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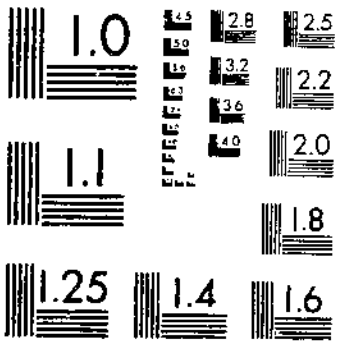


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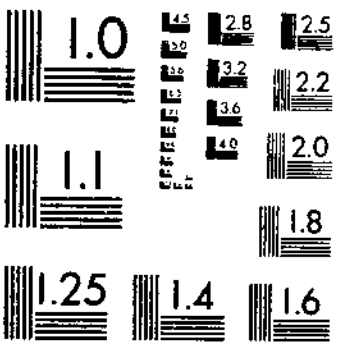


MICROCOPY RESOLUTION TEST CHART
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# ALFALFA SAPONINS

## Studies on Their Chemical, Pharmacological, and Physiological Properties in Relation to Ruminant Bloat

*Technical Bulletin No. 1161*

UNITED STATES DEPARTMENT OF AGRICULTURE
Agricultural Research Service
Washington, D. C.

*April 1957*

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# ALFALFA SAPONINS

## Studies on Their Chemical, Pharmacological, and Physiological Properties in Relation to Ruminant Bloat[1]

## Introduction

Acute tympanites (bloat) of ruminants has steadily become a more serious problem of the livestock industry, despite the fact that research workers of several countries have directed much time and effort toward a solution of the problem. Much of the interest in the past has been directed toward a physical or physiological basis for the disorder, and several theories have been advanced regarding causative factors (*7, 26, 37, 49*).[2] The biochemical and bacteriological approaches to the problem have received little attention until very recently.

The biochemical approach to the pathogenesis of bloat can be directed either toward the animal or toward the plant. Dougherty and Cello (*13, 14*), working on the first approach, have reported a toxic substance in the ingesta from overfed ruminants and from two animals with clinical bloat. The second approach, i. e., searching for biochemical constituents of legumes that could contribute to bloat, is a logical approach and one that warrants full investigations. Three distinct biochemical substances have been incriminated as possible causative factors in acute bloat: Hydrogen cyanide, flavones, and saponins.

Recent studies by several investigators throw considerable doubt on the importance of cyanide in the pathogenesis of bloat (*4, 12*). A flavone, tricin, was isolated from legume juice by Ferguson (*17, 18*). He found that it caused a partial paralysis of the isolated rabbit gut, which led him to postulate that the substance might play some part as a causative factor in bloat. However, the importance of this compound as a causative factor in bloat has been questioned by workers at the Agricultural Research Center, Beltsville, Md., in cooperation with workers of the Western Utilization Research Branch, Albany, Calif. DeEds and coworkers found that quercetin, closely related to tricin, possessed four times the muscle-relaxing powers of tricin. In current studies (1955) at the Agricultural Research Center, Lindahl and coworkers have failed to produce any bloat symptoms when a water suspension of 25 grams of quercetin was administered to five intact sheep. They also failed to find any reduction in ruminal motility or efficiency of eructation when the same dose was administered to two fistulated sheep. On the basis of muscle-relaxing powers, the amount

[1] Submitted for publication July 5, 1956.
[2] Italic numbers in parentheses refer to Literature Cited, p. 81.

of quercetin administered was equivalent to 100 grams of tricin. The tricin content of alfalfa has not been established; however, during isolation experiments conducted at the Western Utilization Research Branch the yield of crystalline tricin was found to be 0.02 percent. In the light of these experiments, it is highly improbable that tricin can contribute to bloat through its muscle-relaxing action. During the preparation of this bulletin, an article appeared in which Ferguson and Terry (*19*) concluded that it was unlikely that flavones are concerned in clinical bloat.

Saponin has been suggested as being a contributing factor in the pathogenesis of ruminant bloat by a number of investigators. Several investigators, including McCandlish (*34*), Olson (*39*), and Quin (*42*), have postulated that saponin alters the surface tension of the ruminal contents and that it might contribute to "frothy bloat" by the entrapment of countless bubbles of the gases of fermentation throughout the ingesta. Henrici (*21*) recently found a saponinlike glucoside in lucerne and *Tribulus* species from pastures in which acute bloat had been a problem, but the compound was absent in the same plants from an area in which bloat did not occur. Although it had been established that two or more saponins could be isolated from alfalfa by Boas and Steude (*3*), Jaretzky (*27*), Jaretzky and Linder (*28*), Henrici (*21*) and Walter, Van Atta, Thompson, and Maclay (*48*), the role of saponin in ruminant bloat remained a matter of speculation until the workers of the Western Utilization Research Branch, Albany, Calif., succeeded in devising procedures whereby sufficient quantities of saponin could be isolated from alfalfa for administration to ruminants. Lindahl, Cook, Davis, and Maclay (*31*) made a preliminary report in 1954 that the oral administration of the isolated composite saponin from alfalfa caused bloat symptoms in ruminants. However, two commercially available saponin preparations isolated from the yucca plant did not produce any bloat symptoms when administered in a like manner to the same animals.

Since the preliminary report was made, a sufficient quantity of alfalfa saponin has been isolated by the Western Utilization Research Branch so that the rather extensive studies that are reported in this bulletin could be directed toward determining the physiological, physical, pharmacological, and toxicological properties of alfalfa saponin when administered to ruminants. Effects of saponin on small laboratory animals and on isolated muscle strips were also studied to see if bloat-producing tendencies could be correlated with other pharmacological action.

## Production of Bloat and Other Symptoms in Intact Sheep by Alfalfa Saponin Administration

By *Ivan L. Lindahl, R. E. Davis,* and *R. T. Tertell*[a]

### Experimental Procedures

All the alfalfa saponin used in the experiments described in this and following sections was isolated by the Western Utilization Research Branch, Albany, Calif. The recovery of the alfalfa saponins was effected by cholesterol addition, us-

[a] The authors express their thanks to R. W. Dougherty, N. Y. State Veterinary College, for his assistance in experiments with animals No. 61 and No. 100.

ing the procedure described in the section entitled "Preparation and Chemistry of Legume Saponins." Unfortunately, all the saponins present in the original plant material are not recovered by this procedure. This incomplete recovery results in an unequal distribution of the various saponins; therefore, the relative proportions of the individual saponins in the recovered product are in some instances lower and in other instances higher than they were in the original plant material. The significance of this fact must not be overlooked in interpreting the following described experiments with animals.

All the animal test work was conducted at the Agricultural Research Center, Beltsville, Md. The majority of the sheep used in the experiments were animals of known bloat susceptibility. This bloat susceptibility was established by drenching the animals with Ladino white clover or alfalfa juice extracts. None of the animals used could be classified as "chronic" bloaters, and none had ever displayed more than a moderate degree of bloat previous to the tests. Very little bloat has occurred under natural grazing conditions at the Agricultural Research Center for several years, and no natural bloat was occurring on any of the pastures at the time of the experiments.

All the experiments but one in 1953 were conducted with animals that were grazing on a single Ladino clover pasture. No cases of natural bloat were observed at any time during the 1953 season, although the pasture contained predominant amounts of Ladino clover and had rather lush growth. Although natural bloat did not occur, it was not difficult to produce bloat symptoms by drenching the animals with either Ladino clover or alfalfa press juice while they were grazing on this pasture. All the experiments during this season were conducted in the pasture, and most of the animals were dosed with the saponin solutions shortly after they had finished their morning grazing.

In 1954 and 1955 many of the animals were removed from the pasture or feed lot and taken to the laboratory before the tests were conducted. In a number of cases there was a time lapse of 2 to 4 hours from the time the animals had access to feed until the experiments were conducted. Considerably more difficulty was experienced in producing bloat symptoms by drenching the animals with legume press juice during the 1954 season than in 1953, although the clover pastures were similar in composition and appearance.

For the intraruminal dosing the saponin and adjuncts were dissolved in approximately a liter of water and the solutions given by stomach tube. The saponin was dissolved in 20 to 50 ml. of sterile water for most of the intravenous dosing, and the solutions injected slowly, using a hypodermic syringe and polyethylene tubing inserted into the jugular vein.

Under our experimental conditions substances used in connection with the saponin, such as dextrose, did not produce bloat symptoms in six animals, which animals were later used in connection with the administration of saponin. Dextrose was used in a number of experiments as a source of a readily fermentable material, because analytical studies at the Agricultural Research Center have shown that Ladino clover is relatively high in reducing sugars during its bloat-producing stages of growth. Sodium formate was used in one experiment, as Doetsch and coworkers (*9*) have shown that formate dissimilates rapidly with the formation of gaseous end products in the presence of ruminal ingesta. It was

also shown that the administration of 3 to 4 times the amount of water that was used in the tests did not produce any bloat symptoms.

Bloat symptons were arbitrarily classified as slight, moderate, or severe. A rating of slight bloat was given when the animal displayed definite ruminal distention over that of the pretest period. A rating of severe was given only in cases of very pronounced ruminal distention, marked discomfort, and with resultant collapse and death of the animal. A rating of moderate was given in cases falling between the two extremes. See figures 1, 2, 3, and 4 for examples of ratings. As we have found that the only satisfactory method of determining intraruminal pressures in sheep is by use of a trocarmanometer (which obviously cannot be used repeatedly on the same animal), no intraruminal pressure measurements were taken unless the animal collapsed.

Observations on ruminal motility as reported in this section were made by visual inspection and pal-

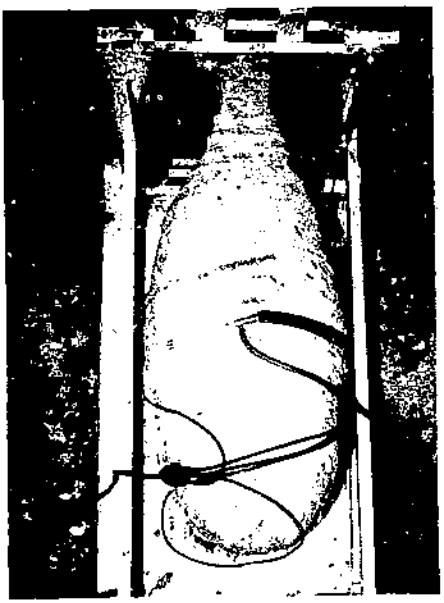


FIGURE 1.—Animal No. 61 before dosing.

FIGURE 2.—Animal No. 61 with slight bloat symptoms.


FIGURE 3.—Animal No. 61 with moderate bloat symptoms.

pation of the left side of the animal. A measurement of ruminal motility in intact sheep by use of a needle inserted into the rumen was not used in these experiments, as preliminary experiments indicated that loss of ruminal gases, clogging of the needle, animal reaction to pain, and resultant injury to the animal were likely to lead to faulty data and conclusions. Eructations were detected either by palpation of the esophageal region or by use of a stethoscope placed over the esophagus. Respiration rates were determined in the most part by counting the movements or by use of a stethoscope. Observations on the respiratory patterns were made by direct tracings, using a pneumograph and ink-writing air tambours.

Blood hemolysis studies were made by centrifuging the blood samples and observing the color of the plasma. Conclusions regarding blood pressure result from direct attempts to measure the pressure or from observations while taking blood samples just before collapse of the animals.

Most of the animals that died as a result of these experiments were autopsied shortly after death, and the results of the gross autopsy and histological examinations are given in the section on toxicity.

## Results and Discussion

Table 1 summarizes the results of 35 experiments in which alfalfa saponin was administered to 23 individual sheep.

### INTRARUMINAL ADMINISTRATION

In the 1953 experiments bloat symptoms were produced in all sheep grazing on Ladino clover by the intraruminal administration of alfalfa saponin. Figures 5 and 6 illustrate one of the reactions from the saponin administration. Figure 6 was taken one hour after dosing

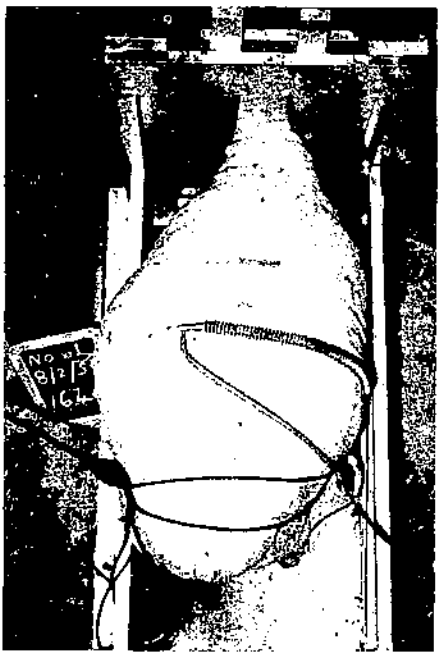



FIGURE 4.—Animal No. 61 with severe bloat symptoms.

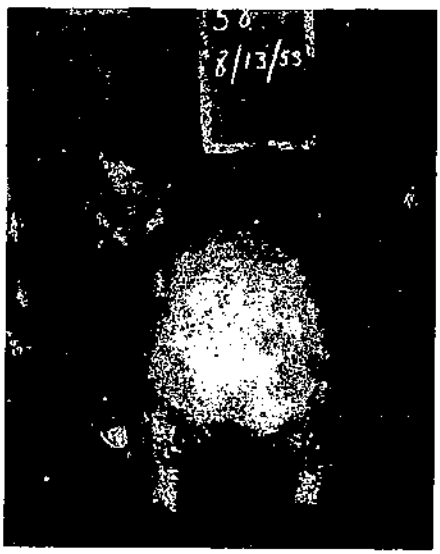



FIGURE 5.—Animal No. 58 before intraruminal administration of alfalfa saponin.

TABLE 1.—*Bloat symptoms resulting from alfalfa saponin administration to intact sheep*

1953 EXPERIMENTS—INTRARUMINAL DOSING

| Date | Animal No. | Pretreatment diet | Alfalfa saponin and method of administration | Degree of bloat | Interval before appearance of bloat symptoms | Duration of bloat symptoms |
|---|---|---|---|---|---|---|
| June 18, 1953 | 36 | Ladino pasture | 55 gm. in 1 liter of water | Severe | Approximately 45 minutes. | Animal treated twice by stomach tube; found dead 8 hours after last treatment. |
| June 25, 1953 | 61 | do | 25 gm. in 1 liter of water | Moderate | Approximately 30 minutes. | 1 to 2 hours. |
| June 30, 1953 | 55 | do | 15 gm. in 1 liter of water | Slight | do | Do. |
| July 1, 1953 | 58 | do | 15 gm. in 1 liter of Ladino clover juice extract. | do | 10 to 15 minutes | Approximately 1 hour. |
| July 2, 1953 | 44 | Grass pasture | 15 gm. in 1 liter of water | None | | |
| Aug. 13, 1953 | 58 | Ladino pasture | 25 gm. in 1 liter of water | Slight (+) | Approximately 30 minutes. | 2 to 3 hours. |
| Do | 44 | do | do | do | do | 1 to 2 hours. |

1954–55 EXPERIMENTS—INTRARUMINAL DOSING

| | | | | | | |
|---|---|---|---|---|---|---|
| June 14, 1954 | 61 | Ladino pasture | 25 gm. in 1 liter of water | Slight | Approximately 30 minutes. | 1 to 2 hours. |
| Do | 60 | do | 25 gm. saponin + 50 gm. dextrose in 1 liter of water. | Moderate | Approximately 20 minutes. | 2 to 3 hours. |
| June 21, 1954 | 55 | do | 25 gm. in 1 liter of water | None | | |
| Do | 27 | do | 25 gm. saponin + 50 gm. dextrose in 1 liter of water. | Moderate | Approximately 30 minutes. | 2 to 3 hours. |
| Do | 101 | do | 50 gm. saponin + 50 gm. dextrose in 1 liter of water. | Severe | do | Collapsed and died in 4 hours. |
| June 30, 1954 | 50 | do | do | do | do | Collapsed and died in 3 hours. |

| | | | | | | |
|---|---|---|---|---|---|---|
| Do | 90 | do | 100 gm. saponin + 50 gm. dextrose in 1 liter of water. | do | Approximately 15 minutes. | Collapsed and died in 1.5 hours. |
| July 8, 1954 | 20 | Grass pasture | 50 gm. saponin + 50 gm. dextrose in 1 liter of water. | Slight | Approximately 20 minutes. | 1 to 2 hours. |
| July 27, 1954 | 95 | do | 100 gm. saponin + 100 gm. formate in 1 liter of water. | Moderate | do | 2 to 3 hours; died 27 hours later. |
| Aug. 2, 1954 | 61 | Ladino pasture | 100 gm. saponin + 50 gm. dextrose in 1 liter of water. | Severe | do | Collapsed and died in 2 hours. |
| Jan. 10, 1955 | 58 | Grass hay | 40 gm. saponin + 50 gm. dextrose in 1 liter of water. | None | | |
| Do | 53 | do | 60 gm. saponin + 50 gm. dextrose in 1 liter of water. | Moderate | Approximately 20 minutes. | 2 to 3 hours. |
| Jan. 24, 1955 | 73 | Alfalfa hay | 60 gm. saponin + 100 gm. dextrose in 1 liter of water. | Slight | Approximately 45 minutes. | 1 hour; died 3 days later. |
| Do | 107 | do | 40 gm. saponin + 100 gm. dextrose in 1 liter of water. | Slight (+) | Approximately 30 minutes. | 1 to 2 hours. |

1954–55 Experiments—Intravenous Dosing

| | | | | | | |
|---|---|---|---|---|---|---|
| Aug. 3, 1954 | 100 | Ladino pasture | 5 gm. saponin by intravenous drip + 50 gm. dextrose in 1 liter of water given intraruminally. | Moderate (+) | Approximately 20 minutes. | Collapsed and died in 1 hour and 15 minutes. |
| Aug. 7, 1954 | 58 | do | 0.5 gm. saponin by intravenous injection + 75 gm. dextrose in 1 liter of water given intraruminally. | None | | |
| Aug. 10, 1954 | 60 | do | 1 gm. saponin by intravenous injection + 50 gm. dextrose in 1 liter of water given intraruminally. | do | | |

TABLE 1.—*Bloat symptoms resulting from alfalfa saponin administration to intact sheep*—Continued

1954–55 EXPERIMENTS—INTRAVENOUS DOSING—Continued

| Date | Animal No. | Pretreatment diet | Alfalfa saponin and method of administration | Degree of bloat | Interval before appearance of bloat symptoms | Duration of bloat symptoms |
|---|---|---|---|---|---|---|
| Aug. 10, 1954 | 27 | Ladino pasture | 2 gm. saponin by intravenous injection + 100 gm. dextrose in 2 liters of water given intraruminally. | Slight | Approximately 30 minutes. | 2 to 3 hours; died 3 days later. |
| Sept. 11, 1954 | 62 | do | 3 gm. saponin by intravenous injection + 50 gm. dextrose in 1 liter of water given intraruminally. | Moderate (+) | do | Collapsed and died in 3 hours. |
| Sept. 12, 1954 | 23 | do | 2.55 gm. saponin by intravenous injection + 50 gm. dextrose in 1 liter of water given intraruminally. | Moderate | Approximately 20 minutes. | 2 to 3 hours; died 4 days later. |
| Jan. 11, 1955 | 64 | Grass hay | 1 gm. saponin by intravenous injection + 50 gm. dextrose in 1 liter of water given intraruminally. | do | do | 2 to 3 hours; died 3 days later. |
| Do | 77 | do | do | None | | Died 3 days later. |
| Do | 60 | do | do | Slight | Approximately 20 minutes. | 2 to 3 hours. |
| Jan. 12, 1955 | 63 | do | 1 gm. saponin by intravenous injection + 50 gm. dextrose and 20 gm. yeast given intraruminally.[1] | Moderate | Approximately 15 minutes. | Do. |
| Do | 37 | do | 2 gm. saponin by intravenous injection + 100 gm. dextrose and 20 gm. yeast given intraruminally.[1] | Moderate (+) | do | Collapsed and died in 6 hours. |

| | | | | | | |
|---|---|---|---|---|---|---|
| Jan. 25, 1955 | 55 | Alfalfa hay | 1 gm. saponin by intravenous injection + 100 gm. dextrose in 1 liter of water given intraruminally. | Moderate | Approximately 20 minutes. | Approximately 2 hours; died 50 hours later. |
| Do | 115 | do | do | None | | |
| Do | 107 | do | 2 gm. saponin by intraperitoneal dosage + 100 gm. dextrose in 1 liter of water given intraruminally. | do | | Died 2 days later. |

[1] The "live" yeast was in the resting stage when administered.

