon the cellulolytic microorganisms of the rumen.

Gas chromatographic analyses for short-chained volatile fatty acids were determined for fermentation media containing clones 11 and 13 and composite samples of five low DMD $F_2$ plants (poor $F_2$) and five high DMD $F_2$ plants (good $F_2$). The data for acetate, propionate, and butyrate are presented in Table 5. Fermentation media of clone 11 and the good $F_2$ composite had higher concentrations of all three acids than their counterparts clone 13 and the poor $F_2$ composite. The differences in fatty acid concentrations were more pronounced in the comparison of $F_2$ samples. The concentrations of acetate and butyrate after six hours were nearly 20% lower in the fermentation medium of the poor $F_2$ plants than in that of the good $F_2$ plants. In the poor $F_2$ sample after 36 hours, levels of acetate, propionate, and butyrate were 16, 14, and 19% lower than the good

Table 4. Cellulose (Solka Floc) degradation during a 24 hour fermentation period by rumen inocula which had been exposed to forage of clones 11 and 13.

| | Percent Dry Matter Disappearance of: | | |
|---|---|---|---|
| Forage Substrate | Cellulose Added at: | | Alfalfa Alone |
| | 0 Hour | 6 Hours After Start | |
| Clone 11 | 22.78 | 30.32 | 39.22 |
| Clone 13 | 21.62 | 14.79 | 31.89 |
| Cellulose (Alone) | 15.21 | | |

Table 5. Concentrations of volatile fatty acids in fermentation media after 6 and 36 hours.

| | μ M/ml of Fermentation Media | | | | | |
|---|---|---|---|---|---|---|
| Substrate | Acetate | | Propionate | | Butyrate | |
| | 6 Hours | 36 Hours | 6 Hours | 36 Hours | 6 Hours | 36 Hours |
| Clone 11 | 97.7 | 179.3 | 47.0 | 68.2 | 9.3 | 20.1 |
| Clone 13 | 87.3 | 161.0 | 45.9 | 63.6 | 8.3 | 17.3 |
| Good $F_2$ | 102.2 | 181.3 | 47.7 | 69.7 | 9.9 | 20.9 |
| Poor $F_2$ | 80.1 | 152.8 | 42.4 | 60.1 | 7.9 | 16.9 |
| Rumen Fluid | 85.8 | | 37.4 | | 7.0 | |

$F_2$ sample. Consequently, during the first six hours of fermentation the greatest suppression of volatile fatty acid production occurred.

Shifts in ruminal bacteria populations were evident from data obtained by analysis of Gram-stained slides of rumen fluid which had been exposed to water extracts of poor and good $F_2$ forage (Table 6). With only a one-half hour exposure to the antimetabolite present in the poor $F_2$ extract, cocci-shaped bacteria, especially Gram-negative cocci bacteria of 40 and 55% were found for rumen fluid samples A and B which had been exposed to the poor $F_2$ extract, whereas the Gram-negative rods were affected to the extent that about a 33% reduction was observed for both rumen samples. The Gram-positive rod bacteria seemed to be the least affected by the antimetabolite of poor $F_2$ plant extracts. Very few Gram-positive cocci were found in any of the slides studied.

After being exposed to water extracts for one-half hour, then associated with a highly digestible forage substrate for six hours, the populations of the various classes of bacteria were regenerated to approximately their original numbers. Therefore, it is evident that the antimetabolite had an immediate, bacteriostatic effect upon certain rumen bacteria species, but this effect was not persistent.

Because of an insufficient amount of forage for each $F_2$ plant, experimental diets of individual $F_2$ plants could not be tested for vole growth responses. Composite samples of $F_2$ plants of both good and poor digestibility ratings were therefore used in vole growth response tests to determine the effect of the antimetabolite of the poor $F_2$ plants upon young voles. Figure 4 illustrates the growth response of three pairs of weanling voles when fed control, good $F_2$, and poor $F_2$ experimental diets.

Table 6. Effect of water extracts of $F_2$ plants of low and high dry matter digestibility upon *in vitro* rumen bacteria populations from two rumen fluid samples. Fermentation fluid was diluted 1:50 with water before fixing onto slides.

Mean Number of Bacteria per Square of Grid

| Time Exposed to Extract | Rumen Fluid A | | | | Rumen Fluid B | | | |
|---|---|---|---|---|---|---|---|---|
| | Gram+ rod | Gram- rod | Gram+ cocci | Gram- cocci | Gram+ rod | Gram- rod | Gram+ cocci | Gram- cocci |
| 0 Hour | 11.2 | 12.8 | 0.2 | 37.2 | 6.0 | 12.8 | 0.8 | 35.8 |
| ½ Hour[1] | | | | | | | | |
| Control | 10.8 | 12.2 | 0.2 | 36.0 | 4.8 | 12.0 | 0.8 | 37.8 |
| Poor $F_2$ extract | 7.8 | 8.4 | 0.2 | 22.6** | 5.0 | 8.2** | 0.2 | 17.0** |
| Good $F_2$ extract | 12.4 | 11.8 | 0.0 | 32.4 | 5.8 | 17.0 | 0.0 | 36.8 |
| 6 Hour[2] | | | | | | | | |
| Poor $F_2$ extract | 11.2 | 15.8 | 0.0 | 33.6 | 6.0 | 11.0 | 0.5 | 32.2 |
| Good $F_2$ extract | 13.4 | 13.8 | 0.2 | 38.0 | 6.8 | 14.5 | 0.2 | 37.0 |

[1] 5 ml of water extract of either poor or good $F_2$ plants was added to 100 ml of rumen fluid and allowed to stand for ½ hour in 39°C water bath. Control was allowed to stand for ½ hour in water bath.

[2] Same treatments as 1 except bacteria sample was centrifuged after ½ hour, resuspended, and added to a high DMD alfalfa--buffer mixture to ferment for 6 hours.

