

CHARACTERIZATION OF CERTAIN
NEGATIVE COMPONENTS LIMITING
NUTRITIONAL EFFICIENCY FROM ALFALFA

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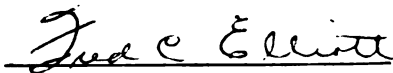
Characterization of Certain Negative Components
Limiting Nutritional Efficiency from Alfalfa

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ABSTRACT

CHARACTERIZATION OF CERTAIN NEGATIVE COMPONENTS LIMITING NUTRITIONAL EFFICIENCY FROM ALFALFA

By

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Alfalfa as a forage legume has been recognized as being highly nutritious with satisfactory protein quality and rich in vitamins and minerals. Alfalfa has been used directly as a forage for animal production and in human consumption and has excellent potential as a source of extracted leaf proteins. The presence of negative nutritional components has reduced its maximum utilization in animal diets. One class of toxic or antimetabolic substances includes the saponins.

Weanling vole bioassays of "Team" alfalfa fed varying saponin levels, as determined by hemolytic activity of water extracts, showed that as saponin content increased, adverse physiological effects, eventually resulting in death, occurred. Histopathological changes included fatty metamorphosis and necrosis of the liver and pancreatic atrophy. Disc electrophoretic studies of liver homogenates revealed changes in liver proteins with increased saponin intake. Postmortem examination of selected organs indicated that cecal weights were increased at all levels of saponin intake when compared to animals fed control diets. Additional assays with low saponin alfalfa cultivars indicated that there were no obvious qualitative differences with

respect to nutritional quality. High saponin cultivars resulted in reduced food consumption, decreased weight gain, and death at the highest levels of intake.

Determination of saponin levels in 3 non-hardy alfalfa cultivars revealed that these were generally low in saponin when compared with winter-hardy cultivars. None of the seedlings sampled in these previously unselected populations were high or very-high in hemolytic activity.

Evaluation of 3rd and 4th cycle alfalfa seedlings selected bidirectionally for saponin showed genetic advance for low saponin to be .70 and .48 for high saponin. Heritability in the broad sense for low saponin populations was 79.69% and 49.38% for high saponin. Intensive testing for hemolytic activity showed that saponin levels increased as the seedlings developed to flowering stages. Saponin content of regrowth was similar to that observed in leaves prior to being cut. High saponin selections had increased numbers of stems and flowered earlier than low saponin selections.

Analysis of 4th cycle F_1 seedlings to determine the genetic nature of saponin inheritance by use of the Jinks-Hayman diallel technique showed that there is epistatic gene action, namely complementary and duplicate genes in F_1 plants with respect to saponin content. There was indication that high saponin is recessive and low saponin is dominant. Heritability in the broad sense was 93.0% for low saponin and 79.0% in the narrow sense. Direct analysis of data for hemolytic activity of 4th cycle F_1 alfalfa seedlings supported results obtained from the diallel analysis indicating that a complex mechanism was involved in the inheritance of saponin with a minimum of 2 genes being involved.

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INTRODUCTION

The world food crisis necessitates the production of high yielding plant foodstuffs of high nutritional quality.

Alfalfa, as a forage legume, has been recognized as being highly nutritious with satisfactory protein quality and rich in vitamins and minerals. Alfalfa has been used directly as a forage for animal production and in human consumption and has excellent potential as a source of extracted leaf proteins. However, the presence of negative nutritional components has reduced its maximum utilization in animal diets. One class of toxic or antimetabolic substances includes the saponins. These have been implicated in causing adverse physiological responses in animals ingesting alfalfa.

This investigation was aimed at observing the physiological effects of alfalfa cultivars with differing saponin levels by using the weanling vole bioassay. Saponin content of non-hardy alfalfa cultivars was determined. Tentative estimates of heritability and genetic advance were made on alfalfa selected bidirectionally for saponin content and observations made on agronomic characteristics. And determinations were made on the nature of inheritance of saponin content with 4th cycle F_1 seedlings.

LITERATURE REVIEW

Alfalfa, a highly nutritious forage legume, has traditionally been used as a dietary constituent for ruminants and, to a limited extent, for humans (Sur, 1961). However, due to the presence of negative nutritional components which affect growth, its positive qualities are not being utilized to the maximum extent.

Criteria for Nutritional Quality

Evaluation of nutritional quality involves consideration of the organism being fed. When assessing forage quality for ruminants, the nutritive value of the plants consumed must be assessed by determining factors which affect the chemical composition of a given plant species, the botanical composition and nutrient content of the animal's diet and the feeding of supplements to correct dietary deficiencies or toxicities (Harris *et al.*, 1969). According to McCullough (1957) the measurement of forage intake and digestibility provides a necessary measure of nutrient and protein available for assimilation in the animal. Animal production is significantly affected by relative changes in dry matter disappearance, DMD, which in turn reflects nutritional changes. Gil *et al.* (1967) stated that the nutritive value of forages is a function of chemical composition and digestibility. Digestion determines the proportion of feed utilized by the animal and controls to some degree the extent and the rate of intake.

Criteria for determining nutritional quality of diets fed to monogastric animals are more complex. Since at least 9 of the 20 or more amino acids that make up proteins have to be provided in the diet, there is not just one requirement of protein but at least 8 separate requirements for individual amino acids. Analyses of nutritional quality are further complicated by the presence of accompanying antimetabolic substances.

Antimetabolites of Alfalfa

Even though the nutritive profile of alfalfa is very good, the presence of toxic and antimetabolic substances has reduced its efficiency as a dietary component. To compensate for these deleterious compounds, reduced amounts of alfalfa are used in animal diets, thereby minimizing the value of available nutrients. Other complicating factors are the possible interactions and associations that these toxic or antimetabolic substances may have with one another.

Vitamin inhibitors. Burnell (1957), using a chick bioassay, showed that approximately one-third of the alpha tocopherol present in alfalfa was available to the chick. Pudelskewicz and Matterson (1960) confirmed the study and isolated compounds in hot ethanol extracts of alfalfa that acted antagonistically to tocopherol. This vitamin E inhibition in alfalfa also occurred in Holstein calves when they were fed dehydrated alfalfa (Eaton *et al.*, 1958).

Uterotropic activity. The uterotropic activity of alfalfa was demonstrated by using the mouse uterine weight assay technique (Pieterse and Andrews, 1956). Intake of estrogens may produce adverse effects in reproduction (Cheng *et al.*, 1957).

Protein inhibitors. The presence of a trypsin inhibitor in alfalfa was first described by Ramirez and Mitchell (1969). They isolated and partially purified a noncompetitive, relatively heat stable factor with trypsin inhibitory activity. A somewhat similar product was observed by Mooijman (1965). He described this as a saponin-amino acid complex.

Jackson and Shaw (1959) found a cholinesterase inhibitor in crude alfalfa extracts. Physiological implications of these in animals are not known. Burns (1963) measured cellulase activity with carboxymethyl cellulose. When using water extracts of alfalfa, inhibitors were present.

Miscellaneous effects of alfalfa antimetabolites. Chury (1969) fed female rabbits fresh alfalfa forage for 36 days, and guinea pigs for 16; rats also had alfalfa included in their diet. He noted depression of ovary function and inhibition of ovulation. Follicular and endometrial cysts formed in guinea pigs which persisted for 4 weeks after alfalfa was withdrawn. The substance responsible for depression of fertility was not an estrogen.

Recently, attention has been given to lipoproteins and protein fractions of alfalfa as causative factors in bloat syndrome (MacArthur *et al.*, 1966).

Saponins. As a group the saponins of alfalfa have been studied more extensively than any other group of toxic substances present in the plant. Very little is known of the purpose they serve in the plant. McNair (1932) reported that saponins, like alkaloids, show a reduction in toxicity from tropical to temperate environments. Also there appears to be a decrease in the number of carbon atoms as saponins become less

toxic. The bitter taste imparted to plants by saponins may have a role in protection against insects and other predators (Birk, 1969).

Saponins in general have been implicated in the production of adverse physiological effects in a wide range of animals. Gorisek (1963) attributed metabolic disturbances in milk cows to the ingestion of beet leaves and other oral saponins. Georgiev (1957) studied the role of saponins in causing vesicle hematuria in cattle. Pernicious anemia in horses due to saponin ingestion was studied by Abderholden and Freil (1909). Dollahite *et al.* (1962) injected cows, rabbits and goats intravenously with *Gutierrezia* sp. saponins. The majority of animals injected died or aborted as a result of the saponin injection. Saponin increased the calcification effects of large doses of vitamin D and blood calcium. Saponin present in *Atalaya hemiglauca* killed 6 healthy horses. The resultant disease (walk-about disease) caused pathological liver changes. The author suggested that various obscure stock diseases may be due to the repeated ingestion of plants containing saponins (Murnane, 1928).

Brune (1961) fed a number of saponins, including those from alfalfa, to young rats. Sublethal doses retarded body weight gains; bone growth was also retarded. In addition, decalcification of bones occurred, probably as a result of impaired calcium absorption.

Effects of alfalfa saponins on poultry. The effects of alfalfa saponins on poultry have received extensive attention. Draper (1948) noted that as levels of alfalfa were increased from 5 to 15 percent in chick diets, there was a significant decline in weight gain. Cooney *et al.* (1948) confirmed the preceding and stated that since other experiment stations have reported better results, they were

inclined to believe that there were differences in alfalfas of similar quality. They concluded that there must be unidentified factor(s) which effectively reduced growth when fed above 10.0 percent. This negative effect could occur by rendering the feed unpalatable or by an actual growth depressing effect. Heywang (1950) noted that in addition to weight gains, egg production was reduced as alfalfa levels were increased in the diet. Peterson (1950) presented evidence that implicated saponins as the causal factor(s). He obtained fractions from aqueous extracts that had hemolytic properties and foaming action. Wilgus and Madsen (1954) fed 100 samples of alfalfa meals of known history; they showed varying response with respect to growth depressing effects. Interestingly, meals containing less than 5 percent alfalfa appeared on occasion to improve early growth markedly, but as the levels were increased, growth was proportionately decreased.

Effects of saponin on the bloat syndrome. Saponins have been reported as a causative agent of bloat in ruminants grazing legume pastures (Quinn, 1943; Henrici, 1952). Olson (1944) stated that animals known to bloat ate plants containing saponins that caused froth and foam in the rumen. Lindhal (1954) implicated alfalfa saponins in the etiology of bloat, by producing it experimentally by intraruminal and intravenous dosing with extracted saponins. They observed an increase in plasma cholesterol values and this was highly correlated with the severity of bloat. The occurrence of bloat was highly correlated with *in vitro* inhibition of muscle respiration produced by a fraction of the forage. Coulson and Davis (1962) observed qualitative differences between bloat producing and non-bloat producing herbage.

Clary *et al.* (1960), studying the alfalfa inhibitor, showed that some alfalfa extracts possessing hemolytic activity did not depress the growth of chicks; other extracts showing weak hemolytic activity did depress growth. There was no correlation necessarily between the saponins of alfalfa and its growth depressing activity. The alfalfa inhibitor was theorized to be a saponin-like compound. Significant amounts of slime production from the degradation of legume saponins by rumen bacteria may be a factor in bloat when animals graze on legume pastures. Guterrez and Davi (1959) isolated both gram-positive and gram-negative organisms capable of attacking and degrading alfalfa saponins.

Relationship of saponins and a respiratory inhibitor. Jackson and Shaw (1959) isolated a respiratory inhibitor of muscle from alfalfa; this inhibitor resembled saponins or a mixture of saponins. Further work resulted in obtaining purified saponin that showed that respiratory inhibition was in the material produced. McNairy (1963) stated that the correlation between a respiratory inhibitor and bloat was not consistent. Chromatography of hydrolysates showed differences in sugars present in samples of the alfalfa cultivars "Vernal" and "Culver." Thus, the quantitative and qualitative levels of alfalfa saponins may be critical in establishing a correlation between the two factors.

Other negative components of alfalfa. Antimetabolic activity of water soluble extracts as manifested by growth inhibition of weanling meadow voles was shown by Schillinger and Elliott (1966). The addition of glycine, aspartic acid and glutamine overcame the growth depressing effects of the voles. Elliott (1963), also using weanling meadow voles, improved nutritional response by supplementing alfalfa diets with niacin.

Incidence of alfalfa saponins. Jaretzky (1934) studied 29 species of *Medicago* for saponin levels: *M. sativa* produced the most saponin; *M. falcata* and its hybrids, *M. arborea*, *echinus*, and *lupulina*, were also high in saponin. Species low in saponin included *lappaceae*, *litoralis*, and *minimum*. He noted that in some species saponins were localized in roots or shoots.

Hansen *et al.* (1963), studying saponin levels, found that "Lahontan" was the lowest in saponin, followed by "Buffalo" and "Vernal." "African", "DuPuits", and "Tourner 501" varied with location. On the average, first cuttings were low in saponin with the second and third cuttings approximately equal. Negative correlations were found between saponin content and yield and saponin content and defoliation. Positive correlations for cutting resulted from low saponin and protein content. However, negative correlations with respect to protein content and saponin level were obtained with "DuPuits." There was a significant negative correlation among variety means resulting from "Lahontan" and "Ranger" which were higher in fiber and lower in saponin. Correlations between fat and saponin among variety means and among cuttings were highly significant and positive. He stated that saponin content might be more easily altered by breeding due to small environmental effects.

Pedersen *et al.* (1967) found differences among saponins from "DuPuits", "Ranger", "Lahontan" and "Uinta" alfalfas in toxicity. "DuPuits" was the most toxic for the chicks. "Lahontan", even though low in toxicity with plant assays, was highly toxic to chicks.

Differences in saponin levels exist between plants, varieties, locations, and cuttings. The mean saponin content, the seasonal average of 4 cultivars--"Buffalo", "Lahontan", "Ranger", and "Vernal"--was between 2-3 percent of the total dry matter. Hardy varieties

appeared to be higher in saponin than non-hardy ones (Hansen *et al.*, 1963).

Characterization of alfalfa saponins. Isolation and fractionation of alfalfa saponins was difficult until paper electrophoretic techniques showed at least 4 constituents to be present and paper chromatography showed 6 (Coulson, 1957, 1958). Lindhal *et al.* (1957) observed that alfalfa saponins were more complex. Later, Van Atta and Guggolz (1958), by using paper chromatography, partially resolved the triterpenoid glycosides. Coulson and Davis (1967), by using even more sophisticated methods, showed 10 to 12 constituents of alfalfa saponins.

Pedersen *et al.* (1966) separated saponin preparations from different varieties into 5 fractions each and found that corresponding fractions of "Lahontan" and "DuPuits" differed in Rf value, color reaction and in biological activity. Using thin layer chromatography, Birk *et al.* (1968) showed that certain alfalfa fractions from root and shoot could be separated into 8 distinct fractions. Root fractions were higher in saponin than those from the top of the plant.

Hydrolysis of mixed alfalfa saponins resulted in a mixture of triterpenoid saponins, sugars and uronic acids (Van Atta, 1962). Among the sugars identified were glucose, arabinose, xylose and, in lesser amounts, galactose, rhamnose and galacturonic acid (Walter, 1954). Differences in type and amount of saponins makes definitive statements difficult about the possible interactions and biological effects that may occur upon ingestion.

Genetic studies involving saponin levels in alfalfa. Because alfalfa saponins have no feed value, low saponin alfalfa would be desirable (Birk, 1969). By plant breeding methods, quality and quantity

of saponins in alfalfa may be modified and thereby increase the potential for alfalfa meal usage; also, better evaluation of alleged anti-metabolic or toxic properties attributed to saponins are possible when distinct populations exist. Varieties with high and low amounts and with stabilized physiological characteristics would be desirable.

