EVALUATION OF ALFALFA PLANTS FOR SAPONINS

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ABSTRACT

EVALUATION OF ALFALFA PLANTS FOR SAPONINS

by Merlyn Jones

Three bio-assays utilizing the hemolytic property and one based on the ichthyotoxic nature of saponin were developed to rapidly and effectively evaluate saponin levels in alfalfa. Hemolytic and minnow determinations were significantly correlated with chemical determinations. The three erythrocyte assays formed the basis of alfalfa saponin investigations.

Saponin was highly heritable in the two populations of Medicago sativa L. studied. Sufficient variation existed in all alfalfa populations studied to facilitate selection for high and low saponin lines. Selection of high and low saponin lines by Erythrocyte Assay I was shown to be effective in four alfalfa populations.

Alfalfa saponin was stable both within the plant and in water extracts. Extracted saponin reacted with cholesterol and lost nearly all its hemolytic

activity. Heat did not affect the hemolytic activity of the same extracts. Extracts from plants low and high in saponin did not exhibit abnormal hemolytic activities when analyzed in mixtures.

Saponin levels in M. falcata changed very little over a two month period of first growth. "Hemolytic values" of leaves were nearly three times as high as those for stems and the top one-third of topgrowth was higher in hemolytic activity than the bottom third.

The organ of saponin synthesis was studied through the use of grafting techniques. Both leaves and roots appear to be capable of synthesizing saponins.

EVALUATION OF ALFALFA PLANTS FOR SAPONINS

By

Merlyn Jones

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Crop Science

1969

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INTRODUCTION

Identification and evaluation of natural plant constituents affecting nutrition should be as important as evaluation of the many factors affecting plant yields. Until recently, applied plant research was directed predominantly toward the improvement of yield, with the exception of cases where very severe animal growth depression or death resulted from certain plant diets.

Alfalfa has been improved in yield and persistency of stand through both breeding and managerial efforts. Improved alfalfa quality, on the other hand, has been achieved primarily through managerial efforts, even though a potential for quality improvement exists through plant breeding. Alfalfa possesses stimulative as well as inhibitive growth properties to many biological systems. Some of the compounds responsible for these properties may be controlled by relatively simple genetic systems. Consequently, rapid shifting in the levels of certain compounds may be realized.

One of the poorly understood and ill-defined chemical components of alfalfa is a group of steroidal and triterpenoid glycosides (saponins) which exhibit a broad spectrum of biological activity. They were identified in alfalfa fifty years ago, but have not received sufficient attention to adequately understand their growth depressing effects on microorganisms, monogastric or ruminant systems, or to appreciate their role in plant metabolism.

Much of the research concerned with biological activity and mode of action of compounds such as saponin is necessarily of an empirical nature. In order to aid this research, alfalfa populations of high and low saponin content should be available. Also, a better understanding of alfalfa saponin distribution within Medicago, distribution within individual plants, and a better knowledge of its properties would be most helpful.

This study was initiated for the purpose of supporting more comprehensive research on alfalfa saponins. Its objectives were to (1) develop rapid, sensitive, and reliable assays for the evaluation of alfalfa saponin, (2) obtain estimates of alfalfa saponin heritability and establish alfalfa base populations of very high and very low saponin content to supply sufficient material for

evaluations in higher organisms, and (3) establish certain properties of alfalfa saponin, their distribution within plants, and the organ or organs where they are synthesized.

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REVIEW OF LITERATURE

Most saponins are nitrogen-free glycosides, each consisting of an aglycone (sapogenin) and one or more sugar units. Many of these diverse compounds possess certain common biological properties. Review articles by Hiller et al. (1966), Heftman (1967a), and Basu et al. (1967) describe many aspects of steroidal and triterpene saponin structure, isolation, purification, chemistry, pharmacology, toxicology, and distribution.

Both of the major saponin groups occur throughout many plant families. Often a plant saponin is actually a mixture of two or more saponins. In a 12 year survey of over 6000 plants representing 208 families and 1397 genera, steroidal saponins were ascertained in a number of monocot and dicot families (Wall et al. 1961a). Of 542 plant species of Malaya, belonging to 295 genera from 89 families, 14% gave a positive saponin reaction (Amarasingham et al. 1964). In Turkmenia 51 of 236 plant species (13 of 48 families) contained saponin Gladkikh et al. 1965). According to Lindner (1946) saponins occur in various genera of the Leguminosae family. It is difficult to discern a pattern of saponin distribution from these studies.

McNair (1932) reported that saponins resemble alkaloids in diminution of toxicity from temperate to tropical climates.

Some saponin-containing plants (spinach, beetroot, and asparagus) are components of the human diet while others are consummed as animal feed. Extracted saponins have found use in such products as soft drinks, soaps, fire extinguishers, confectionaries, and pharmaceuticals. The many demonstrated in-vitro activities of saponin and its detrimental effect on growth of many organisms have prompted legal action to control its use in human foods (George 1965). In spite of numerous investigations the activities and mode of action of saponin in in-vivo monogastric and ruminant animals remains poorly understood.

Many properties of saponins, known to exist at the turn of the century, were described by Sollmann (1942). These include affinity for cholesterol, hemolytic activity, fish and snail toxicant, surface active, mucous membrane irritant, and general solubility in water. The early therapeutic uses of plants containing saponin dating back to the very early 16th century, were based upon effects on absorption, circulation, and hemolysis.

Many important biological activities of saponin have been studied only recently. The antitumoral activity of saponin in rats and mice (Horvath et al. 1967;

and Kupchan et al. 1967) has been investigated only recently. Saponin induced enzyme inhibition has been reported for at least four enzymes (Mircevova et al. 1968; Ishaaya et al. 1965; Ishikawa et al. 1960; and Birk et al. 1963). Cardioactive properties in the frog and guinea pig (Von Blumencron 1941; and Roy et al. 1963), effect on the electrical activity of the rabbit brain (Sokolov 1965), ability to influence atherosclerosis in rabbits and mice (Gladkikh 1965a; and Yefimova et al. 1966), and abortifacient activity in rabbit, goat and cow (Dollahite et al. 1962) are among the many reported activities of saponin.

The antimicrobial activity of saponin is well established (Wolters 1968; Kahl et al. 1966; and Tschesche et al. 1965) and a bioassay for alfalfa saponins was developed on the basis of this activity (Zimmer et al. 1967). Interestingly, however, micro-organism cultures capable of degrading saponin have been observed in silage of Agave (Sanchez-Marroquin et al. 1967) and in the rumen of cattle (Gutierrez et al. 1959).

The role saponins play in plant metabolism and growth, if any, is not known, nevertheless, they have been shown to influence plant development in many ways. An application of saponin solutions to leaves stimulated the development of shoots and roots in Begonia, induced tumors in Hedera helix, influenced chlorophyll

synthesis in Euonymus japonicus, and inhibited root growth in other plants (Benner and Varner 1966). This review also reports a suppression of root hair development in water cress and an acceleration of germination of pea, maize and tomato seeds. Germination is generally inhibited at high saponin concentrations and promoted at low concentrations. Plant growth-regulating activity of some saponins has also been demonstrated (Vendrig 1964).

Distribution of saponins in plant parts is not uniform and maximum levels may occur in different organs of various plant genera at different stages of maturity. Dormant seeds, as well as the tubers, roots, stems, and leaves of Dioscorea contained saponins (Baker et al. 1966). The greatest accumulation occurred in the tubers when the spring shoots were in full bloom. Saponins were detected only in the roots of two Gypsophila species and percent content was highest (as high as 19% of root dry matter) when the first leaves appeared (Kolodzieski et al. 1965). Lowest saponin values of the subterranean organs occurred at flowering in four other plant genera (Drozdz 1964). Differences between the nature of saponins in various plant parts and even in the same part during its development are not uncommon.

Present knowledge of steroidal saponin biosynthesis indicates that cholesterol may be a precursor of certain sapogenins. Cholesterol has been shown to exist in many plant tissues and has been converted by plants to sapogenins and alkaloids (Heftman et al. 1967a). The same review disclosed that the incorporation of radioactive mevalonic acid into sapogenins was higher and qualitatively different in the Dioscorea spiculiflora shoot system than the tubers. Kessar et al. (1968) formed other adducts which readily led to steroidal sapogenins.

Many extraction procedures and evaluation techniques of plant saponins have been utilized. Cholesterol precipitation (Walter et al. 1954), activated carbon-solvent systems (Van Atta et al. 1961b), paper chromatography and electrophoresis (Coulson 1957a), and ion exchange resins and column chromatography (Jackson et al. 1959) have been utilized to isolate saponin fractions. Reaction of extracted saponins with a Lieberman-Burchard reagent provides a chemical assay for saponin (Gestetner et al. 1966; Van Atta et al. 1958a). Quantitative and qualitative evaluation of plant saponins has been based primarily on their biological activities. Many alfalfa and soybean saponin evaluations have employed biological assays similar to those developed for saponins of other sources. Special attention is currently being paid to the biological

properties and physiological activities of compounds found in these two saponin containing plants in order to improve their present and future utilization.