** ($p < .01$) significantly different from control and high DMD sample means.

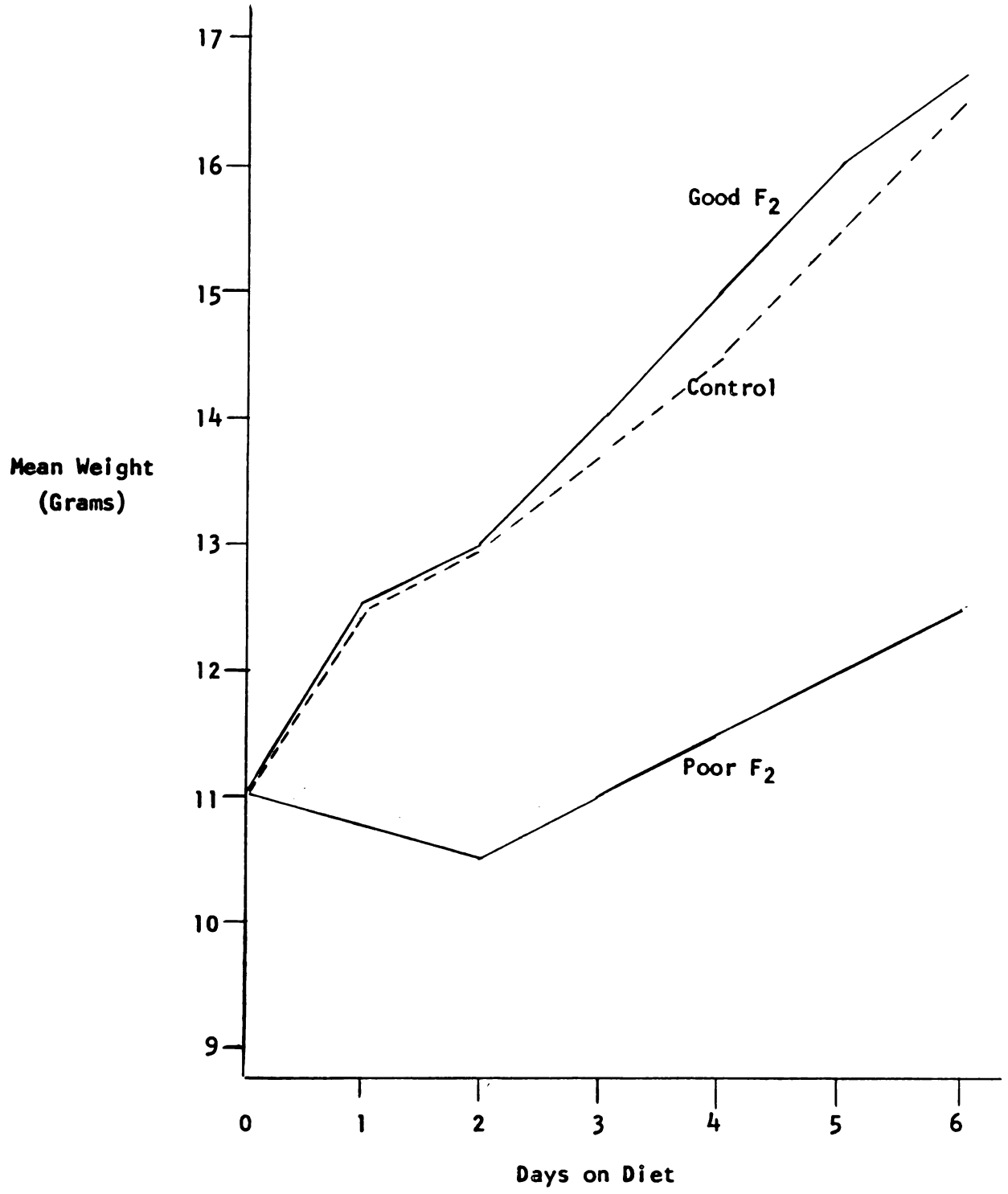



Figure 4. Six-day growth responses of weanling voles fed diets of control, good $F_2$ plants, and poor $F_2$ plants.

The good $F_2$ (High DMD) diets produced an average percent weight gain of 55 as compared to 11 for the poor $F_2$ (low DMD) diets. The good $F_2$ diet stimulated a growth response which was slightly better than the synthetic control diet.

## Effect of Fine-Grinding on Digestibility of Two $F_2$ Plants

The effect of reducing the particle size of forage samples upon dry matter digestibility is shown in Figure 5. Even after the cell wall structures were disrupted by ball-milling, the $F_2$ plant (#48) high in DMD was digested to a much greater extent than the $F_2$ plant (#12) low in DMD after 24 hours of fermentation. Contrary to the expected, digestibility of plant number 48 was reduced by ball-milling, and it was digested only slightly better than plant number 12 for the first 12 hours of fermentation. After 48 hours of fermentation, the percent digestibilities of plants 48 and 12 ball-milled were 31.86 and 24.47, respectively. Of primary significance from these data, $F_2$ plant number 12 of low DMD was not improved in digestibility by fine grinding. This suggests that an antimetabolic factor is primarily responsible for differences in DMD and not a unique lignin-cellulose complex common to low DMD plants.

## Plant Extract Studies

Studies of the effect of water and ethanol plant extracts upon *in vitro* rumen dry matter disappearances (Table 7) indicated that the antimetabolite was present in both the water and ethanol extracts of the poor $F_2$ plants. One milliliter of water extract derived from one-half gram of poor $F_2$ forage reduced dry matter disappearance in every fermentation media to which it was added. When water extracts of poor $F_2$ plants were