Hansen *et al.* (1963) directed attention to possible programs in lowering alfalfa saponins by plant breeding. Pedersen (1967) also showed quantitative differences between varieties and reported higher concentrations of saponins in the leaf and stem. He suggested that saponin content is genetically controlled and modification by selection should not be difficult.

Jones (1969), using *M. sativa* populations, estimated heritability to be "moderate-high." In one population transgressive effects were exhibited. He also stated that the genetic systems controlling saponins were equally well expressed in leaf and stem and concluded that the fixation of low and high saponin populations should be rapid if the estimates of heritability were correct.

Methods of assaying for saponin content. Even though isolation and identification of alfalfa saponins are difficult, adequate *in vitro* techniques for assaying saponin content have been developed. Methods include a gravimetric method for approximate determination, chromatography, foaming, surface tension measurements, formation of molecular compounds with cholesterol and other hydroxy steroids (Van Atta *et al.*, 1961).

The hemolysis test has been used by a number of investigators to identify saponins in alfalfa (Jaretzky, 1943; Peterson, 1950; Jones, 1969). Saponins increase the permeability of the cell wall with the

increased amount of saponin added. When this increased permeability reaches a certain degree, perceptible laking takes place. Also, the lytic action of saponins on red blood cells is probably due to the penetration of lipoid-lipoid-protein constituents of the membrane. Surface activity of saponins may dissolve fatty materials or may denature proteins in the cells' surfaces, leaving holes large enough for hemoglobin molecules to diffuse (Bangham and Horne, 1962).

In addition to chemical analyses, bioassay techniques have been used to determine the presence of saponins in alfalfa (Scardavi and Elliott, 1967). Microbiological methods included the *Trichoderma* sp. test. With increasing concentrations of saponin, inhibition of mycelial growth occurs (Pedersen, 1967; Scardavi and Elliott, 1967). Chick bioassays generally are used to measure growth depression effects of saponins (Cooney *et al.*, 1948; Heywang, 1950; Peterson, 1950). Fish bioassays using *Pimephena* sp. and minnows indicate saponin levels with respect to time required for immobilization to occur (Johnson, 1969; Jones, 1969).

The use of the meadow vole, *Microtus pennsylvanicus*, as a bioassay for forage quality was suggested by Elliott (1963). Because of their small size, fast growth rate, ability to survive on high fiber diets and small daily food intake, they are ideal for evaluating nutritional quality and for determining the presence and effects of toxic or anti-metabolic substances in plants. Elliott (1966) also recognized the necessity of incorporating various analytical procedures to aid in selection of breeder lines. Chemical tests would be employed for initial screening of selections. However, final judgment of nutritional quality should be based on animal performance.

The survey of literature indicates that there is agreement as to the possible negative effects of saponins in alfalfa even though quantitative and qualitative differences may result in discrepancies. Research dealing with inheritance and genetic as well as physiological properties has been limited to date and has been carried out with materials in early cycles of selection. It would be desirable to see what the agronomic and genetic properties of alfalfa selected for both high and low levels of saponin have on nutritional quality.

MATERIALS AND METHODS

I. Nutritive Value of Alfalfa Clones Selected for High and Low Saponin as Determined by *in vitro* Analyses and Weanling Vole Bioassays

Plants used in this study were obtained from *M. glutinosa* and *M. sativa* cultivars "Vernal", "Team", "Saranac", "DuPuits" and a breeding line "Culver"/"DuPuits." All of these populations had been established previously. Harvest of 98 individual clones was made when the plants were in one-tenth bloom. The individually harvested plants were dried at 100 to 110 F, ground to pass through a 1/2 mm screen and stored at approximately 5 C. Plants were weighed at the time of harvest and after drying to get an estimate of yield.

Since this material was to be used in weanling vole feeding studies, it was necessary to get quantitative estimates of saponin levels. For this purpose, a hemolysis test using a standard serial dilution technique was developed. One gram of ground plant material was placed in a paper cup and 8 cc of physiological saline was added to each. After the contents of the cups were mixed, they were left at room temperature for 2 hours. The alfalfa water extracts were filtered through Whatman #2 filter paper into an empty test tube. For each evaluation, a series of 7 tubes was used. Each test tube contained .25 cc saline. One cubic centimeter of concentrated plant extract was placed into the first tube, .5 cc of the contents of the first tube, after mixing, was transferred serially to the 6 additional test tubes. To all the test tubes, a .5 cc saline solution of 2 percent human red

blood cells, Type O+ , was added. The tubes were then shaken and incubated at 37 C for one-half hour in a water bath. The hemolytic activity or saponin content of the sample was the reciprocal of the dilution of the last test tube in which 100 percent hemolysis was observed. After quantitatively determining saponin levels in the individually harvested plants, they were composited according to variety and hemolytic activity so that adequate plant material would be available for feeding trials.

Nitrogen, ash, and moisture were determined according to AOAC methods (1964). Neutral detergent fiber, NDF, acid detergent fiber, ADF, acid detergent lignin, ADL, were determined according to methods described by Van Soest (1963) and Van Soest and Wine (1967). Estimates of hemicellulose and cellulose were obtained by subtracting NDF-ADF and ADF-ADL, respectively. These values represented compository characteristics of cell walls of alfalfa clones with varying hemolytic activity.

Alfalfa composites were further analyzed by the 6-hour *in vitro* Dry Matter Disappearance Test, (6-H-DMD), described by Allinson (1966). This method was a modification of methods of Bowden and Church (1962), Baumgardt *et al.* (1962) and Ingalls (1964). One run of each sample was performed because of small standard deviations between samples (Shenk, 1969).

Amino acid analyses of selected samples were performed on 70-hour acid hydrolysates by separating amino acids by column chromatography and quantitatively determined by automatically recording color intensity produced by the ninhydrin reaction with a Beckman 120 C Amino Acid Analyzer.

After completion of chemical analyses, the diets were formulated. Composition of the experimental and control diets is shown in Table 1. In order that quality determinations reflect only the presence of toxic substances, the diets were adequate for growth and maintenance of the weanling voles. Diets were made up according to methods described by Elliott (1963) and Shenk (1969).

Table 1. Formulation of experimental and semisynthetic control diets for weanling vole bioassays expressed as percent

Ingredient	Experimental Diet	Semisynthetic Control Diet
Alfalfa	75.0	---
Casein ^a	---	14.0
Carbohydrate mix ^b	18.0	34.5
Alpha-cellulose ^c	---	42.5
Vitamin mix ^d	2.0	2.0
Mineral mix ^e	3.0	3.0
Corn oil	---	2.0
Cellulose gum ^f	2.0	---

^a"Vitamin free" casein, 14.5 percent nitrogen, Nutritional Biochemicals Corporation, Cleveland, Ohio.

^bCarbohydrate mix comprised of 50 percent corn starch, 25 percent dextrin, 25 percent sucrose.

^cAlpha-cellulose, Nutritional Biochemicals Co., Cleveland, Ohio.

^dVitamin Diet Fortification Mixture, Nutritional Biochemicals Corporation, Cleveland, Ohio.

^eSalt Mixture W, Nutritional Biochemicals Corporation, Cleveland, Ohio.

^fCellulose gum, type 7HF, Hercules, Inc., Wilmington, Delaware.

In the feeding experiments, weanling voles were used ranging in age from 12 to 14 days and in weight from 11.5 to 13.0 grams. Animals were housed in individual plastic-bottom cages with corncob bedding and nonabsorbent cotton for nesting. Diets were placed in specially designed feeders (Shenk, 1969). Food and water were available *ad libitum* during the experimental period. Daily observations were made on the general appearance and health of the animals. At the end of the experimental period, which was designated as 6 days, weight gains and food consumption were determined.

Experiment 1. Nutritive Value of Alfalfa Composites of the "Team" Variety Containing Varying Levels of Saponin as Determined by Weanling Vole Bioassay

The availability of large amounts of material of "Team" alfalfa made it possible to determine if qualitative differences could be demonstrated in saponin activity when diets were fed to weanling voles.

Alfalfa diets made up from composites of "Team" alfalfa contained 5 levels of saponin. Hemolytic activities of the diets were 2, 4, 8, 16, and 32. There was a twofold difference in saponin concentration between successive diets with a total 32-fold difference between the highest and lowest with respect to saponin concentration. Feeding trials were to last for 6 days with food and water being available *ad libitum*. Daily observations were made on food intake, weight gain, and general appearance of the animals. Control animals were fed a semisynthetic diet containing 14.0 percent casein as the protein source that approximated the composition of the alfalfa diets. At the end of the experimental period tissue samples were obtained, fixed in 10 percent formalin, and studied for histopathological changes. A nested design was used to assign diets to 5 litters totaling 30 weanling voles.

Experiment 2. Effects of Feeding "Team" Alfalfa with Varying Levels of Saponin on the Organ Weights of Weanling Voles

Due to limited amounts of "Team" alfalfa with high saponin levels, only 3 composites were fed to weanling voles. Hemolytic activities of the alfalfa used in the diets were 2, 16, and 64. Semisynthetic diets containing 14.0 percent casein were fed to control animals. Weanling voles were divided into 4 groups of 4 animals each. Daily observations were made on weight gain and food intake. In order to obtain organs from animals that had been on the experimental diets the same number of days, animals were subjected to euthanasia at the end of 3 days or when it appeared that the first mortality would occur. Selected organs were excised, blotted, and weighed.

Experiment 3. Disc Electrophoretic Studies of Liver Extracts from Weanling Voles Fed Alfalfa Composites of "Team" Alfalfa Containing Varying Levels of Saponin

A total of 18 animals representing 3 litters were fed "Team" alfalfa containing 6 levels of saponin. Hemolytic activities of the diets were 2, 4, 8, 16, 32, and 64. In addition, 3 control animals were fed the standard semisynthetic diet. Food and water were available *ad libitum*. After 3 days, surviving animals were sacrificed and livers were immediately removed and quick frozen in dry ice. For purposes of electrophoresis, the livers were homogenized in a tissue grinder and centrifuged. Following centrifugation, the supernatant fluid was removed and analyzed according to methods of Ornstein (1964) and Davis (1964).

Experiment 4. Nutritive Value of Alfalfa Composites Containing Low Levels of Saponin

Alfalfa diets formulated from forage containing low levels of saponin were evaluated for nutritive quality by using the weanling vole. Forage composites with hemolytic activities of 2, 4, and 8 were

designated as low saponin. The cultivars tested included "Vernal", "Culver", "DuPuits", "Saranac" and *M. glutinosa*. Daily observations were made on intake, weight gains and general appearance of animals. A nested design was used to assign diets to 5 litters totaling 20 animals.

Experiment 5. Nutritive Value of Alfalfa Composites Containing High Amounts of Saponin

In order to evaluate the nutritive quality of alfalfa composites of varieties high in saponin, experimental diets of "Vernal", "Saranac", "DuPuits", *M. glutinosa* and a breeding line "Culver"/"DuPuits" were fed to weanling voles. High saponin forage was designated as that with hemolytic activities of 16, 32, and 64. Food and water were available *ad libitum*. Daily observations were made on food intake, weight gain and general appearance of the voles. Postmortem examination was performed to see if gross morphological changes occurred as a result of ingestion of the experimental diets.

II. Saponin Levels of Non-Hardy Varieties

Investigators have mentioned that non-hardy alfalfa varieties appear to be lower in saponin than those that are winter-hardy (Jones, 1969). To verify this, 3 varieties developed from *M. sativa* populations were tested for hemolytic activity. They included "African"-Tillman (Africa), "Mesa Cirsa" (India) and "UC 64", a USDA selection grown at Michigan State University to observe first year growth. None of the varieties had been previously tested for hemolytic activity.

Two hundred seedlings of "African"-Tillman, 205 of "UC 64" and 167 seedlings of "Mesa Cirsa" were grown in the greenhouse. Approximately one month after planting, the seedlings were sampled. Three sets

of fully expanded leaflets were taken from the upper, middle, and lower parts of the plants. These were placed in a 3 mm test tube and dried at 45 C. The plant material was then crushed with a glass rod. Two cubic centimeters of a 2.0 percent saline solution of human red blood cells, Type O+, were added to all tubes. The test tubes were shaken 2 times and incubated at room temperature. Those showing 100 percent hemolysis were designated as very high in saponin. After 45 minutes, the tubes were read again; 100 percent hemolysis at this time was recorded as "high", tubes with no observable lysis were designated as "low". Intermediate levels were designated as "medium-low" or "medium-high" depending on the amount of lysis as evidenced by hemolysis. A cherry-red color resulting from the release of hemoglobin from the destruction of many blood cells was "medium-high", and a slight pink color of the saline as the blood cells settled to the bottom of the tube was read as "medium-low."

III. Determination of Saponin Levels and Observations of Growth Characteristics of 3rd and 4th Cycle Alfalfa Seedlings for High and Low Saponin Content

In order to evaluate progress in the bidirectional selection for saponin content, 300 seedlings each of low and high saponin 3rd cycle selections and 300 seedlings each of high and low saponin selections from the 4th cycle were grown in the greenhouse. Saponin content as indicated by hemolytic activity was determined during the early growth period and during a later vegetative one approximately one month after the initial determination. Hemolytic activity was determined as described in Part II (Saponin Levels of Non-Hardy Varieties).

Heritability estimates and genetic gain were estimated by using the following formulae (Cochran, 1954).

Genetic advance:

$$\Delta G = \frac{\mu_1 \mu_2}{\sigma}$$

where μ_1 = mean of unselected population

μ_2 = mean of selected population

σ = pooled standard deviation

Heritability:

$$h^2 = \frac{\Delta G}{z/b}$$

where $z = f(t) = \frac{1}{\sqrt{2\pi}} e^{-\frac{t^2}{2}}$

$$b = \int_t^{\infty} f(x) dx = \int_t^{\infty} \frac{1}{\sqrt{2\pi}} e^{-\frac{x^2}{2}} dx$$

$$\begin{aligned} z/b &= \frac{f(t)}{\int_t^{\infty} f(x) dx} = \frac{F'(t)}{\int_t^{\infty} F(x) dx} \\ &= \frac{d}{dt} \log \int_t^{\infty} \frac{1}{\sqrt{2\pi}} e^{-\frac{x^2}{2}} dt \end{aligned}$$

The progress realized in any one cycle of selection depends on the amount and nature of genetic variability, the heritability and nature of the screening procedures used. Increases in genetic variability, by providing greater opportunities for selection, allows the establishment of larger selection differentials (Asay, 1970).

Two hundred five plants, from the original 1200 seedlings of the 3rd and 4th cycle selections, were separated for further intensive study. Whenever possible these plants represented all hemolytic classifications from each of the 4 original groups, with emphasis being placed on those that were low in saponin. These plants were evaluated for hemolytic activity 5 more times during the next 4 months. All stages of development, including flowers, were tested.

Saponin content of flowers was determined by removing a completely developed raceme, drying it at 45 C, crushing the flowers with a glass rod and proceeding with the analyses in the usual manner. Hemolytic activity of regrowth was evaluated by cutting the plants after maximum vegetative growth and sampling them again when the regrowth was approximately equivalent to the first growth of the seedlings.

In addition to saponin content, plants were observed for degree of flowering at each sampling. At the final test period, measurements were also made on the number of stems and length of longest stem to see if there were any relationships between these characteristics and saponin levels.