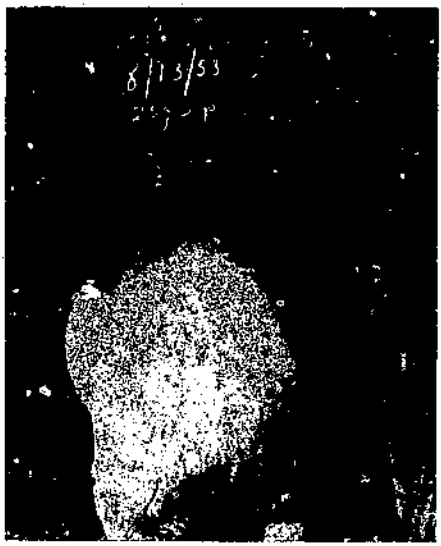



FIGURE 6.—Animal No. 58 one hour after receiving an intraruminal dose of 25 grams of alfalfa saponin.

the animal and thirty minutes after the animal was rated as displaying slight (+) bloat symptoms, illustrating that the reaction was not of a transitory nature. The animal was not allowed to graze during this period. The ability to release gas and reduce ruminal distention in these animals by stomach tube, together with the failure to produce bloat symptoms from the administration of two different saponins from the yucca plant led the authors to state that the distention in these animals appeared to be due to gas retention per se rather than to froth formation (*31*). This response was especially dramatic in the case of animal No. 36 (the animal receiving the largest amount of saponin and displaying the most distention) to treatment with the stomach tube on two occasions.

Experiments conducted on June 14 and June 21, 1954, illustrated that more distinct bloat symptoms might be produced when dextrose was administered along with the alfalfa saponin.

Variations were noted not only between animals in their response to intraruminal administration of alfalfa saponin but also with the same animals at different times. Sheep No. 61 displayed moderate bloat symptoms when given 25 grams of the saponin on June 25, 1953, but only slight symptoms when given the same dose on June 14, 1954. Animal No. 55 displayed slight bloat symptoms after receiving 15 grams of the saponin on June 30, 1953, and no bloat symptoms after receiving 25 grams on June 21, 1954. Animal No. 58 displayed slight bloat symptoms after receiving 15 grams of the saponin in 1 liter of Ladino clover juice on July 1, 1953, slight (+) symptoms after receiving 25 grams on August 13, 1953, and no symptoms after receiving 40 grams of saponin and 50 grams of dextrose on January 10, 1955.

Much greater bloat responses were obtained from the saponin administration when the sheep were pastured on Ladino clover than when their diet was composed of grass pasture, alfalfa hay, or grass hay. In all cases the animals were on a definite diet for several days before treatment with the saponin.

In several of the cases, ruminal motility appeared to be more pronounced just following the intraruminal administration of the saponin solutions. In some cases ruminal motility in sheep grazing on Ladino clover appeared to be active during the early development of the bloat symptoms, but in other cases motility appeared to cease shortly after dosing and before the development of the bloat symptoms.

Other physiological responses that resulted from the administration of large doses of the alfalfa saponin are pointed out in the following case histories.

SHEEP No. 61, August 2, 1954.

Diet: Ladino clover pasture. The animal was removed from the pasture at 12 noon and taken to the laboratory.
Material given: 100 grams of alfalfa saponin and 50 grams of dextrose in 1 liter of water, by stomach tube, at 3 p. m.
Reactions:
See figures 1, 2, 3, and 4. Predose respiration rate was 94 per minute.
3:03 p. m. Respiration rate was 59 per minute.
3:05 p. m. Respiration rate was 72 per minute with an irregular pattern.
3:15 p. m. Respiration rate was 64 per minute.
3:20 p. m. The respiratory pattern was of the Cheyne-Stokes type from this point until collapse.
3:25 p. m. Slight bloat symptoms; no major rumen movements could be detected.
3:40 p. m. Slight (+) bloat symptoms.
3:45 to 4:25 p. m. The animal began to urinate and defecate frequently.
4:00 p. m. The animal displayed moderate bloat symptoms.
4:10 p. m. The animal displayed excessive salivation and mucous secretion.
4:15 p. m. The animal became very restless.
4:45 p. m. The animal became distressed and was unsteady on its feet. The heart rate became accelerated and the blood pressure fell so rapidly that a measurement could not be obtained.
4:47 p. m. The animal collapsed. Intraruminal pressure was 40 mm. Hg, which went up to 60 mm. on inspiration.
4:48 p. m. Respiration was very labored at a rate of 19 per minute.
4:58 p. m. The animal died.
On autopsy it was found that the ruminal ingesta was "frothy" and that the rumen contained a large amount of free gas.

SHEEP No. 90, June 30, 1954.

Diet: Closely grazed Ladino clover-grass pasture. The animal was removed from the pasture at 9:30 a. m.
Material given: 100 grams of alfalfa saponin and 50 grams of dextrose in 1 liter of water, by stomach tube, at 11:29 a. m.
Reactions:
Rumen movements appeared to be very pronounced immediately after dosing.
11:33 a. m. The respiration rate was 72 per minute. The rumen movements were less pronounced than above, but were still more pronounced than in the predose period.
11:37 a. m. Respiration was irregular. Rumen movements could still be detected on visual inspection.
11:45 a. m. The animal displayed slight bloat symptoms. Ruminal motility could not be detected from this point on.
12:05 p. m. A blood sample was taken. No hemolysis could be detected.
12:10 p. m. The animal displayed moderate bloat symptoms and was becoming restless.
12:20 p. m. The animal was very restless.
12:30 p. m. The animal began to urinate frequently.
12:35 p. m. The animal displayed excessive salivation.
12:45 p. m. Respiration was labored and the animal was distressed.
12:55 p. m. The animal displayed severe bloat symptoms. An attempt to take a blood sample was unsuccessful.
1:00 p. m. The animal was going down. An attempt was made to relieve the animal by stomach tube and some gas was released as the animal collapsed.
1:05 p. m. The animal died. Intraruminal pressure was 50 mm. Hg.
On autopsy it was found that the ruminal contents were "frothy" and that the rumen contained a large amount of free gas.

SHEEP No. 50, June 30, 1954.

Diet: Same as for animal No. 90. The animal was removed from pasture at 9:30 a. m.

Material given: 50 grams of alfalfa saponin and 50 grams of dextrose in 1 liter of water, by stomach tube, at 11:27 a. m.

Reactions:

11:30 a. m. Respiration was normal at a rate of 85 per minute. Ruminal motility could be readily detected.
11:40 a. m. Ruminal motility could not be detected from this point on.
11:55 a. m. The animal displayed slight bloat symptoms.
12 noon. The animal displayed slight (+) bloat symptoms. Respiration was irregular at a rate of 75 per minute.
12:10 p. m. The animal began to urinate frequently.
12:45 p. m. The animal displayed moderate bloat symptoms and excessive mucous and salivary secretion.
1:07 p. m. The animal displayed moderate (+) bloat symptoms. Respiration was labored at a rate of 48 per minute.
1:18 p. m. The animal was distressed.
1:45 p. m. Severe bloat symptoms. The animal began to froth at the mouth and nostrils.
2:00 p. m. Animal collapsed. Respiration rate was 25 per minute.
2:30 p. m. The animal died.
On autopsy, the ruminal ingesta was "frothy," but the rumen contained large amounts of free gas.

Although alfalfa saponin has definite toxic effects at the higher levels of administration, as will be pointed out later, rapid collapse and death of the sheep in the above experiments did not occur unless the animal displayed marked bloat symptoms. In all cases where marked bloat symptoms were obtained, the animals displayed frequent urination and defecation, labored respiration, excessive salivation (fig. 7), and extreme discomfort. Immediately prior to collapse, the animals also suffered from a marked drop in blood pressure. The sheep with slight or no bloat symptoms did not display the symptoms listed above, even when they received the same amount of saponin.

## INTRAVENOUS ADMINISTRATION

In order to gain additional information on the action of alfalfa saponin, a number of experiments were conducted in which solutions of the saponin were administered into the jugular vein of sheep. In all the experiments dextrose solutions were given by stomach tube.

As pointed out in table 1, bloat symptoms were produced in 9 of 13 intravenous experiments. Variations among animals were noted,

FIGURE 7.—Excessive salivation and mucous secretion exhibited by animal with marked bloat symptoms.

not only in bloat symptoms but also in other physiological reactions, to this method of saponin administration. Some of the sheep displayed definite nervous reactions following the intravenous dosing, while others displayed little or no reaction. Blood hemolysis could not be detected in some of the animals, even following intravenous injection of up to 1 gram of the alfalfa saponin. There appeared to be no correlation between blood hemolysis and bloat, as some animals bloated without hemolysis and in other cases definite blood hemolysis was detected without any bloat symptoms. A change in the respiratory rate and pattern was noted in most of the animals following the saponin administration. Several of the animals were observed to cough or sneeze frequently as the intraruminal pressure began to build up after either intravenous or intraruminal administration of alfalfa saponin. Gas was invariably expelled by these processes and often seemed to prevent further bloating. Coughing as a process for the elimination of gas from bloated animals has been reported by Wild (*51*) and Cole (*6*).

Two of the case histories of animals receiving intravenous doses of alfalfa saponin are given as follows:

SHEEP No. 100, August 3, 1954.

Diet: Ladino clover pasture. The animal was removed from pasture at 11 a. m.
Material given: 50 grams of dextrose in 1 liter of water, by stomach tube, at 12:05 p. m. and 4.5 to 5 grams of alfalfa saponin by intravenous drip from 12:12 to 12:18 p. m.
Reactions:

12:21 p. m. Ruminal motility and eructations had ceased.
12:30 p. m. The animal began to show bloat symptoms. A blood sample taken at this time displayed slight hemolysis.
12:33 p. m. The animal displayed excessive salivation and became restless.
12:48 p. m. The animal displayed slight (+) bloat symptoms.
12:55 p. m. The animal was depressed and displayed excessive frothing at the mouth.
1:05 p. m. The animal displayed moderate bloat symptoms.
1:10 p. m. The animal displayed muscular tremors and excessive salivation.
1:19 p. m. A rapid acceleration in heart rate and a rapid fall in blood pressure was noted.
1:20 p. m. The animal collapsed with an intraruminal pressure of 30 mm. Hg (fig. 8).
1:26 p. m. The animal died. A blood sample taken at this time showed marked blood hemolysis. On autopsy, the ruminal contents were observed to be less frothy than those of the animals receiving intraruminal administration of the saponin.

SHEEP No. 27, August 10, 1954.

Diet: Ladino clover pasture. The animal was removed from the pasture at 9 a. m.
Materials given: 1 gram of alfalfa saponin by intravenous injection at 1:22 p. m.; 50 grams of dextrose in 1 liter of water, by stomach tube, at 1:23 p. m.; 1 gram of alfalfa saponin intravenously at 1:45 p. m., and 50 grams of dextrose in 1 liter of water, by stomach tube, at 1:48 p. m.
Reactions:

1:24 p. m. Muscular tremors were observed immediately after the first dose.
1:32 p. m. Ruminal motility and eructation rate and volume appeared to be normal.
1:35 p. m. Ruminal motility was still active.
1:45 p. m. A blood sample taken at this time displayed slight hemolysis.
1:46 p. m. The animal displayed nervous symptoms immediately following the second dose of saponin.

SHEEP No. 27, August 10, 1954—Continued

Reactions—Continued

1:49 p. m. The animal passed bloody urine. Major ruminal motility could not be detected at this time.
2:12 p. m. The animal began to show bloat symptoms. No eructations were detected during a 7-minute observation period.
2:30 p. m. The animal displayed slight bloat symptoms.
3:05 to 3:15 p. m. No eructations could be detected; however, the animal coughed several times during this period and passed ruminal gas while coughing.
3:42 p. m. The animal appeared to be alert and the bloat symptoms were beginning to subside.
4:30 p. m. The animal displayed no bloat symptoms and was released to a pasture.
7:15 p. m. to 7:45 p. m. The animal was observed to be grazing.
August 11, 1954. The animal appeared to be relatively normal.
August 12, 1954. The animal appeared to be relatively normal.
August 13, 1954. The animal displayed labored respiration and died at 2:30 p. m. On autopsy, the lungs showed severe congestion.

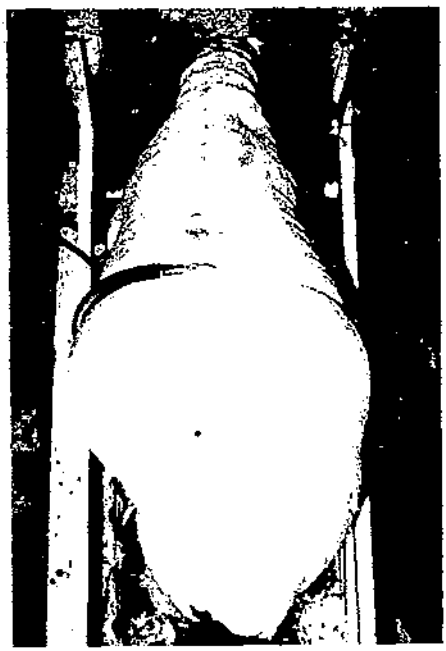


FIGURE 8.—Animal No. 100, with moderate (+) bloat symptoms after receiving an intravenous dose of alfalfa saponin.

The intravenous administration of alfalfa saponin led to the eventual death of the majority of the animals and will be discussed in the section on toxicity of the saponin. The significance of the intravenous experiments to clinical bloat is a matter of speculation; however, the experiments illustrate that alfalfa saponin has properties other than its surface tension action that could contribute to bloat.

## EXPERIMENTS WITH OTHER PLANT SAPONINS

In a number of experiments conducted in similar fashion to those with alfalfa saponin, intraruminal administration of up to 100 grams of two saponin preparations isolated from the yucca plant and available commercially have failed

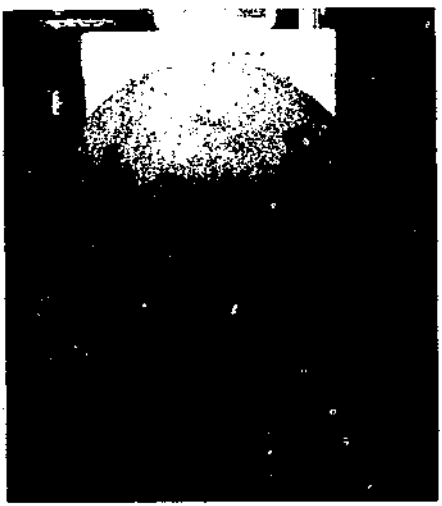


FIGURE 9.—Bloat symptoms produced from intravenous injection of quillai saponin.

to produce any bloat symptoms in sheep. The intravenous administration of 1 gram of one of these saponins (labeled as toxic) also failed to produce any bloat symptoms or toxic effects in four different experiments.

In another experiment, two sheep were each given an intravenous dose of 1 gram of quillai (*Quillaja saponaria*) saponin. One animal displayed marked bloat symptoms (fig. 9) and collapsed within 5 hours of dosing. The second animal did not display any bloat symptoms but died within 2 days after dosing.

The quillai saponin, like the alfalfa saponin, contains a triterpenoid nucleus, while the yucca saponin contains a steriod nucleus.

## Effect of Alfalfa Saponin and Some Other Plant Materials on Ruminal Motility and Eructation

By *Ivan L. Lindahl, R. W. Dougherty,* and *R. E. Davis*

DeEds and coworkers[4] of the Western Utilization Research Branch, USDA, found that alfalfa saponin modified the contractions of isolated intestinal muscle in a manner that differed from the actions of a commercially available saponin. Alfalfa saponin, in sufficient concentration, caused a loss of contraction and tonus within a short period of time. Parsons (*40*) also reported that alfalfa saponin inhibited the motility of isolated gut. In view of the above findings and observations made on intact animals during the experimental production of bloat symptoms, a number of experiments were conducted to determine the effect of alfalfa saponin and some other plant materials on ruminal motility and eructation. Colvin and coworkers (*8*) have reported that the intraruminal administration of alfalfa saponin to sheep would result in decreased ruminal motility.

### Effect of Intraruminal Administration of Saponin and Legume Press Juices on Ruminal Motility

#### EXPERIMENTAL PROCEDURES

Ten mature sheep and one steer, each fitted with permanent ruminal fistulas, were used in this phase of the study. The various diets that the animals received previous to the experiments included mixed pasture, which was largely Ladino clover, grass pasture, alfalfa hay, and alfalfa meal.

Numerous experiments with sheep were conducted at the Agricultural Research Center over a period of a year, using several different lots of the isolated alfalfa saponin, three commercially available nonlegume saponins, and press juices from alfalfa and Ladino clover.

A continuous feed kymograph with ink-writing air tambours was used in all the experiments. The air tambours used in most of the experiments employed a large disk covered with sensitive rubber. In all cases a direct recording of intraruminal pressure changes was made by connecting the air tambours with an open tube inserted through the stopper of the fistula cannula. At the beginning of the experiments the tambours and ink-writing pens were adjusted so that the intraruminal pressure changes resulting from respiratory movements would be recorded. This was done to insure a recording of

[4] Personal communication. See section entitled "Some Pharmacological Effects of Alfalfa Saponin on Nonruminants and on Isolated Muscle Strips."

the intraruminal pressures resulting from slight ruminal contractions. All dosing was done through a second tube inserted into the fistula stopper. A flexible rubber tube attached to the delivery tube was employed to direct the dose material into the ruminal contents.

The saponin was dissolved in approximately 1 liter of water and was administered in one of three ways: As a single dose given in a few minutes; as a split dose; or, in small increments over an extended period of time.

In some experiments, air or carbon dioxide gas was also introduced into the rumen through the dosage tube. A wet test gas meter was used to measure the flow of gas and the rate was marked on the tracing by a signal magnet.

In preparation of the legume press juices, the following steps were taken: (1) The legume material was frozen by storing it at a temperature of −30° F., (2) the material was then thawed rapidly, (3) passed through a meat grinder, and (4) the juice pressed out of the ground material, using a modified Carver hydraulic press and 10,000 pounds per square inch of pressure on the cake.

## RESULTS

*Alfalfa Saponin.*—Considerable variation was noted in the effect of alfalfa saponin administration on ruminal motility among animals and with the same animals on different days.

Figures 10 and 11 illustrate a contrast between the reaction of two sheep to the same amount of alfalfa saponin (25 gm.) given in the same manner, on the same day, and when they were receiving the same diet. Ruminal motility was markedly reduced in the case of animal No. 118 (fig. 10) within a few minutes following dosing, but little or no effect was observed in animal No. 232 (fig. 11).

Another definite contrast between the reaction of a different pair of sheep is illustrated in figures 12 and 13. Both animals were receiving the same predose diet and were treated in the same manner. In both cases, a dose of 25 grams of alfalfa saponin and 50 grams of dextrose was administered into the rumen. The ruminal motility was traced for a period of 15 to 20 minutes and then a second dose of 25 grams of dextrose was given. The administration of the first dose of alfalfa saponin and dextrose resulted in a rapid reduction in ruminal motility in animal No. 776 (fig. 12) and a slight buildup of intraruminal pressure. The second dose of dextrose resulted in an additional increase in intraruminal pressure.

Sheep No. 92 (fig. 13) responded in a much different manner. Ruminal motility appeared to be increased for about 7 minutes immediately following the first dose and a definite increase in the frequency of ruminal contractions was noted following the second dose of dextrose. In a later experiment, the intraruminal administration of 25 grams of alfalfa saponin along with 25 grams of dextrose and 25 grams of egg albumin to sheep No. 92 (see fig. 26) resulted in a definite but transitory reduction in ruminal motility.