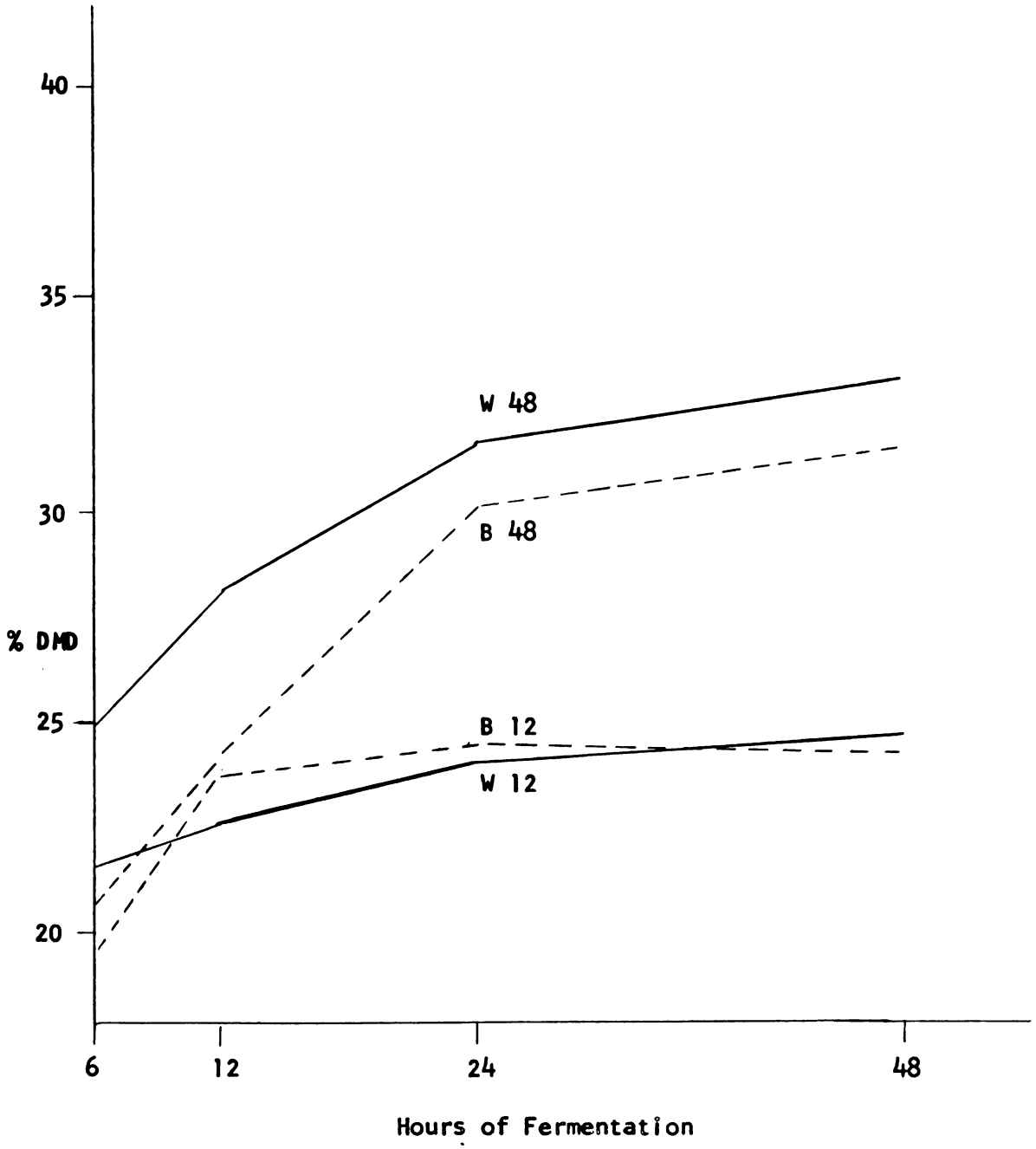



Figure 5. The effect of grinding method (Wiley milled (W) vs Ball milled (B)) upon DMD of $F_2$ plants of high and low DMD.

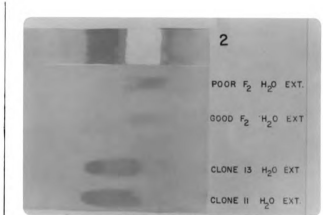
Table 7. The influence of one milliliter of cold-water and ethanol extracts of $F_2$ plants upon six-hour DMD of parents, $F_1$ and $F_2$ plants.

| Source of Forage | | No Extract Control | Six-hour % DMD: Water Extracts of $F_2$ Plants of: Low DMD | % DMD Change | High DMD | % DMD Change |
|---|---|---|---|---|---|---|
| Parents | Clone 11 | 28.82 | 26.38 | -2.44 | 28.69 | -0.13 |
| | Clone 13 | 23.81 | 21.51 | -2.30 | 24.22 | +0.41 |
| $F_1$ | Bulk sample | 28.96 | 27.40 | -1.56 | 28.50 | -0.46 |
| $F_2$ | Plant No. 38 | 30.94 | 28.42 | -2.52 | 30.70 | -0.24 |
| | 14 | 28.52 | 25.32 | -3.20 | 28.25 | -0.27 |
| | 43 | 27.94 | 19.74 | -8.20 | 25.71 | -2.23 |
| | 45 | 28.20 | 24.82 | -3.38 | 27.98 | -0.22 |
| | 31 | 24.62 | 21.61 | -3.01 | 23.85 | -0.77 |
| | | | Ethanol Extracts of $F_2$ | | | |
| $F_2$ | 45 | 28.20 | 26.20 | -2.00 | 28.79 | +0.59 |

added to fermentation media containing various $F_2$ plants, reductions in percent dry matter disappearances ranged from 2.52 to 8.20, as compared to 0.22 to 2.23 for fermentation media of the same $F_2$ plants with water extracts of good $F_2$ plants added. Substantial reductions in dry matter disappearance due to additions of poor $F_2$ water extracts were also observed when forage of parents and $F_1$ plants were used as substrate of fermentation media. The ethanol extract of poor $F_2$ plants contained sufficient antimetabolite to reduce dry matter disappearance of $F_2$ plant no. 45 by 2%, in contrast to a 0.59% increase in dry matter disappearance when the ethanol extract from good $F_2$ plants was added.

Water extracts of parental clones, poor $F_2$ plants, and good $F_2$ plants were chromatographed on a descending BAW solvent system then stained with bromcresol green (Figure 6). A marked difference in density and mobility of stained areas was noted for extracts from parental and $F_2$ plants. For the parental extracts the stained areas had rf values of .29 whereas the rf values for $F_2$ extracts were .20.

Figure 6. Chromatogram profiles of water extracts of parents, good $F_2$ plants, and poor $F_2$ plants. The chromatogram was developed on a BAW (5:1:2.5) solvent system and stained with bromcresol green.



The bromcresol green positive areas of chromatograms of poor and good $F_2$ water extracts were different in intensity of stained area, with the area on the chromatogram of the poor $F_2$ water extract being more intensely stained. Both these areas were eluted with water and rechromatographed on a BAW solvent system. On the second chromatogram, the area stained with bromcresol green on the chromatogram of poor $F_2$ extract separated into two distinct areas, denoted $B_1$ and $B_3$ with rf values of .16 and .27, respectively (Figure 6). In contrast, only one stained area ($B_3$) was observed on the second chromatogram of the eluate from the good $F_2$ chromatogram.

Using the same plant extracts and solvents as above, chromatograms were developed and sprayed with a modified Dragendorff's reagent. In this case, one stained area ($D_1$) was observed with a rf value of .29. When this area was eluted from the chromatograms of $F_2$ plant extracts and rechromatographed, one Dragendorff's reagent stained area was again observed with a rf value of .31.

Each of the above mentioned areas ($B_1$, $B_3$, and $D_1$) of the poor $F_2$ chromatogram was eluted from full-sized unstained chromatograms and tested in the <u>in vitro</u> bioassay of dry matter disappearance (Table 8). Two concentrations of the substances associated with these stained areas were tested. The concentrations varied by the amount of water extract (750$\lambda$ vs 1500$\lambda$ ) applied to the original chromatograms. From the data of Table 8, the effect of concentration of substances in areas $B_1$ and $B_3$ of the poor $F_2$ extract is evident. Of the three eluates from chromatograms of poor $F_2$ water extracts tested, $B_1$ had the greatest influence upon dry matter disappearance. At the higher concentration, it reduced

Table 8. Antimetabolic effect of eluates of water extract of low DMD $F_2$ plant separated by paper chromatography using a BAW (5:1:2.5) solvent and stained with bromcresol green and modified Dragendorff's reagent.

| Substrate | Control | % Dry Matter Disappearance After Six Hours | | | |
|---|---|---|---|---|---|
| | | Poor $F_2$ | | | Good $F_2$ |
| | | $B_1$[a] | $B_3$[b] | $D_1$[c] | $B_3$ |
| $F_2$ Plant No. | | | | | |
| 43[1] | 27.94 | 27.00 | 27.84 | 27.75 | 27.74 |
| 11[2] | 24.43 | 21.11 | 21.70 | 24.09 | 24.18 |
| 38[2] | 29.82 | 25.92 | 26.64 | 29.73 | 29.93 |

1 200 λ eluated from chromatogram streaked with 750 λ of water extract of low DMD $F_2$ plants.