IV. Inheritance of Alfalfa Saponins as Determined by F_1 Seedlings

For studying the inheritance of saponin in alfalfa, the diallel technique was employed (Jinks, 1954; Hayman, 1959). Six parent lines comprised of *M. glutinosa* and *M. sativa* cultivars of "Culver", "Culver"/"Culver"/"Vernal", "Hardy Moapa", "MSB", a USDA selection, and L₄909 selected in previous cycles for low saponin were crossed in all possible combinations.

F_1 seeds were harvested and one-half of the diallel along with parent seeds were planted in the greenhouse. The F_1 seedlings were sampled 3, 7, and 10 weeks after planting and hemolytic activity determined in the usual manner. A genetic analysis was made by the Jinks-Hayman W_r , V_r regression method for each determination and also for the pooled data. For the analyses, variances of the arrays (V_r) and the covariance of each array (W_r), with the non-recurrent parents were calculated and the regression of the latter on the former was fitted. A regression slope of unity indicates that the basic genetic

assumptions of the analysis have been met. Deviations from the slope of unity means that one or more of the basic assumptions underlying the technique have not been met. Compensatory procedures set forth by Oakes (1967) and Mather (1968) were used in interpretation of the data when the assumptions did not hold true.

The following formulae were used in the calculation of statistics:

$$Vr = 1/4 D - 1/4 Fr + 1/4 H_1 + [E + \frac{1}{\sum n-1}]n$$

$$Wr = 1/2 D - 1/4 Fr + E/N$$

$$Vol_1 = 1/4 D + 1/4 F + 1/4 H_1 = 1/4 H_2 + [E + 1/2 (n-2) \sum d_i^K - n^2]$$

$$Volo = \hat{D} + E$$

$$H_1 = Volo - 4 Wolo_1 - \frac{(3n-2)}{n} E$$

$$Fr = 2 Volo = 4 Vol_1 \frac{2(n-2)}{n} E$$

$$H_2 = 4 Vil_1 = 4 Vol_1 - 2 E$$

$$K = \frac{(Mol - Mil)}{H_2}$$

where:

Vil_1 = sum of variance of each array

$Volo_1$ = sum of covariance of each array with non-recurring parents

Vol_1 = variance of array mean

$Volo$ = variance of parents

Fr = interaction between dominance and additive genetic effects as measured by covariance

D = variance due to additive genetic effects

H_1 = variance due to dominant gene action

H_2 = variance due to dominant gene action - $H_1 [1-u-v]^2$

u = frequency of positive genes

v = frequency of negative genes

K = minimum number of effective factors involved

To complement the diallel analyses technique, direct analyses of the frequencies were made to see if a genotype could be formulated that would explain the observed ratios. A chi-square, goodness of fit test using Yates' correction $\chi^2 = \sum_{i=1}^n \frac{(E_{i1} - O_{i1} - .5)^2}{E_{i1}}$ was used to statistically evaluate the significance of accepting the hypotheses. The Yates' Correction was used because of the small number of observations in some classes.

RESULTS AND DISCUSSION

I. Nutritive Value of Alfalfa Clones Selected for High and Low Saponin as Determined by *in vitro* Analyses and Weanling Vole Bioassays

Experiment 1. Nutritive Value of Alfalfa Composites of "Team" Variety Containing Varying Levels of Saponin as Determined by Weanling Vole Bioassay

In order to evaluate the physiological effects of alfalfa with varying amounts of saponin on weanling vole growth, 5 composites of "Team" alfalfa with hemolytic activities of 2, 4, 8, 16, and 32 were formulated. Prior to being incorporated into diets, the alfalfa meals were evaluated for chemical composition. Amino acid analyses of diets containing varying saponin levels are shown in Table 2.

Amino acid analyses of the 3 alfalfa composites show that there was an increase in aspartic acid and proline in alfalfa meals with hemolytic activities of 8 and 32. The highest saponin meal showed reduction in alanine, leucine, isoleucine, and methionine. The differences noted in amino acid concentrations with changes in hemolytic activity are difficult to ascertain. Wilson and Tilley (1965), using ion exchange chromatography, showed no differences in amino acid content related to stages of growth. No significant differences in amino acid composition could be related to species, variety or maturity (Gerloff *et al.*, 1965; Chibnal *et al.*, 1963). Differences between species appeared to be no more significant than those within a species. Methionine appeared to be the amino acid that varied the most. The presence of negative nutritional components in the alfalfa meals makes

Table 2. Amino acid analyses of "Team" alfalfa composites with hemolytic activities of 2, 8, and 32

Amino acid	Hemolytic Activity 2	Hemolytic Activity 8	Hemolytic Activity 32
	gm residue/ 100 gm sample	gm residue/ 100 gm sample	gm residue/ 100 gm sample
Lysine	1.22	1.10	.98
Histidine	.55	.00	.50
Arginine	.82	.78	.74
Aspartic	3.76	4.94	5.75
Threonine	.42	.39	.40
Serine	.17	.14	.15
Glutamic	2.40	1.32	2.14
Proline	.96	1.37	1.30
Glycine	1.21	1.12	1.01
Alanine	1.46	1.55	.64
Cysteine	.00	.00	0.00
Valine	1.65	1.73	1.57
Methionine	.26	.23	.19
Isoleucine	1.19	1.15	.97
Leucine	1.84	1.39	1.54
Tyrosine	.78	.82	.78
Phenylalanine	1.37	1.42	1.34
Tryptophan	0.00	0.00	0.00

correct evaluation of protein quality difficult as reflected by amino acid analyses. If the saponin effect is not overwhelming, interactions between the two may result in an additive negative effect.

Results of chemical analyses of "Team" composites used in the feeding experiments are shown in Table 3.

Table 3. Chemical composition of "Team" alfalfa composites used in determining growth response of weanling voles

Number of clones/composite	Hemolytic Activity	Average Yield (gm)	Ash %	Ether Extract (%)	Protein (%)
6	2	85.00	7.81	2.61	20.96
6	4	95.25	7.10	2.52	19.74
7	8	109.43	8.10	2.68	20.58
9	16	113.83	7.69	2.83	20.43
4	32	98.75	6.70	3.10	20.74

No significant differences existed between the composites with respect to protein content and saponin level. The same holds true with ash and ether extract content (Table 3). Hansen and Kohler (1961) and Hansen *et al.* (1963), studying saponin content in 5 varieties at 8 different locations, found that in 3 consecutive harvests saponin was significantly correlated with protein, ash and fat. However, Anderson and Pedersen (1971), working with 21 strains of alfalfa, showed data that indicated that no significant differences existed with respect to saponin content and protein even though there may be significant differences among the 6 varieties in protein. Increases in yield are seen as hemolytic activity of the alfalfa composites increases. Observations at the time of harvest indicated that this increase was reflected

by more and longer stems, rather than an increase in the number of leaves. Significant negative correlations between hay yield and saponin content were reported by Hansen *et al.* (1963).

Results of chemical determinations for cell wall constituents and 6 hour dry matter disappearance are shown in Table 4.

Table 4. *In vitro* and chemical values of "Team" alfalfa composites with varying hemolytic activity (values are expressed as percent)

Hemolytic Activity of Composite	6H-DMD	NDF	ADF	ADL	Hemi-cellulose	Cellulose
2	48.84	39.78	31.82	5.69	7.96	26.13
4	46.47	42.85	34.89	6.04	7.96	28.85
8	45.80	39.32	33.87	6.17	5.45	28.42
16	47.12	40.73	34.62	6.36	6.11	28.26
32	45.43	37.81	32.53	5.44	5.28	28.09

6H-DMD = six hour dry matter disappearance test

NDF = neutral detergent fiber

ADF = acid detergent fiber

ADL = acid detergent lignin

Hemi-cellulose = NDF - ADF

Cellulose = ADF - ADL

Results of the 6H-DMD indicate that at high saponin concentrations there is a reduction of digestibility of alfalfa forage. This is probably indicative of adverse effects of saponin on the cellulolytic rumen bacteria used in the fermentation. Schillinger (1965) reported the presence of antimetabolites of alfalfa capable of modifying the bacterial

flora of the fermentation media. Results of gram stains of the media showed shifts in the bacterial population. Bacteria capable of utilizing saponins with the production of slime have been described by Hungate *et al.* (1955). Schillinger concluded that an antimetabolic factor rather than a lignin-cellulose complex found in the low DMD plants was the primary cause for differences in DMD. Analyses of the "Team" composites for cell wall composition showed the only consistent change with respect to hemolytic activity to be with hemi-cellulose content. There were no other significant changes in composition as the level of saponin increased in the alfalfa meals. Reduction of hemi-cellulose with a resulting effect on nutritive value has been alluded to by Shenk (1969). However, there is no conclusive evidence that hemi-cellulose content is directly involved with nutritional quality of forages. Poor growth response of weanling voles fed alfalfa diets with low 6H-DMD at 48.0 percent meal in the diet was attributed to low protein. Subsequent research by Shenk and Elliott (1970) showed that at those protein levels, voles would be expected to give a better response. In addition, differences in weight gains when forages high and low in 6H-DMD were fed was attributed to compositional differences. Since the presence of antimetabolites was not determined, these cannot be ruled out as causative factors for the poor responses noted.

After completion of chemical tests, alfalfa composites were made up into diets and fed *ad libitum* to weanling voles. Average daily weight gains are shown in Figure 1. At the 2 highest saponin levels, death occurred as early as 2 days after the voles were placed on the diets. By the end of the experimental period of 6 days, there was 100 percent mortality in both of those groups. Reduced food intake, as

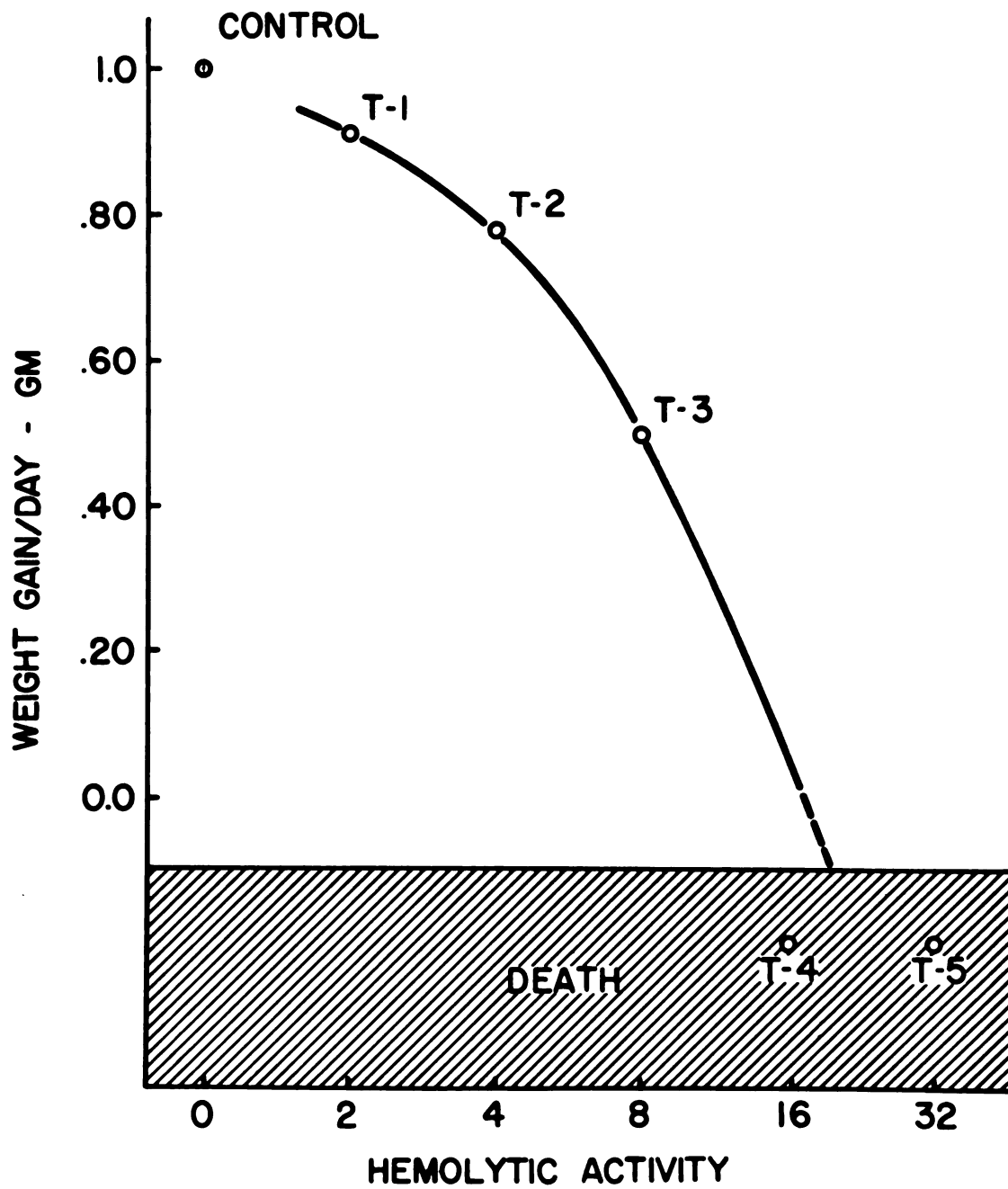


Figure 1. Growth responses of weanling voles fed composites of "Team" alfalfa containing different levels of saponin.

well as a generally poor appearance, was noticed within 24 hours after initiation of the experiment. The reduction of food intake could have been caused by either a disagreeable odor or by the objectionable taste of the diets.

Analysis of variance table for effects on average daily weight gains of voles on diets with 3 saponin levels where no mortalities occurred are shown in Table 5.

Table 5. Analysis of variance table for effects on average daily weight gain of weanling voles fed "Team" alfalfa composites with hemolytic activities of 2, 4, and 8

	df	S.S.	M.S.	F
Total	19	38.94	---	---
Block	4	2.00	.5	1.538 ^{n.s.}
TMT	3	33.04	11.01	33.87 ^{**}
Error	12	3.90	.325	---

^{**}Significant $P < .01$.

From the analysis of variance table, there were highly significant effects with regard to weight gains as a result of ingesting diets containing increasing amounts of saponin. The LSD test of the control diet and the 3 saponin containing ones showed that there were significant differences in total weight gains between voles on diets with hemolytic activities of 2 and 4. Highly significant effects were seen when weight gains of control animals were compared with voles fed diets of hemolytic activities of 4 and 8, and also between diets with hemolytic activities of 2 and 8, and 4 and 8. There were no significant differences in weight gains of voles on the control diet and the diet with a hemolytic activity of 2.

Histopathologic examination of tissues from weanling voles fed "Team" alfalfa diets with hemolytic activity of 8 showed evidence of fatty degeneration of the liver and some additional liver degeneration eventually leading to necrosis. The fatty changes were located in the central areas of the lobules. Other degenerative changes affected all parts of the lobule. These were characterized by granularity of the cytoplasm of the cells, swelling of the cells and loss of normal arrangement of liver cords so that sinusoids were completely obliterated (Figures 2, 3, and 4). Atrophy of the pancreas was observed in tissues of weanling voles fed "Team" alfalfa diets with hemolytic activities of 16 and 32. In addition to smaller pancreas size, the acini were widely separated and appeared to be smaller than normal (Figures 5, 6, and 7).