Some animals displayed a much more variable reaction to repeated intraruminal administration of alfalfa saponin than others. Intraruminal administration of alfalfa saponin to sheep No. 776 (figs. 12 and 14) and to sheep No. 118 (fig. 10) always resulted in a definite reduction in ruminal motility; however, the speed of the reaction and the extent of the reduction varied from day to day. Other animals varied from displaying little or no reaction to marked reduction in mo-

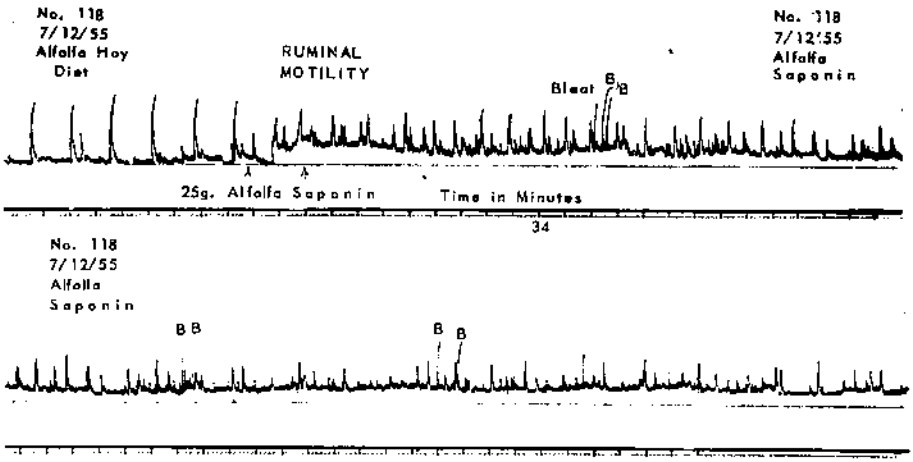


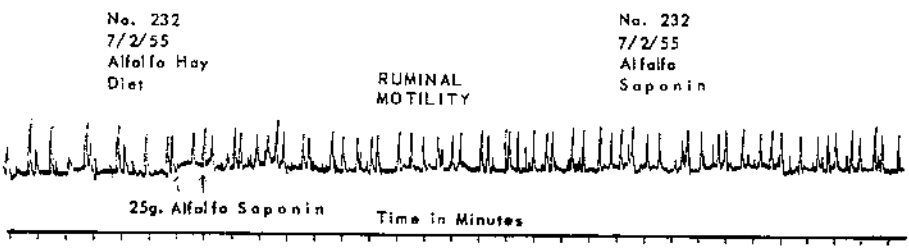

FIGURE 10.—Reduction in ruminal motility in sheep following intraruminal administration of 25 gm. of alfalfa saponin. *B*=bleat.

tility on repeated administration of the saponin.

The reasons for the variation among sheep and the same sheep on different days in respect to alfalfa saponin administration and reduction in ruminal motility are not clear at the present time. The differences in reactions cannot be adequately explained by previous dietary regimens, different lots of the saponin, or method of introduction, because definite reduction in ruminal motility was obtained at one time or another on all dietary regimens, with all lots of saponin, and with all methods of intraruminal administration.

Figure 14 illustrates a result obtained from introducing carbon dioxide gas into the rumen along with alfalfa saponin. Twenty-four grams of alfalfa saponin dissolved in 500 ml. of water was introduced into the rumen in two doses. Carbon dioxide gas was introduced at a rate of approximately 1 liter per minute. The administration of the first 15 grams of alfalfa saponin resulted in an immediate cessation of the major ruminal contraction and an increase in the frequency and depth of respiration. Although there was also a reduction in the amplitude of the "eructation contractions," i. e., the contraction associated with eructation, the animal was still able to eructate this large amount of gas without any increase in intraruminal pressure. The second dose (9 grams) of the saponin resulted in a further decrease in the amplitude of the "eructation contraction," an im-

FIGURE 11.—Negative response of ruminal motility in sheep following administration of 25 gm. of alfalfa saponin.



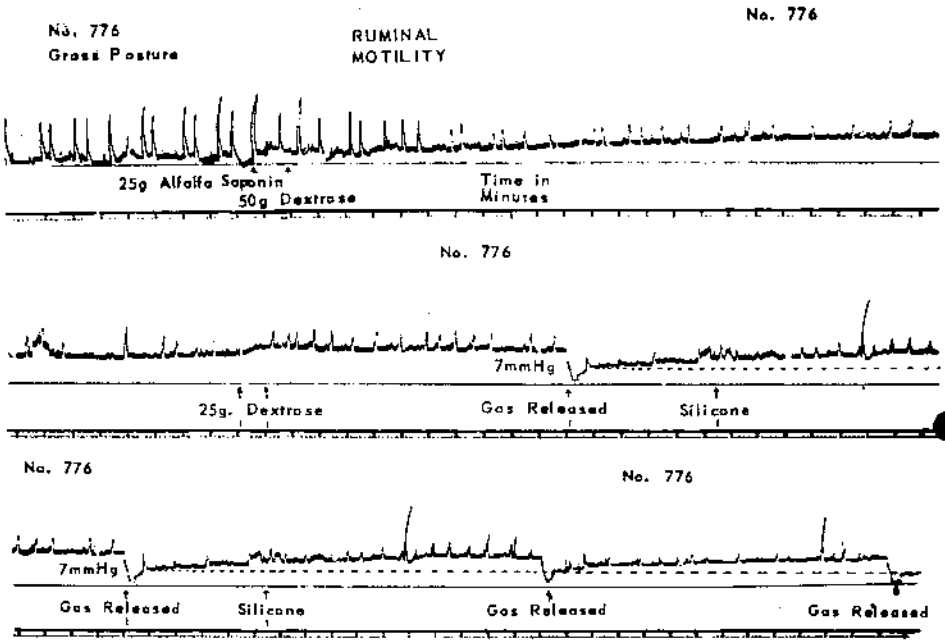



FIGURE 12.—Reduced ruminal motility and increased intraruminal pressure in sheep following intraruminal administration of alfalfa saponin and dextrose. Note negative response following intraruminal administration of a methyl silicone preparation.

pairment in efficiency of eructation, and an increase in intraruminal pressure.

Additional experiments conducted with other sheep using sublethal levels of alfalfa saponin revealed that it was difficult to build up excessive intraruminal pressure by the introduction of carbon dioxide gas or air into the rumen at a rate of a liter per minute, even where ruminal motility was severely impaired. In some cases when ruminal motility was severely impaired, there appeared to be an almost constant passage of gas from the esophagus after intraruminal pressure had built up to a few millimeters of mercury pressure.

Although normal eructation is a well coordinated mechanism involving muscular actions of the rumen, reticulum, cardia, diaphragmatic sphincter, and esophagus (*15*, *50*), recent cinefluorographic studies by Dougherty and Meredith (*15*) show that eructation can occur in the absence of ruminal or reticular motility, provided that the cardia is not covered by ingesta. Weiss (*50*) reported similar findings in his studies on the effect of small levels of potassium cyanide (prussic acid) on the efficiency of eructation.

Possible reasons why the introduction of gas into the ruminal contents containing alfalfa saponin did not result in sufficient froth to block eructation in the absence of ruminal motility will be offered in a following section.

Two experiments were conducted with the steer. In the first experiment, when the animal was grazing on grass pasture and receiving 6 pounds of oats per day, the administration of 100 grams of alfalfa saponin along with 400 grams of dextrose resulted in a marked decrease in ruminal motility and a

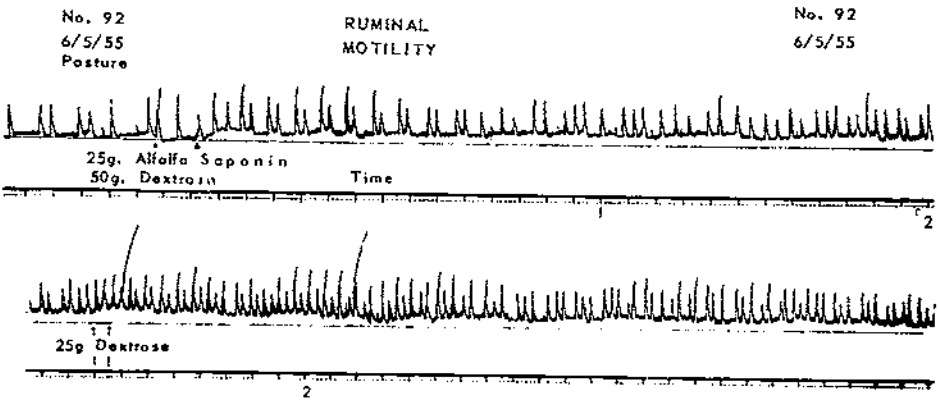



FIGURE 13.—Increased ruminal motility in sheep following intraruminal administration of alfalfa saponin and dextrose.

slight increase in intraruminal pressure (as measured by an elevation of the base line of the motility tracing). In the second experiment, when the animal was grazing on a pasture containing predominant amounts of Ladino clover, the intraruminal administration of 175 grams of alfalfa saponin in a split dose (100 gm. and 75 gm.) resulted in no detectable effect on ruminal motility during a 2.5-hour observation period.

*Nonlegume Saponins.*—The intraruminal administration of 35 grams of a commercially available saponin from the yucca plant (labeled as nontoxic) to a sheep that had shown definite responses to alfalfa saponin did not reduce ruminal motility on three different occasions. The same result was obtained from the intraruminal administration of 25 grams of a second yucca saponin preparation (labeled as toxic) to four different sheep. Compare figures 10 and 15.

On the other hand, intraruminal administration of 25 grams of quillai (*Quillaja saponaria*) saponin to a sheep resulted in a marked reduction in ruminal motility and an increase in intraruminal pressure.

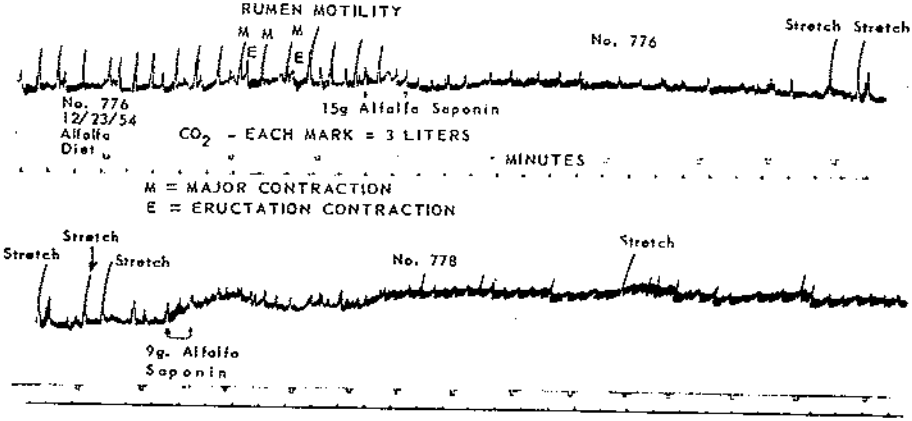



FIGURE 14.—An effect of intraruminal administration of alfalfa saponin on ruminal motility and efficiency of eructation in sheep.

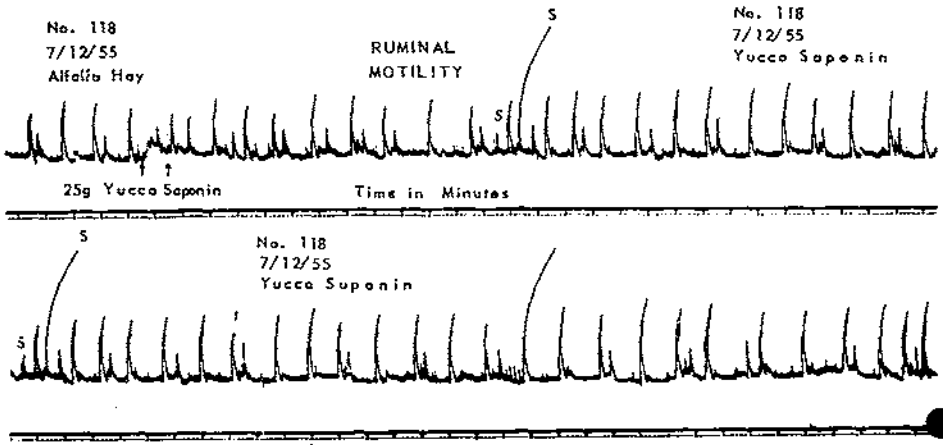



FIGURE 15.—Negative response in sheep of ruminal motility to intraruminal administration of a yucca saponin preparation.

*Legume Press Juice.*—Variation was noted in the effect of intraruminal administration of legume press juices on ruminal motility of sheep. This variation was expected in view of the results obtained with alfalfa saponin administration. In addition to animal variations, the legume press juice also varied according to the stage of maturity of the legume and other factors.

However, the intraruminal administration of press juices from alfalfa and Ladino clover did result in reduced ruminal motility in a number of cases. This reduction in motility was similar to that obtained from alfalfa saponin administration. But this result does not necessarily imply that the saponin content of the juices was the only constituent present which had an effect on motility.

Figures 16 and 17 illustrate responses obtained from intraruminal administration of alfalfa press juice. The same animal was used in both cases and had been receiving an alfalfa hay diet previous to the experiments. In the first case (fig. 16) the administration of the alfalfa juice resulted in a marked and prolonged reduction in the amplitude of the major ruminal contraction. In the second case, the administration of 1,800 ml. of alfalfa juice and 25 grams of dextrose resulted in (1) a reduction in motility, (2) an unusual motility pattern, and (3) an increase in the frequency of ruminal contractions as the sheep bloated. The motility pattern shortly after dosing resembled a particular type of motility noted by workers of the Agricultural Research Center after dosing some intact animals with legume

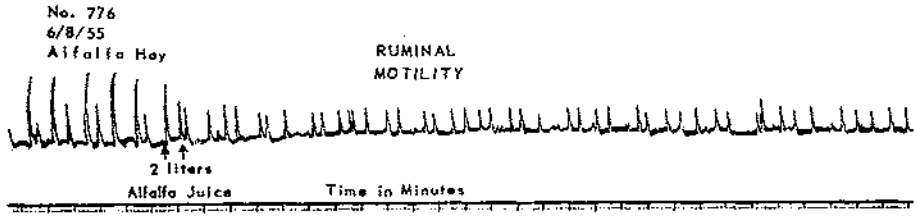



FIGURE 16.—Reduced ruminal motility in sheep following the intraruminal administration of alfalfa press juice.

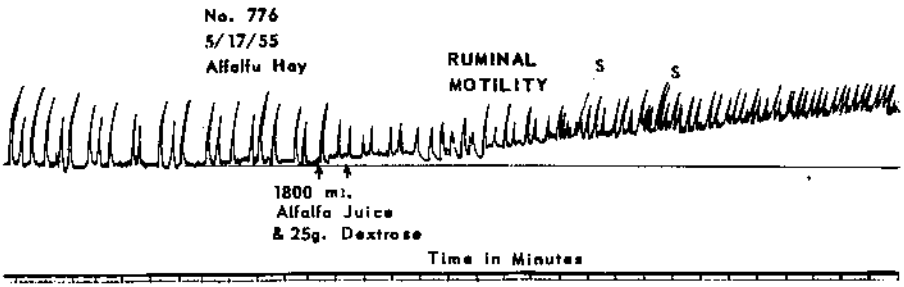



FIGURE 17.—Ruminal motility in sheep during the development of bloat symptoms resulting from intraruminal dosing with alfalfa press juice and dextrose. S=sneeze.

juices and alfalfa saponin. Ruminal movement, on visual inspection, appeared to begin much lower on the side of the animal than is detected during regular ruminal contractions. Although this type of motility might appear to be more pronounced than normal, the tracing indicates that it may be caused by an abnormality during the relaxation phase rather than during the contraction phase of the ruminal motility cycle.

In this experiment the animal developed "frothy" bloat symptoms that disappeared rapidly after the introduction of a methyl silicone preparation into the rumen, but the froth did not fill the rumen to a level sufficient to block the open tube to the recording tambour.

Figure 18 illustrates a pronounced reduction in ruminal motility in a sheep following the intraruminal administration of 1,875 ml. of Ladino clover press juice. There was also a slight increase in intraruminal pressure. Parsons and coworkers (*41*) have recently reported that the introduction of 8 pounds of Ladino clover juice into the rumen of a steer inhibited eructation but did not result in a noticeable reduction in ruminal contractions.

To correlate these observations definitely with clinical or experimental bloat is not simple, as illustrated by a comparison of figures 16 and 17. In these two experiments with alfalfa press juice, ruminal motility was more active when the sheep bloated than when it did not.

## Effect of Intravenous Administration of Alfalfa Saponin on Ruminal Motility

### EXPERIMENTAL PROCEDURES

Five experiments were conducted in this phase of the study, using a different animal for each experiment. All the sheep were mature and had been fitted with permanent ruminal fistulas previous to the

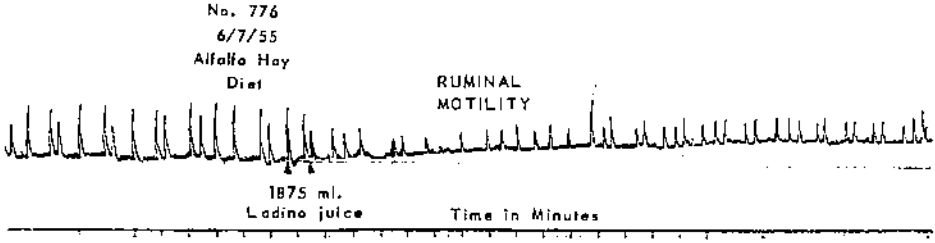



FIGURE 18.—Reduced ruminal motility in sheep following intraruminal administration of Ladino clover press juice.

tests. The tracings were made in a fashion similar to those described under the studies on intraruminal administration of saponin. The first experiment was conducted at the Agricultural Research Center. The remainder of the experiments were conducted at the New York State Veterinary College. The details of each experiment are given in the following discussion.

## RESULTS AND DISCUSSION

*Experiment 1.*—The animal used in this experiment had been grazing on a pasture that was predominantly Ladino clover. A 0.75-gram dose of alfalfa saponin in aqueous solution was injected into the jugular vein, using a hypodermic syringe. Immediately after this dose, the ruminal motility was markedly reduced but recovered in approximately 6 minutes and remained normal for the next 15 minutes. During the next 30 minutes the strength of the contractions was gradually reduced and the intervals between contractions increased. Fifty minutes after the first dose, an additional 0.25 gram of the saponin was given, as above. After the second dose, the strength of the contractions decreased at an accelerated rate and the intervals between contractions became farther and farther apart until all motility ceased an hour later.

*Experiment 2.*—The sheep used in this test had been receiving a diet of poor quality, coarse grass hay previous to the test. A total of 0.94 gram of alfalfa saponin was administered into the jugular vein over a period of 25 minutes. The solution (47 ml. of aqueous solution containing 0.02 gram saponin per ml.) was injected at a uniform rate by using an infusion pump. Figure 19 illustrates the complete cessation of ruminal motility, with impaired efficiency of eructation, obtained in this experiment.

*Experiment 3.*—The conditions of this experiment were identical to those of experiment 2, except that the dose was given to the animal in 15 minutes. Ruminal motility decreased rapidly and ceased by the time 0.6 gram of saponin had been administered. One hour and 20 minutes later, the sheep displayed definite bloat symptoms, although it had not had opportunity to eat for about 3 hours.

*Experiment 4.*—The conditions of this experiment were similar to those of 2 and 3, except that 0.75 gram of saponin was administered to the sheep over a period of 14 minutes. Little change was noted in the ruminal motility during the first 15 minutes after completion of the dose, but during the next 10 minutes all motility ceased and eructation was blocked, resulting in a buildup of intraruminal pressure.