2 200 λ eluated from two chromatograms each streaked with 1500 λ of water extract of low DMD $F_2$ plants.

a $B_1$--Bromocresol green stained area with rf value of .16

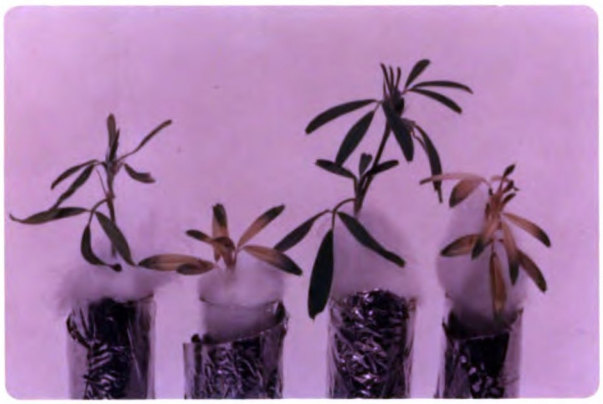
b $B_3$--Bromocresol green stained area with rf value of .22

c $D_1$--Modified Dragendorff's reagent stained area with rf of .29

percent dry matter disappearance of $F_2$ plants no. 11 and no. 38 by 3.32 and 3.90, respectively, whereas the eluate from $B_3$ reduced percent dry matter disappearance in these same plants by 2.78 and 3.18. The $D_1$ eluate did not noticeably affect dry matter disappearance at either concentration; likewise, the $B_3$ eluate from the chromatogram of the good $F_2$ water extract was ineffective in reducing dry matter digestibility.

Figure 7A shows the alfalfa cuttings after 48 hours in water, 3% amaranth, water extracts of good (high DMD) $F_2$ and poor (low DMD) $F_2$ plants. A plant extract:water dilution of 1:15 was used. The extract of poor $F_2$ plants produced a chlorotic condition in the cutting. The chlorosis developed progressively from the base of the leaves to the tips. In contrast to the chlorotic symptoms produced by the extract of the poor $F_2$ plants, the extracts of the good $F_2$ plants produced no noticeable symptoms. Cuttings in the water control remained turgid throughout the duration of the experiment. After 10 hours, the amaranth dye had been translocated throughout the cutting, thus indicating that the transpiration stream of the cuttings was active.

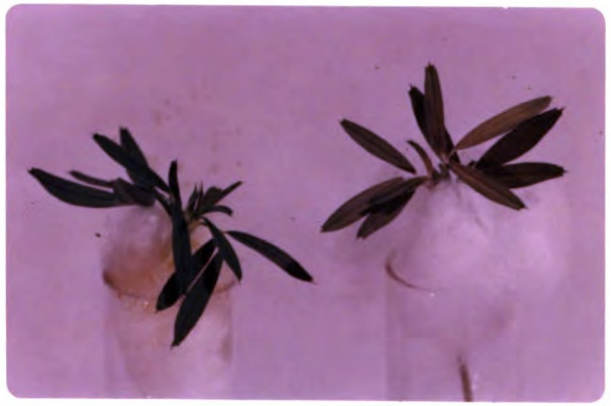
The development of chlorosis of the leaves is illustrated in Figure 7B at 48 hours after the start of experiment. No symptoms from the good $F_2$ extract was noted with dilutions of 1:10 or greater. However, at high concentrations of both water extracts (>10%), wilting of the cuttings occurred after 48 hours. Microscopic analysis indicated the wilting to be due to vascular plugging of the shoot. Cuttings pictured in Figure 7B show the wilted cutting in a 1:7 dilution of good $F_2$ extract, whereas at the 1:15 dilution no wilting occurs.



A. (1) and (3) good $F_2$ extract: water dilution of 1:7 and 1:15. (2) and (4) poor $F_2$ extract:water dilutions of 1:7 and 1:15.

B. (1) water (2) amaranth dye (3) good extract, 1:15 dilution (4) poor extract, 1:15 dilution.

C. (1) poor extract 1:37 dilution--44 hours (2) poor extract 1:37 dilution glycine, glutamine, and aspartic acid added--44 hours.



D. (1) poor extract 1:75 dilution--44 hours (2) poor extract 1:75 dilution glycine, glutamine and aspartic acid added--44 hours.

Figure 7. Effect of water extracts of poor $F_2$ and good $F_2$ plants upon alfalfa shoot cuttings.

Antidote Studies

Additions of a complete vitamin mixture, a B-vitamin mixture, nicotinamide, and nicotinic acid were made to fermentation media containing $F_2$ plants of high DMD (#47 and #49) and low DMD (#11 and #21) (Table 9). None of the vitamin additions were successful in completely overcoming the suppressed digestibility of plants 11 and 21. However, the B-vitamin mix was beneficial to the digestibility of plants 47, 49, and to a lesser extent 11, since their percent dry matter disappearances were increased by 1.73, 2.33, and 0.36 over the control. Both nicotinamide and nicotinic acid additions caused a definite reduction in dry matter disappearance.

Further studies of possible antidotes for suppressed digestibility in $F_2$ plants included several amino acid groups, coenzymes DPN and DPNH, and adenine (Table 10). Although a general beneficial response to the additions of all amino acid groups was observed, amino acid group A increased digestibility of the low DMD samples significantly better than the other amino acid groups. In fact, the dry matter disappearance of the $F_2$ plants low in DMD was increased to approximately the level of the $F_2$ plants high in DMD. Amino acid groups B and C produced similar responses in $F_2$ samples of both low and high DMD and actually increased digestibility of the high DMD $F_2$ more than group A.

The coenzymes DPN and DPNH were also effective in increasing digestibility of the low DMD $F_2$ plants, especially at the 10 mg concentration. Only the oxidized coenzyme was tested with the high DMD $F_2$ sample, but very little response was noted. The DPNH coenzyme produced a beneficial response at each of the three concentrations (1, 4, and 10 mg) used. However, adenine, a precursor of DPN, reduced the digestibility of the forage to which it was added at both the 3 and 10 mg levels.

Table 9. Effect of vitamin additions upon percent dry matter disappearance of various $F_2$ plants.

| Vitamin Additions: | % Dry Matter Disappearance After Six-Hours | | | |
|---|---|---|---|---|
| | $F_2$ Plant Number | | | |
| | 47 | 49 | 11 | 21 |
| None (Control) | 29.04 | 30.30 | 24.66 | 23.39 |
| Complete Vitamin Mix[a] | 31.88 | 29.93 | 24.65 | 23.72 |
| B-vitamin Mix[b] | 30.77 | 32.63 | 25.02 | 22.81 |
| Nicotinamide[c] | 27.98 | | | 20.97 |
| Nicotinic Acid[c] | | 28.76 | 21.89 | |

[a] 4 mg vitamin Fortification Mix (NBC) per g of alfalfa

[b] 10 ml/g of alfalfa contained: nicotinic acid, 100 mg; thiamine HCl, 50 mg; riboflavin, 25 mg; pantothenic acid, 50 mg; pyridoxin HCl, 25 mg; choline Cl, 1000 mg; inositol, 500 mg; folic acid, 5 mg; and biotin, 1 mg dissolved in 500 ml of distilled water.