Alfalfa Saponins

Evaluations of the energy, protein, fiber, mineral, fat, and vitamin content of alfalfa have been useful criteria for quality, but in many cases could not be closely correlated with biological responses. The search for other factors affecting alfalfa nutritive utilization has revealed the presence of estrogens (Bickoff *et al.* 1957a), purine derivatives (Bickoff *et al.* 1968b), and saponins (Lourens *et al.* 1951).

Although an extract from alfalfa possessing the properties of saponins was obtained early in the century (Jacobson 1919), little research was directed toward understanding the biological and chemical properties of alfalfa saponins until recently. As many as seven to ten alfalfa saponins have been isolated using paper chromatographic techniques (Lourens 1961; and Coulson 1962b) and several biological activities have been attributed to these water-soluble compounds.

Biological activities, extraction, purification, and characterization of alfalfa saponins are reviewed by Scardavi *et al.* (1967). Articles on saponin inhibition of seed germination (Guenzi *et al.* 1964; Pedersen 1965; and Megie *et al.* 1967), control of the causative fungus

of Avocado root rot (Zentmyer et al. 1967), association with ruminant bloat (28), and the development of an assay based on a saponin sensitive fungus (91) concur with observations cited in the review by Scardavi et al. (70). Other investigations as to the cause of ruminant bloat, however, indicate that alfalfa saponins are probably not solely responsible for this condition (Lindahl et al. 1957; Stifel et al. 1968; and McArthur 1964).

The many activities of alfalfa saponins are thought to be primarily the action of triterpenoid saponins. Extraction and purification methods have been based primarily on water or alcohol extracts and the property of saponins to be adsorbed on charcoal, or combined with cholesterol. Detection and characterization of alfalfa saponins have been based on reactions with Liebermann-Burchard reagent, inhibition of the respiration of rat diaphragm, chick and fungus growth inhibition, hemolytic activity, melting point, specific rotation and spectographic information.

Pedersen et al. (1967b) was able to confirm the report of Hanson et al. (1963) which demonstrated quantitative differences between varieties. He also reported higher saponin concentrations in the leaf than the stem and quantitative changes within varieties sampled throughout one growing season. This is not surprising as quantitative changes within seasons have

been observed in other saponin containing plants (3, 45, 15). In the same report Pedersen *et al.* (62) demonstrated the existence of qualitative alfalfa saponin differences in varieties by the use of chicks, fungus, and lettuce seed germination assays. *M. sativa* and *M. falcata* are reportedly two of the highest saponin containing species of the *Medicago* genus (Jaretzky 1940).

Saponin biosynthetic activity of the *Dioscorea spiculiflora* shoot system was greater than the roots (Bennett *et al.*1965) and leader shoots were given as possible sites of saponin synthesis in two other *Dioscorea species* (3). Nevertheless, saponins occurred throughout the plants. Saponins occur in all alfalfa organs; roots, leaves, stems, and flowers (Morris 1965; 1961; and Pedersen 1967b), but may be qualitatively different. One site of synthesis is unlikely if saponins cannot diffuse through living cells or across grafts as was the case in *Calendula officinalis* (Fischer et al. 1951).

Heat processing of extracted soybean saponins decreased hemolytic activity, but had no influence on saponin within heat treated seed (8). Heating saponins of other sources did not change inflammatory or hemolytic properties, however, treatment with cholesterol decreased hemolytic, but not inflammatory properties (Richou *et al.* 1965). Cholesterol had no

influence on hemolytic activity of a group of acid saponins or a saponin extracted from Chelidonium majus L. (Ruyssen et al. 1946; and Kwasniewske 1958).

Genetic Studies

The general and/or specific combining ability (gca/sca)- as determined by Griffing's (26) analysis of dialled systems - of a number of quantitative characters in alfalfa have been studied recently. Significant effects for gca existed for alfalfa forage yield (Porceddu 1968; and Daday 1967). Likewise, gca of the following M. sativa plant characteristics was highly significant; estrogen content, leaf/stem ratio, protein content, in-vitro dry matter digestibility, cell wall constituents, acid detergent lignin, and acid detergent fiber (Gil et al. 1967; and Stuthman 1967). Buker (1963) reported significant gca for percent leaves. Breeding for improved methionine levels may also be feasible (Singleton et al. 1952).

Two reports have directed attention to possible progress in lowering alfalfa saponin levels through plant breeding (29, 62). An estimate of saponin heritability exists for one crop, Dioscorea floribunda. This plant is cultivated for yields of steroidal saponins used in the synthesis of steroidal drugs (Martin et al. 1967). Clones were compared for fresh

weight, dry weight, precent sapogenin, and total sapogenin. Percent sapogenin was the only trait with an estimate of heritability which would be useful.

Erythrocyte Assay

Saponins, as determined by hemolytic activity, have been reported in many plant families (22). Alfalfa saponins possess hemolytic activity (48), however, this activity has not been used as a major screening criterion in alfalfa saponin studies.

The hemolytic activity of saponins has been attributed, by some researchers, to a chemical reaction of saponins with cholesterol of the cell membrane (Joos *et al.* 1967b, and Schmidt-Thome *et al.* 1950). Not all hemolytic saponins, however, form cholesterides (Joos 1966a; Kwasniewski 1958; and Ruyssen *et al.* 1946). Complete hemolysis occurred at concentrations equal to or lower than would be needed to form a single monolayer on the cell surface (Gorter *et al.* 1931; and Granick 1949). In one study (Kesten *et al.* 1928) hemolysis was inversely proportional to cell concentration and directly proportional, through the greater portion of the reaction, to the square of saponin concentration. With a given saponin, hemolytic velocity varies with the source of the erythrocyte, being low for man and high for sheep (71). Rate of saponin hemolysis of different normal human blood samples

was essentially the same under constant conditions (Elbel et al. 1934; and Kesten 1928). Erythrocyte resistance to saponin hemolysis is about the same, irrespective of source, at equal saponin and erythrocyte concentrations (Jung et al. 1950).

Richou et al. (66) found no relation between hemolytic activity, lethal dose in mice, and inflammatory effect of different lots of saponin. Lindahl et al. (48) also reported no definite correlation between blood hemolysis and other physiological actions of alfalfa saponins, however, Awe et al. (1950) found that the surface tension and hemolytic indices of extracts of certain roots and fruit had parallel variations. Vacek et al. (1962) showed that correlations existed between foam number, superficial tension, hemolytic activity, and toxicity after subcutaneous administration of four saponins.

Fish Assay

Fish were used early in the 20th century to evaluate toxic effects of certain plant extracts (Priess 1911). An "equation of toxicity" was developed for surface-tension lowering poisons and successfully tested on minnows 90 - 100 mm in length of Alburus lucidus in 1942 (Macovski et al. 1942). A fish assay was proposed as a simple method for verifying the presence of saponin in water extracts of

ichthyotoxic plants (Meyer 1942) and a fish index, based upon Lebistes reticulatus, was later used to evaluate saponin extracts from a Brazilian shrub (Wasicky et al. 1949). Saponins were found to control predaceous fish in shrimp ponds in Taiwan (Tang 1961).

Chemical Assay

A procedure described by Wall et al. (1952b) for extraction, isolation, and identification of steroidal sapogenins has been employed in the isolation of alfalfa saponins (50, 91). A gravimetric determination of total alfalfa saponin was developed by Van Atta et al. (81). The latter method of extraction has been used to study alfalfa saponins as related to variety, cutting, and location variables (29), and to compare chemical and biochemical assays (62).

MATERIALS AND METHODS

Plant material was obtained primarily from eight alfalfa populations of three Medicago species (M. sativa, M. falcata, and M. glutinosa). Only one of the populations had been previously selected for saponin content.

Populations of three diallel crosses (two of Vernal and one of Culver parentage), two USDA releases (MSA and MSB), DuPuits, M. falcata (Russian source 22506), and M. glutinosa (Russian source 29003) were transplanted on 0.914 m centers in the summer of 1965 at East Lansing, Michigan on Conover loam with a pH of 6.5. Phosphorous levels were adequate for good alfalfa growth, however, potassium levels were low. A selected Culver x Vernal population was established in the same area in 1966. Harvests of individual clones were made at first bloom on June 10, 1967 and 1968. Following air drying, leaf-stem separations were made on much of the 1968 harvested material. Field and greenhouse materials were dried at 43 - 47 C, ground to pass through a 1 mm screen, and stored at 0 - 6 C.

Greenhouse materials were of two types; 1. established field clones selected and transferred as potted plants to the greenhouse for further study and 2. seedlings grown from selected crosses.