*Experiment 5.*—The conditions of this experiment were the same as those in 2, 3, and 4, with 0.75 gram of saponin being administered to the animal over a period of 3.5 minutes. Little or no effect was detected in ruminal motility or efficiency of eructation during an observation period of 1.5 hours.

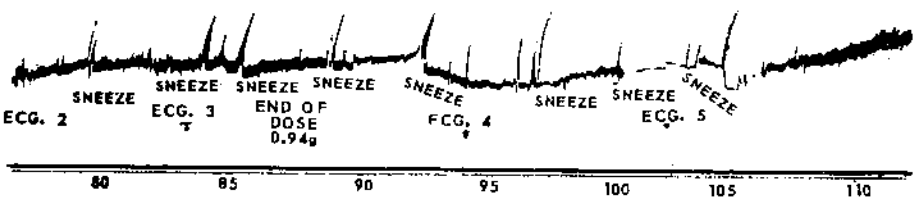



FIGURE 19.—Effect of intravenous administration of 0.94 gm. of alfalfa saponin on ruminal motility in sheep. *EOG*=electrocardiogram taken.

## Effect of Administration of Saponin into the Duodenum and Small Intestine, or Both, on Ruminal Motility

### EXPERIMENTAL PROCEDURES

A mature sheep, which had been fitted with permanent ruminal and duodenal fistulas, was used in all the experiments conducted in this phase of the study. Polyethylene tubing was passed into the small intestine via the duodenal fistula or placed in the duodenum for the administration of the saponin solutions. Direct recordings of the ruminal and intestinal motilities were made by using ink-writing air tambours connected to the respective fistulas. The preliminary experiment was conducted at the New York State Veterinary College, when the animal was receiving a diet of poor-quality grass hay. After the preliminary experiment, the animal was moved to the Agricultural Research Center, Beltsville, Md., and put on a constant diet of alfalfa hay. During the experiments conducted at the Agricultural Research Center, carbon dioxide gas was introduced into the rumen at a rate of approximately 1 liter per minute. This was done to standardize the ruminal motility and eructation pattern. All water-soluble test materials were dissolved in 50 ml. of water and introduced through the duodenal fistula in the same manner and in approximately the same length of time.

### RESULTS AND DISCUSSION

In the preliminary experiment, alfalfa saponin was administered to the sheep in two doses of 0.6 gram each. The first dose of 0.6 gram (15 ml. of solution containing 0.04 gram per ml.) was introduced into the small intestine over a period of 6 minutes, using an infusion device. The second dose of 0.6 gram was introduced in the same manner 42 minutes later. The first dose resulted in an immediate decrease in ruminial motility and an increase in intestinal motility (fig. 20). The increase in intestinal motility was apparent for 10 to 12 minutes, starting by the time 0.4 gram of saponin had been given. Ruminal motility was still reduced and intestinal motility was almost nil when the second dose was given. Midway through the second dose period, intestinal motility increased for a period of about 4 minutes and then decreased rapidly. Ruminal motility was still severely reduced over the predose pattern when the tracing was interrupted 30 minutes later. Two hours later, both ruminal and intestinal motilities approached the predose pattern.

Experiments with different lots of composite alfalfa saponin have resulted in patterns similar to that illustrated in figure 21. In all

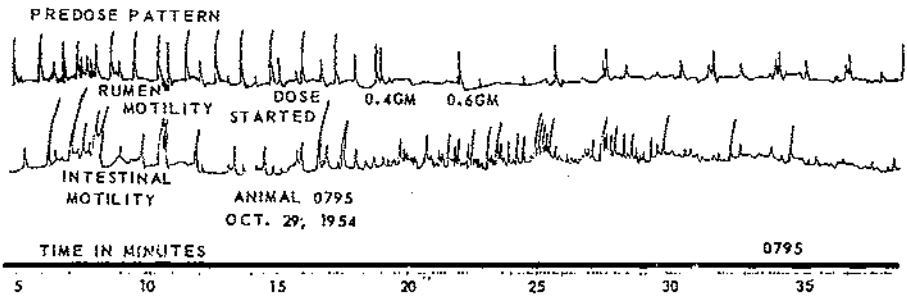



FIGURE 20.—Effect of intestinal administration of 0.6 gm. of alfalfa saponin on ruminal and intestinal motility in sheep.

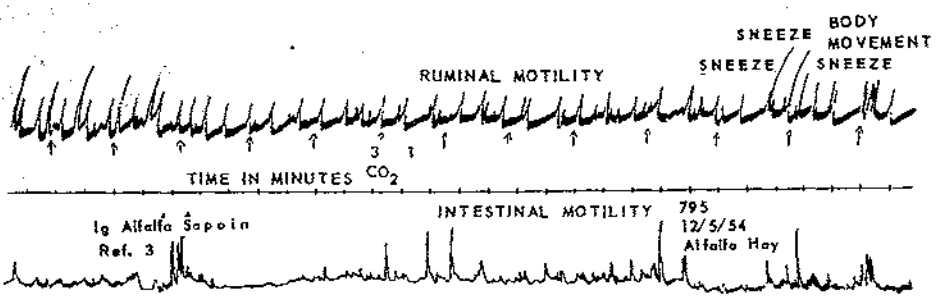



FIGURE 21.—Typical result of intestinal administration of composite alfalfa saponin on ruminal and intestinal motility in sheep. Distance between arrows indicates time taken to introduce 3 liters $CO_2$ into rumen.

cases, the major ruminal contraction has been rapidly and markedly reduced in amplitude when 1 gram of alfalfa saponin has been introduced into the intestine of sheep. Intestinal motility has varied somewhat among experiments but usually has increased, for at least a short time interval, following the dosing. Repeated experiments with one of the saponins from the yucca plant have resulted in no reduction in ruminal motility, as illustrated in figure 22. In three experiments, dosage with 1 gram of a sodium salt of a Ladino clover saponin has not resulted in any reduction of ruminal motility, as illustrated in figure 23. The introduction of 1 gram of quercetin in water suspension also resulted in no reduction in ruminal motility. When 1 gram of quercetin was dissolved in 40 ml. of propylene glycol and introduced into the intestine of the animal, ruminal motility was affected in a manner similar to that produced by alfalfa saponin. However, in a repeated trial, 40 ml. of propylene glycol introduced into the intestine also reduced ruminal motility.

Figure 24 is given to illustrate the effect of quercetin dissolved in propylene glycol on intestinal motility in a sheep. The pronounced and prolonged increase in intestinal motility is a different result than would be expected from experiments with this compound on isolated intestinal strips.

The mechanism by which ruminal motility is affected so rapidly by the introduction of a small amount of alfalfa saponin into the intestine is not evident. A postulation for this reaction is given in another section of this bulletin entitled "Some Pharmocological Effects of Alfalfa Saponin on Nonruminants and on

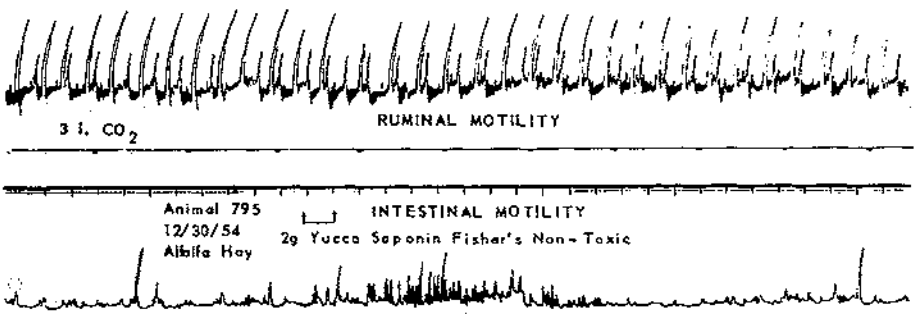



FIGURE 22.—Typical reaction resulting from the intestinal administration of a yucca saponin in sheep.

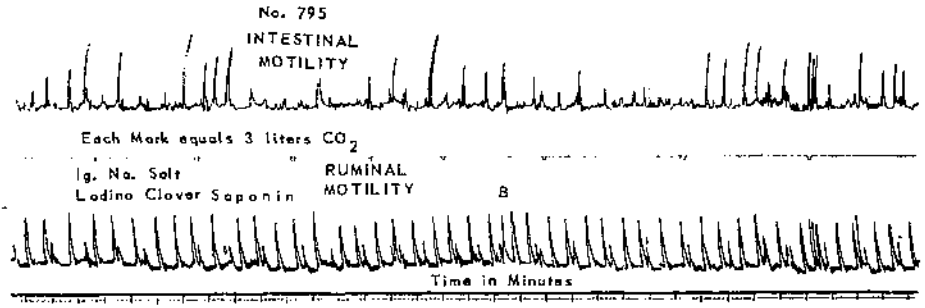



FIGURE 23.—Typical reaction resulting from the intestinal administration of a sodium salt of a Ladino clover saponin in sheep.

Isolated Muscle Strips." It appears to be significant that the results of the intestinal administration of alfalfa saponin and other compounds are in agreement with the results obtained by other methods of administration.

All the experiments above were conducted with a single animal. It could be questioned whether the sheep had intestinal injuries resulting from fistulation, which could have affected the results. However, the animal had remained in excellent health during the experiments conducted over several months in addition to dropping and raising a normal lamb during the same period.

## Cinefluorographic Studies

### EXPERIMENTAL PROCEDURES

Two lambs weighing approximately 50 pounds each were used in this phase of the study. The general procedures developed by Dougherty and Meredith (*15*) were employed in these experiments. The experiments were conducted with the cooperation of staff members of the Department of Radiology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y., using the equipment assembled by that school.

One lamb was given a toxic level of alfalfa saponin and the second was given a moderate but sublethal level of saponin. In each experiment the animals were given an oral dose of barium sulfate, insufflated with a mixture of 40 percent carbon dioxide and 60 percent methane (initial pressure to 40 mm. Hg, and then reduced to 20 mm. Hg). The flouroscopic image of the normal action of reticular motility and eructation was recorded on 35-mm. movie film. After motility

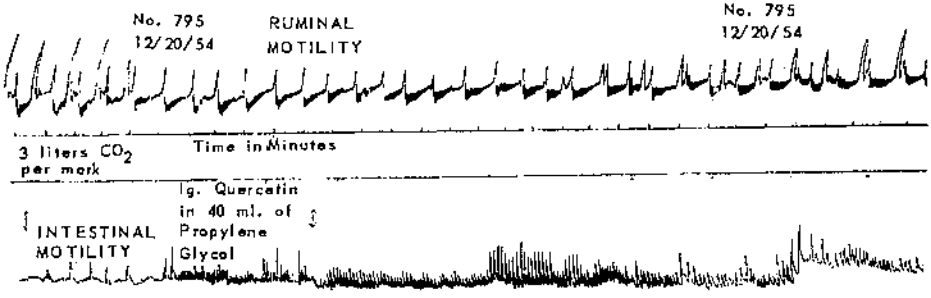



FIGURE 24.—Effect of quercetin dissolved in propylene glycol on ruminal and intestinal motility in sheep.

under these conditions was recorded, the alfalfa saponin, dissolved in 1 liter of water, was introduced into the rumen through the fistula. The ruminal motility was traced by regular means until the rumen became quiescent. At this point the animals were treated as above except for dosage with saponin, and a second recording of reticular motility and eructation was made.

## RESULTS AND DISCUSSION

*Experiment 1.*—This animal was given a lethal dose of alfalfa saponin of 50 grams. Projection of the films, which were taken 38 minutes after the saponin administration, revealed that all ruminal and reticular motility had ceased and that eructation was completely inhibited. Deglutition and intestinal motility, however, were not inhibited. That eructation was inhibited while deglutition and intestinal motility were not inhibited would indicate that the inhibition of eructation was caused by a direct effect of the alfalfa saponin on the central nervous system rather than to a direct effect on the stomach and esophageal musculature.

*Experiment 2.*—The second animal was given 15 grams of the alfalfa saponin. Projection of the film, which was taken 11 minutes after the saponin administration, revealed that the rumen was quiescent and that contraction of the reticulum was incomplete. Eructation was attempted but was partially inhibited. Projection of a second film, which was taken 40 minutes after the first, revealed a quiescent rumen, slight recovery of reticular motility, and some, but still inefficient, eructation.

## Summary

Intraruminal, intravenous, and intestinal administration of alfalfa saponin to sheep resulted in reduced ruminal motility. Considerable variation was noted among animals and with the same animals at different times in their reaction to intraruminal administration of alfalfa saponin. Reasons for this variation are not apparent at the present time. Variations were also noted among animals in their reaction to intravenous administration of alfalfa saponin. Intestinal administration of small amounts of alfalfa saponin resulted in a rapid reduction in the amplitude of the major ruminal contraction. Although some of the effect of alfalfa saponin on ruminal motility may be explained on the basis of a direct action on the ruminal musculature, other reactions noted in the above studies cannot be readily explained on that basis. In some cases, ruminal motility was reduced immediately following intraruminal dosing, but in other cases ruminal motility was reduced only after a timelag of several minutes.

The reactions noted in the above experiments also indicate that certain muscular actions are inhibited more readily than others. The major ruminal contraction was more vulnerable to the alfalfa saponin than the "eructation contraction." It also appears that ruminal motility is affected more readily than efficiency of eructation.

Intraruminal administration of two saponin preparations from the yucca plant (a steroid-type saponin) did not reduce ruminal motility, while a third saponin of the triterpenoid type (like alfalfa saponin) did result in decreased motility.

The intraruminal administration of press juices from alfalfa and Ladino clover also resulted in reduced ruminal motility in sheep. The reaction was similar to that obtained with alfalfa saponin; however, this does not imply that

saponin was the only factor involved. Variation was also noted in the effect of legume press juices on ruminal motility. Part of this variation was undoubtedly caused by the animals and part by variability in the press juices. Although legume press juices can result in reduced ruminal motility, experimental data are presented which indicate that motility can be relatively active during the development of bloat symptoms following the administration of legume press juices.

Cinefluorographic studies illustrate that alfalfa saponin can inhibit motility of the reticulum and exert a direct effect on eructation.

## In Vitro and In Vivo Experiments With Saponin in Relation to Froth Formation and Stability

By *Ivan L. Lindahl* and *R. E. Davis*

Several investigators have postulated that saponin alters the surface tension of the ruminal contents and contributes to the development of frothy bloat (*34, 39, 42*). Recent studies reported by Hungate and coworkers (*24*), Johns (*29*), Smith and others (*45*), Weiss (*49*), and Jacobson and Lindahl (*25*) indicate that the formation of froth is more complex than can be readily explained by a single factor.

A diversity of opinions has existed in regard to the importance of froth formation and how it contributes to bloat. Cole and coworkers (*6*), in criticism of the foam theory, stated that they tapped a number of cows bloated on alfalfa pasture without encountering a case in which a free gas pocket was absent, and concluded that it was difficult to understand how foaming could cause bloat when a free gas space exists. However, Cole (*5*) has recently stated that more and more evidence is accumulating to substantiate the view that foaming is an important factor in causing bloat on legume pastures. Smith (*44*) has reported that frothy bloat produced under his conditions could not be relieved by stomach tube. Lindahl and coworkers (*31*) stated in a preliminary report on the production of experimental bloat with alfalfa saponin that the bloat symptoms appeared to be caused by gas retention, per se, rather than to froth formation, because free gas could be released from the animals with a stomach tube. However, Jacobson and Lindahl (*25*) have recently reported that when taking ruminal samples from animals with frothy bloat (produced with a diet similar to that used by Smith) gas repeatedly escaped when the curvature of the stomach tube was up during placement of the tube into the rumen. Weiss (*49*) has also reported that in most cases of frothy bloat produced under his experimental conditions free gas escaped from the fistula on opening. Only in a few cases did the ingesta froth to such an extent as to fill the whole rumen and exude from the fistula on removal of the stopper.

Clark (*3*), in treating natural cases of frothy bloat by injecting antifoaming agents directly into the rumen, found that when eructation did not occur spontaneously, the gas was easily removed by stomach tube. Johns (*29*) has recently stated that frothing is probably a matter of degree and that it seems likely that the foam is never so stable as to retain all of the gas without a slow release to a gas pocket, even in the most severe cases. Other reports in the litera-

ture indicate that little, if any, free gas is found in some severely bloated animals.

The following studies were undertaken to determine some of the relationships of alfalfa saponin to froth formation.

## In Vitro Experiments on the Effect of Saponin and Protein on Froth Stability

### EXPERIMENTAL PROCEDURES

Preliminary in vitro experiments indicated that alfalfa saponin, in the presence of glucose, could stabilize the "frothy" ruminal contents of cattle receiving feed lot diets (*35*).[5] The procedure for measuring ingesta volume increase, as reported by Jacobson and Lindahl (*35*), was used in the following tests to compare the effects of alfalfa saponin, two saponin preparations from the yucca plant, a water-soluble protein (egg albumin), and a combination of the protein and alfalfa saponin on froth formation and stability of unstrained ruminal contents from animals receiving feed lot diets. Some additional experiments were conducted with the saponins, using ruminal contents taken from cattle grazing on a grass pasture.

During the actual tests, a rather large sample of ruminal contents was drawn by stomach tube. The samples were well mixed, and 200 ml. of the ruminal contents were delivered into a 500-ml. graduated cylinder. Three grams of dextrose were added to each cylinder. One cylinder served as a control. All the test materials were dissolved in sufficient water so that 5 ml. of solution contained 0.1 gram of the material. The respective test materials were then added to the other cylinders. After thoroughly mixing the contents of the cylinders, the cylinders were placed in a water bath at 39° C. for an hour. At the end of 1 hour, the cylinders were removed from the water bath and the total volume of the contents recorded. The original volume of ruminal contents plus the volume of the additives were subtracted from the total. The increase in volume divided by 2 was recorded as percentage of ingesta volume increase. The contents of the cylinders were then stirred, using 24 rotations with a heavy stirring rod. The material on the sides of the cylinders was pushed down to the new level of the contents, using a cork stopper that just fitted into the cylinder. A second reading was then taken and corrected as above. This increase in volume was again divided by 2 and recorded as percentage of stable volume increase.

Eight head of cattle from feed lot studies with varying bloat records were used in the first phase of the study. In these studies, conducted from July 18 through July 26, 1955, four ruminal samples were taken from each animal during the period. Two samples were taken before the morning feedings and two before the afternoon feedings. Ruminal samples, however, were taken only once during any particular day from a single animal. Each animal was rated for the degree of bloat exhibited 1 hour after feeding and given a numerical rating ranging from 0 to 5. In the tables of results, the average daily bloat index is given for each of the animals during the tests. As the morning and afternoon samples were not taken on the same days, the bloat index as given in the tables vary slightly.

Four head of cattle were used in the pasture studies. These animals

[5] JACOBSON, D. R. BIOCHEMICAL, PHYSICAL, AND BACTERIOLOGICAL FACTORS INVOLVED IN FROTHY (FEED LOT) BLOAT. 1956. [Unpublished doctor's thesis. Copy on file in Library, Univ. of Md., College Park.]

were grazing on a grass pasture that contained only small amounts of legumes. Each animal was sampled twice, but only one sample was taken from a single animal on any one day.

## RESULTS AND DISCUSSION

The results of the experiments with the feed lot cattle are given in tables 2 and 3, and the results with the pasture cattle in table 4. In vitro experiments cannot duplicate the conditions existing in the rumen; however, the degree of correlation between the stable volume increase values of the control samples (ingesta plus dextrose) taken before feeding and the average daily bloat indexes is encouraging. These observations are similar to those reported by Hungate and coworkers (*24*) in studies on legume pasture bloat. The total volume increase values do not have the same degree of correlation, indicating that the stability of the froth is far more important than the total froth formation. The values obtained on the samples taken before the morning feeding are higher and more consistent than those taken in the afternoon.