[c] 4 mg/g of alfalfa

Table 10. Effects of three groups of amino acids, DPN, DPNH, and adenine, upon six-hour dry matter disappearance of two $F_2$ samples differing markedly in % DMD.

| | % Dry Matter Disappearance After Six Hours | | | |
|---|---|---|---|---|
| | $F_2$ Composite Sample of | | | |
| | Low DMD Plants | % DMD Change | High DMD Plants | % DMD Change |
| Control | 23.80 | | 30.66 | |
| *Amino Acid Group | | | | |
| A | 31.22 | +7.42 | 32.72 | +2.06 |
| B | 26.98 | +3.18 | 34.58 | +3.92 |
| C | 27.74 | +3.94 | 33.76 | +3.10 |
| DPN 10 mgm | 27.90 | +4.10 | 30.90 | +0.24 |
| DPNH 1 mgm | 27.04 | +3.24 | | |
| 4 mgm | 26.10 | +2.30 | | |
| 10 mgm | 27.83 | +4.03 | | |
| Adenine 3 mgm | 23.20 | -0.60 | 29.57 | -1.09 |
| 10 mgm | 22.62 | -1.18 | 29.82 | -0.84 |

*Amino Acid Groups: A glycine, aspartic acid, glutamine (10 mg of each)

B arginine HCl, L--cystine, L--isoleucine, L--lysine HCl, L--serine, L--tyrosine, L--valine (10 mgm of each)

C L--alanine, L--leucine, L--proline, L--tryptophan (10 mgm of each)

The amino acids which engroup produced increases in digestibility were assayed alone or in combinations of two or three (Table 11). The mixture of glycine, glutamine, and aspartic acid again proved to be the most successful antidote for low dry matter disappearance. This group once again increased the level of digestibility of the low DMD $F_2$ sample to that of the high DMD $F_2$ sample, raising the former 7.40% in digestibility. When added to the high DMD $F_2$ sample, this amino acid group reduced dry matter disappearance.

While a very successful antidote in combination, glycine, glutamine, and aspartic acid could not produce a similar effect when added alone or in pairs. However, in all cases except one, an increase in digestibility was observed, the exception being the combination of aspartic acid and glutamine which reduced digestibility by 1.51%. Glycine and aspartic acid when added in combination increased digestibility by 4.43% while the three amino acids together increased it 7.40%. In general, amino acid additions to the $F_2$ sample of high DMD had a detrimental effect, with only glycine alone and glycine plus aspartic acid slightly increasing digestibility.

The remaining amino acids tested did not increase digestibility of the poor $F_2$ plants to the extent of the aspartic acid, glycine, and glutamine group although all except tryptophan promoted slight increases in digestibility. Tryptophan, a precursor to niacin, suppressed dry matter disappearance in every fermentation flask to which it was added.

When glycine, glutamine, and aspartic acid (2 mg of each/4 ml of water) were added to poor $F_2$ extract:water diltuions of 1:37 and 1:75 in alfalfa shoot bioassays, a retardation in the development of chlorosis

Table 11. Responses to amino acids in six-hour dry matter disappearance tests of two $F_2$ samples differing in DMD ratings.

| | % Dry Matter Disappearance in Six Hours | | | |
|---|---|---|---|---|
| | High DMD $F_2$ | % DMD Change | Low DMD $F_2$ | % DMD Change |
| Control | 33.30 | | 25.93 | |
| Glycine (Gly) | 35.04 | + 1.74 | 26.56 | + 0.63 |
| Aspartic Acid (Asp) | 28.43 | -5.13 | 27.24 | + 1.31 |
| Glutamine (Glut) | 30.61 | -2.69 | 28.34 | + 2.41 |
| Gly Asp | 33.88 | + 0.58 | 30.30 | + 4.43 |
| Gly Glut | 31.90 | -1.40 | 28.67 | + 2.74 |
| Asp Glut | 31.06 | -2.24 | 24.42 | -1.51 |
| Gly Asp Glut | 31.78 | -1.52 | 33.33 | + 7.40 |
| Alanine Leucine | | | 27.54 | + 1.62 |
| Arginine Tryptophan | | | 27.76 | + 1.84 |
| Valine Serine | | | 26.98 | + 1.05 |
| Tyrosine isoleucine cystine | | | 27.71 | + 1.79 |
| Tryptophan 1 mg | 31.86 | -1.44 | 25.34 | -0.59 |
| 4 mg | 33.04 | -0.26 | 25.08 | -0.85 |
| 10 mg | 29.58 | -3.88 | 24.92 | -1.01 |

was observed. Figures 7C and 7D illustrate the effect of the amino acid additions to poor $F_2$ extract:water dilutions of 1:37 and 1:75, respectively after 44 hours. However, the amino acids retarded chlorosis for only 60 hours; after this time chlorosis developed in the cuttings. Also, a concentration effect of a water extract of poor $F_2$ plants can be seen by comparing the cuttings with no amino acids added in Figures 7C and 7D.

Experimental diets of clone 13, known to produce poor responses in weanling vole specific growth tests, were antidoted with niacin (nicotinic acid) and an amino acid mixture of glycine, aspartic acid, and glutamine and fed to voles from the same litter (Figure 8). When the amino acid group was added, the growth of the voles was significantly increased. Average percent weight gains of two pairs of sibling voles fed clone 13+ amino acid mix and clone 13 alone were 33 and 2 respectively. In contrast, on the clone 13 diet with niacin added, the average percent weight gained was only 7. The response of the voles of this litter to the synthetic control diet was superior to all experimental diets.

## Chemical Analyses of Forage

Table 12 contains the mineral analyses of the parents grown in the field and the composite samples of low and high DMD $F_2$ plants grown in the greenhouse. Even though the contents of Na, Mn, and Fe were noticeably different for the $F_2$ samples, they were still within the range determined by Loper and Smith (1961) for succulent alfalfa. The higher mineral content of the $F_2$ plant samples reflects their succulent stage of growth at harvest. Crampton (1957) also points out that mineral content of forages is innocuous to rumen bacteria.