Evaluation of Alfalfa Saponin

Hemolytic activity and ichthyotoxicity, were the basis of the four assays in this study for evaluating individual plants.

A. Erythrocyte Assay I

Crude saponin extracts of alfalfa were prepared by soaking 2 g of ground leaf, stem, or whole plant samples in 23 ml of physiological saline solution for 2 hr. (The saline extracts were not different in hemolytic activity from distilled water extracts made isotonic with salt after extraction.) The mixture was then filtered through two layers of Kimwipes tissue and the filtrate collected and refrigerated at 2 C. Specific amounts of the extracts were added to a 2% human erythrocyte solution. The erythrocyte solution was prepared by centrifuging citrated human blood at 5,900 g for five min, washing the collected red blood cells twice, and adding 20ml to 980 ml of physiological saline solution.

Ten tubes, each containing 1 ml of the erythrocyte preparation, were used in a series to evaluate each plant

extract. One ml of the plant extract was added to tube #1, mixed, and 1 ml transferred with a 1 ml syringe to tube #3. The contents of tube #3 were mixed and transferred, as above, through tubes #5, 7, and 9. One ml of a diluted plant extract (1.3 ml plant extract + 0.7 ml saline solution was placed in tube #2, mixed, and transferred in the same manner through tubes #4, 6, 8, and 10. The series was placed in a water bath (37 C) for 30 min, removed from the bath and allowed to remain at room temperature for 15 - 20 min before reading. A series containing no saponin was included periodically to aid percent hemolysis estimates.

This preparattion differed from a standard dilution series. Plant extract concentrations in the reaction mixture were obtained by transferring one-half the initial plant extract-erythrocyte mixture through a series of tubes containing only erythrocyte preparation. In this series the plant extract concentration decreased and, unlike the standard series, the concentration of erythrocytes increased. This allowed a rapid method for obtaining concentration gradients and the results did not differ significantly from those obtained in a standard series.

The lowest concentration of plant extract causing complete hemolysis was termed "titer". Complete hemolysis was defined as absence of erythrocyte sedimentation at the bottom of the test tube. Titer values were established

by recording the last tube containing completely hemolyzed erythrocytes and estimating the percent hemolysis occurring in the adjacent tube. The "hemolytic value" was calculated as

$$\frac{\text{volume of 2\% erythrocyte preparation}}{\text{volume of undiluted extract at titer concentration}}.$$

These proportions for tubes #1 - 10 respectively were 1.00/1.00, 1.00/0.65, 1.50/0.50, 1.50/0.33, 1.75/0.25, 1.75/0.16, 1.88/0.13, 1.94/0.06, 1.94/0.04, and the resulting "hemolytic values" were 1.0, 1.5, 3.0, 4.6, 7.0, 10.8, 15.0, 23.1, 31.0, and 47.7 respectively. In a few instances the plant extracts did not have sufficient hemolytic activity to cause lysis in tube #1. These samples were freeze-dried and reconstituted to increase the extract concentration as much as fivefold. Two hemolytic determinations were made for each sample.

Modifications in preparing plant samples for the hemolytic assay included the use of fresh and frozen plant samples. Water extract of fresh plant material was obtained by chopping 7 g of alfalfa leaves with 50 ml saline solution for 1.5 min at high speed in a Waring blender and filtering the resulting mixture through two layers of Kimwipes. Plant juices were also collected by freezing a small fresh sample and after thawing, placing it under 2,270 kg pressure in a Carver press. Extracts from samples thus prepared were analyzed in the same manner as the extracts of dried material.

B. Erythrocyte Assay II

Sample size was limiting in one of the studies. As little as 30 - 40 mg of dried alfalfa leaves was available. In order to accurately evaluate the hemolytic activity of this size sample, microscopic observations were made of the erythrocyte-extract solutions. Saline extracts of alfalfa leaves (1 part saline solution : 0.017 parts alfalfa leaf) were obtained using a 2 hr soaking period. This extract was filtered through one layer of Whatman #1 filter paper and added to an 0.08% human erythrocyte preparation in a micro concave slide. Different volume combinations of plant extract and erythrocyte preparation were used to obtain the desired erythrocyte preparation:plant extract ratios. Pipettes of 10 - 100 λ capacity allowed proper volume combinations. The reaction mixture was stirred twice during the ½ hr reaction period and covered at other times to avoid evaporation losses.

A dilution series contained ten erythrocyte-extract mixtures and included erythrocyte preparation: plant extract ratios from 2.0:1.0 to 1.0:0.07. In most cases this series of ten mixtures included the desired hemolytic range (0 - 100% hemolysis). If the hemolytic range was not included, ratios adequate to include this range were prepared. After a ½ hr incubation period the erythrocytes were observed at a magnification of 125 X to determine the degree of lysing. Titer values were

established by recording the last preparation containing completely hemolysed erythrocytes and estimating the percent hemolysis occurring in the adjacent preparation. Erythrocyte preparation:plant extract ratios as small as 1.0:6.0 and as great as 1.0:0.02 were prepared. Two hemolytic determinations were made for each plant extract.

C. Erythrocyte Assay III

An abbreviated form of Assay I was developed to screen large numbers of alfalfa plants for hemolytic activity. Sample size ranged from 2 - 7 leaflets (approximately 23 - 28 mg) depending upon leaflet size. One sample was collected from each plant in an area of young, fully expanded leaves. Leaflets were placed in 3 ml test tubes, dried, crushed with a glass rod, and 2 ml of 1% human erythrocyte preparation added. Each tube was shaken twice in the following 20 min and visually rated for degree lysing after ½ and 4 hr. Samples causing complete hemolysis in the first ½ hr were rated as having high hemolytic activity and those causing no hemolysis after 4 hr were rated very low in activity. Only plants evaluated as being very low in saponin were re-evaluated.

D. Fish Assay

Locally available fathead (Pimephales promelas) minnows were purchased. The minnows were sized, 4.0 - 4.5 cm minnows saved, and held at 20 C for no more than two days. Alfalfa crude saponin extracts for the minnow assay were prepared by soaking 2 g ground alfalfa leaves in 23 ml distilled water for 120 min. The mixture was then filtered through two layers of Kimwipes tissue and the filtrate collected and refrigerated at 2 C.

One minnow was placed in a 360 g wax paper cup containing 90 ml distilled water (20 C) for each plant assay. If the minnow appeared calm and healthy 5 min after its transfer, 10 ml of the alfalfa extract was added. The minnows were closely observed for the duration of the assay (180 min maximum). The time at which the minnow became immobilized was recorded. At least two determinations were made for each plant extract.

E. Chemical Assay

Alfalfa leaves were chemically analyzed for total saponin by the method of Van Atta et al. (81). The leaf samples were finely ground so 3 g of plant material were used rather than the suggested 15 g and proportionally smaller volumes of liquids were used to prepare the extract solution. One determination was made for each leaf sample.

The above assays are the basis for all saponin evaluations to be described.

F. Other

Griffing (26) Model II, Method 2 was used to partition mean squares for crosses into general and specific combining ability. Individual plants from within crosses were used to estimate the within family variance. Estimates of heritability were calculated according to the formula

$$\frac{2\hat{\sigma}^2_g}{2\hat{\sigma}^2_g + \hat{\sigma}^2_s + \hat{\sigma}^2_e} .$$

Tips of alfalfa stems (1.0 - 1.5 cm) selected for high or low hemolytic activity were grafted onto stems of selected plants. The grafting procedure was the same as reported by Gorz et al. (24) in which Melilotus alba Desr. scions were grafted onto stems of the same species. Thirteen M. sativa plants from four different populations were selected as source material. Scions of high and low hemolytic activity were grafted onto stocks of both high and low activity. A limited number of scions were grafted back onto the plant from which they were removed. As many as six grafts were made of each graft combination with a total of more than 250 grafts. Scions were harvested after attaining maximum growth and the leaves analyzed by Erythrocyte Assay III.

RESULTS AND DISCUSSION

The erythrocyte assays provided a means of better studying the nature of saponins in alfalfa. Each erythrocyte assay was a rapid, sensitive, and reproducible method of evaluating hemolytic, water soluble alfalfa saponins. The degree of lysing was visually observed in each of the assays with no difficulty because distinct breaking points existed between lysed and non-lysed preparations, even at similar plant extract concentrations.

Erythrocyte Assay I

A typical range of hemolytic activity for Assay I is presented in Figure 1. Each "Set" was the dilution series of one plant extract. Tubes were numbered from left to right. The titer of "Set A" was between tubes #1 and 2. Erythrocytes were completely lysed only at the highest leaf extract concentration (tube #1). Saponins were not present in sufficient quantity in tubes #2 - 10 and intact erythrocytes settled. The titer of "Set B" was between tubes #5 and 6. Very high hemolytic activity existed in the extract of "Set C." The titer was between tubes #9 and 10. Between 40 - 70% hemolysis occurred in tube #10.