TABLE 2.—*Total ingesta volume increase of ruminal contents from feed lot cattle*

SAMPLES TAKEN BEFORE THE MORNING FEED

| Animal No. | Average bloat index | pH of ruminal contents | Ingesta volume increase: Control | + 0.1 gm. alfalfa saponin | + 0.1 gm. yucca saponin 1 | + 0.1 gm. yucca saponin 2 | + 0.1 gm. egg albumin | + 0.1 gm. egg albumin and 0.1 gm. saponin |
|---|---|---|---|---|---|---|---|---|
| | | | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* |
| 41 | 0.00 | 7.4 | 47.5 | 101.2 | 40.0 | 47.5 | 72.5 | 96.2 |
| 22 | .50 | 7.3 | 48.7 | 81.2 | 52.5 | 86.2 | 133.7 | 103.7 |
| 34 | 1.00 | 7.1 | 140.0 | 131.2 | 72.5 | 72.5 | 140.0 | 162.5 |
| 27 | 1.50 | 6.5 | 78.7 | 108.7 | 87.5 | 93.2 | 77.0 | 125.0 |
| 26 | 1.75 | 7.2 | 102.5 | 90.0 | 91.2 | 96.2 | 92.5 | 112.5 |
| 40 | 2.20 | 7.0 | 38.7 | 46.2 | 56.2 | 53.7 | 49.7 | 50.0 |
| 32 | 2.50 | 7.1 | 117.5 | 112.5 | 106.2 | 128.7 | 135.0 | 120.0 |
| 42 | 3.00 | 7.0 | 105.0 | 138.7 | 140.0 | 128.7 | 127.7 | 110.0 |
| Average | | 7.1 | 84.8 | 101.2 | 80.8 | 88.3 | 103.5 | 110.0 |

SAMPLES TAKEN BEFORE THE EVENING FEED

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 41 | 0.25 | 6.5 | 28.7 | 77.5 | 22.5 | 27.5 | 57.5 | 96.2 |
| 22 | 1.50 | 6.2 | 41.2 | 68.7 | 35.0 | 33.7 | 73.7 | 71.2 |
| 34 | 1.50 | 6.3 | 70.0 | 71.2 | 73.7 | 67.5 | 60.0 | 80.0 |
| 27 | 1.75 | 7.0 | 50.0 | 68.7 | 50.0 | 43.7 | 63.7 | 66.2 |
| 40 | 2.25 | 6.5 | 61.2 | 70.0 | 27.5 | 58.7 | 83.7 | 81.2 |
| 32 | 3.00 | 5.9 | 65.0 | 75.0 | 55.7 | 65.0 | 80.0 | 80.0 |
| 42 | 3.00 | 6.0 | 66.2 | 75.0 | 80.0 | 68.7 | 77.5 | 98.7 |
| 26 | 4.50 | 6.4 | 40.0 | 46.2 | 40.0 | 55.0 | 52.5 | 55.0 |
| Average | | 6.4 | 52.8 | 69.1 | 47.8 | 52.5 | 68.6 | 78.6 |

TABLE 3.—*Total stable volume increase of ruminal contents from feed lot cattle*

SAMPLES TAKEN BEFORE THE MORNING FEED

| Animal No. | Average bloat index | pH of ruminal contents | Stable volume increase | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Control | +0.1 gm. alfalfa saponin | +0.1 gm. yucca saponin 1 | +0.1 gm. yucca saponin 2 | +0.1 gm. egg albumin | +0.1 gm. egg albumin and +0.1 gm. saponin |
| | | | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* | *Percent* |
| 41 | 0.00 | 7.4 | 11.2 | 68.7 | 21.2 | 25.0 | 18.7 | 73.7 |
| 22 | .50 | 7.3 | 15.0 | 66.2 | 18.7 | 36.2 | 92.5 | 46.2 |
| 34 | 1.00 | 7.1 | 22.5 | 81.2 | 26.2 | 18.7 | 51.2 | 126.2 |
| 27 | 1.50 | 6.5 | 52.5 | 105.0 | 60.0 | 57.5 | 110.0 | 121.2 |
| 26 | 1.75 | 7.2 | 42.5 | 46.2 | 21.2 | 25.0 | 36.2 | 71.2 |
| 40 | 2.20 | 7.0 | 32.5 | 43.7 | 53.7 | 48.7 | 45.0 | 46.2 |
| 32 | 2.50 | 7.1 | 48.7 | 92.5 | 65.0 | 40.0 | 53.7 | 112.5 |
| 42 | 3.00 | 7.0 | 68.7 | 128.7 | 68.7 | 63.7 | 108.7 | 87.2 |
| Average | | 7.1 | 36.7 | 79.0 | 41.8 | 39.4 | 64.5 | 85.6 |

SAMPLES TAKEN BEFORE THE EVENING FEED

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 41 | 0.25 | 6.5 | 7.5 | 25.0 | 7.5 | 7.5 | 8.7 | 26.2 |
| 22 | 1.50 | 6.2 | 8.7 | 41.2 | 10.0 | 7.5 | 65.0 | 56.2 |
| 34 | 1.50 | 6.3 | 30.0 | 65.0 | 38.7 | 37.5 | 50.0 | 71.2 |
| 27 | 1.75 | 7.0 | 15.0 | 43.7 | 21.2 | 16.2 | 55.0 | 55.0 |
| 40 | 2.25 | 6.5 | 31.2 | 37.5 | 18.7 | 33.7 | 50.0 | 50.0 |
| 32 | 3.00 | 5.9 | 46.2 | 52.5 | 35.0 | 40.0 | 72.5 | 62.5 |
| 42 | 3.00 | 6.0 | 61.2 | 68.7 | 58.7 | 50.0 | 50.0 | 96.2 |
| 26 | 4.50 | 6.4 | 18.7 | 31.2 | 18.7 | 37.5 | 42.5 | 37.5 |
| Average | | 6.4 | 27.3 | 45.6 | 26.1 | 28.7 | 49.3 | 56.9 |

The higher values obtained from morning samples can be at least partially explained by the fact that the afternoon samples contained more entrapped gas and consequently less liquid ruminal contents than the morning sample. The pH values were obtained immediately after drawing the ruminal contents. The pH values were not correlated with the froth stability and were in agreement with the observation of Hungate and others (*24*) that foam production and bicarbonate content of the ruminal contents were not correlated and that foam production was not caused directly by a difference in pH of the ruminal contents. The difference between the effect of the alfalfa saponin and the yucca saponins on foam stability was very pronounced even though the yucca saponins possessed strong foaming characteristics. The differences obtained between ruminal samples from different animals in the response to added saponin, albumin, and the combination of the saponin and albumin illustrate that froth stability cannot be determined by a single factor. The differences in the total stable froth, after the

TABLE 4.—*Results of the effects of saponins on the stabilization of ruminal contents of cattle on grass pasture*

| Animal No. | Increase in volume | | | |
|---|---|---|---|---|
| | Control | +0.1 gm. alfalfa saponin | +0.1 gm. yucca saponin 1 | +0.1 gm. yucca saponin 2 |
| PERCENTAGE OF TOTAL VOLUME INCREASE | | | | |
| | *Percent* | *Percent* | *Percent* | *Percent* |
| 33 | 43.7 | 97.5 | 43.7 | 45.0 |
| 35 | 52.5 | 96.2 | 40.6 | 60.0 |
| 37 | 42.5 | 85.0 | 38.8 | 42.5 |
| Bull | 37.5 | 62.5 | 35.0 | 37.5 |
| Average | 44.0 | 85.3 | 37.0 | 46.2 |
| PERCENTAGE OF STABLE VOLUME INCREASE | | | | |
| 33 | 6.2 | 41.2 | 7.5 | 5.0 |
| 35 | 13.7 | 28.7 | 6.8 | 12.5 |
| 37 | 7.5 | 35.0 | 7.5 | 8.8 |
| Bull | 3.7 | 23.5 | 7.5 | 6.2 |
| Average | 7.8 | 32.1 | 7.3 | 8.1 |

addition of the same level of saponin, obtained from the pasture and feed lot animals again illustrate that alfalfa saponin can be only one of the factors in stable froth formation.

The significance of the results with the egg albumin in actual bloat is only a matter of speculation at this time. This substance was used because a suitable plant protein was not available. The tests with the protein were conducted because protein and protein degradation products have been postulated as playing a part in froth formation (*29*). We have also found that legume press juices contain considerable amounts of heat coagulable protein that is either water soluble or in colloidal suspension. In addition, legume pastures contain high levels of protein during periods of lush growth.

## In Vivo Experiments With Alfalfa Saponin and Egg Albumin in Relation to Froth Formation

### EXPERIMENTAL PROCEDURES

In vitro experiments have shown that although alfalfa saponin has strong surface active qualities, introduction of air or gas from an open tube into an aqueous solution results in a layer of foam on top of the solution rather than a stable froth existing throughout the solution. However, when gas is introduced in minute quantities over a larger area, such as passing the gas through a sintered glass funnel, a very stable froth consisting of small bubbles results. Attempts to produce a frothy condition in the rumen of animals grazing on grass pastures by passing gas through a sintered glass surface were not successful, probably because of a relatively localized area within the

rumen. The addition of alfalfa saponin and dextrose alone to animals grazing on grass or mixed pastures or consuming hay appeared to be inadequate to result in appreciable stable froth formation. However, frothy ingesta was obtained in animals that had been grazing on Ladino clover pastures when alfalfa saponin and dextrose were administered. In view of the results obtained in the in vitro experiments with a combination of alfalfa saponin and egg albumin, three experiments were conducted in which these materials along with dextrose were slowly introduced into the rumen of fistulated sheep, followed by additional dextrose and sodium formate. Ruminal motility was traced by the same procedures as described in a previous section. The first two experiments were conducted with sheep that had been grazing on a pasture which contained only small amounts of clover. The third experiment was conducted with an animal on an alfalfa hay diet.

## RESULTS AND DISCUSSION

Figure 25 illustrates the results of the first experiment with sheep. An aqueous solution containing 25 grams of alfalfa saponin, 25 grams of albumin, and 25 grams of dextrose was introduced into the rumen during a time interval of approximately 17 minutes. Seven minutes later, the administration of a second solution containing 25 grams of dextrose and 25 grams of sodium formate was started. The second solution was given over a 15-minute period. Approximately 7 minutes later the intraruminal pressure was 10 mm. Hg. Fifty grams of sodium

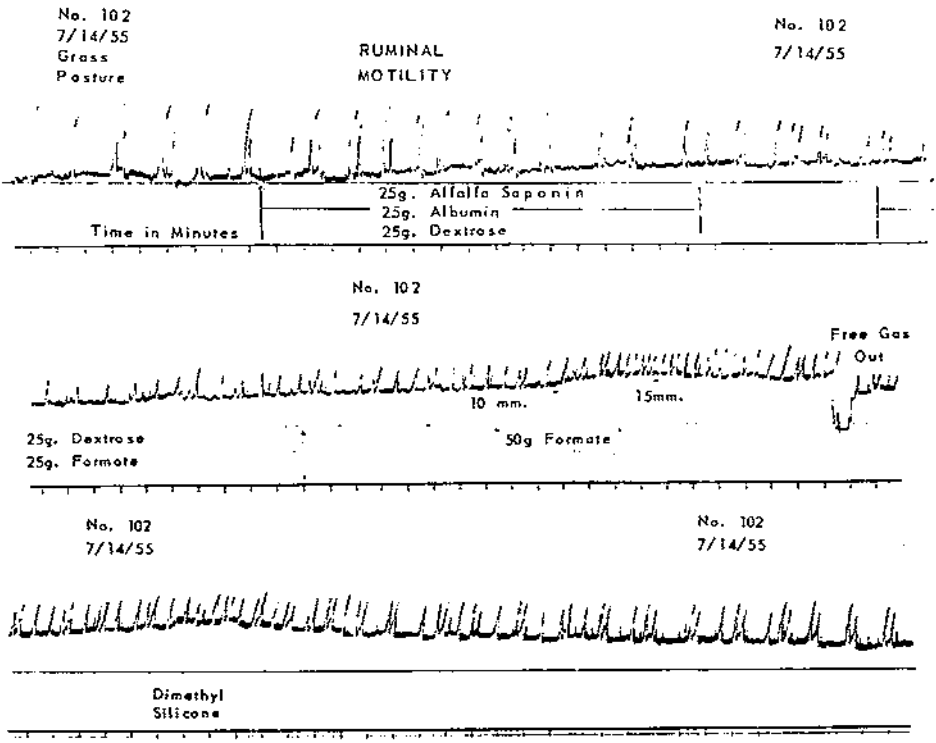



FIGURE 25.—Ruminal motility in sheep from first in vivo froth experiment.

formate were then introduced into the rumen over a period of approximately 5 minutes. Shortly after this dose, the intraruminal pressure had built up to 15 mm. Hg. Shortly thereafter, the manometer was disconnected and the free gas released from the rumen. This resulted in a drop of approximately 5 mm. Hg pressure; however, the pressure continued to build up and was back to the original level within a few minutes. A dimethyl silicone preparation introduced into the rumen at that time resulted in a rapid decline in the intraruminal pressure. The amplitude of the major ruminal contraction was reduced in this experiment, but the frequency of the "eructation contractions" increased as the pressure increased.

The second in vivo froth experiment was conducted in a similar manner. Figure 26 illustrates the results of this experiment. Twenty-five grams of alfalfa saponin, 25 grams of albumin, and 25 grams of dextrose were introduced into the rumen over a period of approximately 18 minutes. The introduction of sodium formate was begun in approximately 11 minutes. Fifty grams of this material was

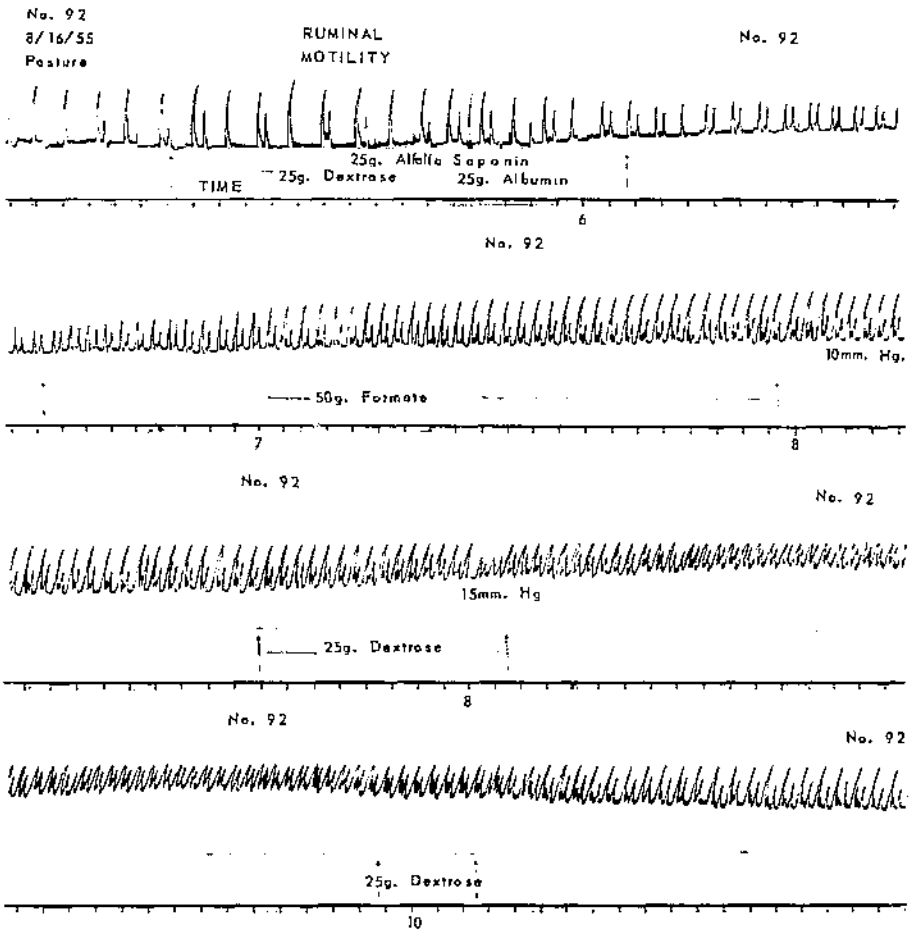



FIGURE 26.—Ruminal motility in sheep from second in vivo froth experiment.

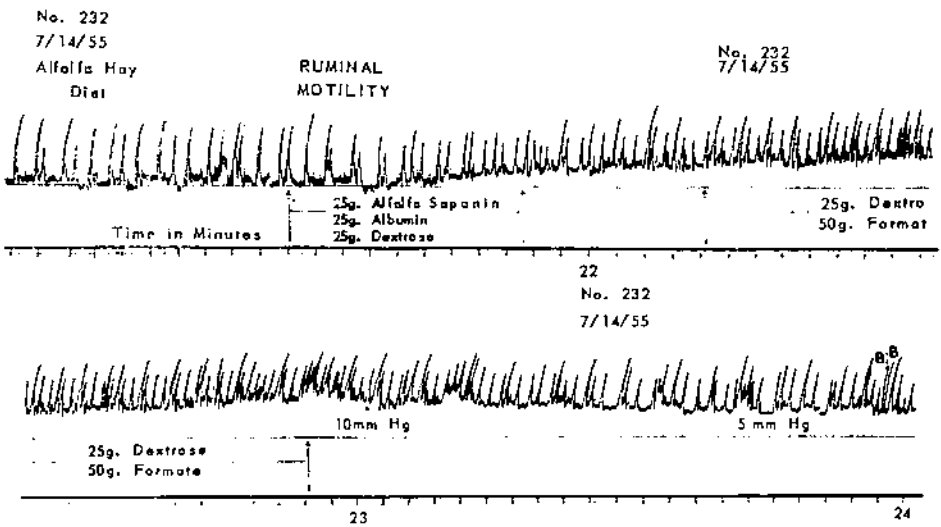



FIGURE 27.—Ruminal motility in sheep from third in vivo froth experiment.

given over a period of approximately 30 minutes. The sodium formate was then followed by an additional 25 grams of dextrose. At the end of this dose the intraruminal pressure had increased to 15 mm. Hg and continued to increase over the next 18 minutes. An additional 25 grams of dextrose given at that time did not result in an additional increase in pressure; instead, the pressure began to decrease and continued to do so. Except for a short period following the administration of the saponin, ruminal motility was very active and pronounced; this was especially true during the increase in intraruminal pressure. The apparent reduction in amplitude of contraction at the height of the intraruminal pressure is not a true reduction but is caused by an inadequacy of the recording system.

The results of the third in vivo froth experiment are illustrated in figure 27. This sheep had been receiving an alfalfa hay diet previous to the experiment. In this experiment, a dose of 25 grams of alfalfa saponin, 25 grams of albumin, and 25 grams of dextrose was followed by 25 grams of dextrose and 50 grams of sodium formate. Intra-

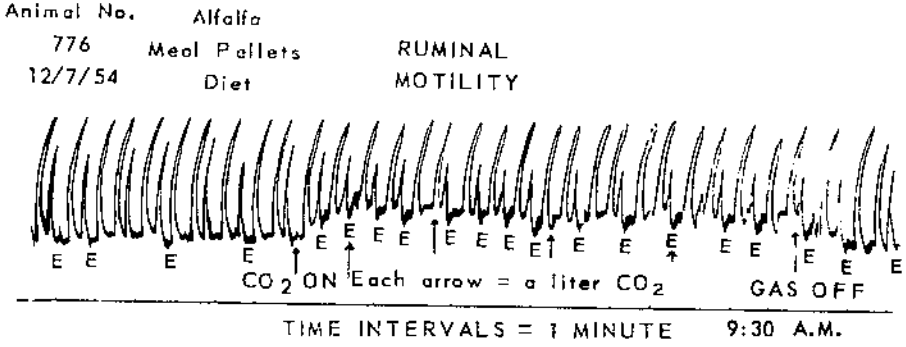



FIGURE 28.—Rapid response of eructation reflex to gas pressure stimulus in sheep. *E*=eructations.