Pathological changes of the liver and pancreas result in serious physiological disturbances in the animal. The liver not only stores reserve sugars but is essential for transformations which are necessary before some of the sugars can be utilized by the body. In addition, the liver is involved in protein metabolism and is the major site of amino acid deamination. The functions of the pancreas include furnishing of pancreatic juice, a major constituent being trypsin, a powerful proteolytic enzyme which breaks down proteins into amino acids. Also, it elaborates substances and plays an essential role in the regulation of carbohydrate metabolism (Copenhaver and Johnston, 1958).

The results obtained from the *in vitro* chemical analyses and feeding trials indicate that the adverse effects on weight gain in weanling voles fed "Team" alfalfa composites of varying hemolytic activities was due to the effects of antimetabolic substances in the alfalfa. Even though amino acid analysis showed differences in the amino acid composition of 3 of the diets containing 3 levels of saponin, the deaths

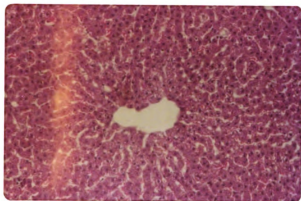


Figure 2. Photomicrograph of a liver section from weanling voles on a semisynthetic casein diet. H & E x 200.

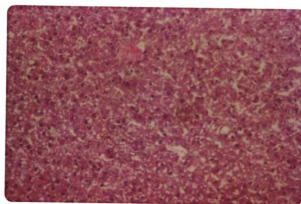


Figure 3. Photomicrograph of a liver section from a weanling vole fed "Team" alfalfa with hemolytic activity of 8. Note fatty degeneration. H & E x 200.

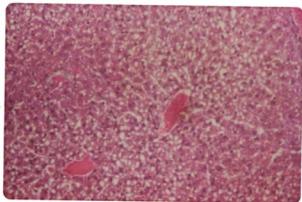


Figure 4. Photomicrograph of a necrotic liver section from a weanling vole fed "Team" alfalfa with hemolytic activity of 8. H & E x 200.

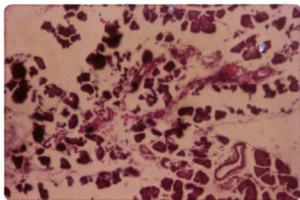


Figure 5. Photomicrograph of a pancreas section from a weanling vole on a semisynthetic casein diet. H & E x 50.

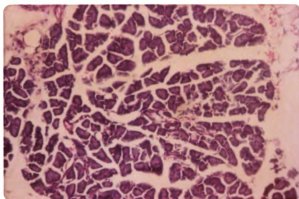


Figure 6. Photomicrograph of a pancreas section from a weanling vole fed "Team" alfalfa with hemolytic activity of 16. H & E x 200.

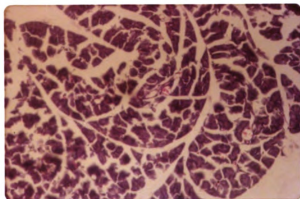


Figure 7. Photomicrograph of a pancreas section from a weanling vole fed "Team" alfalfa with hemolytic activity of 32. H & E x 200.

which resulted cannot be directly attributed to deficiencies in essential amino acids. Chemical composition of the diets with respect to ash and protein can also be ruled out as a cause of poor growth response. Even though differences in ether extract occurred as diets contained higher amounts of saponin, this would not be a critical factor in affecting weight gain. The same appears to be the case when cell wall components are considered. Alfalfa diets formulated from composites of "Team" alfalfa with high levels of saponin, as indicated by hemolytic activity, caused reduction in weight gain, resulting in death at the highest levels of intake. Death was probably due to the cumulative effects of saponins alone or in conjunction with other negative nutritional components of the diets.

Experiment 2. Effects of Feeding "Team" Alfalfa Composites with Varying Levels of Saponin on Organ Weights of Weanling Voles

Ingestion of alfalfa composites with high *in vitro* hemolytic activity caused a toxic response characterized by reduced food intake, weight loss and finally death. Similar results due to toxicity have been obtained by feeding rats soybeans and navy beans (Hewitt *et al.*, 1970). Phadke and Sohonie (1962), feeding field beans to rats, reported that weights of hearts and kidneys increased significantly as compared with organs of control animals on a casein diet. Similar findings were reported by Kakade *et al.* (1965) feeding navy beans to rats.

Effects on the growth rate of weanling voles as a result of ingesting the "Team" alfalfa composites were similar to those seen in the previous experiment (Table 6).

Table 6. Effects of "Team" alfalfa composites on weight gains of weanling voles after 3 days

Alfalfa Composite	Hemolytic Activity	Weight Gain (gm)	Intake (gm)
"Team"	2	+2.5	3.6
"Team"	16	+0.5	2.8
"Team"	64	-1.1	1.0

Weanling voles on casein control diets appeared normal at the end of the 3 days, after which time the animals were sacrificed. However, voles on alfalfa composite diets of hemolytic activity 16 and 64 were emaciated, had rough hair coats and were hunched.

The effects of diets on different organ weights are shown in Table 7.

The data indicate that weights of the cecum were increased at all levels of saponin intake as compared to cecal weights of animals on the casein diet. This increase could have been the result of the type of diet. Cecal contents of voles on the alfalfa diets were consistently more viscous than those of control animals. It is difficult to account for the cecal weights considering the reduced food intake of voles on the alfalfa diets, especially since the ceca appeared to be full. Feeding trials with commercial saponins incorporated into semisynthetic diets gave similar results (Marcarian, 1969). However, both gross observation and histopathological examination did not reveal any abnormalities. The distention of the cecum was attributed to an unknown mechanism that resulted in a type of paralysis that prevented the ingesta from moving down the digestive tract (Keahey, 1969). At

Table 7. Organ weights of weanling voles obtained at autopsy (expressed as percent of body weight)

Organ	Control	Hemolytic Activity-2 wt.gm.	Hemolytic Activity-16 wt.gm.	Hemolytic Activity-64 wt.gm.
Heart	0.65 ± .04	.71 ± .13	.55 ± .03	.48 ± .07
Liver	4.61 ± .52	5.20 ± .37	4.32 ± .73	2.94 ± .20
Stomach	3.74 ± 1.54	2.97 ± .33	3.71 ± 1.45	5.73 ± 1.70
Spleen	0.19 ± .04	.16 ± .007	.11 ± .010	.05 ± .020
R.Kidney	0.62 ± .06	.70 ± .002	.60 ± .040	.59 ± .031
L.Kidney	0.61 ± .09	.67 ± .020	.56 ± .091	.58 ± .020
R.Adrenal	0.030 ± .004	.031 ± .001	.030 ± .001	+.010 ± .001
L.Adrenal	0.020 ± .004	.022 ± .003	.023 ± .003	.022 ± .004
Cecum	0.746 ± 2.33	9.78 ± 1.96	12.32 ± 3.54	12.77 ± 2.05

hemolytic activities of 16 and 64, weights of heart, liver and spleen were significantly less than organ weights of control animals.

Differences in organ weights at the highest level of saponin intake may be due partly to the reduced food intake which resulted in a small amount of available amino acids for growth. However, it cannot be ruled out that growth inhibition effects could be related to saponins or other antimetabolic substances with cumulative effects that interfere with amino acid availability or cause amino acid deficiencies.

Experiment 3. Disc Electrophoretic Studies of Liver Extracts From Weanling Voles Fed Composites of "Team" Alfalfa Containing Varying Levels of Saponin

Electrophoretic techniques have been used quite extensively for clinical purposes (Smith, 1960; Gronwall, 1959; Ribeiro, 1955). These

techniques have proven reliable in aiding diagnosis of many metabolic disorders (Brent, 1954; Hardwicke, 1953; Smith, 1960). However, to date, electrophoresis has not been used in the study of alfalfa saponins on monogastric animals. Cheeke and Oldfield (1970) have been the only investigators to study the effects of alfalfa saponins on liver proteins, these being *in vitro* determinations only, because of limiting amounts of saponin available.

Representative disc electrophoretic gels of liver homogenates from weanling voles fed alfalfa with varying saponin levels are shown in Figure 8. Electrophoretic patterns from livers of control voles show at least 3 major bands and at least 3 minor bands. However, as the amount of saponin was increased, distinct changes in the nature of the electrophoretic bands occurred. The interpretation of effects of saponin at the highest levels of intake are complicated by the reduced food intake which would consequently adversely affect protein synthesis and therefore result in abnormal patterns. Gels of liver homogenates of voles on alfalfa diets with the least saponin show changes also in liver proteins when compared with the controls. Since food intake was comparable with voles on control diets, alterations in liver protein synthesis were probably due to antimetabolic substances in the diet. In T-1, where the hemolytic activity was 2, the "Team" composite with the smallest amount of saponin, the weight gains were equal to the control, and metabolic changes were evident, as reflected by alterations in the electrophoretic bands. Whether this is characteristic of all alfalfa or merely those containing saponin is difficult to say.

Cheek and Oldfield (1970), studying the effects of alfalfa saponins on liver homogenates and mitochondria from rats, found that alfalfa saponin is bound to the succino-oxidase enzyme system and suggested



Figure 8. Representative disc electrophoresis patterns from homogenates of livers from weanling voles on a casein diet and "Team" alfalfa diets with varying saponin content.

the possibility of cumulative effects *in vivo*. Inhibition of enzymes concerned with the citric acid cycle would have significant effects on the efficiency of nutrient utilization and growth in animals.

Inhibitory effects of alfalfa on enzymes associated with mitochondrial energy transport and oxidative phosphorylation have also been reported by Schillinger and Elliott (1966). Addition of coenzymes NAD and NAD⁺⁺ produced increases in dry matter disappearance in F₂ alfalfa composites with poor growth promoting abilities.

To establish precisely the relationships between alfalfa saponins and other fractions with inhibitory enzymatic *in vivo* effects, methods utilizing electrophoretic and histochemical techniques should be utilized. This would lead to more meaningful conclusions regarding metabolic disturbances caused by ingestion of alfalfa saponins.

Experiment 4. Nutritive Value of Alfalfa Composites with Low Hemolytic Activity

Qualitative differences of alfalfa saponins have been demonstrated by the use of paper chromatographic and electrophoretic techniques (Birk *et al.*, 1968; Pedersen, 1963). In addition, Cooney *et al.* (1948) stated that alfalfas of similar quality exhibited differences in physiological effects in chicks with respect to growth response. Results of feeding weanling voles "Team" alfalfa indicated that toxic or antimetabolic effects were enhanced when diets with high hemolytic activity were fed. In order to study effects of low saponin alfalfa, 4 varieties with low hemolytic activity were composited and fed *ad libitum* for 6 days.

Chemical composition of alfalfa composites is shown in Table 8.

Results of chemical tests of alfalfa composites with equivalent hemolytic activity were in agreement with data obtained in Experiment 1.

Table 8. Chemical composition of alfalfa composites with low hemolytic activity

Cultivar	# Clones/ Composite	Hemolytic Activity	Yield (gm)	Ash (%)	Ether Ex- tract (%)	Protein (%)
"Vernal"	7	2	135.0	8.36	2.55	20.84
"Culver"/ "DuPuits"	6	2	88.0	8.04	2.14	20.74
<i>M. glutinosa</i>	6	2	57.6	7.64	2.43	19.50
"Saranac"	2	2	69.0	7.54	2.03	19.44

With the exception of the variety "Vernal", average clonal yields were low, as was previously indicated.

Values obtained for the evaluation of cell wall components are shown in Table 9.

Digestibility of the 4 alfalfa samples with similar hemolytic activities was approximately equivalent. These values were in agreement with those observed for the "Team" cultivar at the same saponin level. Hemi-cellulose content of the composites was fairly uniform with the exception of "Vernal." The lignin content of "Vernal" was approximately the same as "Team" at the same hemolytic activity.

Results of feeding weanling voles alfalfa composites low in saponin are shown in Table 10.

With the exception of the *M. glutinosa* composite, weight gains of weanling voles on low saponin diets were comparable to animals on a semisynthetic casein diet. Even though good growth was observed on these diets, it cannot be categorically stated that low saponin content had no detrimental effects on growing animals. Vacek (1962) has reported that increased weight gains were noted in animals on saponin diets.

Table 9. Values for chemical composition of cell wall components of alfalfa composites with low hemolytic activity

Cultivar	Hemolytic Activity	DMD	NDF	ADF	ADL	Hemi-cellulose	Cellulose
"Vernal"	2	49.75	39.06	33.36	5.74	5.78	27.62
"Culver"/"DuPuits"	2	48.11	41.37	34.70	6.24	6.67	28.46
<i>M. glutinosa</i>	2	48.84	39.78	31.82	5.69	6.96	26.13
"Saranac"	2	48.80	43.14	36.54	6.03	6.60	30.51

DMD = dry matter disappearance

NDF = neutral detergent fiber

ADF = acid detergent fiber

ADL = acid detergent lignin

Hemi-cellulose = ADF - ADL

Cellulose = NDF - ADF

Table 10. Effects of low saponin alfalfa composites on growth of weanling voles

Cultivar	Hemolytic Activity	Average Daily Weight Gain (gm)	Average Daily Intake (gm)
"Vernal"	2	.90	3.25
"Culver"/"DuPuits"	2	.95	3.5
<i>M. glutinosa</i>	2	.73	4.1
"Saranac"	2	1.0	3.47
Control	---	.93	3.9

However, since no long-term studies were made regarding effects of long-term ingestion of saponin, no conclusions can be made. Increased absorption of nutrients as a result of saponin ingestion may have a beneficial role in weight gains initially observed. Not until large amounts of alfalfa with negligible amounts of saponin are available can the true effects of saponin ingestion be known. Also, the reduced gains of voles on *M. glutinosa* diets indicate that there may be qualitative differences in saponin within varieties. Whether these differences are related to or are independent of other toxic or antimetabolic substances in alfalfa is not known.

The results of this experiment indicate that low saponin alfalfa composites produce weight gains in weanling voles that are similar to control animals. Qualitative differences in composites may occur that result in inconsistent responses when diets containing them are fed to test animals.

Experiment 5. Nutritive Value of Alfalfa Composites with High Hemolytic Activity

Diets comprised of clones with the 2 highest saponin levels were fed to weanling voles to evaluate growth response as related to alfalfa composites with high hemolytic activity (Table 11).

The protein content, ash, and ether extract concentrations were independent of saponin levels. Average yields of the 3 varieties were in agreement with those observed in high saponin composites of "Team" alfalfa. Here again, high saponin composites resulted in a greater increase in stems than in number of leaves.

Composition of cellular components of high hemolytic activity composites is shown in Table 12.

Table 11. Chemical analyses of alfalfa composites high in hemolytic activity

Cultivar	# Clones/ Composites	Hemolytic Activity	Average Yield (gm)	Ash (%)	Ether Ex- tract (%)	Pro- tein (%)
"Vernal"	3	32	115	7.76	2.10	19.23
<i>M. glutinosa</i>	2	32	94	8.19	2.78	20.38
"Saranac"	5	32	114	7.43	2.56	21.32
"DuPuits"	1	64	54	7.48	2.81	20.56

Table 12. Composition of cellular components of alfalfa composites with high hemolytic activity (values are expressed as percent)

Cultivar	Hemolytic Activity	NDF	ADF	ADL	Cellulose	Hemi-cellulose
"Vernal"	32	42.68	33.25	4.36	29.02	9.43
<i>M. glutinosa</i>	32	40.43	31.00	6.04	24.96	9.43
"Saranac"	32	38.24	32.53	5.44	27.09	5.71
"DuPuits"	64	41.86	29.80	4.52	25.28	12.06

NDF = neutral detergent fiber

ADF = acid detergent fiber

ADL = acid detergent lignin

Cellulose = ADF - ADL

Hemi-cellulose = NDF - ADF

Considerable variability existed among the different varieties with respect to cellulose content. Values for hemi-cellulose were higher than those obtained for lower saponin composites of "Team" alfalfa.