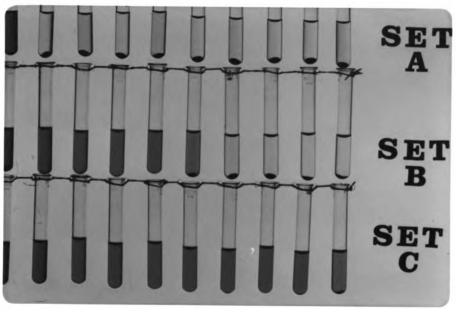

Figure 1. Ten tube dilution series of three plant extracts evaluated for hemolytic activity by Erythrocyte Assay I. "Set A" - low saponins, "Set B" - average saponins, and "Set C" - high saponins.

The ten tube hemolysis series had a "hemolytic value" range of 1 - 48. "Hemolytic values" as low as 0.2 have been recorded after concentrating plant extracts of low activity by freeze-drying. Saponin preparations possessing "hemolytic values" greater than 48 could easily be evaluated by continuing the dilution series beyond tube #10. Values ranging from 0.4 to 45.0 are reported in Table 1; over a hundredfold difference in hemolytic activity.

Larger populations of alfalfa plants could be evaluated by Assay I by modifying the harvesting and sampling procedures. Water extracts of plants freshly harvested and homogenized in a Waring blender exhibited the same order of hemolytic activity as dried, ground, and water extracted plants. Similar activity also resulted from plant juices collected from fresh frozen samples. Erythrocyte Assay III would be adequate for screening most plant populations for saponin but Assay I could also be effectively employed by using only five tubes in the hemolysis series (tubes #1,3, 5,7, and 9).

Erythrocyte Assay II

Low concentrations of erythrocytes were used for this assay (0.08% by volume) and plant extracts were filtered through filter paper to facilitate observation of erythrocytes under the microscope. The break between complete and no hemolysis was nearly as distinct as in Assay I.

A gradation of hemolysis usually occurred (from 0 - 100%) within three preparations of a series. Figures 2, 3, and 4 illustrate this gradation (0, 50, and 100% hemolysis respectively).

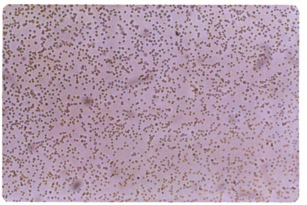

Figure 2. Micrograph of 0% hemolysis at an erythrocyte preparation:plant extract ratio of 1.00:0.85.

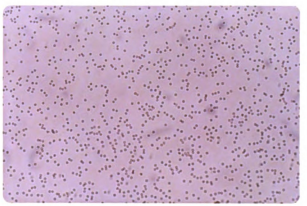

Figure 3. Micrograph of 50% hemolysis at an erythrocyte preparation:plant extract ratio of 1.00:1.00.

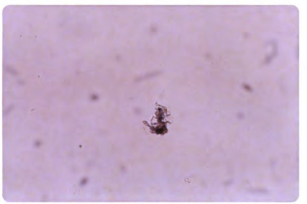

Figure 4. Micrograph of 100% hemolysis at an erythrocyte preparation:plant extract ratio of 1.00:1.15.

In this case gradation of hemolysis occurred at erythrocyte preparations: plant extract ratios of 1.00:0.85, 1.00:1.00, and 1.00:1.15 respectively. Ratios as low as 1.00:5.00 wererequired to obtain complete hemolysis in some samples of low saponins and as high as 1.00:0.027 to cause complete hemolysis in samples with very high saponins. Over eighty alfalfa samples were evaluated by this assay and a representative portion of them re-evaluated by Erythrocyte Assay I and III to compare results.

Erythrocyte Assay III

Over 1500 greenhouse seedlings and established field clones were evaluated by Erythrocyte Assay III in the spring of 1969. The analysis of individual plants is illustrated in Figure 5. A vertical series of three tubes formed a time sequence for one plant. The horizontal series "Set A" is all ten plant extract-erythrocyte preparation mixtures after ½ hr. "Set B" is the same set of mixtures after 2 hr and "Set C" after 4 hr. Plant extracts 2,3,5,6,7,9, and 10, from the left of the horizontal series, exhibited no hemolytic activity after ½ hr. Complete hemolysis had already occurred in plant extracts 1,4, and 8 after the ½ hr reaction period. Only plant extracts 3,6, and 9 exhibited no hemolysis after the 2 hr reaction period and these same extracts demonstrated no hemolytic activity after the

4 hr period ("Set C"). The plants of this series were classified as follows; plants 1,4, and 8 --high saponins; 3,6, and 9--very low saponins; and 2,5,7, and 10--moderate saponins.

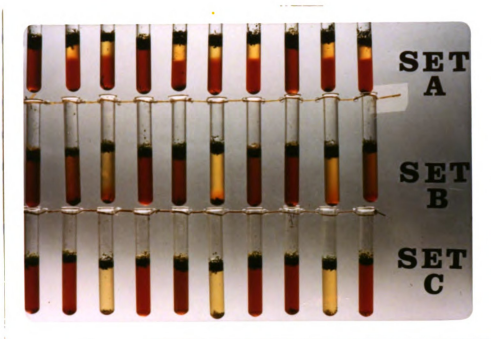

Figure 5. Hemolysis time sequence of ten alfalfa leaf samples analyzed by Erythrocyte Assay III. Horizontal series-Sets A, B, and C- are hemolysis after ½, 2, and 4 hours of ten plant samples arranged in vertical rows.

Plant sample preaparation was minimal for this assay, however, it was sufficient to allow the saponins of the alfalfa leaf to express themselves as hemolytic agents. Hemolytic activity of whole uncrushed leaflets was very low, but crushing the dried leaflets slightly, even with the fingers, was sufficient to allow the same

hemolytic activity as if the sample was dried and ground through a mill.

Comparison of Erythrocyte Assays

Information obtained through Assay I forms the basis of the studies reported. Assays II and III were developed to allow evaluations of very small and numerous samples. Although the assays have the use of hemolytic activity in common, each has its unique characteristics.

Thirty samples were prepared and analyzed per day with Assays I and II. Over 200 plants could be evaluated in one day by one individual with Assay III. The variance of determinations was very low. Standard deviation on a single determination basis of one preparation of 14 samples was 0.3 and 0.5 hemolytic units respectively for material analyzed by Assay I and II. Larger variations would be expected in Assay III because the sample size was not strictly controlled.

When values from Assay II were adjusted to the common basis of "hemolytic value" a lower range of values was obtained than with Assay I. The highest "hemolytic value" from Assay II would have been 7.0 and the lowest would have been less than the lowest recorded in Assay I. This reduced activity is due in part to filtering of the extract, but is thought to be caused primarily by the increased surface area provided by the micro concave

slide. As reaction mixtures are spread thinly over a large surface the hemolytic activity of surface-active saponin will decrease considerably (Ponder 1948).

Assay III values compared well with those of Assay I. If hemolysis did not occur in Assay III after 4 hrs it would have a "hemolytic value" less than 3. "Hemolytic values" less than 3 would also be considered low in Assay I.

In spite of differences in actual "hemolytic values" the three assays, rated plants in the same relative order.

Fish Assay

The biological activity of saponins was readily expressed by using the simple materials and methods of the fish assay. Defining the time of assay termination was important for consistent and meaningful results. The assay may be terminated when the fish becomes immobilized or dies. Immobilization was reported in the study because it varied less between determinations and was more easily observed than death. Time from immobilization until death may range from 1 - 15 min depending upon toxicity of the sample. Standard deviation on a single determination basis of 20 samples evaluated for minnow toxicity was 3.9 minutes. The period from assay initiation to minnow immobilization ranged from 10 min to termination of the assay (180 min)(Table 1).

A 0.0135% solution of commercial saponin (Nutritional Biochemicals Corporation) immobilized the minnows in 8 - 9 min.

Local California shiner minnows and female Gambusia fish have also been successfully used to detect alfalfa saponins.

Comparison of Erythrocyte Assay I, Fish and Chemical Assays

Values of each assay for alfalfa leaf saponins (Table 1) were correlated with the other two assay values (Table 2). Correlation coefficients for transformed fish and erythrocyte and non-transformed chemical values (-0.91, 0.90, and -0.81) as well as non-transformed fish and erythrocyte values indicated that the two saponin assay procedures accurately described alfalfa populations for crude saponin levels. The square root transformation of erythrocyte values improved only slightly the correlation between erythrocyte and chemical assays.

High correlation coefficients have also been obtained by evaluating alfalfa stem and whole plant samples with the three saponin assays. The fish assay was not as highly correlated with the chemical determination as was Erythrocyte Assay I and it lacked other assay qualities possessed by the erythrocyte assay.