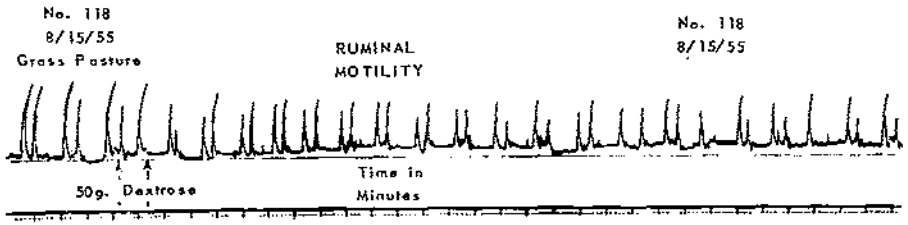



FIGURE 29.—Effect of intraruminal administration of dextrose on ruminal motility in sheep.

ruminal pressure increased rather steadily until it reached 10 mm. Hg pressure and then declined rapidly. Ruminal motility was very active throughout this experiment. In the absence of mechanical or physiological blockage of eructation, it is difficult to build up intraruminal pressure either through the introduction of relatively large amounts of gas into the rumen, as illustrated in figure 28, or by the use of the gas formers such as dextrose and formate, as illustrated in figures 29, 30, and 31. An effect of introducing 25 grams of albumin and 50 grams of dextrose into the rumen is illustrated in figure 32.

In view of our failure in a number of experiments to produce ruminal distention or increase intraruminal pressure by the introduction of gas formers, such as dextrose or formate, into the rumen and of the active ruminal motility exhibited by two of the animals, it is concluded that the increase in intraruminal pressure resulted from the formation of sufficient froth to interfere with eructation.

## In Vivo Experiments with Alfalfa Saponin and Alfalfa Press Juice in Relation to Froth Formation

### EXPERIMENTAL PROCEDURES

In order to study further the interaction of alfalfa saponin and other plant constituents in relation to froth formation, the following series of three experiments was conducted.

Alfalfa press juice was prepared from a sample of alfalfa that had not produced bloat symptoms when fed to cattle in the freshly cut state. The press juice was prepared immediately preceding each of the three experiments, according to the procedures outlined in the section on ruminal motility.

A single fistulated sheep was used in the experiments. The animal was grazing on a pasture containing large amounts of grass and some Ladino clover. In each of the experiments the animal was removed from the pasture 2 to 3 hours before the experiments were conducted.

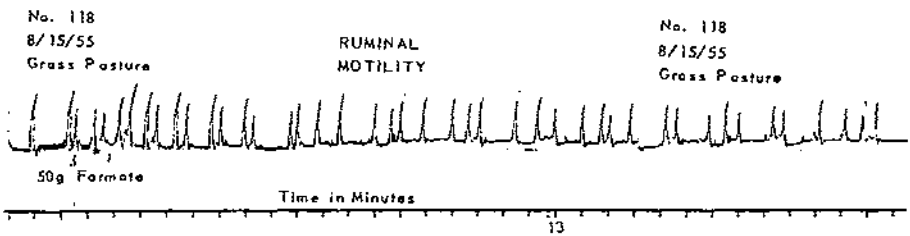



FIGURE 30.—Effect of intraruminal administration of sodium formate on ruminal motility in sheep.

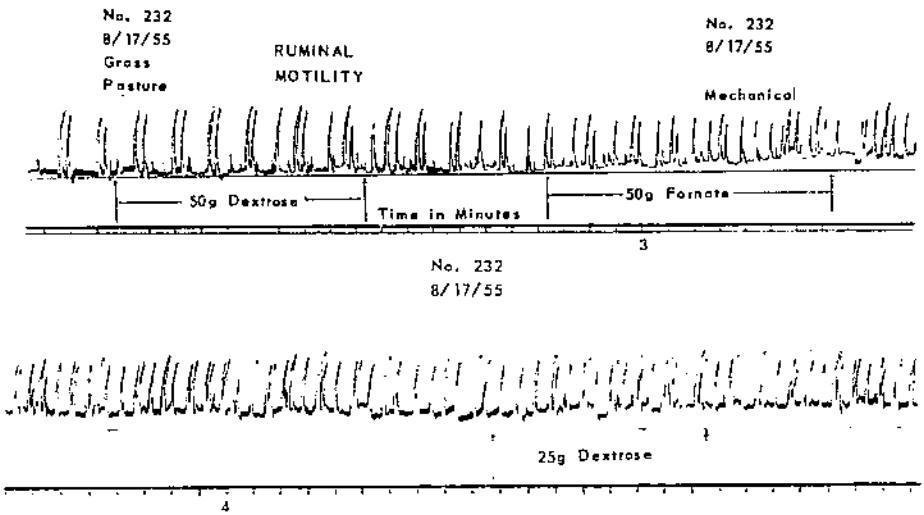



FIGURE 31.—Intraruminal administration of dextrose, formate, and dextrose on ruminal motility in sheep.

Ruminal motility was recorded by using the previously described techniques, and intraruminal pressure was recorded by using a manometer connected to the fistula of the animal.

In the first experiment, 1,900 ml. of the alfalfa press juice was introduced into the rumen of the sheep and the control record obtained.

In the second experiment, conducted on the following day, 300 ml. of a water solution containing 15 grams of alfalfa saponin and 50 grams of dextrose was added to 1,600 ml. of the alfalfa press juice, and the mixture introduced into the rumen of the animal, as above.

In the third experiment conducted 3 days later, 2 liters of the alfalfa press juice was slowly heated to 90° C. A copious precipitate which contained considerable amounts of protein was obtained. The juice was then filtered and cooled to body temperature. Just previous to administration of the juice to the animal, 15 grams of alfalfa saponin and 50 grams of dextrose were added to the solution.

Methyl silicone was used in the second and third experiments in order to show the effect of an antifoaming agent on the experimental bloat symptoms.

Where it was possible to distinguish clearly between the major ruminal contractions and the "eructation contractions," the frequency and amplitude of the contractions were measured and recorded by 5-

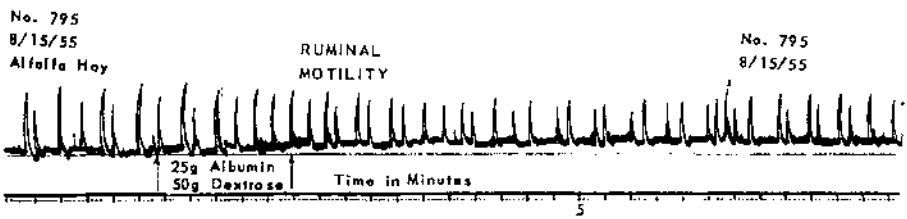



FIGURE 32.—Effect of intraruminal administration of albumin and dextrose on ruminal motility in sheep.

minute periods. The predose amplitude measurements were given an arbitrary index value of 100.

## RESULTS AND DISCUSSION

The results of the three experiments on the sheep are given in table 5. These experiments together with the experiments in which alfalfa saponin and egg albumin were combined are preliminary in nature, but these experiments indicate that an interaction between saponin and protein may exist in the formation of frothy ingesta. Readily fermentable carbohydrates, such as dextrose, also appear to aid in the formation of froth. Whether this action is limited to more rapid gas formation or whether dextrose has other properties that contribute to stable froth formation remains to be elucidated by further experimentation.

In all the experiments, a definite increase in the frequency of the

TABLE 5.—*Results of introduction of alfalfa press juice, saponin, and dextrose into rumen of fistulated sheep*

| Period | Total ruminal contractions | Major ruminal contractions | | Eructation contractions | | Intraruminal pressure |
|---|---|---|---|---|---|---|
| | | Frequency | Amplitude | Frequency | Amplitude | |
| | | *No. per minute* | *Index* | *No. per minute* | *Index* | |
| **First Experiment—1,900 ml. Alfalfa Press Juice** | | | | | | |
| Predose | 6 | 0.8 | 100.0 | 0.4 | 100.0 | Normal. |
| 1st postdose | 9 | 1.0 | 77.6 | .8 | 100.0 | Slight increase. |
| 2d postdose | 10 | 1.2 | 77.6 | .8 | 100.0 | Do. |
| 3d postdose | 9 | 1.0 | 77.6 | .8 | 100.0 | 5 mm. Hg. |
| 4th postdose | 9 | 1.0 | 77.6 | .8 | 100.0 | Do. |
| 5th postdose | 9 | .8 | 95.7 | 1.0 | 100.0 | Decreasing. |
| **Second Experiment—1,600 ml. Alfalfa Press Juice Plus 15 gm. Alfalfa Saponin and 50 gm. Dextrose** | | | | | | |
| Predose | 8 | 1.2 | 100.0 | 0.4 | 100.0 | Normal. |
| 1st postdose | 13 | 1.4 | 69.4 | 1.2 | 140.7 | Rapid increase. |
| 2d postdose | 11 | 1.2 | 60.2 | 1.0 | 123.2 | Do. |
| 3d postdose | 12 | | | | | 10 mm. Hg. |
| 4th postdose | 13 | | | | | 14 mm. Hg and increasing. |
| 5th postdose [1] | 11 | | | | | Began to decrease. |
| 6th postdose | 9 | | | | | Decreasing. |
| 7th postdose | 9 | 1.2 | 49.2 | .6 | 100.0 | Do. |
| 8th postdose | 6 | .6 | 49.2 | .6 | 100.0 | Do. |
| **Third Experiment—Alfalfa Juice Minus Protein Plus Saponin and Dextrose** | | | | | | |
| Predose | 7 | 1.0 | 100.0 | 0.4 | 100.0 | Normal. |
| 1st postdose | 13 | 1.4 | 46.0 | 1.2 | 100.0 | Slight increase. |
| 2d postdose | 12 | | | | | 5 mm. Hg. |
| 3d postdose | [2] 7 | | | | | 7 mm. Hg. |
| 4th postdose | 8 | | | | | Do. |
| 5th postdose [1] | 8 | | | | | Do. |
| 6th postdose | 7 | .6 | 27.2 | .8 | 71.4 | Decreasing. |

[1] Methyl silicone introduced into the rumen.
[2] All contractions reduced in strength.

"eructation contraction" was observed, and in the second experiment in the series with the alfalfa press juice, an increase in the amplitude of the "eructation contraction" was also noted as the intraruminal pressure was increasing. In all the experiments in the alfalfa press juice series, the major ruminal contraction was reduced in amplitude following the dosing and it was markedly reduced in the experiment in which the heat precipitable protein was removed from the alfalfa press juice. The amplitude of the "eructation contraction" was reduced in only one of the experiments in the press juice series. These observations are in agreement with the experiments on the effect of alfalfa saponin on ruminal motility, i. e., that the major ruminal contraction is more readily affected by saponin than the "eructation contraction." Another observation is that a greater reduction in ruminal motility was observed in the last experiment in the alfalfa press juice series than in the second experiment in which a more pronounced bloat reaction was obtained. This observation is in agreement with the previously described experiments with legume press juices, as illustrated in figures 16 and 17.

Johns (*29*) and Hancock (*30*) have reported that ruminal movements increase in frequency and intensity during the early stages of frothy pasture bloat. Jacobson and Lindahl (*25*) have reported that ruminal motility increased in frequency during the development of frothy feed lot bloat. The observations on ruminal motility as reported in this section at least partially substantiate these observations; however, a number of fundamental questions still remain to be answered.

Ruminal motility is quite complex, especially when motility of the rumen and reticulum are considered together. The observations, as reported in this bulletin, illustrate that certain ruminal contractions appear to be more readily affected by the action of alfalfa saponin and legume press juices than others. It appears that the amplitude of the major ruminal contraction can be reduced while the frequency of the "eructation contraction" can be increased. Studies by Weiss (*50*) and others (*10*, *23*, *30*), together with a number of experiments conducted by the authors, have shown that in normal animals ruminal contractions and eructation rates vary with intraruminal pressure. Increased intraruminal pressure leads to an increase in the frequency of the "eructation contraction" and the rate at which eructation occurs. This is controlled apparently by a reflex mechanism. Weiss (*50*) indicated that the efficiency of the reflex may vary among animals. On the other hand, substances such as atropine, sodium carbonate, and potassium cyanide, in addition to saponins, can cause ruminal atony or paralysis. Animals also vary in their reaction to drugs, some being more susceptible than others. Dougherty (*11*) has found that ruminal motility could be stimulated by increasing intraruminal pressure, after ruminal paralysis was induced by atropine administration. Weiss (*50*) induced total ruminal paralysis in sheep by dosing the animals with either sodium carbonate or potassium cyanide, and then increased intraruminal pressure by introducing air into the rumen. He found that the "increased intraruminal pressure stimulated the eructation reflex so that the forward moving eructation contractions reappeared rhythmically, although the backward moving ruminal contractions remained inhibited." Weiss also found that these "eructa-

tion contractions" were markedly increased if eructation was inefficient and high intraruminal pressures were sustained.

It was pointed out in a previous section that a reduction in ruminal motility or even ruminal paralysis does not in itself lead to ruminant bloat, provided that the muscular actions of the cardia and esophagus are not inhibited. However, in the presence of frothing of ruminal contents, impaired ruminal and reticular motility could well be contributory to the development of bloat. The cinefluorographic studies illustrate that the alfalfa saponins also can affect the motility of the reticulum and the muscular actions of the esophagus in addition to acting on ruminal motility.

Some important questions remain to be answered in relation to ruminal-reticular motility and ruminant bloat. Although it appears that there is a ruminal contraction associated with eructation and that the frequency of this contraction varies with intraruminal pressure, the hypothesis as advanced by Weiss that this "eructation contraction" is forward moving in nature as contrasted to the backward moving "major ruminal contraction" is questioned by some workers. Also, it has not been confirmed that the stimulus for the "eructation contraction" originates from pressure receptors in the dorsal blind sac. It is important that the exact nature of the "eructation contraction," including its innervation, be definitely established and the relationship of this "eructation contraction" to contractions of the reticulum, reticulo-ruminal fold, and other parts of the ruminant stomach be determined.

Until more data are available, an important question is: Can the contractions of the reticulum and reticulo-ruminal fold be quite inefficient while the frequency of the "eructation contraction" is increased? It would appear that a reduction in the strength of the contractions of the reticulum and reticulo-ruminal fold would contribute to difficulty in clearing the cardia of frothy ingesta. In addition to the need for more fundamental data on the mechanism of eructation in relationship to ruminal motility, definite and precise measurements of the motility of the rumen, reticulum, and reticulo-ruminal fold during the actual development of clinical bloat should be attempted.

Another question that needs to be answered is whether or not absorption of physiologically active constituents is as rapid in frothy bloat as it is when the rumen is free of stable froth.

## Measurement of the Free Gas Pocket in an Animal with Frothy Bloat

### EXPERIMENTAL PROCEDURES

The following experiment was conducted in order to determine the amount of free gas that could be found in the rumen of a steer bloating as a result of froth formation. The animal had been fitted with a permanent-type ruminal fistula and had received a bloat-producing diet for several months previous to the test. The diet used was similar to that used by Smith and coworkers (*45*) and Lindahl and Davis (*32*), and identical to that reported by Jacobson and Lindahl (*25*). In previous experiments it had been found that ruminal motility was active in this animal during the development of bloat symptoms.

During the experiment the intraruminal pressure was measured (1 hour after the morning and afternoon feeding periods) by using a mercury manometer attached to a tube running through the fistula

plug and into the rumen. Then a stomach tube of 1-inch inside diameter and with a natural curvature was directed into the rumen and toward the dorsal blind sac. When the stomach tube filled with ingesta, it was removed and a second intraruminal pressure measurement was obtained, as above. The remaining free gas was then slowly released by opening the pinchcock on the fistula tubing until froth began to come out of the tube. At that time, a third measurement of the intraruminal pressure was obtained.

## RESULTS AND DISCUSSION

The results obtained in the experiment are given in table 6. Although these results were obtained with an animal with a particular type of frothy bloat, they do illustrate that, at least under some conditions, a considerable amount of free gas can be found in the rumen along with a large amount of froth.

TABLE 6.—*Free gas in the rumen of a steer with frothy bloat*

| Date | Initial intraruminal pressure | Intraruminal pressure after gas was released by stomach tube | Intraruminal pressure after release of remaining free gas |
|---|---|---|---|
| | *Mm. Hg* | *Mm. Hg* | *Mm. Hg* |
| Aug. 19, 1955 a. m. | 20 | 14 | 2 |
| p. m. | 30 | 12 | 2 |
| Aug. 22, 1955 a. m. | 18 | 8 | 4 |
| p. m. | 16 | 4 | 2 |
| Aug. 23, 1955 a. m. | 18 | 8 | 2 |
| p. m. | 16 | 8 | 2 |
| Aug. 24, 1955 a. m. | 16 | 6 | 0 |
| p. m. | 6 | 2 | 0 |
| Aug. 25, 1955 a. m. | 20 | 8 | 2 |
| p. m. | 8 | 2 | 0 |
| Aug. 26, 1955 a. m. | 16 | 2 | 0 |
| p. m. | 20 | 8 | 2 |
| Aug. 27, 1955 a. m. | 26 | 10 | 8 |
| p. m. | 28 | 16 | 8 |
| Aug. 28, 1955 a. m. | 22 | 8 | 6 |
| p. m. | 24 | 8 | 4 |
| Average | 19.0 | 7.8 | 2.8 |

## SUMMARY

In vitro and in vivo experiments indicate that alfalfa saponin can contribute to the formation of and to the stabilization of froth of ruminal ingesta. However, it is also quite evident that alfalfa saponin is not the only factor involved in stable froth formation, either experimentally or in clinical bloat. That a possible interaction may exist among saponin, proteins, and carbohydrates in the formation of froth needs further investigation. In addition to the contributions made by the plants to froth formation, it is quite evident from recent experimental work and from the occurrence of clinical bloat that one or more animal factors are also involved.

It also appears that frothy ingesta can contribute to or cause bloat symptoms in some cases without filling the entire rumen.

Certain questions are raised concerning ruminal motility and frothy bloat resulting from the use of physiologically active materials in the production of the bloat symptoms.

## Some Pharmacological Properties of Alfalfa Saponin When Administered to Sheep

By *R. W. Dougherty* and *Ivan L. Lindahl*

The following described experiments were undertaken to gain an insight into some of the pharmacological properties of alfalfa saponin when administered to ruminants. With the exception of some general observations and blood studies made at the Agricultural Research Center, Beltsville, Md., the experiments were conducted at the New York State Veterinary College, Ithaca, N. Y., using western range sheep. These sheep were receiving a rather poor quality grass hay at the time of the experiments and, to our knowledge, had never grazed on legume pastures.

### Experimental Procedures and Results

#### BLOOD STUDIES

*Hemolysis.*—One of the properties of the saponins in general is their ability to hemolyze blood. Sollmann (*46*) states that the hemolytic action of saponins may be prevented by cholesterol, but that the cholesterol protection does not extend to the "central saponin actions." No correlation was found between bloat symptoms due to saponin administration and blood hemolysis in a number of experiments conducted at the Agricultural Research Center; i. e., bloat symptoms were obtained without blood hemolysis, and blood hemolysis was obtained without bloat symptoms. Although intravenous injection of saponin was more prone to result in hemolysis than intraruminal administration, the injection of up to 1 gram of the alfalfa saponin failed to produce hemolysis in some cases and only slight hemolysis, which disappeared rapidly, in other cases. In still other cases, rather marked hemolysis was noted.

An experiment was designed to show the effects of hemolysis and also to show the effects of removing an equal or even larger number of red cells than could have been lost in the degree of hemolysis caused by the administration of alfalfa saponin. A range wether weighing 110 pounds was used in the in vitro-in vivo hemolysis experiment. Five hundred ml. of blood were withdrawn from the jugular vein in a period of 3 minutes. The blood was laked with 600 ml. of distilled water and then injected into the jugular vein, using the continuous drip method. The injection was started 15 minutes after the end of the wirhdrawal period and was continued for 56 minutes. Ruminal motility was recorded throughout the experiment. Figure 33 shows the marked decrease in ruminal motility following the blood withdrawal. Forty-five minutes after the completion of the transfusion of hemolyzed blood, the animal passed blood-tinged urine. Beginning approximately 45 minutes after the transfusion and for several minutes thereafter the animal displayed muscular tremors. During the period of tracing, the ruminal contractions and respiration were considerably slower than in the pretreatment pattern. When the animal was checked 7 hours after the completion of the transfusion, it

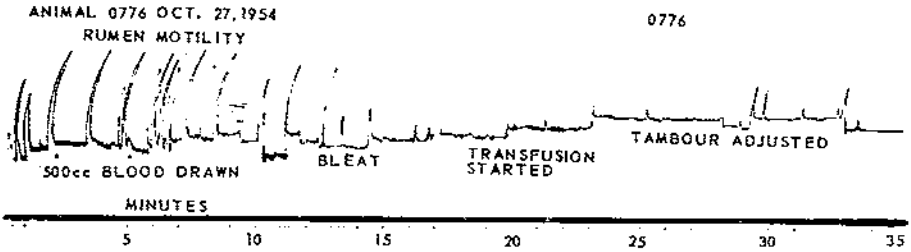



FIGURE 33.—Marked decrease in ruminal motility following the withdrawal of 500 ml. of blood from the jugular vein of a sheep.

appeared to be relatively normal. The animal was checked closely over the next several days and at no time did it display any signs of discomfort or abnormalities. Several months after the experiment the animal continued to be in good health.