Results of feeding weanling voles diets with high hemolytic activity are shown in Table 13.

Table 13. Growth response of weanling voles fed alfalfa diets with high hemolytic activity

Cultivar Composite	Hemolytic Activity	Average daily intake (gm)	Average daily wt. gain (gm)	No. of surviving voles after 6 days	No. of days after initiation of expt. when mortality occurred
"Vernal"	32	3.5	-1.5	0/4	3,3,2,3
<i>M. glutinosa</i>	32	2.2	+ .5	1/4	3,4,3
"Saranac"	32	3.2	-1.1	0/4	2,2,3,5
"DuPuits"	64	2.7	- .5	0/4	2,2,2,4
Control	---	4.8	+1.0	4/4	---

Data from the feeding experiments indicate no qualitative effects with respect to growth response in alfalfa diets with high hemolytic activities. With the exception of one vole on a *M. glutinosa* diet with hemolytic activity of 32, mortality consistently occurred at higher levels of intake. In addition, food consumption was reduced. The bitter taste of the diets, when saponin levels were high, could have accounted for this. Bitter flavor of forage extracts from leaf protein has been attributed to the presence of inorganic ions, especially zinc (Buchanan, 1968). Jennings (1949) stated that flavor differences are slight between species. However, with alfalfa meals, those with high saponin consistently gave a bitter taste when placed on the tip of the

tongue. On a whole plant basis, zinc concentration alone would not be adequate to give such a bitter taste. It is possible that saponins in conjunction with other factors are responsible.

Mortality occurred as early as 2 days after voles were placed on diets. Generally, weanling voles had a rough coat and appeared to be hunched up 24 hours after being placed on high saponin diets. Post-mortem examination showed no gross lesions. However, there was virtually no body fat and some edema was noted in the gastrointestinal tract.

In addition to the above diets, composites of "Team", "Culver" and "MSB" alfalfa were fed to weanling voles. These diets were also high in saponin as evidenced by hemolytic activities of 32 and 64. Because of limitations of material, only 2 replications were available. However, 100 percent mortality was observed.

From the results of this experiment, alfalfa composites high in saponin resulted in a poor growth response when compared to control animals on a semisynthetic diet and also to low saponin composites. *In vitro* hemolytic activity appears to be a good measure of over-all toxicity of alfalfa saponins in monogastric animals, either acting alone or in conjunction with other antimetabolites.

II. Saponin Levels of Non-Hardy Alfalfa Varieties

The possibility of complex genetic interactions leading to instability for low saponin in progeny from selections evaluated previously for hemolytic activity necessitated the search for other potential sources of parental material that could be incorporated in a low saponin synthetic. Based on observations by McNair (1932) and Jones (1969) which reflected the opinion that non-hardy alfalfas were lower in

saponin than hardy ones, 3 non-hardy cultivars were evaluated for hemolytic activity.

Saponin levels as indicated by hemolytic activity for 3 non-hardy alfalfa cultivars, "African"- "Tillman", "Mesa Cirsa", and "UC 64", are shown in Figure 9. No plants were high or very-high in saponin content, with the greatest percentage being low. These 3 populations were previously neither selected for saponin content, nor tested for saponin levels. Jones (1969) found that generally unselected "Vernal", "Culver", and "MSB" populations had normal saponin distributions when individual plants were classified. "DuPuits" and "Saranac" populations were skewed to the high saponin side.

McNair (1932) studied over 80 families of plants containing saponins. The families ranged from those found in tropical and subtropical areas to temperate environments. Generally, those plants and varieties grown in the tropics had lower saponin concentrations, with some having none at all. His studies showed that the molecular weights of saponins were higher in the temperate areas. This included both highest and lowest mean molecular weights. Also, saponins from tropical areas had fewer carbons and hydrogen atoms per molecule.

Recent investigations in saponin structure have shown that alfalfa saponins are triterpenes, the aglycone containing 30 carbons with the rest being hexoses (Steiner and Holzen, 1955; Boiteau *et al.*, 1964; Birk, 1969). Since the aglycone portion of the molecule generally remains fixed with respect to molecular weight, larger molecular weights of saponins in winter-hardy cultivars can be due to the presence of additional carbohydrates, or as a result of complexing with amino acids. The relationship between saponin content and cold hardiness is unclear. Winter hardiness in alfalfa appears to involve interactions between

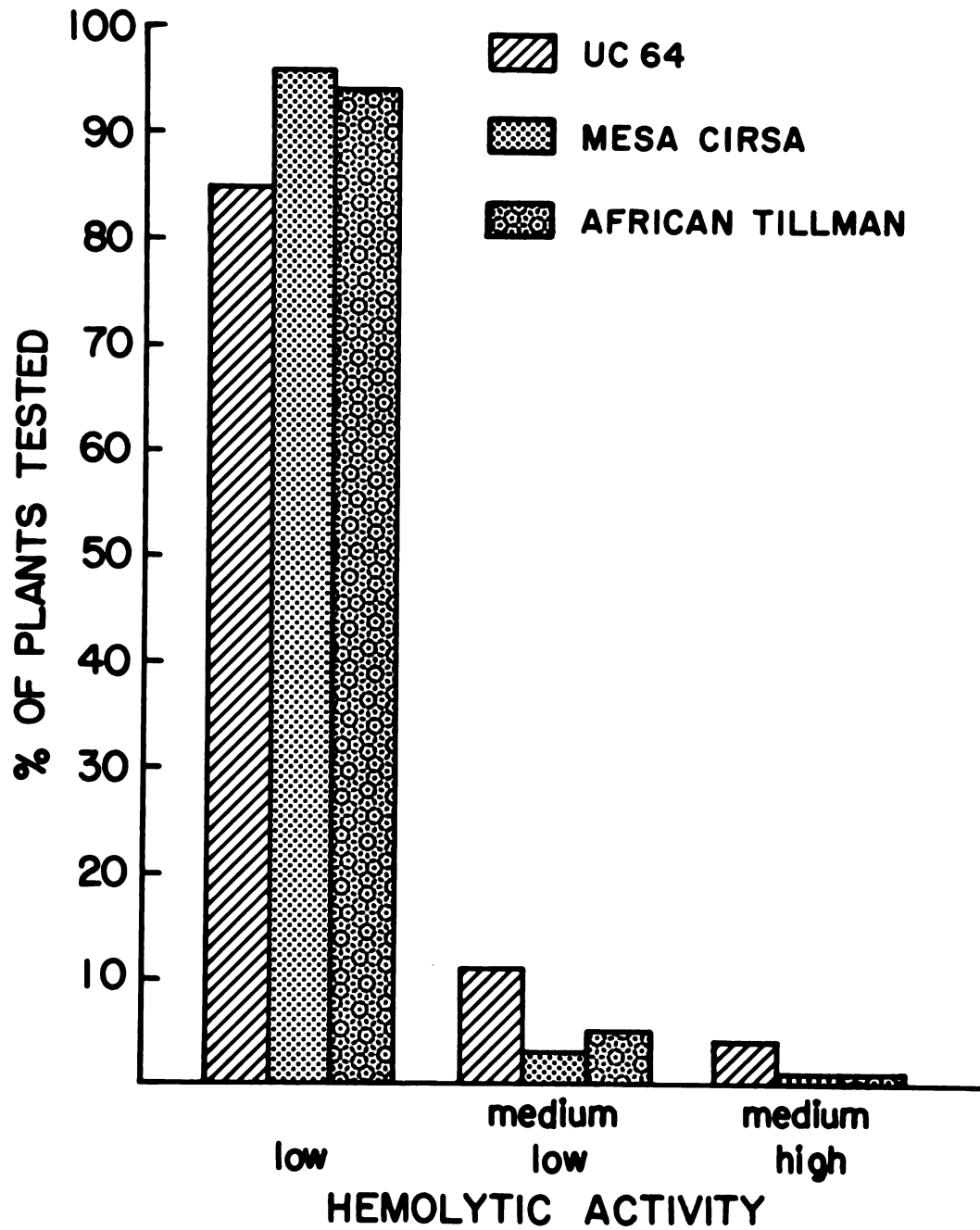


Figure 9. Distribution for saponin content of 3 non-hardy alfalfa varieties.

proteins and carbohydrate reserves within the plants (Smith, 1960; Bula *et al.*, 1954; Jung and Smith, 1960). Associations between saponins and amino acids have been demonstrated by Mooijman (1965). In addition, Marker *et al.* (1947) have shown that in *Agave sp.* and *Yucca sp.* sapoginin fractions increase in complexity during the winter. Krider and Wall (1954) observed that "saponases", specific enzymes capable of hydrolyzing saponins, are present in the plant. Saponases of alfalfa may, as a result of hydrolysis, release either amino acids or carbohydrates necessary for winter survival.

From a plant breeding point of view, the presence within winter-hardy cultivars of individual plant selections low or lacking saponins indicates that genes for saponin may not be essential for plant survival. If winter-hardy plants are of equal agronomic quality with high saponin selections, physiological mechanisms for saponin concentrations and saponin hardness may be independent.

III. Determination of Saponin Levels and Observation of Growth Characteristics of 3rd and 4th Cycle Alfalfa Selected for High and Low Saponin Content

Forage legumes are indirectly evaluated by animal productivity. The goal of the plant breeder, therefore, should be concerned with changing the plant to improve animal productivity. Saponins of alfalfa have been shown to have a deleterious effect on growth. The availability of a sensitive *in vitro* analysis enables selection of clones with extreme and intermediate levels of saponins.

The purpose of this study was to evaluate 3rd and 4th cycle alfalfa seedlings for saponin content in order to determine progress in selection. Since selection for saponin content has been bidirectional, it was possible to have both low and high saponin populations to test.

Figure 10 shows the results obtained. In low saponin populations there were a few plants very high in saponin, but during the 4th cycle, there was a reduction, with skewing toward the low saponin side. The high saponin selections, on the other hand, did not show a dramatic shift to the high side.

From the frequency distribution, Figure 11, heritability estimates and genetic advance were determined. Heritability in the broad sense is defined as the ratio of total genetic variance to the phenotypic variance (Asay, 1971). Heritability in the broad sense, for low saponin populations was 79.69 percent and 49.38 percent for high saponin. Genetic advance for low saponin was .70 and, for high saponin .43. The heritability estimates obtained were different than those reported by Jones (1969). Using a diallel between 7 "Vernal" clones, he determined heritability to be 90.2 percent of the whole plant. The high value seen could reflect the increase in heritability that occurred with mild inbreeding, since only one variety was used in the diallel. The lower values obtained from the synthetic populations for low and high saponin may indicate that some complexity exists in the genetic nature of inheritance for saponin.

The results obtained during the analysis of the 1200 seedlings of 3rd and 4th cycle material indicated that there was a surprisingly high number of high and very-high saponin plants in the low saponin selections and a large number of low saponin plants in the high saponin group. Since determination of saponin content was done at a very early stage of development, it was decided to follow some of the plants intensively for saponin levels as the plants developed. From previous observations, plants in the "high" range rarely changed in hemolytic activity to a lower value; because of this, the majority of plants

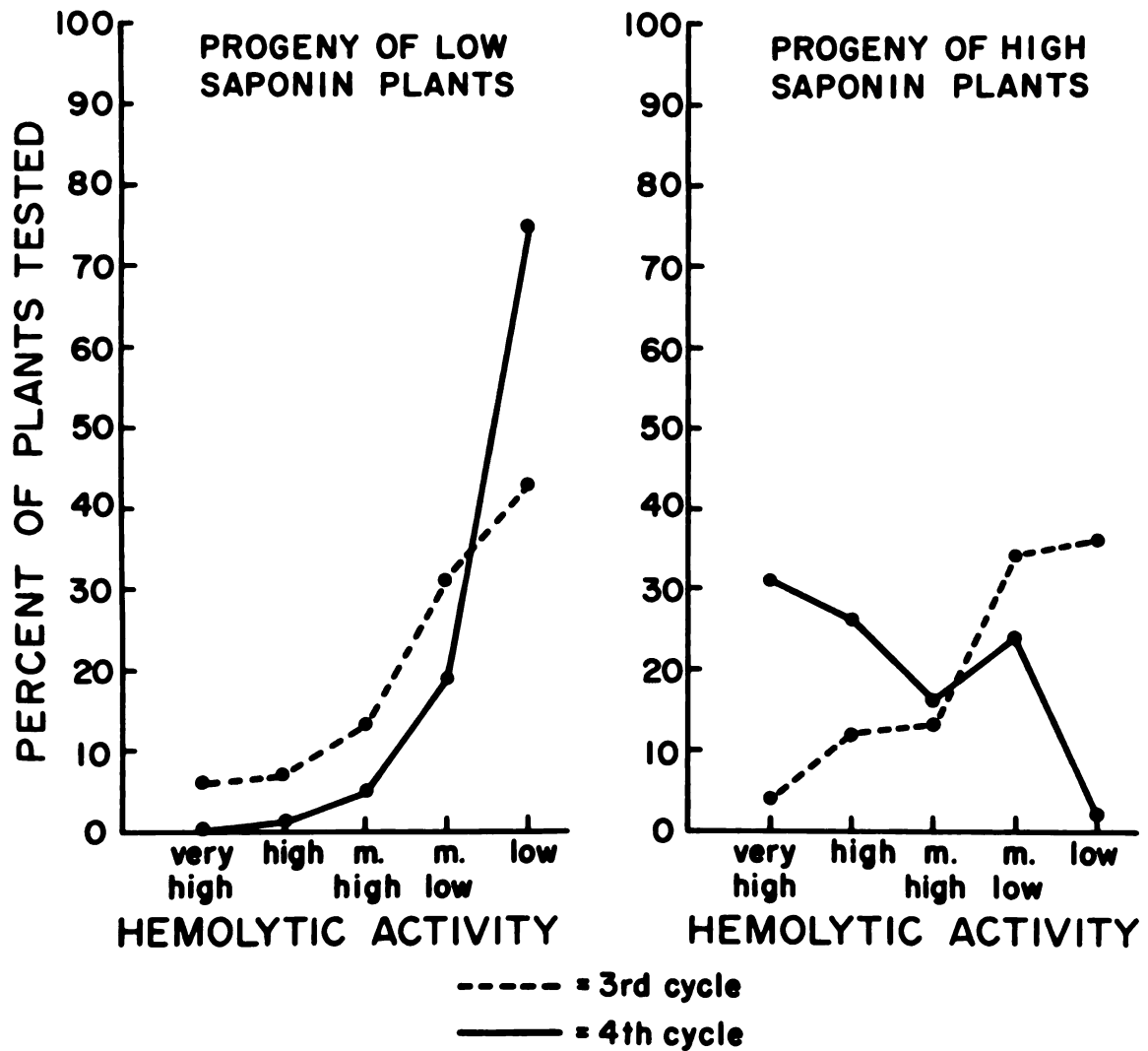


Figure 10. Hemolytic activities of 3rd and 4th cycle alfalfa plants selected for low and high saponin.