However, the availability of human blood or a uniform source of minnows may be the major criterion for selecting between the two assays.

Table 1. Erythrocyte, minnow, and chemical assay values of leaf samples from twenty alfalfa plants.

"Hemolytic value"	Plant extract toxicity to minnows: min required for immobilization	Chemical determination: mg crude saponin/3 g sample
X	Y	Z
45.0	11	78
31.0	22	69
28.0	14	62
26.0	10	72
26.0	12	70
21.0	14	56
19.0	30	48
16.0	20	46
16.0	34	48
16.0	39	31
7.0	78	38
7.0	40	43
7.0	58	46
7.0	105	45
7.0	117	44
3.0	180	35
2.0	180	39
1.9	180	33
0.6	180	26
0.4	180	20

Table 2. Correlation coefficients for three assays of crude saponin extracts of alfalfa leaves.

	$\sqrt{Y}$	Z
$\sqrt{X}$	-0.91	0.90
X	-0.82	0.89
$\sqrt{Y}$		-0.81
Y		-0.76

The erythrocyte assay may be affected by presence of a substance(s) or condition(s) altering the nature or rate of hemolysis. Accelerators or inhibitors of hemolysis could produce reversible or irreversible effects on the cells of the hemolytic system or affect the properties of the hemolysin. Sugars, proteins, electrolytes, pH, and temperature are some of the factors which may affect the rate or degree of hemolysis. Variations in these or other factors, however, were not major deterrents to the reliability of the erythrocyte assay.

The fish assay would be affected less likely by moderate fluctuations of the above variables. Both the erythrocyte and fish assays undoubtedly reflected qualitative as well as quantitative saponin differences, however, for these materials studied, the quantitative

aspect had the greatest influence on assay values.

Distribution and Heritability of Saponin in Alfalfa

Studies evaluating the presence, distribution, and inheritance of saponins in diverse populations of the plant kingdom have most often been directed toward identifying specific species able to yield saponin of high pharmaceutical value. Consequently, plant species containing high levels of steroidal saponin have been studied more completely than those containing a predominance of the triterpene types. Saponin containing plants which may have negative nutritional effects on animals consuming them, such as alfalfa, have not received adequate attention.

Past saponin evaluations of alfalfa have relied primarily upon *M. sativa* for source material. Many characteristics of saponin from this source are similar to those of other plant saponins. Plant saponin surveys based upon these common characteristics indicated that its presence often occurs in many species of a genus containing saponin. Therefore, its distribution in *Medicago* species would also be expected to be rather broad.

Individual whole plants of three *Medicago* species and three *M. sativa* varieties were sampled in 1967 and 1968 and analyzed for crude saponin by Hemolytic Assay I (Table 3). Differences of saponin content within species

was much greater than differences between. M. sativa exhibited the greatest range of "hemolytic values" of the three species, however, it was also the most thoroughly sampled. The mean "hemolytic value" of M. falcata was double that of the other two species.

Of the varieties, Vernal, Culver, and DuPuits plants possessed "hemolytic values" of 103, 16, and 3 fold differences, respectively. DuPuits exhibited the highest and Culver the lowest mean values.

Table 3. "Hemolytic value" ranges and means of three Medicago species and three M. sativa L. varieties.

Species or variety	Number of plants	"Hemolytic Value" range	mean
M. glutinosa	40	3.0-15	7
M. falcata	40	3.0-35	16
M. sativa	250	0.2-31	6
DuPuits	30	5.0-15	9
Culver	50	1.0-16	4
Vernal	80	0.3-31	6

M. dzawkhetica as well as other plant introductions and varieties have also been sampled and shown to contain saponin. The level of saponin was not always as high as that of the three species sampled in detail. Moapa, a variety of M. sativa developed from plant introductions

from Africa, was shown to be very low in saponin on the basis of the fish and Erythrocyte assays.

The only available information regarding the inheritance of saponin, based upon a study conducted in Puerto Rico on Dioscorea floribunda (50), revealed that the heritability of percent sapogenin was "moderate." The heritability of saponin in alfalfa was investigated in the present study by evaluating the saponin content of individual plants from three M. sativa populations. Estimates of saponin heritability were found to be "moderate - high."

One-half of the diallel crosses within Vernal and Culver parentage were harvested in 1967; two years after establishment. The parents were part of a comprehensive alfalfa quality study but had not been selected previously for saponin content. Six Vernal parent and five Culver parent clones formed the basis for each of the diallel crosses. At least ten plants were established (1965) for each of the resulting 15 Vernal and 10 Culver crosses. Ten randomly chosen Vernal crosses were harvested as bulks and up to twelve plants from each of six crosses were harvested and analyzed individually for an estimate of within family variance. Six Culver crosses were randomly chosen and harvested as bulks and the within family variance estimated from six to ten plants from each of five crosses harvested and analyzed individually.

The "hemolytic values" of six Vernal parents ranged from 1.3 - 25 and those of 5 Culver parents from 1.4 - 7. The gca was highly significant in both populations (Table 4). The sca was also significant in both populations (5% level in Culver and 1% level in Vernal).

Table 4. Analysis of variance and estimates of saponin heritability of two M. sativa L. populations.

	df		SS		MS	
Source	Vernal	Culver	Vernal	Culver	Vernal	Culver
gca	5	4	320.15	30.01	64.03**	7.50**
sca	15	10	139.74	5.10	9.32**	0.51*
error	51	39			0.41	0.20

	Vernal	Culver
Estimate of heritability	0.60	0.79

* significant at 5% level
** significant at 1% level

A major portion of the highly significant sca in Vernal was due to the transgressive effect exhibited by progeny of two combinations; V 51 X V 214 and V 37 X V 214 (Table 5). The progeny of these combinations as well as V 37 X V 132 had mean "hemolytic values" lower than either parent. A trend toward plants of lower saponin content than would be expected from parental levels existed in this population. Over 45% of all individual plant values from this Vernal parentage were lower than either parent; less than 4% were higher.

Table 5. Mean progeny "hemolytic values" of Vernal alfalfa parentage.

	V 7	V 214	V 132	V 51	V 46	V 37
V 7	1.4	7.7	5.5	3.0	1.4	3.9
V 214		24.6	8.0	6.3	4.7	7.9
V 132			6.3	6.3	3.6	5.5
V 51				7.0	3.9	7.0
V 46					1.3	3.6
V 37						8.3

This trend did not exist in the Culver population (Table 6). Only one progeny mean value fell outside the parental values, C 58 X C 13, and it was higher than expected. Only 7% of the plant values were lower than either parent, but 28% were higher.

Table 6. Mean progeny "hemolytic values" of Culver alfalfa parentage.

	C 44	C 5	C 13	C 268	C 58
C 44	2.2	3.1	3.1	1.4	3.0
C 5		7.0	5.9	3.0	4.7
C 13			3.0	2.3	5.0
C 268				1.4	3.0
C 58					3.9

Progeny of a diallel cross between Vernal plants previously selected for growth characteristics under different fertility conditions formed the basis of a more exhaustive study of saponin inheritance in alfalfa. Seven parent clones formed the basis of the diallel crosses. Ten plants of every combination of one-half of this diallel were harvested on June 10, 1968 and leaf-stem separations made. Samples were analyzed by Erythrocyte Assay I. The gca of leaves, stems, and whole

plant were highly significant (Table 7). In no case was the sca significant. The estimates of heritability were high; leaf 0.903, stem 0.916, and whole plant 0.902. This indicated that the genetic system controlling saponin levels in alfalfa was expressed equally well in the leaf and stem. Mean progeny leaf, stem, and whole plant "hemolytic values" are reported in (Table 8).

Table 7. Analysis of variance and estimates of saponin heritability in leaf, stem, and whole samples of a diallel cross between seven Vernal clones.

Source	df	SS			MS		
		leaf	stem	whole	leaf	stem	whole
gca	6	765.10	105.07	374.37	127.52**	17.51**	62.40**
sca	21	52.15	7.33	30.87	2.48	0.35	1.47
error	189				3.00	0.27	1.31

	leaf	stem	whole
Estimate of heritability	0.903	0.916	0.902

** significant at 1% level

Table 8. Mean progeny leaf, stem, and whole plant "hemolytic values" of Vernal alfalfa parentage.

Mean progeny leaf "hemolytic values".

	H1	H3	H4	L1	L3	L4	H5
H1	15.0						
H3	14.6	21.0					
H4	16.2	19.4	17.0				
L1	9.2	14.8	16.6	8.9			
L3	10.2	12.7	11.6	6.5	4.6		
L4	6.0	11.8	7.5	3.3	3.8	1.1	
H5	11.9	10.5	10.6	4.4	3.7	3.5	3.0

Mean progeny stem "hemolytic values".