Whether the decreased ruminal motility was caused by lowered blood pressure and a subsequent decrease in splanchnic blood flow or by a direct effect of the decreased oxygen-carrying capacity of the blood, or both, could not be determined from this experiment. In an attempt to answer these questions, an experiment was designed to remove small amounts of blood at regular intervals until a large amount had been removed and to replace the volume with the same volume of blood extender. A range wether weighing approximately 110 pounds was used in this experiment. Three hundred ml. of blood was withdrawn from the jugular vein in 6 steps of 50 ml. each over a period of 20 minutes. Each time 50 ml. of blood was withdrawn, an equal volume of plasma volume expander (6 percent dextran in an isotonic NaCl solution) was introduced into the jugular vein. Ruminal motility was traced by using an ink-writing air tambour attached to a permanent ruminal fistula of the animal. After completion of the experiment, the amplitude of the major ruminal contraction was measured from the tracing and the frequency of the contractions counted.

The results as given in table 7 indicate that the reduction in

TABLE 7.—*Effect of removing 300 ml. of blood from a sheep at regular intervals and replacing the blood by an equal volume of plasma on ruminal contraction and the frequency of contractions*

| Time | Amplitude index (major contraction) | Frequency of contractions (major contraction) |
|---|---|---|
| Pretreatment | 100 | 1 per 1.25 minutes. |
| After drawing 50 ml. | 100 | 1 per 1.50 minutes. |
| After drawing 100 ml. | 90 | Do. |
| After drawing 150 ml. | 90 | 1 per minute. |
| After drawing 200 ml. | 80 | 1 per 1.50 minutes. |
| After drawing 250 ml. | 80 | Do. |
| After drawing 300 ml. | 55 | 1 per 2.50 minutes. |
| 15 minutes after taking 300 ml. | 60 | 1 per 1.67 minutes. |
| 30 minutes after taking 300 ml. | 60 | 1 per 1.25 minutes. |
| 45 minutes after taking 300 ml. | 60 | 1 per 1.67 minutes. |

ruminal motility was not caused by the reduction in blood volume and pressure alone. Additional experiments along this line are desirable.

*Blood Cell Counts.*—Five experiments were conducted as described below.

*Experiment 1* was conducted at the New York State Veterinary College. A normal blood sample was obtained from a sheep and then 0.75 gram of alfalfa saponin was introduced into the jugular vein during a period of 3.4 minutes. No blood hemolysis was detected in any of the samples (table 8).

TABLE 8.—*Effect of introduction of 0.75 gram of alfalfa saponin in jugular vein on blood cell count of sheep*

| Sample | Packed cell volume | White blood cells | Red blood cells |
|---|---|---|---|
| | *Percent* | | *Million* |
| Predose | 33 | 5, 850 | 7. 11 |
| 8 minutes after dose | | 2, 750 | 9. 90 |
| 42 minutes after dose | 31 | 2, 450 | 9. 28 |
| 71 minutes after dose | 28 | 1, 800 | 8. 85 |

The results of the differential counts shown in table 8 are as follows:

| *Sample* | *Blood constituents* |
|---|---|
| Normal (predose) | Eosinophils, 4 percent; basophils, 2 percent; band, 9 percent; segmenters, 24 percent; monocytes, 4 percent; and lymphocytes, 57 percent. |
| Second | Monocytes, 28 percent; and lymphocytes, 72 percent; very pronounced leucopenia. |
| Third | Lymphocytes, 100 percent; microscopic examination of the smear revealed only 20 leucocytes. |
| Fourth | 15 to 20 minutes were required to find 6 leucocytes. |

*Four additional experiments* on sheep, conducted at the Agricultural Research Center, confirmed the above results; i. e., an increase in the red blood cell count and a decrease in the white blood cell count always followed intravenous injection of the saponin.

*Blood Histamine and Glucose Levels.*[6]—The following experiment was conducted to determine if intravenous administration of alfalfa saponin resulted in histamine release or affected the blood glucose levels.

Over a 15-minute period 0.94 gram of alfalfa saponin was administered into the jugular vein of a mature sheep. The solution (47 ml. containing 0.02 gm. per ml.) was injected at a uniform rate by using an infusion pump. Local anesthesia (Procaine) was used before the insertion of the polyethylene delivery tube into the jugular vein. The following negative results were obtained: Predose—no blood histamine and 43 mg. glucose per 100 ml. plasma; after the administration of 0.6 gram of saponin and cessation of ruminal motility—no blood histamine and 45 mg. glucose per 100 ml. plasma; after the administration of 0.94 gram saponin—no blood histamine; and, 12 minutes after the complete

[6] The blood histamine determinations were made in A. L. Neal's laboratory, Department of Biochemistry and Nutrition, Cornell University, and the blood glucose determinations were made by Augusto Vallenas, New York State Veterinary College.

dose of saponin—no blood histamine and 45 mg. glucose per 100 ml. plasma.

## STUDIES ON THE EFFECT OF ALFALFA SAPONIN ON RESPIRATION AND OXYGEN CONSUMPTION

Observations made during a number of experiments at the Agricultural Research Center indicated that either intraruminal or intravenous administration of alfalfa saponin to sheep resulted in a change in their respiratory rate and rhythm. In general, the saponin administration resulted in an increased respiratory rate for several minutes following the dose, then the pattern became irregular and in some cases developed into Cheyne-Stokes respiration. In two cases (described under the experiments on the cardio-vascular system) in which lethal doses of alfalfa saponin were given, respiratory failure preceded cardiac failure.

To obtain additional data on the effect of alfalfa saponin on respiration and oxygen consumption, the following described experiment was conducted. The trachea of a ewe that weighed approximately 100 pounds was cannulated under local anesthesia, and the oxygen consumption measured by using a Benedict-Roth metabolism apparatus. A 12-minute predose pattern was obtained, and then 0.75 gram of alfalfa saponin was introduced into the jugular vein over a 32-minute period. The saponin solution (0.02 gm. per ml.) was introduced at a uniform rate by using an infusion pump. The oxygen consumption and respiration rate were obtained during the dose period and for the next 57 minutes. The results are given in table 9, with the predose oxygen consumption given an arbitrary value of 100.

Figure 34 illustrates the change in the respiratory rate and rhythm following the administration of the 0.75 gram of saponin shown in table 9.

## STUDIES ON THE EFFECT OF ALFALFA SAPONIN ON THE CARDIO-VASCULAR SYSTEM

To determine the action of alfalfa saponin on cardiac action and blood pressure, the following experiments were conducted.

*Blood pressure.*—A western range ewe, weighing approximately

TABLE 9.—*Respiratory rate and relative oxygen consumption by a ewe during and after 0.75 gram of alfalfa saponin was introduced into the jugular vein*

| Time | Respiratory rate | Relative oxygen consumption |
|---|---|---|
| | | *Value* |
| Predose | 36 per minute | 100 |
| First 3 minutes of dose period | 53 per minute | 76 |
| Next 4 minutes | 49 per minute; irregular | 105 |
| Next 5 minutes | 47 per minute; irregular | 109 |
| 2.5 to 9.5 minutes after end of dose | 58 per minute; irregular | 87 |
| 9.5 to 15.5 minutes after end of dose | 49 per minute; irregular | 105 |
| 18 to 24 minutes after end of dose | 48 per minute; irregular | 99 |
| 24 to 30 minutes after end of dose | 56 per minute; irregular | 87 |
| 33 to 45 minutes after end of dose | Too rapid to determine | 102 |
| 45 to 57 minutes after end of dose | do | 99 |

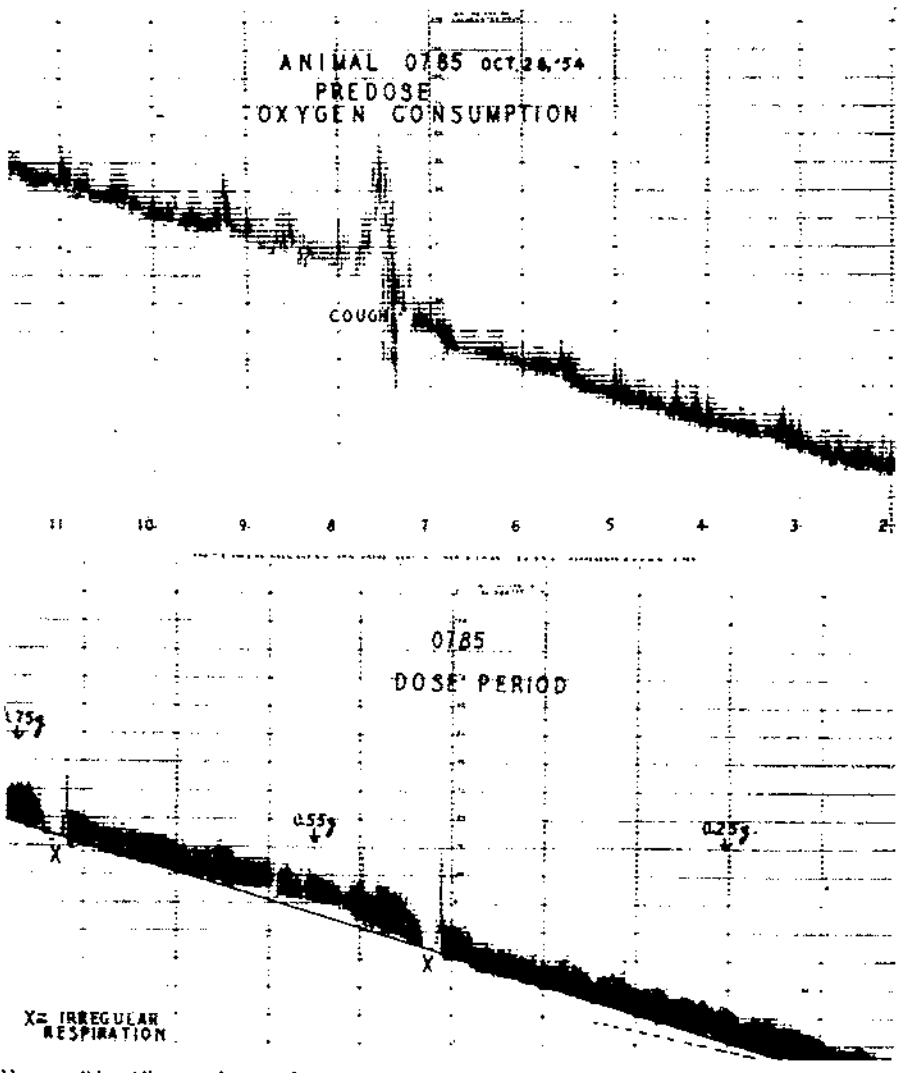



FIGURE 31. Change in respiratory rate and rhythm of ewe following alfalfa saponin administration.

70 pounds, was anesthetized by the use of Nembutal. A 20-gage needle was inserted into the carotid artery, and the blood pressure recordings were made, using a Sanborn Electromanometer and Viso-Cardiette. A total of 2 grams of alfalfa saponin in aqueous solution (1 ml. contained 0.01 gm.) was injected into the saphenous vein in 5 doses, using a hypodermic syringe. The size and frequency of the doses along with their effect on blood pressure are given as follows:

The predose blood pressure averaged 125 mm. of Hg. After the injection of 0.25 gram of the saponin, the pressure dropped to 75 mm. but returned to the predose level in approximately 45 seconds. A second injection of 0.25 gram was given in 1 minute and 22 seconds

after the first; this dose again resulted in a momentary drop in pressure with rapid return to normal. A third dose of 0.5 gram of saponin was given in 1.5 minutes after the second. The drop in pressure was less pronounced than with the first two doses (from 125 to 90 mm. of Hg) and again was of short duration. A fourth injection of 0.75 gram was given 2 minutes and 15 seconds after the third. The blood pressure dropped to 75 mm. shortly after the dose and fluctuated between 65 and 75 mm. for the next 7.5 minutes, at which time the final injection of 0.25 gram was given. The final injection resulted in a slight depression in the pressure for a few seconds. During the next 3 minutes the blood pressure remained fairly constant at approximately 70 mm. and then began to fluctuate preceding death of the animal. Death of the animal occurred approximately 10 minutes after the final dose with respiratory failure preceding cardiac failure by 2 to 3 minutes.

*Cardiac action.*—A total of 0.91 gram of alfalfa saponin was administered into the jugular vein of a wether (weight, 90 lb.). The saponin solution (47 ml. containing 0.02 gm. saponin per ml.) was injected at a uniform rate over a period of 25 minutes by using an infusion pump. Local anesthesia (Procaine) was used before insertion of the polyethylene delivery tube into the jugular vein. An ink-writing air tambour connected to the permanent ruminal fistula of the animal was used to trace ruminal motility. Electrocardiographic tracings were obtained by using a Sanborn Viso-Cardiette with three leads. Predose heart rate was 100 to 110 per minute; the following results were obtained.

The second electrocardiogram was taken 8 minutes after starting the dose period, or when 0.3 gram of saponin had been administered. Ruminal motility was decreasing and the respiratory rate was increasing at this point. The heart rate was 75 to 80 per minute and all cycle components with the exception of the T wave were of somewhat greater amplitude than the predose pattern. The third electrocardiogram was taken 7 minutes later, or when a total of 0.6 gram of saponin had been administered. Major ruminal motility had ceased and the respiratory rate was rapid. The heart rate was now 67 to 73 per minute and leads 1 and 2 showed stronger deflections in all components. The fourth electrocardiogram was taken 7 minutes later, or when a total of 0.82 gram of saponin had been administered. Major ruminal motility had ceased, respiration was rapid, and the animal was sneezing frequently. The heart rate was 65 to 70 per minute and all leads showed stronger deflection of all components.

The fifth electrocardiogram was taken 8 minutes after the end of the dose period. Heart rate was 65 to 70 per minute and the electrocardiograms were similar to No. 4. The sixth electrocardiogram was taken 8 minutes later; the records were similar to Nos. 1 and 5 with heart rate being 70 to 75 per minute. The seventh record was made 23 minutes later; intraruminal pressure had built up to 8 mm. of Hg and additional $CO_2$ plus $CH_4$ was introduced into the rumen to bring the pressure up to 20 mm. of Hg. The electrocardiographs showed a decrease in the T-P interval with a heart rate of 95 to 100 per minute. The eighth record was obtained 15 minutes later. Lead 2 still showed stronger deflection of all components, and the heart rate was 110 per minute. The ninth record was obtained 33 minutes later. Ruminal

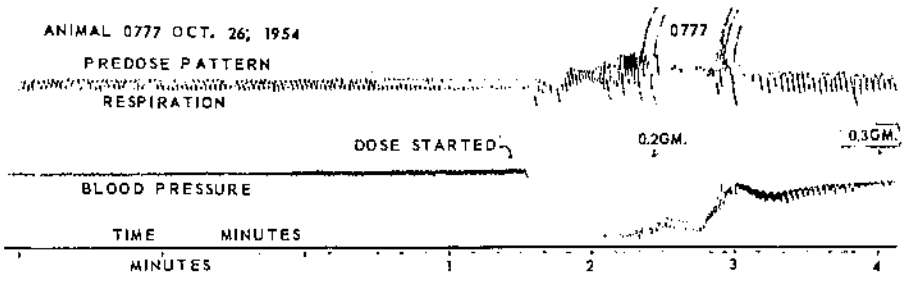



FIGURE 35.—Predose and beginning of dose respiratory and blood pressure record of wether.

motility had begun to show activity and the heart rate was 120 per minute with the amplitude of all components greater, with a shorter S-T interval. The last record was obtained 51 minutes later. Ruminal motility was still improving, and the electrocardiographs resembled the predose pattern with a heart rate of 110 per minute.

*Blood pressure and cardiac action.*—A wether, weighing approximately 95 pounds, was anesthetized with Nembutal. The carotid artery was cannulated and a direct tracing of the blood pressure obtained by using an ink-writing mercury manometer. Respiration was recorded with a pneumograph and ink-writing air tambour, and the electrocardiographic tracings were made with a Sanborn Viso-Cardiette. A total of 1.78 grams of alfalfa saponin in aqueous solution (0.02 gm. per ml.) was administered into the cannulated saphenous vein.

The first dose of 0.94 gram was administered over a period of 13 minutes with an infusion pump, but owing to mechanical difficulty the first 0.2 gram was given in 1 minute and the rest of the dose during the next 12 minutes. Three minutes were taken to refill the injection apparatus and to start the second dose: this dose consisted of 0.84 gram and was given over a period of 19 minutes. The total dose time was 35 minutes. The predose and beginning of the dose pattern are shown in figures 35 and 36. The respiration rate was 29 per minute, the blood pressure 125 mm. of Hg, and heart rate 120 per minute. The injection of the first 0.2 gram of saponin resulted in a dramatic drop in blood pressure and change in the respiration pattern, as illustrated in figure 35; however, the blood pressure returned to 110 mm. Hg within a minute. By the time 0.6 gram of saponin had been given, the respiration pattern was rather constant with a rate of 34 per minute, and blood pressure was averaging 90 mm. Hg, as shown in figure 37.

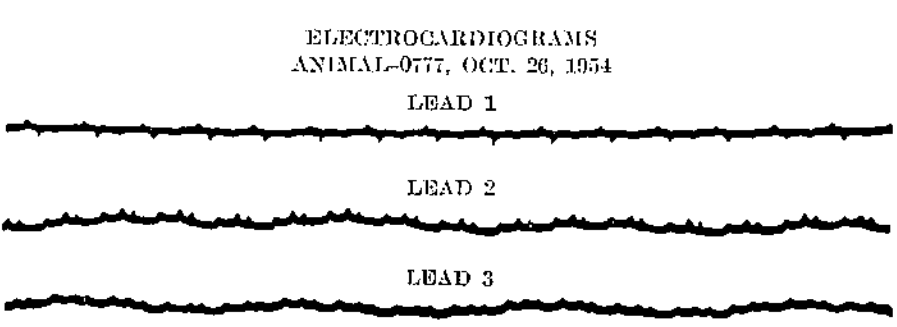



FIGURE 36.—Predose electrocardiogram of wether.

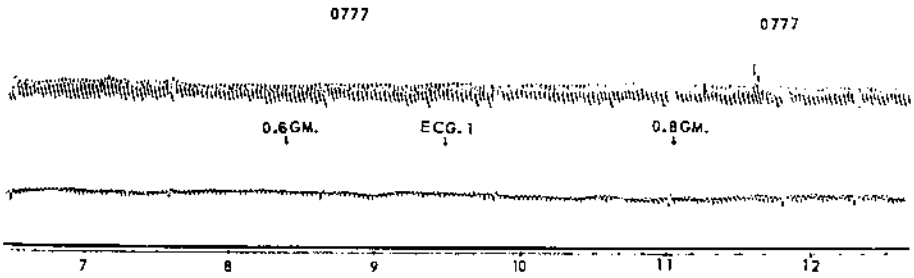



FIGURE 37.—Respiration pattern and blood pressure of wether at the time when 0.6 gm. and 0.8 gm. of saponin had been administered.