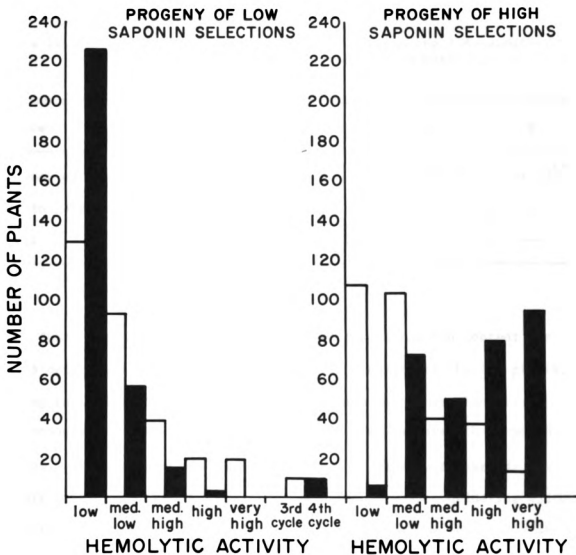


Figure 11. Distribution for hemolytic activity of 1200 3rd and 4th cycle alfalfa plants selected bidirectionally for saponin content.

selected for the intensive study were ones initially low, medium-low or medium-high in hemolytic activity. Here, too, the sampling method included mature leaves from different parts of the plants to give an over-all estimate of saponin within the plant. Analysis of variance for changes in saponin content over the 4 months of sampling is shown in Table 14.

Table 14. Analysis of variance for saponin content and 5 determinations of hemolytic activity in selected alfalfa seedlings

Source	df	S.S.	M.S.	F
Between categories	4	837.75	209.43	119.79**
Within categories	1000	1748.32	1.74	---
Total	1004	2586.07	---	---

** Significant $P < .0005$.

There were highly significant differences in saponin content for the 5 determinations. Changes in hemolytic activity of the 203 plants, during the 5 additional estimations, are shown in Figures 12-12e. The changes in hemolytic activity are quite dramatic over the 4-month period. There were significant differences in saponin content as the plants developed. Time of sampling was critical when considering which selections were to be used in future cycles. Deceptive low values as a result of sampling too early would give an overestimate of low selections that could be used. Ineffective screening procedures will, by reducing the size of the selection differential, offset the positive effects of high heritability and genetic variability (Asay, 1971).

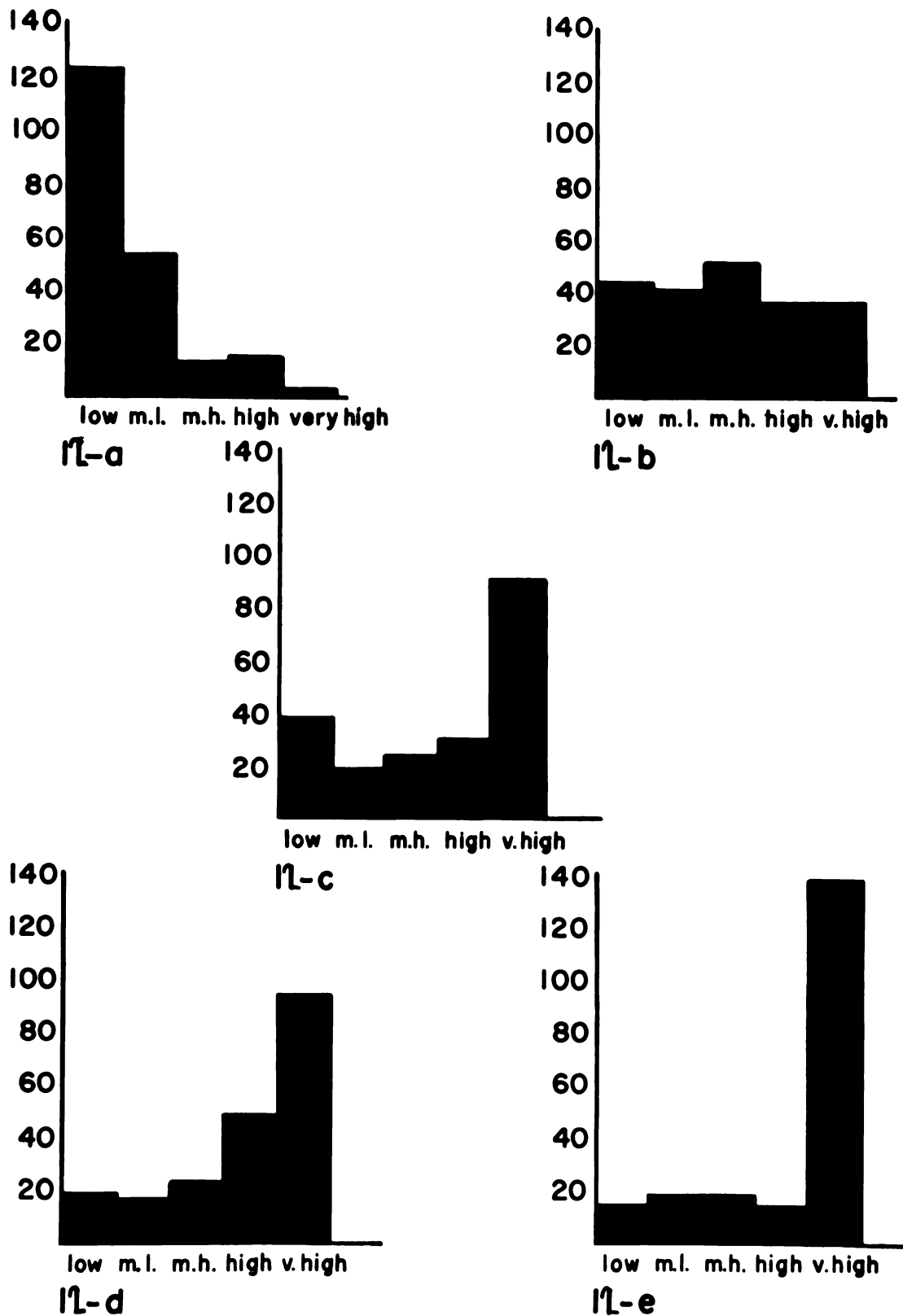


Figure 12. Number of plants in each classification for saponin content over 5 estimations of hemolytic activity.

Consistent changes in hemolytic activity to higher values were unexpected, especially since Jones (1969) reported that even though stem saponin levels were constant, leaves showed a decrease in saponin content. Also, he stated that saponin levels remained constant during the first growth. Hanson *et al.* (1963) reported that saponins in the plant are generally higher in the second cutting. However, they did not follow saponin concentrations during the early development of the plants. The discrepancies of the data can possibly be explained by the nature of the materials used. For purposes of analysis, Jones (1969) used 4 asexually propagated *M. falcata* clones, while the material used in this study was comprised of an alfalfa synthetic made up of at least 5 parents. Complex genic interactions as a result of hybridization may have been responsible for the changes in hemolytic activity as the plants developed.

During the testing period, it became apparent that plants highest in saponin began to flower first. Therefore, during the course of the experiments, the nature of flowering was observed and recorded. Table 15 shows the analysis of variance table for saponin level and flowering.

Table 15. Analysis of variance for flowering and saponin levels of selected alfalfa seedlings

Source	df	S.S.	M.S.	F
Between categories	4	448.18	112.04	94.53**
Within categories	1000	1185.27	1.18	---
Total	1004	1633.46	---	---

** Significant $P < .005$.

There was a highly significant relationship between flowering and saponin level. Changes in flowering with respect to changes in hemolytic activity are shown in Table 16. Those plants highest in saponin

Table 16. Flowering trends as related to saponin content in 170 alfalfa plants over a 4-month testing period for hemolytic activity

Saponin Level	Test Period	Stage of Flowering		
		Bud No. plants	Early Flower No. plants	Full Flower No. plants
Low	2	0	0	0
Medium-low		0	5	1
Medium-high		0	4	0
High		0	1	6
Very-high		0	3	0
Low	3	0	0	3
Medium-low		0	0	2
Medium-high		0	0	3
High		0	1	5
Very-high		1	3	9
Low	4	0	0	3
Medium-low		0	0	4
Medium-high		0	0	5
High		0	0	8
Very-high		0	1	33
Low	5	0	0	5
Medium-low		0	0	12
Medium-high		0	0	6
High		0	0	7
Very-high		0	2	90

flowered earlier than low saponin selections. Interestingly, hemolytic activity of flowers from selected plants within this group were generally high in saponin. There was no correlation between low saponin content of vegetative parts of the plant and flowers. Marker *et al.* (1947) and Marker and Lopez (1959) have shown that the sapogenin fraction of plants undergoes changes with time. Young *Agave* and *Yucca* plants have fewer sapogenins than older ones. This has led Hendricks (1960) to hypothesize that the flower hormone is either a steroid or isopernoid. He also suggested that sterols might be solubilized for transport by glycosidation. The increase of sapogenins in *Agave* and *Yucca* could correspond to increases in saponin levels in alfalfa as the plant matures. Drodz (1962) found *Primula officinalis* roots and rhizomes had the highest saponin content during the flowering phase. Since roots of alfalfa have been shown to produce saponin, there may be some relationship between those of the roots and flowers. Translocation of one or more of these complex substances may account for differences reported in plant parts.

In addition, Bonner *et al.* (1963) have presented evidence to support the assumption that the flower hormone is a steroid or other polyisopernoid. Since alfalfa saponins are isopernoid in structure, their presence could possibly be a result of either synthesis or breakdown of polyisopernoids. The presence of "saponases" or related compounds in alfalfa may be responsible for varying saponin levels seen within and between cultivars.

Simple correlations between saponin content and flowering, number of stems and length of longest stem are shown in Table 17.

Table 17. Simple correlations between saponin level and flowering, number of stems, and length of longest stem in alfalfa seedlings

Flowering	.2862 ^{**}
Number of stems	-.1628 [*]
Length of longest stem	.2572 ^{**}

^{*}Significant $P < .05$.

^{**}Significant $P < .01$.

There were significant relationships between saponin levels and flowering, number of stems and stem length. Table 18 shows measurements of stem length at the termination of the experiment.

Table 18. Relationships between stem length and saponin levels of selected alfalfa seedlings

Saponin Level	Mean Length	Shortest Stem	Longest Stem
Low	16.93"	10"	23"
Medium-low	20.44"	13"	27"
Medium-high	20.18"	15"	28"
High	19.69"	8"	28"
Very-high	22.28	9"	36"

The biological significance of the relationships seen between saponin content of alfalfa and number of stems and stem length is unclear. Either direct or indirect effects of saponin biosynthetic pathways may be responsible for the changes. Physiological implications of lowering or raising saponin content in alfalfa are unknown since the

synthesis and function of saponins within the plant have been so poorly elaborated. Before comprehensive studies can be undertaken, stable populations, both low and high in saponin, must be available in order that proper comparisons can be made.

IV. Inheritance of Alfalfa Saponins as Determined by F_1 Seedlings

In an attempt to study the inheritance of alfalfa saponins, the Jinks-Hayman diallel technique was employed. The diallel cross method of determining the genetical properties of a group of lines is based upon the assumptions that parents are homozygous, no multiple allelism exists, genes are independently distributed between the parents and that diploid segregation occurs (Jinks, 1954; Hayman, 1954). Despite alfalfa being an autotetraploid, the information obtained from the diallel table generated by diploid parents can still be extracted even if parent lines are autotetraploids. The first step in the analysis was to test the variability among the entries. The results of the analysis of variance for saponin levels of 4th cycle F_1 and parents in a 6 parental diallel cross are shown in Table 19.

Table 19. Analysis of variance for saponin content in alfalfa as measured by hemolytic activity on F_1 generation of a 6x6 parent diallel cross

Source	Degree of Freedom	Sum of Squares	Mean Square	F
Rep	2	20.5711	10.2856	67.2701 ^{***†}
Entry	20	12.0229	.6011	3.9313 ^{***}
Error	40	6.1109	.1529	---
Total	62	36.6871	---	---

†)*** = significant difference at the α -level of .005.

There were highly significant differences among the 21 entries. This supports the validity of the genetic information obtained from the present analysis. The genetic nature of this trait was worked out with the Jinks-Hayman diallel analysis technique. Data from 3 determinations in the F_1 generation were treated separately and also pooled for diallel analysis, treating each determination as a replication. The data for each determination, as well as pooled values, are shown in Tables 20, 21, 22, and 23. the variances and covariances of arrays, as well as array means, are shown.

With statistics V_r and W_r , the W_r , V_r graph was plotted and the limiting parabola was constructed using the theoretical relationship $W_r = \sqrt{V_r V_p}$. The graphical demonstration shows the degree of dominance, dominance of parents and additional information about the genetic relationships among the parents. Study of the graphical analyses indicates that at least one of the basic assumptions underlying the diallel technique has not been met because the slope of the regression line deviated from unity. The calculated value of the minimum number of effective factors shows that control of saponin content appears to be under the control of multiple genes. Interpretation of data when interaction among non-allelic genes occurs have been discussed by Mather (1967). Complementary gene action causes the W_r/V_r line to be concave upwards with the middle point of the line falling below the regression line and causing the mid-point to move to the right end of the line. Duplicate gene action, on the other hand, raises the middle-point above the line and moves it to the left causing the line to be concave downwards and the middle-point clustered to the left. According to Mather, the variance of the segregating

Table 20. Saponin levels as determined by hemolytic activity for the first set of a 6x6 diallel in the F₁ generations (parents included)

Array Number	Parent	1	2	3	4	5	6	Vr	Wr	Mean
1	<i>M. glutinosa</i>	<u>1.53</u>	1.0	1.72	1.20	1.71	1.18	.4632	.0666	1.3900
2	"Culver"/"Culver"/ "Vernal"	1.0	<u>1.0</u>	1.18	1.0	1.0	1.05	.0260	-.0219	1.0383
3	L ₄ 909	1.72	1.18	<u>1.0</u>	1.0	1.21	1.0	.3895	.2761	1.1850
4	"Vernal"	1.20	1.0	1.0	<u>1.04</u>	1.38	1.0	.1219	.0487	1.1033
5	"MSB"	1.71	1.0	1.21	1.38	<u>1.0</u>	1.0	.4109	.2680	1.2167
6	"Hardy Moapa"	1.18	1.05	1.0	1.0	1.0	<u>1.0</u>	.0260	.0735	1.0383

Table 21. Saponin levels as determined by hemolytic activity for the second set of a 6x6 diallel in F₁ generation (parents included)

Array Number	Parent	1	2	3	4	5	6	V _r	W _r	Mean
1	<i>M. glutinosa</i>	<u>2.80</u>	1.22	2.31	1.92	2.35	1.68	1.9704	1.0861	2.0467
2	"Culver"/"Culver"/ "Vernal"	1.22	<u>1.63</u>	1.81	1.31	1.82	1.31	.3643	-.0105	1.5167
3	L ₄ 909	2.31	1.81	<u>1.92</u>	1.30	2.21	1.91	.6322	.7917	1.9100
4	"Vernal"	1.92	1.31	1.30	<u>1.91</u>	2.58	1.22	1.4091	1.1780	1.7119
5	"MSB"	2.35	1.82	2.21	2.58	<u>3.27</u>	1.86	1.4398	1.0938	2.3483
6	"Hardy Moapa"	1.68	1.31	1.91	1.22	1.86	<u>2.54</u>	1.1411	.7011	1.7533

Table 22. Saponin levels as determined by hemolytic activity for the third set of data of a 6x6 diallel in F₁ generation (parents included)

Array Number	Parent	1	2	3	4	5	6	Vr	Wr	Mean
1	<i>M. glutinosa</i>	<u>3.0</u>	2.75	3.50	2.32	4.38	2.67	2.7241	1.7861	3.1033
2	"Culver"/"Culver"/ "Vernal"	2.75	<u>1.72</u>	2.0	1.68	2.38	1.84	.8449	1.0336	2.0483
3	L ₄ 909	3.50	2.0	<u>2.12</u>	1.56	3.57	2.17	3.6449	2.5701	2.4767
4	"Vernal"	2.32	1.68	1.50	<u>2.21</u>	2.78	2.17	1.0636	2.0972	2.1100
5	"MSB"	4.38	2.30	3.57	2.78	<u>3.64</u>	2.75	2.9037	1.8019	3.2367
6	"Hardy Moapa"	2.67	1.84	2.17	2.17	2.75	<u>2.43</u>	.5929	1.1302	2.3383

Table 23. Saponin levels as determined by hemolytic activity for the pooled data of a 6x6 diallel in F₁ generation (parents included)

Array Number	Parent	1	2	3	4	5	6	Vr	Wr	Mean
1	<i>M. glutinosa</i>	<u>7.32</u>	4.98	7.02	5.43	6.36	5.46	4.5151	3.6472	6.0950
2	"Culver"/"Culver"/ "Vernal"	4.98	<u>4.35</u>	4.98	3.99	5.10	4.20	1.1343	2.0187	4.6000
3	L ₄ 909	7.02	4.98	<u>5.04</u>	3.78	6.99	4.86	8.3628	7.5712	5.4450
4	"Vernal"	5.43	3.99	3.78	<u>5.16</u>	6.72	4.38	6.0132	6.5160	4.9100
5	"MSB"	6.36	5.10	6.99	6.72	<u>7.89</u>	5.61	4.9741	4.1089	6.4450
6	"Hardy Moapa"	5.46	4.20	4.86	4.38	5.61	<u>5.97</u>	2.5302	.7278	5.0800

families is increased with complementary gene interactions; the reverse appears to be true of duplicate gene action. He concluded that selection for a single optimum phenotype can favor duplicate but not complementary interactions. Complementary interactions could be sometimes favored by selection toward 2 or more optima.