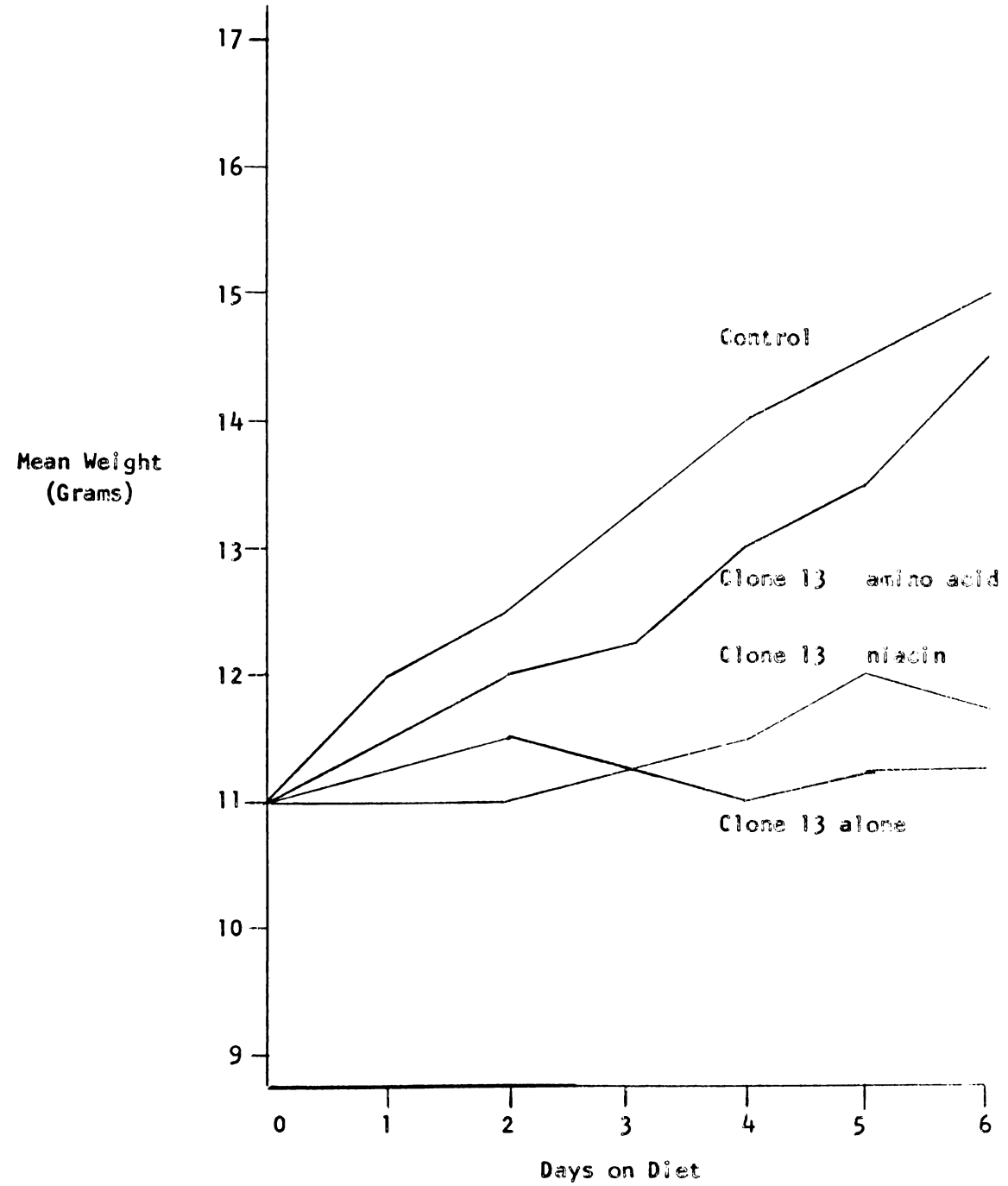



Figure 8. Six-day growth responses of weanling voles fed diets of clone 13 alone, with glycine, glutamine, and aspartic acid added, and with niacin added.

Table 12. Mineral and chemical analyses of parents (field, 1964) and $F_2$ plants of high and low DMD (greenhouse, 1965).

Elemental Content Expressed as:

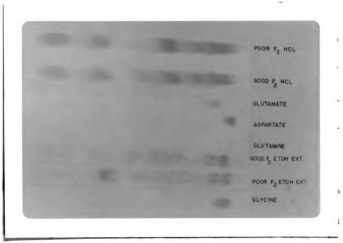
| Sample | % of Dry Weight | | | PPM | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | P | Ca | Mg | Na | Mn | Fe | Cu | B | Zn | Al |
| Clone 11 | 0.49 | 1.22 | 0.40 | 256 | 26 | 118 | 12.0 | 29.5 | 34 | 76.5 |
| Clone 13 | 0.47 | 1.14 | 0.33 | 141 | 22 | 78 | 10.2 | 31.9 | 24 | 58.6 |
| Poor $F_2$ | 0.30 | 1.82 | 0.30 | 299 | 119 | 726 | 11.0 | 36.8 | 34 | 107.7 |
| Good $F_2$ | 0.32 | 2.02 | 0.29 | 528 | 63 | 504 | 8.4 | 47.0 | 24 | 64.2 |

| Sample | % of Dry Weight | | | |
|---|---|---|---|---|
| | Ash | Crude Fiber | Ether Extract | Protein |
| Clone 11 | 7.89 | 25.88 | 2.03 | 25.00 |
| Clone 13 | 7.55 | 27.41 | 1.64 | 25.00 |
| Poor $F_2$ | 12.04 | 19.75 | 1.99 | 19.81 |
| Good $F_2$ | 9.18 | 22.79 | 2.03 | 20.38 |

The chemical composition of parents and $F_2$ plants is also found in Table 12. These data do not suggest a logical explanation for differences in bioassay responses. While crude fiber content varied somewhat, being higher in high DMD $F_2$ plants and clone 13 forage, protein was similar for compared samples. Differences in composition between parental clones and the $F_2$ samples can be accounted for by the differences in age.

Amino acid contents were estimated from chromatogram profiles of protein hydrolysates and ethanol extracts of poor $F_2$ (low DMD) and good $F_2$ (high DMD) samples (Figure 9). A BAW solvent system was used to develop the chromatograms and ninhydrin was used to stain the profiles. Neither the chromatographic profile of poor $F_2$ hydrolysate or ethanol extract suggests a deficiency of an amino acid which might account for differential responses found in the bioassays. All chromatograms were compared to known solutions of glycine, glutamine, glutamic acid, and aspartic acid.

Figure 9. Chromatograms of HCl hydrolysates and ethanol extracts of poor $F_2$ and good $F_2$ plants with glycine, glutamine, glutamic acid, and aspartic acid standards.



## DISCUSSION

A heritable antimetabolite affecting nutritive value was found in diploid Medicago falcata alfalfa. The antimetabolic principle was indicated by bioassays and chemical analyses. Observation of symptomatic responses of the antimetabolite in small animals, microorganisms, and plant cuttings did not reveal the antimetabolite to be organism-specific. Instead, an antimetabolite affecting a broad spectrum of organisms seems to be involved.

Even though a conclusive pattern of inheritance of the antimetabolic effect was not determined in this study, data from $F_2$ plants of the cross of two heterozygous parents (clones 11 and 13) show segregation for the antimetabolic principle in dry matter digestibility studies. Since the data from the same $F_2$ plants grown under field and greenhouse conditions were closely correlated, the possibility of a differential environmental influence was virtually eliminated. The presence of a completely dominant or recessive gene which controls the occurrence or absence of the antimetabolic components is not evident in either the $F_1$ or $F_2$ population. Instead, at least two genes appear to be involved and the data suggest that they have an additive genetic effect. The transgressive segregation of some of the $F_2$ plants from the parents and the concentration of $F_2$ plants between the parental means support this hypothesis. Furthermore, no single antidote of the antimetabolic effect was found; and the adverse effect was completely overcome in dry matter digestibility determinations only when glutamine, aspartic acid, and glycine were added in combination. Two main limitations of the genetic interpretation of these

results are the relatively small $F_2$ population and the heterozygous condition of the parents.