	H1	H3	H4	L1	L3	L4	H5
H1	5.8						
H3	6.9	7.9					
H4	5.4	5.4	5.8				
L1	2.7	4.1	3.6	3.0			
L3	4.0	3.6	3.0	2.0	0.7		
L4	2.6	3.8	1.7	1.0	0.7	0.2	
H5	4.4	4.0	2.7	1.4	1.1	0.8	1.0

Table 8 (cont'd.)

Mean progeny whole plant "hemolytic values".

	H1	H3	H4	L1	L3	L4	H5
H1	10.4						
H3	10.0	15.5					
H4	10.7	13.3	11.6				
L1	5.9	9.8	10.2	6.8			
L3	7.0	8.5	7.7	4.4	2.8		
L4	3.5	7.9	4.8	2.1	2.4	0.7	
H5	8.5	6.9	5.6	2.8	2.4	2.1	2.0

In this population one-third of the leaves and whole plants, and one-fourth of the stems were higher or lower in saponin content than either of the parents. Unlike the previous Vernal population, leaf, stem, and whole plant values falling outside parental values were equally divided between those which were higher and those which were lower.

Saponin levels of leaf and stem were significantly correlated ($t_{.01}$), however, the correlation (0.74) was less than might be expected (Table 9). "Leaf saponin/stem saponin" values ranged from 0.8 - 45. Stem saponins averaged one-third that of the leaves. In spite of the fact that both stem and leaf saponin were significantly

correlated ($t_{.01}$) with the "leaf/stem ratio", 0.21 and 0.20 respectively, the correlations were not sufficiently high to hamper selection for leafy plants of low saponin content.

Table 9. Correlation coefficients of leaf and stem "hemolytic values" and leaf/stem ratios.

	leaf saponin	leaf/stem ratio
stem saponin	0.74**	0.20**
leaf saponin		0.21**

** significant at 1% level

Sufficient variation in saponin content existed in all alfalfa populations studied to allow selection for high and low saponin lines. Progeny from plants selected for low or high saponins should be lower or higher in saponins than the average non-selected population. Fixation of this character at a low level should be rapid if the estimates of heritability were accurate. Likewise, high saponin levels should be realized in progeny of plants selected for high saponin content.

These observations were found to be correct. Over 1500 plants of 2nd. cycle saponin selection or unselected populations were analyzed by Erythrocyte Assay III in

1969. Most of the plants were greenhouse seedlings resulting from 1968-1969 crosses.

Plants were rated as low, medium, or high in saponins on the basis of hemolytic activity exhibited by leaflets from young seedling plants or the 2 - 4 leaflets from established plants required to obtain a leaf sample of 23 - 27 mg. Parental combinations and saponin levels of their progeny are presented in Table 10.

Unselected Vernal, Culver, and MSB populations had normal saponin distributions when individual plants were classified as low, medium, or high. The DuPuits population was skewed toward high saponins. The distribution of all progeny from selected low and high combinations, however, was very skewed (Figure 6). Nearly 79% of the progeny from selected low plants were classified "low" and over 86% of the progeny from selected high plants were classified "high".

The distribution of saponins in unselected Saranac (Table 10) was skewed to the high side. This is not surprising as Saranac is closely related to DuPuits. The other variety, Team, was developed for weevil resistance. One parent of the California increase had previously been selected for low saponins. This selection was apparently very effective for lowering the level of saponins.

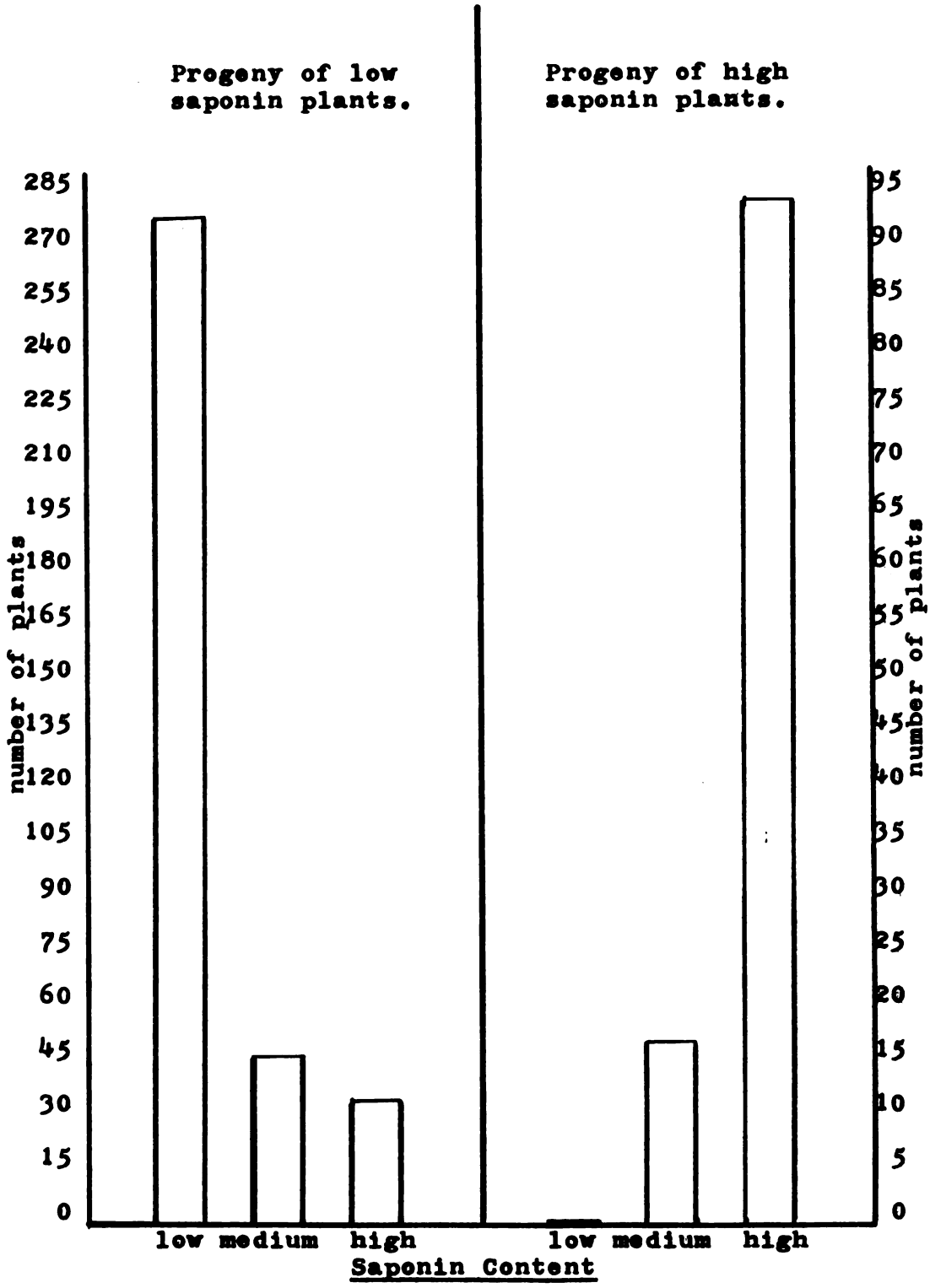

Figure 6. Distribution of progeny from plants selected for low and high saponin.

Table 10. Saponin levels of progeny from selected low or high saponin parents, two unselected varieties, and one partially selected alfalfa increase.

Source	Low	Medium	High
Low saponin--2nd. cycle			
C1 / C/V 1	22	2	5
C 2 / C/V 2	23	4	4
C 2 / C/V 3	20	5	3
V 1 / V 2	24	3	1
V 3 / V 4	14	11	2
V 5 / V 4	20	1	0
V 6 / V 1	24	3	3
V 7 / V 6	10	4	4
C/V 2 / C/V 4	25	0	0
C/V 5 / C/V 6	13	1	0
B 1 / B 2	22	2	1
B 1 / B 3	19	6	1
B 3 / B 1	19	6	1
B 3 / B 2	22	0	1

Table 10 (cont'd.)

	Low	Medium	High
High saponin--2nd. cycle			
$\frac{B\ 4}{B\ 5}$	0	2	25
$\frac{B\ 5}{B\ 4}$	0	4	21
$\frac{V\ 8}{D\ 1}$	0	7	22
$\frac{D\ 1}{C\ 3}$	0	2	27
Varieties previously unselected for saponin			
Saranac	8	23	46
Team	21	28	53
Alfalfa increase partially selected for saponin			
$\frac{L_4 909}{N\ 529}$ F_2 increase	266	56	39

V - Vernal D - DuPuits
C - Culver B - MSB

Some Properties of Alfalfa Saponins

Due to the diversity of compounds classified as saponins, different biological activities and chemical properties would be expected of saponins from various sources. Cholesterol influences the hemolytic activity of some saponins, but not all. Heat is also variable in its influence on hemolytic activity of different saponins. It is possible that the effect of these two factors may even vary from plant to plant in a heterozygous polyploid plant such as alfalfa.