Analysis of the V_r/W_r graphs for the 3 determinations and pooled data shows that there is epistatic gene action, namely complementary and duplicate genes in the F_1 plants with respect to saponin content. Even though there is a scattering of points, Figure 13 shows that there is a complementary gene effect. The improper position of array 1 can be accounted for by the apparent heterozygosity for saponin content in that variety. There is also an indication that high saponin is recessive due to the position of arrays 1 and 5 on the right-hand portion of the regression line. Figure 14 shows a change in the genetic interactions as the plants develop. Duplicate gene action was predominant at this stage. Here, too, parents high in saponin were located in the part of the curve that represented recessiveness. The third determination again shows a good indication that high saponin is genetically recessive, with the low saponin parents located on the left of the regression line (Figure 15). The pooled data (Figure 16) show a balance between complementary and duplicate interactions. The position of parents 1 and 5, now at the middle of the regression line, gives further indication of the ambidominant nature of the genetic interactions. Since the original data show heterozygosity among some of the parents, another of the basic assumptions of the diallel analysis technique has not been met. Because of

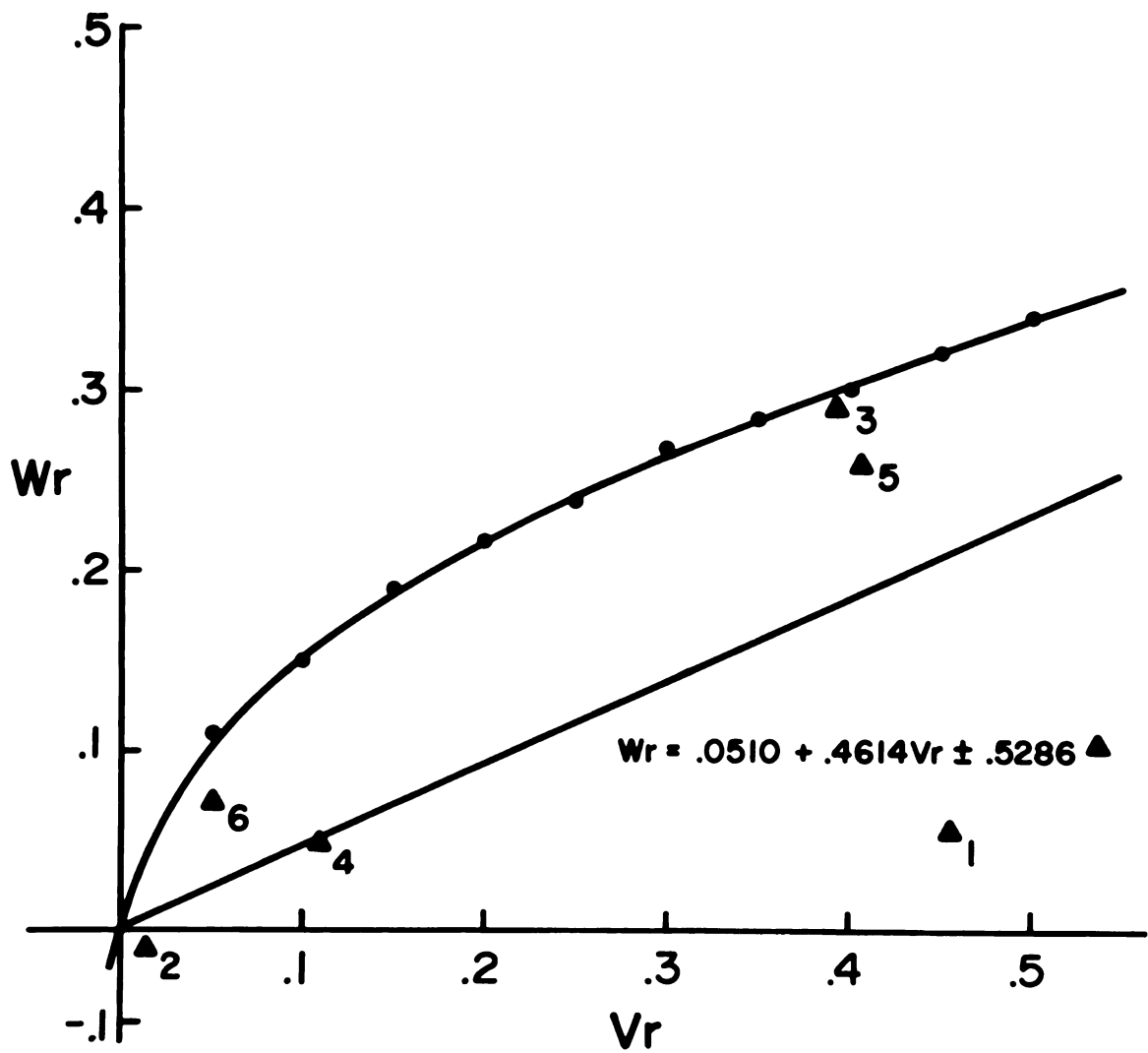


Figure 13. Wr/Vr graph for saponin content in F_1 generation alfalfa at the first determination of hemolytic activity.

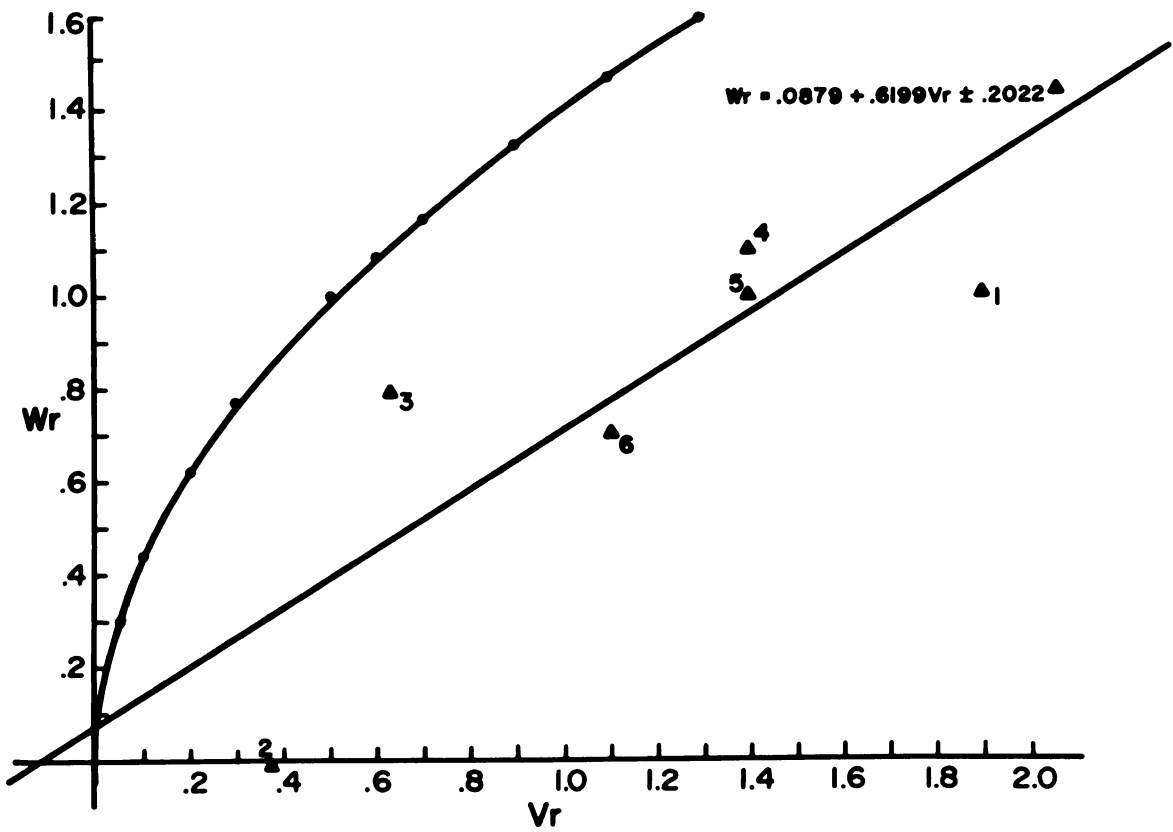


Figure 14. W_r/V_r graph for saponin content in F_1 generation alfalfa at the second determination of hemolytic activity.

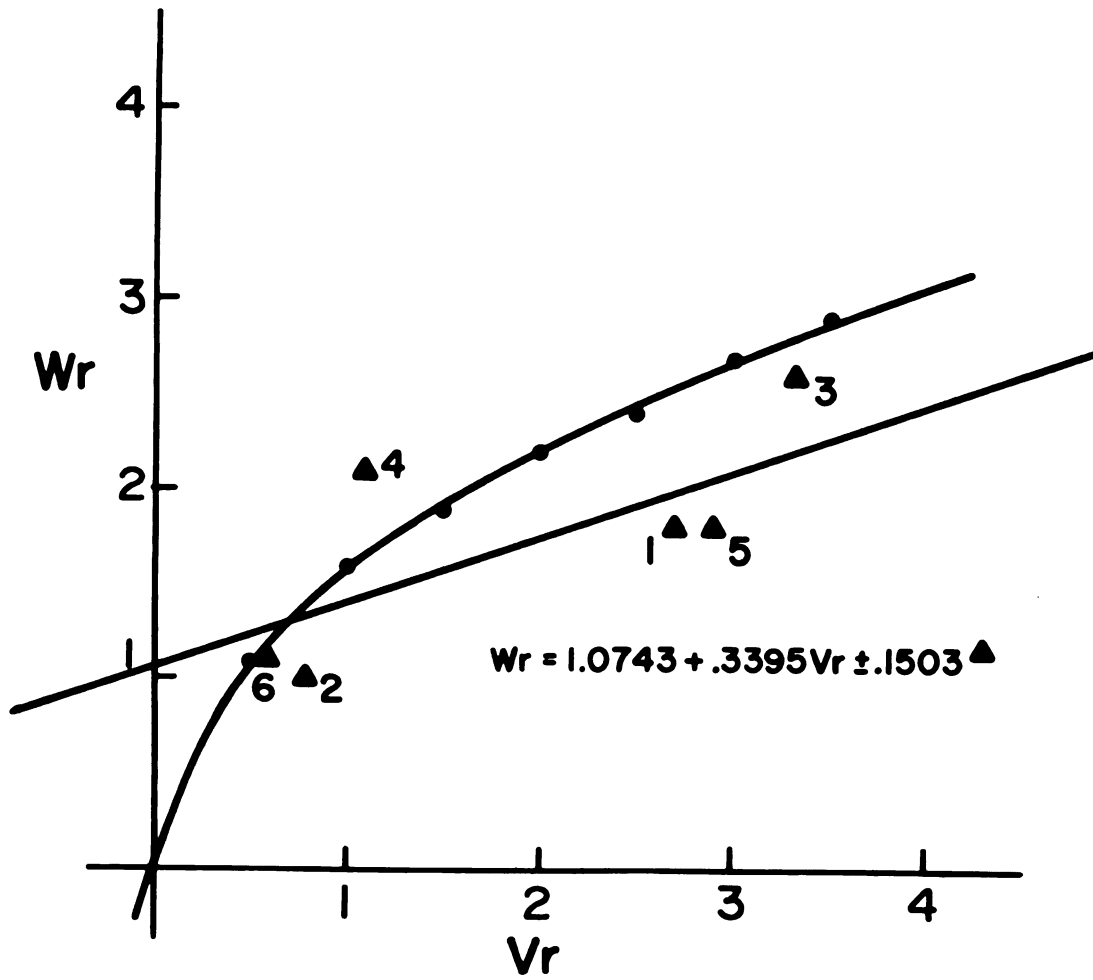


Figure 15. W_r/V_r graph for saponin content in F_1 generation alfalfa for the third determination of hemolytic activity.