In screening alfalfa plants for antimetabolic components, the combination of a specific growth response test of weanling meadow voles and *in vitro* rumen dry matter disappearance tests was very satisfactory. Results from these tests were complementary and gave an accurate demonstration of the nutritive value of an individual plant. The minimal requirement for forage, 75 g for duplicated vole tests and 8 to 10 g for *in vitro* rumen fermentations, also is a major asset since individual first-year alfalfa plants rarely yield more than 250 g of forage.

The experimental diets of weanling voles were synthesized so that all dietary essentials were supplied except protein. The alfalfa meal was expected to provide enough protein to support the young vole's rapid growth. Variations in vole growth response could not therefore be readily associated with dietary deficiencies of vitamins, minerals, or carbohydrates, especially since voles fed control diets containing the same supplementation responded by gaining, on the average, approximately 40% of their weight in six days. Also, crude protein analyses of alfalfa meals did not reveal differences of sufficient magnitude to account for the growth response variations. However, antimetabolic substances occurring in the alfalfa could account for the differences in vole growth rates by hindering the efficient utilization of the available dietary components.

It is realized that *in vitro* dry matter digestibility measures many dynamic factors in forage quality and combines them into a single numerical value. Therefore, before it was possible to directly associate

differences in dry matter digestibility to the occurrence of an antimetabolite in the forage, other factors which also influence digestibility ratings had to be considered. Physical handling of forages was standardized to reduce any bias in regard to harvesting, drying, milling, or storing. Chemical analyses of the plants did not reveal marked differences in composition. Individual plants were tested with at least two different samples of rumen inoculum to minimize inherent differences in rumen fluid.

When differences in dry matter digestibility between clones 11 and 13 were established, studies were initiated to determine the site of action of the clone 13 antimetabolite. It had been noted that the final pH readings of fermentation media containing clone 13 were consistently lower than those of clone 11. Gas chromatographic analyses of the filtrates of fermentation media indicated a relationship between lower volatile fatty acid production and lower pH. The fermentation media of clone 13 was lower in concentrations of acetic, propionic, and butyric acids after 6 and 36 hours. These analyses were repeated for $F_2$ plant samples of low and high digestibility, and the results were comparable although the differences in volatile fatty acid production were more extreme.

Inasmuch as the main products of cellulolytic rumen bacteria are short-chained volatile fatty acids, these findings suggested a difference in cellulose degradation between clones 11 and 13 and between $F_2$ samples of low and high digestibility. Two possible explanations for this are (1) a direct influence of an antimetabolic factor upon the cellulolytic bacteria of the rumen inoculum or (2) the presence of a cellulose-lignin

complex in clone 13 and low DMD $F_2$ plants which interferes with cellulose breakdown by rumen bacteria.

To elucidate the first of these possibilities, the cellulolytic capability of rumen fluid which had been associated with clones 11 and 13 was studied. Data from these studies showed that clone 13 forage altered the rumen inoculum so that it had a reduced capacity to degrade cellulose (Table 4). Since the available evidence indicates that cellulase enzymes are closely associated only with live bacteria and are highly labile when free of the bacterial cell, the steps of cellulose degradation could be noticeably hindered by a bacteriostatic agent selective for certain cellulolytic bacteria. Estimates of bacterial populations after exposure to water extracts of alfalfa plants thought to contain antimetabolites suggest that a selective bacteriostatic action may be involved (Table 6). The antimetabolite had a short-term effect upon cellulolytic bacteria species since after the rumen bacteria had been associated for one-half hour with a water extract containing the antimetabolite, then centrifuged, and introduced to an alfalfa forage of high digestibility, the bacteria population was restored to its original status.

Of the four types of bacteria classified, the Gram-negative cocci seemed to be affected more adversely. Dehority (1960) and El-Shazly *et al.* (1961) concluded the Gram-negative cocci and rod-shaped bacteria are responsible for a large portion of the cellulose digestion which occurs in the rumen.

The data clearly indicate that the antimetabolic effect occurs during the first hours of *in vitro* rumen fermentations. Differences in

dry matter digestibility after six hours were not magnified after 36 hours of fermentation. Also, certain populations of rumen bacteria were affected during the first half hour of association with the anti-metabolite of the low DMD $F_2$ plants. Perhaps the antimetabolite itself is degraded by ruminal bacteria to a non-toxic molecule.

Crampton (1957) described various ways that the cellulose digestion rate can be retarded. He proposed that circumstances evolving from the forage material interfere with activity of rumen microflora and suggested excessive lignification as a primary factor. Fine-grinding, by disrupting plant cell walls, allows bacterial enzymes to penetrate into regions from which they may normally be excluded because of the protective effect of lignification or of the crystallinity in cellulose structure. The possibility that differential lignification or cell wall composition was responsible for observed differences in nutritive value was virtually eliminated when ball-milled $F_2$ plants of high and low DMD had similar dry matter disappearances to samples which were Wiley-milled only. Chemical analyses of alfalfa meals also showed no consistent differences between plants with regard to crude fiber content.

Critical inferences arise from marked differences between plant selections in dry matter digestibility and in volatile fatty acid production in rumen fermentation studies. Any consistent reduction in digestibility of a forage will seriously affect animal performance. McCullough (1959) reported that differences of 5% digestibility in forages will, on the average, produce highly significant changes in animal responses. Much attention recently in animal nutrition research has been given to increasing the voluntary feed consumption. However, it is generally concluded that until improvement in forage digestibility

is realized, little success will result since poorly digested forage has a longer rumen retention time and therefore delays the hunger reflex. Consequently, the differences of 12 to 15% digestibility between $F_2$ plants of the clone 11 x clone 13 cross become more meaningful. Inasmuch as ruminants are dependent upon absorbed volatile fatty acids for approximately 40% of their digested energy, the forage from plants of poor digestibility and lower volatile fatty acid production directly affect the animal's energy production and balance, thus influencing the production of meat and milk.

Results from *in vitro* rumen fermentations and alfalfa cutting bioassays indicated that the antimetabolite was present in the cold-water extracts of alfalfa plants of low nutritive value. When the water extract of low DMD plants was added to fermentation media containing forage of either the parents, $F_1$ plants, or $F_2$ plants, the dry matter digestibility was reduced by 2 to 8%. Water extracts from plants of high and intermediate digestibility had little effect on dry matter digestibility. The water extracts of low DMD $F_2$ plants reduced dry matter digestibility 13 to 15 times more than did the water extracts of high DMD $F_2$ plants.

Paper chromatographic analyses using a BAW solvent system indicated differences in the mobility of substances stained with bromcresol green in parent and $F_2$ water extracts. The area stained with bromcresol green was more mobile in the parents (rf = .29) than in the $F_2$ water extracts (rf = .20). Also, the stained area of the poor $F_2$ sample was found to actually contain two areas ($B_1$ and $B_3$) which were separated by rechromatographing the eluates of the original chromatogram. This was not true for the stained area of the chromatogram of good $F_2$ extract; only one area ($B_3$) was observed on the second chromatogram.