Crude saponin extracts from nineteen Vernal leaf and stem samples (1st. crop 1968) were analyzed by Erythrocyte Assay I for heat lability and the forming of cholesterides (Table 11). The samples were drawn from six crosses designated A - F. Individuals within these crosses were recorded as subscripts. Cholesterol, when added as 2% of the crude saponin extract and heated momentarily to 80 C, effectively decreased the hemolytic activity to one-tenth its original value. Heating the crude saponin extracts at 120 C for 20 min did not decrease hemolytic activity. Hemolytic activity of low saponin samples actually increased under these conditions.

Although hemolytic activity of all alfalfa saponin extracts studied was affected only slightly by heat, other biological activities of saponins may be affected to a greater extent. This was demonstrated in an

Table 11. Effect of cholesterol and heat treatment on the hemolytic activity of crude saponin extracts of Vernal alfalfa stem and leaf samples.

Source	"Hemolytic value"		
	extract	extract + heat	extract + cholesterol
A L	29	29	1.5
B_1L	29	27	1.5
C_1L	27	23	1.5
C_2L 1/	27	23	1.5
B_2S	14	17	1.5
E_1L	9	9	1.5
D_1L	8	8	0.8
D_2L	8	11	0.8
C_3S	7	7	1.5
C_4S	7	7	0.8
B_3S	7	7	0.6
E_2L	3	4	0.4
F L	2.8	3	0.5
D_3S	1.6	2	0.3
D_4S	1.6	2	0.3
E_3L	1.1	3	0.3
E_4L	1.2	3	0.0
E_5S	0.3	0.7	0.0
E_6S	0.3	0.5	0.0

Table 11 (cont'd.)

L - leaf S - stem
1/ Source materials identified with the same letter are different plants resulting from the same cross.

experiment in which Trichoderma sp. grew normally in the presence of some crude saponin extracts treated with heat but grew only poorly in other heat treated extracts.

Alfalfa saponins are reasonably stable; both in the plant and in water extracts. Plants harvested and stored under a variety of conditions retained their original hemolytic activity. Drying temperature; speed of drying; and storage temperature, light, and period did not cause major changes in hemolytic activity of saponin extracts. Fresh plant samples were stored at 5 C for over one week with no deleterious effect on the contained saponins. Water extracts of crude saponin were stored for five days in waxed paper cups at 5 C with no change in hemolytic activity. The activity of extracted saponins is apparently not altered over a period of time by other factors in the extract.

The effect that extracts from different plants might have upon extracted saponins was studied in two experiments (Table 12). Crude extracts from different plants were mixed in the proportions 3:1, 1:1, or 1:3 and hemolytic activity of the mixture, based on

Table 12. Expected and actual hemolytic activity of crude saponin extracts of alfalfa whole plant, stem, or leaf samples.

Plant	"Hemolytic value"	Expected "Hemolytic value"
Experiment 1		
A	18.0	
B	1.3	
C	13.9	
1/4A+3/4B	4.3	5.4
1/2A+1/2B	8.9	9.6
3/4A+1/4B	11.0	13.8
1/2B+1/2C	6.4	7.6
D	1.3	
E	17.0	
F	39.0	
1/4D+3/4E	11.0	13.1
1/2D+1/2E	7.0	9.2
3/4D+1/4E	3.8	5.3
1/4D+3/4F	30.0	30.9
1/2D+1/2F	19.0	20.4
3/4D+1/4F	9.8	10.9
1/4E+3/4F	29.1	33.9
1/2E+1/2F	27.1	28.2
3/4E+1/4F	19.0	22.6
Experiment 2--source material was the same as reported in Table 12.		
1/2A L+1/2C_1 L	29.0	28.0
1/2A L+1/2E_5 S	14.0	14.6
1/2A L+1/2E_3 L	14.0	15.0
1/2A L+1/2D_3 S	14.0	15.3
1/2C_1 L+1/2C_3 S	15.0	17.0
1/2B_3 S+1/2B_1 L	15.0	18.0
1/2E_2 L+1/2C_2 L	15.0	15.0
1/2B_3 S+1/2B_2 S	10.0	10.5
1/2B_2 S+1/2E_5 S	7.0	7.1
1/2B2 S+1/2D_4 S	7.0	7.8
1/2E_1 L+1/2E_4 L	3.8	5.1
1/2E_1 L+1/2E_6 S	4.2	4.6
1/2B_3 S+1/2F L	3.4	4.9
1/2E_6 S+1/2D_1 L	4.6	4.1
1/2D_2 L+1/2E_3 L	3.0	4.5

Erythrocyte Assay I, was compared with the expected activity. Expected activity was calculated only on the basis of dilution. The mixture of two extracts possessing "hemolytic values" 15 and 5, if mixed in equal portions, should be 10.

In the first experiment "hemolytic values" of the mixtures were slightly, but significantly ($t_{.01}$) lower than expected values. This difference could have been due to factor(s) in the extract of lower activity which decreased the activity of the higher. It could also have been due to the loss of some saponins during the mixing of extracts. The surface active nature of saponins have created problems of this type in the past.

Greater care was taken to protect against transfer losses of saponins in the second experiment. The hemolytic activity of the mixtures remained consistently and significantly ($t_{.05}$) lower than expected. These differences, however, were smaller than those of experiment 1 and were not of an important magnitude. The discrepancy between actual and expected "hemolytic values" may be explained as the loss of saponins due to lack of perfect transfer procedures.

Mixtures of crude saponin leaf and stem extracts, when properly reconstituted to represent the whole plant, exhibited the same hemolytic activity as the whole plant. Mixtures of leaf extracts from closely or more distantly related plants had little or no effect on their

combined expected hemolytic activity. The same lack of hemolytic stimulation or inhibition of mixtures was observed for stem X stem and stem X leaf mixtures.

Seasonal Trend and Areas of Topgrowth Concentration of Alfalfa Saponins

Selected alfalfa plants grown in the field or greenhouse under a variety of environmental conditions were consistently placed in the same order on the basis of saponin content. This was the case irrespective of the type of sample preparation.

In order to study seasonal trend of saponin levels in different alfalfa genotypes several clones of four M. falcata plants, previously selected only for other quality factors, were analyzed for saponins over a two-month period of 1968 first growth. As many as three plants were harvested on the first sampling date and as few as one on the last date. Leaf-stem separations were made and the dried and ground samples analyzed for saponins by Erythrocyte Assay I.

The four selected plants contained "medium to medium-high" levels of saponins and although P 21 and P 49 generally contained less saponins than P 48 and P 11 (Table 13) the differences were not of an important magnitude. Leaf and stem samples from the four selections exhibited somewhat different trend patterns of saponins over the two-month period. "Hemolytic values" which

Table 13. Leaf and stem "hemolytic values" from four asexually propagated *M. falcata* plants harvested over a two-month period.

Source	Harvest date 1968						
	5-4	5-14	5-24	6-3	6-13	6-23	7-4
P 11							
leaf	12.5	7.0	16.0	14.5	13.7	13.9	9.8
stem	4.2	2.6	4.6	3.0	3.0	2.6	3.0
P 21							
leaf	7.0	7.0	12.5	7.5	11.7	13.9	13.9
stem	4.2	3.4	3.0	2.6	1.6	1.5	3.0
P 48							
leaf	11.3	9.8	12.5	13.5	13.7	15.0	8.9
stem	6.4	4.6	3.8	3.6	3.1	4.2	3.0
P 49							
leaf	7.0	9.8	10.8	9.3	11.3	9.8	7.0
stem	1.3	2.3	1.5	1.5	1.5	1.5	1.5

Stage of maturity

vegetative	5-4, 5-14
early bud	5-24
mid bud	6-3
1st. flower	6-13
full flower	6-23
early seed pod	7-4

differed by as much as 1.6 were significantly different (Tukey's w-procedure), however, differences of this magnitude were not considered meaningful or important. There was no significant difference in "hemolytic values" between clones of the same selection on any given harvest date.

An "over-all trend" in alfalfa saponin content from very early growth through full flower was apparent when the average leaf and stem "hemolytic values" were taken for each date (Figure 7). Ten days after first harvest the average "hemolytic value" decreased from 9.3 to 8.4. From May 24 - June 23 saponin content remained relatively high and finally decreased on the last harvest date, July 4. Average stem saponin levels remained rather constant throughout the sampling period, however, there was a trend toward decreasing levels from first to last harvest. The leaf:stem "hemolytic value" ratio increased from first harvest through June 13 and then decreased at the last harvest. Ratio values from first through last harvest were 2.3, 2.6, 4.1, 4.3, 5.5, 5.3, and 3.8 respectively.