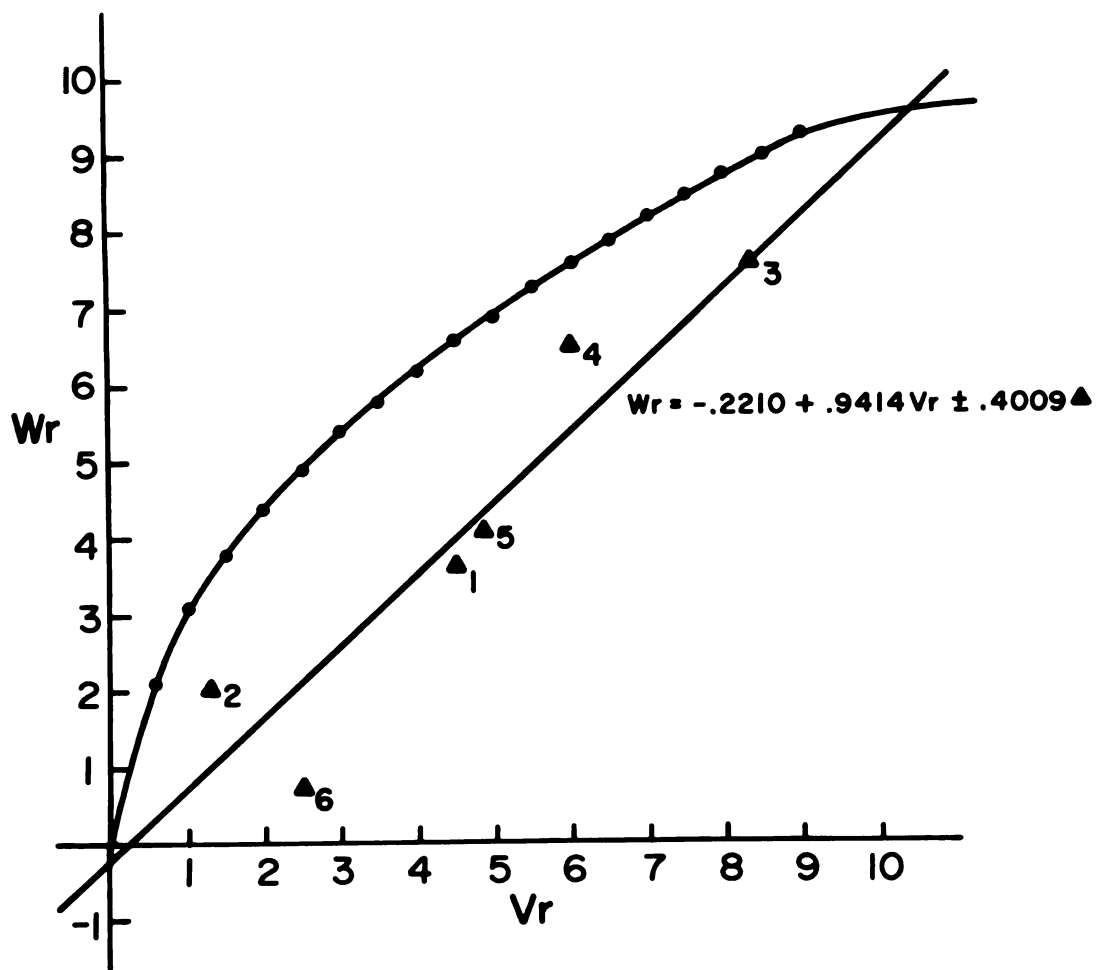


Figure 16. W_r/V_r graph for saponin content of pooled data for F_1 generation alfalfa.

these 2 conditions, analyses set forth by Jinks and Hayman would be misleading. To compensate for this, statistical analyses were carried out according to the methods set forth by Oakes (1967). This provides for valid estimates of heritability and dominance when heterozygosity is present in parents used in the diallel technique. Estimates of genetic parameters of F_1 on the diallel cross for saponin content are shown in Table 24. Heritability in the broad sense was 93 percent for low saponin and 79 percent in the narrow sense. The value for heritability in the broad sense was different than that obtained from data

Table 24. Estimates of genetic parameters of F_1 of a 6x6 diallel cross for saponin content

Parameter	Determination			
	1	2	3	Pooled Values
Fr	.0300	.0106	0	.2034
D	.0456	.3963	.4778	1.7977
H_1	0	0	0	.4077
H_2	0	0	.1666	.3058
h^2 broad	1	1	1	.9316
h^2 narrow	1	1	.7416	.7946
K	0	0	.3484	1.725

with the 3rd and 4th cycle seedlings where heritability in the broad sense was estimated to be 76.69 percent. However, this increase in heritability is not unusual since it is expected that heritability will increase due to additive gene effects. Heritability in the narrow sense for low saponin content was 79.46 percent. Jones (1969) determined estimates of saponin heritability for 2 *M. sativa* populations, "Vernal"

and "Culver". Broad sense heritability for "Vernal" was 60 percent and, for "Culver", 79 percent. Estimates of heritability for a diallel cross between 7 "Vernal" clones was 90 percent for the whole plant.

The nature of dominance is shown by $\frac{H^2}{D}$. As $\frac{H^2}{D} = .191$, where there is partial dominance. This is also verified by the W_r/V_r graphs. Further verification of the graphical analysis is shown by the F_r value. A small F_r value indicates a slight degree of epistasis. From the k value a minimum of 2 genes are probably involved in saponin content in alfalfa.

In an attempt to further elucidate the mechanism of inheritance of saponin content, the data were studied directly to see if a genotype could be hypothesized that would explain the observed ratios of each level. It must be noted that the populations studied were not large enough to permit categorical statements to be made concerning the genotypes of the parents. However, valid estimates concerning them can be made which would provide useful information to the plant breeder.

There is strong information that saponin levels increase during the development of the plant. From Table 25, it can be seen that in all cases of the F_1 plants, saponin content increased in each determination even though 4 of the 6 parents initially appeared to be homozygous for low saponin. Of the F_1 plants, 6 combinations were low in saponin initially and the rest were mostly low. During the second and third determinations of hemolytic activity, saponin increased significantly. For purposes of analysis, data obtained from the first determination were used because a more accurate estimate could be made on the initial activity observed without being thrown off by the higher values in the other determinations. After analysis of the results, ratios were postulated first for low and medium-low saponin content. Tables 26 and 27

Table 25. Percentage of parent and F1 plants in each saponin level classification for 3 determinations as measured for hemolytic activity

	"Hardy Moapa"			"MSB"			"L ₄ 909"			"Culver"/ "Culver"/ "Vernal"			"Vernal"			<i>M. glutinosa</i>
	Rep	1	2	3	Rep	1	2	3	Rep	1	2	3	Rep	1	2	3
Saponin level																
Very high	0	0	5	0	18	50	0	0	12	0	5	5	0	8	13	0
High	0	21	8	0	36	9	0	19	7	0	5	5	0	18	13	7
Medium-high	0	34	29	0	14	9	0	7	4	0	5	14	0	8	4	7
Medium-low	0	24	30	0	18	18	0	22	3	0	22	36	4	8	28	33
Low	100	21	20	60	14	14	100	52	44	100	63	40	96	58	42	53
	"L ₄ 909" x "Vernal"			"L ₄ 909" x "Hardy Moapa"			"Hardy Moapa" x "Vernal"			"Vernal" x "Culver"/"Culver" /"Vernal"			"MSB" x "Vernal"			"L ₄ 909" x "MSB"
Saponin level	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1
Very high	0	0	5	0	4	17	0	0	4	0	0	0	0	8	22	0
High	0	5	0	0	10	10	0	0	4	0	0	14	0	15	8	0
Medium-high	0	0	5	0	4	17	0	0	0	0	9	0	8	23	0	0
Medium-low	0	15	20	0	39	13	0	22	37	0	14	27	23	38	62	21
Low	100	80	70	60	43	43	100	78	56	100	77	59	69	15	8	79
	"Culver"/"Culver" /"Vernal"			"Culver"/"Culver" /"Vernal"			"MSB" x <i>M. glutinosa</i>			"Culver"/"Culver" /"Vernal"			"Vernal" x <i>M. glutinosa</i>			"L ₄ 909" x <i>M. glutinosa</i>
Saponin level																
Very high	0	0	9	0	0	5	0	0	50	0	0	4	0	4	8	8
High	0	9	9	0	5	0	0	0	36	0	21	8	0	4	4	5
Medium-high	0	18	0	0	0	16	21	36	7	0	8	17	8	16	24	5

show the test of goodness to fit to the theoretical ratios using chi-square statistics. Cursory examination of ratios for saponin levels

Table 26. Goodness of fit test for observed and expected numbers of low and medium-low saponin F_1 alfalfa plants

Combination	Frequency in Each Class			X ²	P
	Observed	Expected	Assumed Ratio		
	Low Medium-Low	Low Medium-Low			
"Culver"/ "Culver"/ "Vernal" x L ₄ 909	9 : 2	8.8 : 2.2	4:1	.0476	.80-.90
L ₄ 909 x "MSB"	11 : 3	11.2 : 2.8	4:1	.0393	.90-.95
"MSB" x "Vernal"	9 : 3	9.6 : 2.4	4:1	.0529	.95-.98

Table 27. Goodness of fit test for observed and expected numbers of low and medium-low saponin F_1 alfalfa plants with *M. glutinosa* as a parent

Combination	Frequency in Each Class			X ²	P		
	Observed		Assumed				
	Low	Medium-Low					
	Low	Medium-Low	Low	Medium-Low	Ratio		
"Hardy Moapa" x <i>M. glutinosa</i>	14	: 2	12.0	: 4	3:1	.8520	.40-.58
"Vernal" x <i>M. glutinosa</i>	22	: 1	17.3	: 5.7	3:1	2.381	.1 -.25
L4909 x <i>M. glutinosa</i>	15	: 3	13.5	: 4.5	3:1	.2995	.50-.70
"MSB" x <i>M. glutinosa</i>	7	: 4	8.3	: 2.7	3:1	.8291	.40-.50
"Culver"/"Cul- ver"/"Vernal"x <i>M. glutinosa</i>	10	: 7	12.8	: 4.2	3:1	1.278	.20-.30

indicated that a complex mechanism was in operation as indicated by changes in hemolytic activity as the plant matured. It was hypothesized that a minimum of 2 genes were operative for the F_1 plants because of the ratios with respect to low and medium-low saponin. Each of the 2 genes is of a recessive nature. If the genotypes of parents giving low saponin are assumed to be $aaaaBbbb$, nulliplex in A and simplex in B, with Bb showing incomplete dominance, phenotypic ratios of 3:1 and 4:1 can be obtained with tetraploid inheritance. Chi-square values were compatible with theoretical expectations. Low saponin genotypes would be bb and Bb; only those plants with BB genotype would be medium-low in saponin content. *M. glutinosa* appears to be an exception to this. The genotype of *M. glutinosa* is thought to be $AaaaBBbb$, a simplex in A and duplex in B, which would segregate as 1/12 each of AaBB, Aabb, aaBB, aabb plus 1/3 each of AaBb and aaBb. Combinations with low saponin parents (ex. L_4909) would have the number of large genes as shown below:

<i>M. glutinosa</i> L_4909	Gene frequency					
	$\frac{1}{AaBB}$	$\frac{4}{AaBb}$	$\frac{1}{Aabb}$	$\frac{1}{aaBB}$	$\frac{4}{aaBb}$	$\frac{1}{aabb}$
aaBb	4	3	2	3	2	1
aabb	3	2	1	2	1	0

The possible combinations with the cross *M. glutinosa* x L_4909 are shown in Table 28.

Since diallel analysis indicated that at least 2 genes were possible for regulating saponin in plants, and the nature of the ratios obtained, a third completely dominant gene "c" responsible for high and very-high saponin was postulated (Table 29). Since *M. glutinosa* consistently showed high saponin values, it was assumed that this parent was homozygous for the third gene, cccc, a nulliplex. On the other hand,

Table 28. Possible saponin levels resulting from combinations with *M. glutinosa* as a parent

Saponin level	No. of Large Genes	Expected Ratio	Expected Frequency	Observed Frequency
Low saponin	2,3,4	17	13.4	15
Medium-low saponin	1	6	4.8	3
Medium-high saponin	0	1	.8	1
$\chi^2 = .5549$ $P = .9-.75$				

"Hardy Moapa", because of its low saponin, was thought to be CCCC, a quadriplex. The other parents, L₄909, MSB, and "Culver"/"Culver"/"Vernal", were postulated to be CCcc, a duplex, and "Vernal", Cccc, a simplex. Ratios were hypothesized that would be compatible to an inheritance determined by about 3 genes, A, B, and C. Chi-square values and probabilities were compatible with theoretical expectations (Table 29).

Combinations in the third gene between *M. glutinosa* and L₄909 would result in the following possibilities:

	L ₄ 909		
<i>M. glutinosa</i>	CC	Cc	cc
cc	ccCC	Cccc	cccc

Comparisons between "Vernal" and "Hardy Moapa" would result in the following combinations:

	"Vernal"	
"Hardy Moapa"	Cc	cc
CC	CCCc	CCcc

Table 29. Goodness of fit test for observed and expected numbers of non-high and high saponin F_1 alfalfa plants

Combination	Frequency in Each Class		Assumed Ratio	χ^2	P
	Observed	Expected			
	High Non-High	High Non-High			
"MSB" x "Vernal"	1 : 12	1.1 : 11.9	1:11	.1884	.50-.70
"Vernal" x <i>M. glutinosa</i>	2 : 23	4.2 : 20.8	1:5	.8190	.30-.46
L ₄ 909 x <i>M. glutinosa</i>	3 : 19	3.7 : 18.3	1:5	.0166	.80-.90
"Culver"/"Cul- ver"/"Vernal" x <i>M. glutinosa</i>	5 : 19	4.0 : 20.0	1:5	.0637	.70-.90

Results of the diallel analysis and the direct analysis of the ratios gives evidence that inheritance of low saponin is indeed a complex phenomenon. The change in saponin content observed as the plants develop is different from what Jones (1969) reported. He noted that a decrease in saponin levels occurred as the plants developed. To account for the increase in saponin content, it is postulated that when gene *c* is present in the homozygous dominant condition, the system is complete for immediate expression for high saponin regardless of the other 2 genes present, because the "*c*" locus is epistatic over the 2 loci. As a saponin end product under the heterozygous condition in the "*c*" locus, high saponin is not immediately expressed; however, as the plant develops, as the number of dominant genes, "*c*" increases, high saponin levels are achieved. Precursors are converted by the necessary enzyme actions to formation of saponin in the plant, i.e.,

? ?

Enzyme A → B → C → ... saponin → P.

Since biochemical changes depend on enzymatic activity which, in turn, are controlled by gene action, it is possible that one dose of gene c is enough for the suppressive action toward the other 2 loci, A and B.

The direct analysis of ratios appears to be in agreement with the hypothesis that at least 2 genes are operative and that both duplicate and complementary gene action occur. To the plant breeder working toward development of low saponin varieties, proper understanding of the complexity of the problem is emphasized.

SUMMARY AND CONCLUSIONS

This study was undertaken in an attempt to characterize quality aspects of alfalfa cultivars associated with saponin levels as determined by *in vitro* hemolytic activity.

Weanling vole bioassays of "Team" alfalfa fed varying saponin levels showed that as saponin content increased, adverse physiological effects, eventually resulting in death, occurred. Histopathological changes included fatty metamorphosis of the liver and pancreatic atrophy. Disc electrophoretic studies of liver homogenates showed changes in liver proteins with increased saponin intake. Postmortem examination of selected organs indicated that cecal weights were increased at all levels of saponin intake where compared to controls. Additional assays with low saponin alfalfa cultivars indicated that there were no obvious qualitative differences with respect to nutritive quality. High saponin cultivars resulted in reduced food consumption, decreased weight gain, and death at the highest levels of intake.

Determination of saponin levels in 3 non-hardy alfalfa cultivars revealed that these were generally low in saponin when compared with winter-hardy cultivars. None of the seedlings sampled in these previously unselected populations were high or very-high in hemolytic activity.

Evaluation of 3rd and 4th cycle alfalfa seedlings selected bidirectionally for saponin showed genetic advance for low saponin to be .70 and .48 for high saponin. Heritability in the broad sense for low

saponin populations was 79.69% and 49.38% for high saponin. Intensive testing for hemolytic activity showed that saponin levels increased as the seedlings developed to flowering stages. Saponin content of regrowth was similar to that observed in leaves prior to being cut. High saponin selections had increased numbers of stems and flowered earlier than low saponin selections.

Analysis of 4th cycle F_1 seedlings to determine the genetic nature of saponin inheritance by use of the Jinks-Hayman diallel technique showed that there is epistatic gene action, namely complementary and duplicate genes in F_1 plants with respect to saponin content. There was indication that high saponin is recessive and low saponin is dominant. Heritability in the broad sense was 93.0% for low saponin and 79.0% in the narrow sense. Direct analysis of data for hemolytic activity of 4th cycle F_1 alfalfa seedlings supported results obtained from the diallel analysis indicating that a complex mechanism was involved in the inheritance of saponin with a minimum of 2 genes being involved.

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