A suppressive effect upon digestibility of alfalfa forage was noted only when eluates of the first and second areas ($B_1$ and $B_3$) of poor $F_2$ extracts stained with bromcresol green were added to the fermentation media. Eluates from stained areas of chromatograms of other water extracts did not affect digestibility.

These observations combined with the distribution of the $F_2$ population for dry matter disappearance suggested that the antimetabolic activity of the poor $F_2$ plants was unlike that of the parent clone 13. This difference may be attributed to either an accumulation of a more potent concentration of an antimetabolite in the poor $F_2$ plants or the elaboration of an antimetabolite of different chemical nature. Also, some of the antimetabolic activity of poor $F_2$ plants is associated with a specific area of paper chromatograms developed with a BAW solvent.

Characterization of the antimetabolic factors present in diploid M. falcata began by antidoting experimental diets of the low nutritive value parent, clone 13, with niacin. Minimal responses were noted in several six-day growth tests. However, massive dosages of niacin were unable to completely overcome the suppressed growth response of clone 13 as Elliott (1964) had noted in diets comprised of Vernal alfalfa. This suggested that an antimetabolic principle other than that found in Vernal alfalfa was involved in clone 13. Schillinger and Elliott (1964) reported that responses from individual alfalfa plants in specific growth tests of weanling voles were also more variable in a Vernal population than in a diploid M. falcata population. Thus, it appears that the two Medicago species elaborate different antimetabolites.

The most effective antidote for the antimetabolite in clone 13 in the vole growth tests was a group of amino acids consisting of aspartic

acid, glycine, and glutamine. Although the amino acid additions did not fully restore the vole growth response of clone 13 to that of clone 11, they did increase daily weight gains noticeably.

The success of antidoting low digestibility in certain $F_2$ plants with aspartic acid, glutamine, and glycine is unexplained at this time. Their effect upon dry matter disappearance was not a general one since they had very little effect upon digestibility of plants of good nutritive value. Also, no deficiencies of amino acids were detected in poor $F_2$ plants.

Partial recovery from the antimetabolic effect occurred when the coenzyme DPN was added to the fermentation media. Since the above three amino acids are directly involved in adenine synthesis, a precursor to DPN, it appeared that the antimetabolite site of action was adenine synthesis. However, additions of adenine to the fermentation media containing alfalfa of low nutritive value had no beneficial effect on digestibility. This does not eliminate adenine synthesis as the antimetabolic site of action, inasmuch as adenine has to be degraded before being incorporated into bacterial cells. Possibly it is catabolized to different end products than the DPN molecule and its end products are incapable of overcoming the antimetabolic effect.

The hypothesis of adenine synthesis as the site of action for the antimetabolite may also be supported by the fact that the combination of aspartic acid and glycine overcame the suppressed dry matter digestibility second only to the mixture of aspartic acid, glycine, and glutamine. Glycine and aspartic acid actually become part of the adenine molecule during adenine biosynthesis; therefore, a hindered adenine synthesis is

more apt to respond to these amino acids. Of the fourteen recognized enzymatic reactions involved in synthesizing adenine from ribose-5-phosphate, glutamine provides amide groups for two reactions, a glycine molecule is attached to a substituted ribose-phosphate in another reaction, and the aspartic acid molecule is incorporated in two other reactions.

The beneficial role of amino acid additions might be linked to volatile fatty acid content of fermentation media. Bryant and Robinson (1963) found that a considerable portion of dietary protein and amino acids are broken down to $NH_3$, $CO_2$, and volatile fatty acids. Levels of volatile fatty acids were found to be reduced with plants of poor digestibility; and since amino acids are readily converted to fatty acids by rumen microorganisms, the subsequent increase in fatty acid concentration after the addition of the amino acids could serve as a stimulation to microbial breakdown of plant material. Bryant and Robinson (1962) observed that acetate was important in the nutrition of many ruminal bacteria and in fact stimulated several species of cellulolytic bacteria when in high concentration. This probable explanation of the antidoting effect is, however, somewhat refuted by the ineffectiveness of the other amino acids to completely overcome suppressed digestibility. Amino acids such as alanine, valine, and leucine can be readily converted to propionic and isovaleric acids, two fatty acids shown by Wegner and Foster (1960) to be essential for ruminal bacteria.

Additions of nicotinamide and nicotinic acid, vital components of the DPN molecule, were antagonistic to digestibility of all clones in this test. Porter (1961) indicated that rumen bacteria are capable of synthesizing an adequate nicotinic acid supply and are not dependent

upon exogenous sources. Therefore, levels of toxicity must have been reached when more niacin was added to the fermentation media. Tryptophan, a precursor of nicotinic acid in bacteria, also caused reduced digestibility of forages, further substantiating the niacin-toxicity statement above.

Results of alfalfa shoot assays, while surprising, illustrated that the antimetabolic principle can adversely affect the very plant that elaborated it. Although only gross symptoms of the antimetabolic effect were recorded, it was quite evident that the alfalfa cuttings were being altered physiologically by the development of a characteristic chlorotic pattern. An antidote effect was again indicated by glutamine, glycine, and aspartic acid when the expression of the chlorotic condition was delayed for approximately thirty hours in the leaves of shoots immersed in water extracts from $F_2$ plants with poor nutritive value.

## CONCLUSIONS

These studies provided the following conclusions:

1. Certain alfalfa plants contain antimetabolic substances which interfered with the growth of weanling voles and dry matter digestion by rumen microorganisms.
2. The antimetabolic principle found in alfalfa plants of low nutritive value but not in plants of high nutritive value reduced the cellulolytic activity of rumen bacteria as indicated by lower volatile fatty acid production and less cellulose digestion.
3. Water extracts of alfalfa plants of low nutritive value contained antimetabolic agents which markedly reduced the number of Gram-negative cocci bacteria present in the rumen inoculum. In contrast, water extracts of plants of high nutritive value did not affect the rumen bacteria.
4. Water extracts of plants of low nutritive value suppressed dry matter digestibility, but water extracts of plants of high nutritive value had no suppressive effect on digestibility.
5. Water extracts of plants of low nutritive value caused the development of chlorotic symptoms of alfalfa shoots after 44 hours, whereas shoots in extracts from plants of high nutritive value did not develop chlorosis.
6. Reductions in dry matter digestibility and cellulolytic activity occurred in the first six hours of association between plants of low nutritive value and rumen inoculum.

7. Glycine, glutamine, and aspartic acid when added engroup were an effective antidote of the antimetabolic effect.
8. The coenzymes DPN and DPNH partially antidoted the antimetabolite effect of plants of low nutritive value.
9. Materials ineffective as antidotes of the antimetabolic effect were vitamin mixtures, niacin, adenine, and combinations of amino acids other than glycine, glutamine, and aspartic acid.
10. The genetic system which controls the occurrence of the antimetabolite appeared complex.

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