Unlike other factors contributing to forage quality, saponin levels remained rather constant throughout first growth. During the same period percent leaves decreased from 72 to 42 and whole plant cell wall constituents increased from 24 to 51 %; lignin increased from 3.7 to 9.0%, and cellulose increased from 15% to 32%.

Figure 7. Hemolytic trend of M. falcata leaves and stems over a two month first growth period and trend of percent leaves for the same populations.

Leaf saponins were primarily responsible for whole plant saponin levels. Saponin distribution, however, was not always the same in younger and older leaves. Saponin content, as analyzed by Erythrocyte Assay I, was generally higher in the upper portion of four M. sativa plants (Table 14). These greenhouse grown plants were harvested at full flower and divided into top, center, and bottom leaf and stem samples. Flowers were hand separated from all samples.

The bottom one-third of DuPuits and Vernal leaf samples exhibited only two-thirds as much hemolytic activity as the younger leaves of the samples from the top. Differences between samples from the top and bottom did not exist in the other two plants. In all cases stem samples displayed a trend of decreasing hemolytic activity from top to bottom. The top stem samples were from 1.8 to 2.9 times as high in hemolytic activity as samples from the bottom.

Table 14. "Hemolytic values" of stem and leaf samples from three different positions of four *M. sativa* plants.

Plant source	Sample position	Sample type	"Hemolytic value"
DuPuits	top	leaf	31.0
		stem	17.0
	center	leaf	29.1
		stem	14.2
	bottom	leaf	22.0
		stem	5.8
Culver			
Vernal	top	leaf	16.0
		stem	6.7
	center	leaf	----1/
		stem	5.2
	bottom	leaf	15.0
		stem	3.0
Vernal	top	leaf	45.0
		stem	13.5
	center	leaf	32.0
		stem	9.8
	bottom	leaf	27.1
		stem	7.0
MSB	top	leaf	22.0
		stem	7.0
	center	leaf	28.0
		stem	5.0
	bottom	leaf	20.0
		stem	3.8

1/ missing value

Organ of Saponin Synthesis

Evaluation of research describing areas of alfalfa saponin concentration led to the question "what is the organ of synthesis?" The approach selected was similar to that of Gorz et al. (23) in which the translocation of coumarin across a graft was studied. Although saponins are found throughout the alfalfa plant, leaves and roots were considered to be the two organs most likely involved.

Alfalfa plants of only very high or low saponin content were selected for this investigation in order to accentuate any changes in saponin levels.

Even after considerable experimentation with different types of grafts only 62 of 250 grafts were successful. Grafts made on the same plant were no more successful than those between different varieties, however, certain plant combinations had a higher percent of successful grafts than others. As many as six and as few as none resulted from the six attempted for each graft combination.

Due to the limited grafting success only those with two or more successful grafts were reported. The successful grafts yielded between 0.03 and 0.17 g dried leaves. The development of Erythrocyte Assay II made possible the evaluation of these very small plant samples.

Leaf "Erythrocyte Assay II hemolytic values" of parent plants in this study ranged from 0.7 to 26.0 (Table 15). Hemolytic activity of leaves resulting from the successful grafts was higher than either parent in three combinations, lower than either parent in two combinations, and intermediate in the remaining nine. The intermediate combinations demonstrated hemolytic activity near the average of the two parents involved. Hemolytic activities deviated from expected results. In spite of the unexpected results, special consideration of certain combinations allowed a better understanding of saponin distribution in individual alfalfa plants.

The stock was primarily responsible for the "hemolytic values" of leaves from scions which fell outside parent values. Leaves from scions grafted onto "V 1" were consistently higher in hemolytic activity than either parent (combinations 1 and 2). When this parent was grafted as the scion, however, "hemolytic values" were intermediate to those of the two parents (combination 3). In combination 6 the scion grown on "V 5" stock produced leaves higher in activity than either parent, but again, "V 5" as the scion produced leaves with activity intermediate to the parents.

Table 15. "Erythrocyte Assay II hemolytic values" of leaves from forty-seven grafts and of the stock and scion parents.

Combination	Plant source	"Hemolytic value" parent	"Hemolytic value" individuals within one combination	"Hemolytic value" average
1	V/C	17.0		
	V 1	23.0	31,32,35,30	32.0
2	C 1	4.0		
	V 1	23.0	24,25,27,29,29,22	26.0
3	V 1	23.0		
	C 1	4.0	13,17,16,17,17	16.0
4	C 2	20.0		
	V 2	2.0	13,4,8,7	8.0
5	V 3	20.0		
	V 2	2.0	10,8	9.0
6	V 4	3.0		
	V 5	5.0	6,8,14	9.3
7	V 5	5.0		
	V 4	3.0	4,4	4.0

Table 15 (cont'd.)

Combination	Plant source	"Hemolytic value" parent	"Hemolytic value" individuals within one combination	"Hemolytic value" average
8	C 1 / V/C	4.0 / 17.0	12,16	14.0
9	V/C / C 1	17.0 / 4.0	13,13,15	13.7
10	V 6 / V/C	26.0 / 17.0	15,13,16	14.7
11	V 4 / V/C	3.0 / 17.0	10,9,9,15	10.8
12	V 7 / V 8	14.0 / 1.5	1.0,0.3,0.2,0.9	0.6
13	V 9 / V 10	1.0 / 20.0	8,11,20	13.0
14	V 11 / V 10	6.0 / 20.0	20,18	19.0

An exception to this specific stock effect is demonstrated in combinations 8, 10, and 11 in which "V/C" is the common stock. In one case the average leaf "hemolytic value" of the scion is lower than either parent, however, in the other two combinations the scion values are intermediate to the parents. When "V/C" was the scion source (combination 9) "hemolytic values" were again intermediate. The only other apparent specific stock effect was that of "V 8" in combination 12.

In those combinations in which scion leaves were intermediate in "hemolytic values" to either parent neither the stock nor scion exhibited a controlling force. Scion leaf "hemolytic values" were, in most cases, near the average of the parents.

The question of which plant organ, root or leaf, might be the more logical area of alfalfa saponin synthesis was not resolved. Both the root and leaf probably produce saponins. If only one of these organs produced saponin the distribution of these compounds in the leaves of high and low scions would have been much different.

In this study leaves of scions were as much as 4.5 times as active as leaves from the stock parent and as much as 13 times as active as leaves from the scion parent, but still intermediate in activity to the parents.

Also, leaves of scions were less than one-half as active as leaves from the stock parent in certain instances and nearly one-half as active as the leaves from the scion parent in others, yet intermediate in activity to the parents.

The graft itself may have increased hemolytic activity somewhat. Scions grafted directly back to their parent produced leaves with hemolytic activity somewhat greater than the parent. This increase was as great as 10 "hemolytic value units," however, it never changed a low value into a medium or a medium to a high saponin value. This increase and other small increases of leaf hemolytic activity over parent plants may be due to a younger average age of scion leaves than parent leaves.

Another variable which may have influenced the hemolytic activity of scion leaves was the traversing of a graft by saponins. This variable, however, could not explain all cases of the diverse results.

On the bases of the data presented and the above discussion, this study of organ of synthesis should serve primarily as added direction for future investigations.

CONCLUSIONS

1. Hemolytic activity of saponins in alfalfa may be used as the basis of assays of hemolytic saponin content of individual alfalfa plants
2. Erythrocyte Assay I, an adaptation of other hemolytic assays, is effective for rapidly and accurately evaluating the saponin content of dried, fresh, or frozen alfalfa samples.
3. Erythrocyte Assays II and III were further adaptations of Erythrocyte Assay I and allowed evaluations of small samples and large numbers of samples.
4. Chemical and biological (fish assay) evaluations agree with erythrocyte assay results.
5. Accelerators or inhibitors of hemolysis, if present in the reaction mixture, do not alter the realiability of the three erythrocyte assays.
6. Alfalfa saponins change very little in hemolytic activity under various storage conditions.
7. Differences of saponin content within alfalfa species is much greater than between species.
8. General combining ability for saponins is highly significant in *M. sativa* populations.

9. Variation in alfalfa saponin content within populations and saponin heritability are sufficiently large to facilitate selection for high and low saponin lines.
10. Alfalfa leaves contain about three times as much saponins as stems.
11. Estimates of heritability of M. sativa leaf and stem saponins are nearly identical.
12. Saponins of alfalfa form cholesterides resulting in a major decrease in hemolytic activity.
13. Based on their hemolytic activity, alfalfa saponins are not heat labile.
14. The hemolytic activity of mixed alfalfa plant extracts is not greatly different from what would be expected on a strict dilution basis.
15. Leaf and stem M. falcata saponin levels do not change greatly over a two-month first growth period.
16. There is a trend toward higher saponin content in younger alfalfa leaves and stems than in older.
17. Both alfalfa leaves and roots are likely areas of saponin synthesis.

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