The electrocardiograms taken shortly after 0.94 gram of saponin was given are shown in figure 38. The heart rate had decreased to 70 per minute. At the end of the first dose (0.94 gm.) the blood pressure averaged 75 mm. Hg. and respiratory rate was 40 per minute. Note the changing respiration pattern at the start of the second dose, shown in figure 39. The second electrocardiograms are shown in figure 40. The heart rate was 62 per minute at this time. The respiratory pattern had changed again (fig. 41) by the time the third electrocardiogram (fig. 42) was taken, but there was little change in the blood pressure.

The blood pressure had decreased to approximately 110 mm. of Hg and the respiration rate had decreased to 30 per minute at the time the fourth electrocardiogram was taken (figs. 43 and 44). Respiratory failure preceded cardiac failure by about 5 minutes (fig. 43).

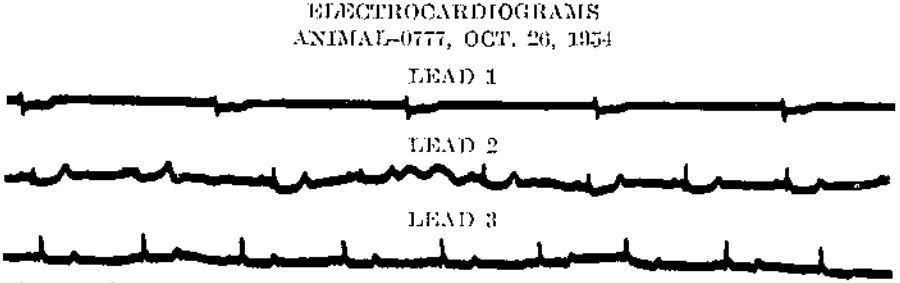



FIGURE 38.—Electrocardiogram No. 1 after administration of saponin: Increase in *T-P* period over predose electrocardiogram.

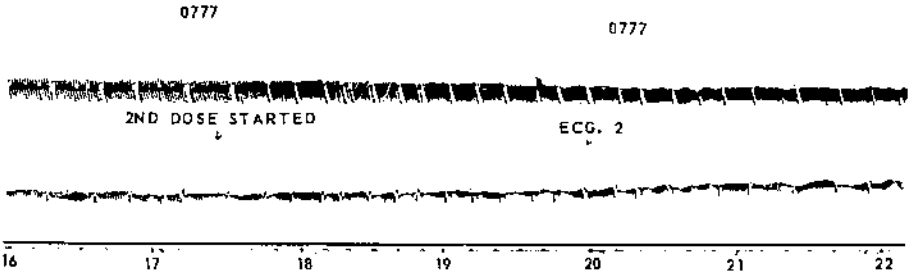



FIGURE 39.—Respiration pattern and blood pressure at the beginning of the second dose of saponin.

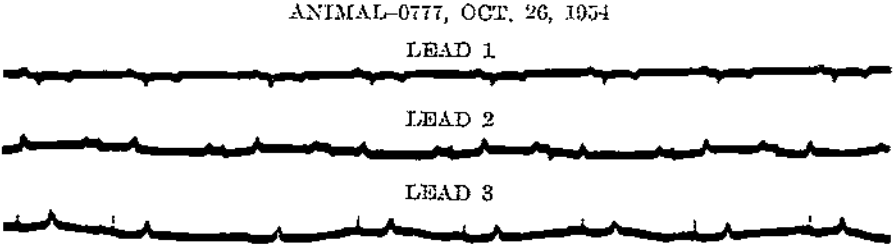



FIGURE 40.—Electrocardiogram No. 2 after saponin dose: Increase in $T$ wave and in $T$–$P$ interval.

Starting within 1 minute after the respiratory failure, the terminal electrocardiograms as shown in figures 45 through 51 were taken in rapid succession.

saponins are rather general in nature, having rather pronounced actions on the cardiovascular, nervous, and digestive systems. No system is singled out by these drugs.

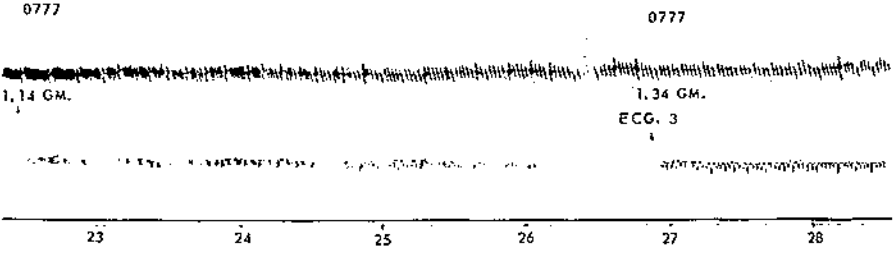



FIGURE 41.—Respiration and blood pressure at time of third electrocardiogram.

## Discussion

Two outstanding facts are evident in studying the pharmacological data: The extracted composite saponins of alfalfa are extremely active; and they are very toxic, depending on the dosage and site of administration. Actions of alfalfa

Their actions do not permit classification under any known group. In some respects they are parasympathomimetic in action; in other respects they are not.

Another rather important finding is that the degree of hemolysis can be responsible for only a small part of the general symptomatology.

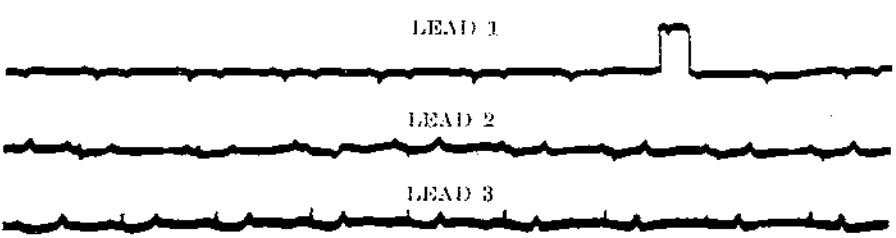



FIGURE 42.—Third electrocardiogram after administration of saponin: Heart rate was 70 to 75 per minute; note the unusual $QRS$ on lead 2.

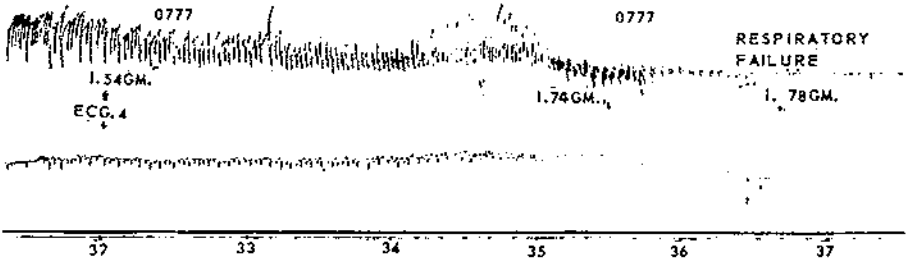



FIGURE 43.—Respiratory pattern and blood pressure at the time of fourth electrocardiogram.

Blood counts show two things: (1) There is an apparent hemoconcentration as shown by the relative rise in red cell counts, and (2) the leukopenia is a neutropenia. The causes for these changes are certainly not clear. Histological examination did not reveal any noticeable damage to the bone marrow.

The effects on respiration with little or no change in oxygen consumption indicate that there may have been a direct action on the respiratory centers. The subsequent anoxia in the later stages of experimental saponin bloat may have eventually led to some depression of the cardiac center, but the histopathological changes in the heart must have been partially caused by direct action of the saponins on the myocardium.

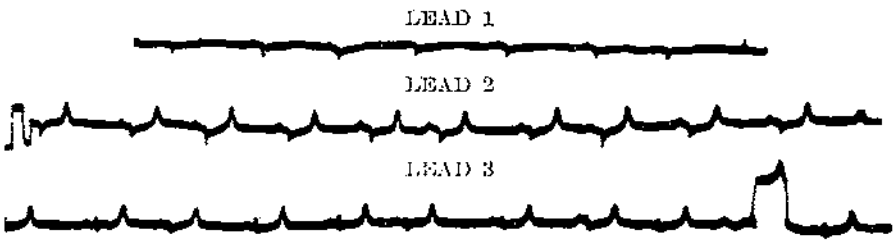



FIGURE 44.—Fourth electrocardiogram after saponin dose: Heart rate was 80 to 85 per minute; large *T* wave on leads 2 and 3.

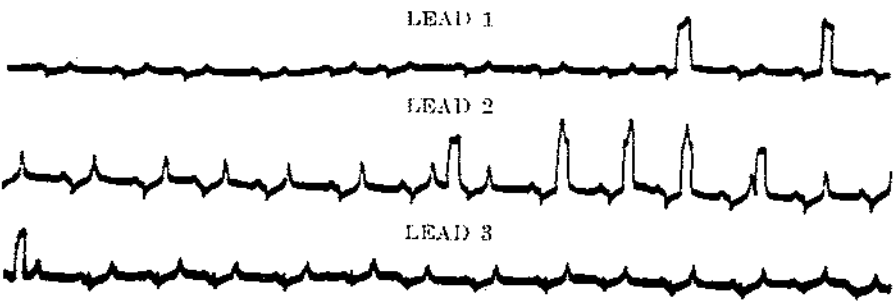
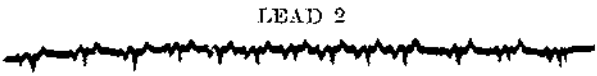



FIGURE 45.—Fifth electrocardiogram: Heart rate 100 per minute; large *T* wave on lead 2; decrease in *T*–*P* interval from previous electrocardiogram.

ELECTROCARDIOGRAMS
ANIMAL–0777, OCT. 26, 1954

LEAD 1

LEAD 2

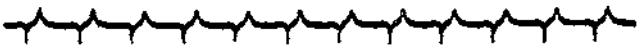


LEAD 3

FIGURE 46.—Sixth electrocardiogram: Heart rate 150 to 175 per minute; large inverted *P* wave; marked shortening of all intervals; some irregularities in beats.

ELECTROCARDIOGRAMS
ANIMAL–0777, OCT. 26, 1954

LEAD 1



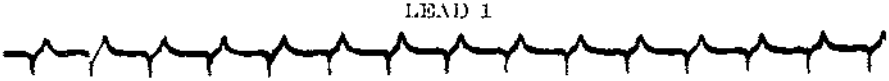
LEAD 2

LEAD 3

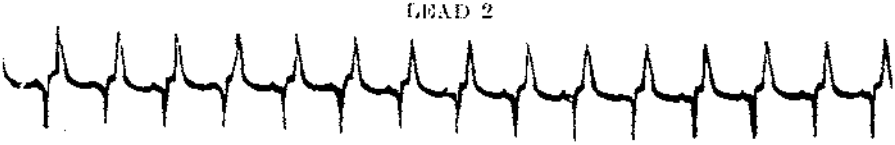
FIGURE 47.—Seventh electrocardiogram: Heart rate 125 to 130 per minute; lead 2 shows unusual conformation of *QRS* complex; very short *S–T* interval.

ELECTROCARDIOGRAMS
ANIMAL–0777, OCT. 26, 1954

LEAD 1



LEAD 2



LEAD 3

FIGURE 48.—Eighth electrocardiogram: Heart rate 120 to 130 per minute; extreme amplitude of *T* wave, lead 2.

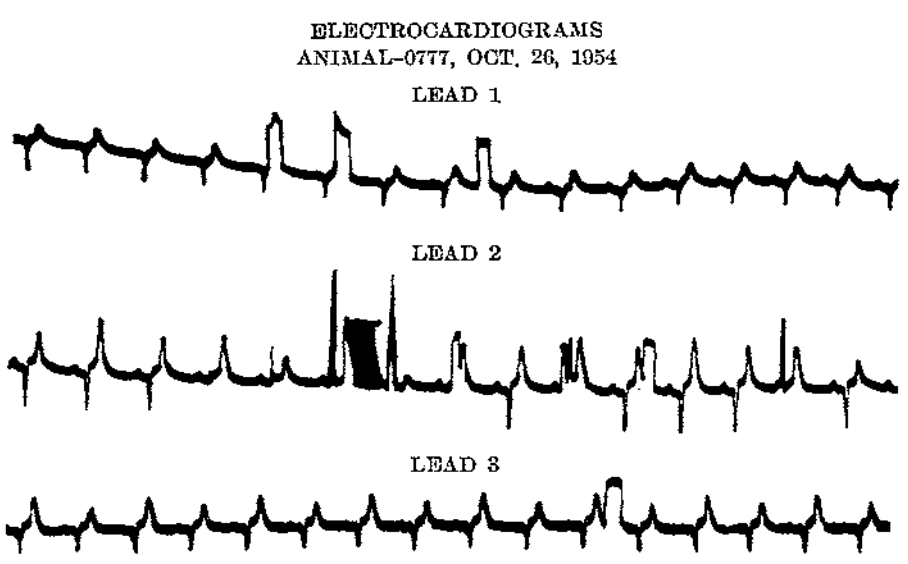



FIGURE 49.—Ninth electrocardiogram: Heart rate 120 per minute; irregularities in *QRS* complex, lead 2.

Whether or not the various physiopathological reactions in experimentally induced saponin bloat and in clinical bloat are similar is an important question and more information on this subject is needed. Very little physiological data are available in histories of clinical bloat, and the difficulties encountered in obtaining this kind of information are obvious.

Aside from their possible role in the etiology of bloat, the legume saponins are sufficiently interesting to merit extended pharmacological studies. These studies should definitely be continued and expanded.

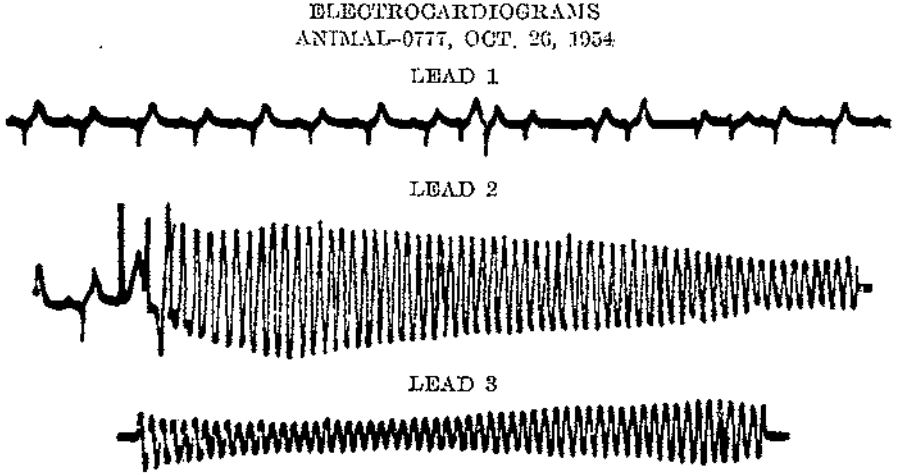



FIGURE 50.—Tenth electrocardiogram: Lead 1—extra systole and irregularities in frequency of beats; leads 2 and 3—all similarity to normalcy gone; going into fibrillation.

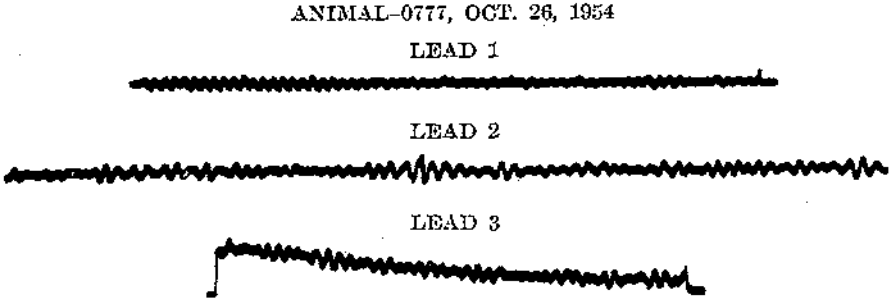



FIGURE 51.—Eleventh electrocardiogram: Fibrillation, resulting in circulatory collapse.

## Toxicity of Saponins When Administered to Ruminants

By *Ivan L. Lindahl, W. T. Shalkop, G. E. Whitmore, R. E. Davis,* and *R. T. Tertell*

Sollmann (*46*) states that many plant saponins are so poorly absorbed that they are relatively harmless when taken orally and that they are from ten to a thousand times more toxic when administered intravenously than when given orally.

In the previously described experiments, a number of reactions were observed which indicated that the composite alfalfa saponin could be absorbed from the gastro-intestinal tract of sheep and that the saponin was rather toxic.

In order to complete the study on the effect of alfalfa saponin administration to ruminants, a compilation of the toxecological, pathological, and histological findings on the animals used in the previously described tests was made and certain additional tests were conducted in order to round out the information.

### Toxicity of Alfalfa Saponin

Such factors as limited supplies of alfalfa saponin, complications arising from the production of severe bloat in some cases, and carryover effects make the determinations of the minimum lethal oral dose of alfalfa saponin for sheep a complex problem. Such studies with cattle are not feasible at the present time, owing to the time-consuming and expensive procedures employed in the isolation of the saponin.

On a single-dose basis, it appears that the minimum lethal oral dose of alfalfa saponin for mature sheep, weighing from 100 to 140 pounds, is approximately 50 to 60 grams. Intraruminal doses of from 25 to 40 grams of alfalfa saponin have been given to 15 mature sheep without production of any apparent permanent effects. Intraruminal doses of 50 to 55 grams of alfalfa saponin resulted in severe bloat in 3 cases when it was administered to mature sheep that had been on Ladino clover pasture. One animal was treated twice by using a stomach tube to relieve the gas pressure, but the bloat reoccurred and the animal was found dead within 8 hours following the last treatment. The other 2 animals were not treated for bloat and died within 3 to 4 hours after dosing. The administration of the same amount of saponin to an animal that had been grazing on a grass pasture resulted in only slight bloat symptoms, and the animal displayed no distress symptoms up to

27 hours after dosing. This animal was then killed, so that its internal organs could be compared with those from animals that had collapsed with acute bloat symptoms. The administration of 60 grams of the alfalfa saponin to a mature ewe on a grass hay diet resulted in no apparent permanent effect, while a similar animal on an alfalfa hay diet succumbed in 3 days following the dose.

The administration of intraruminal doses of 100 grams of alfalfa saponin to two sheep that had been on Ladino clover pasture resulted in severe bloat symptoms, with collapse and death of the animals within 2 hours. The same dose given to a mature wether that had been on grass pasture resulted only in moderate bloat, and death of the animal did not occur until 27 hours after dosing. The first two animals displayed bloat distress symptoms preceding death. The other animal displayed symptoms suggestive of central nervous system disturbances, such as incoordination and muscular spasms, for 3 to 4 hours preceding death. The supplies of alfalfa saponin have been too limited to determine the effect of continued administration of moderate amounts of the saponin over a period of time, and the observations made on animals that have received repeated doses are too limited to warrant any conclusions at the present time.

The composite alfalfa saponin appears to be from 50 to 60 times more toxic when given intravenously than when given orally, as the intravenous administration of 1 gram of the saponin to mature sheep resulted in the death of 7 out of 9 animals within 2 to 7 days following the dosing.

Rapid death did not occur following intraruminal dosing of any of the animals, unless the animal displayed marked bloat symptoms, indicating that stresses or increased rate of absorption from intraruminal pressure apparently contributed to the sudden collapse of the animals.

The cause of death in acute bloat has not been determined. A view held by many workers that death is caused by anoxia resulting from physical impairment of circulation and respiration has not been substantiated as the only factor involved. In recent studies, Dougherty, Meredith, and Barrett (*16*) found that insufflation of the rumen of sheep would result in a rapid increase in cerebospinal fluid pressure, which would drop when the pressure in the rumen was relieved. Their studies on the general physiological effects of intraruminal insufflation indicated that other systems are involved and that the physiopathological picture is complex. Some workers, especially Dougherty (*12*) and Olson (*39*), have questioned the idea that death results entirely from physical factors. Parsons and coworkers (*41*) reported that 4 pounds of Ladino clover press juice (made from clover obtained from a pasture where acute bloat was a problem) given orally to a mature ewe resulted in acute bloat and death of the animal in 11 minutes. They also stated, "The observation in the field on acute (fatal) bloat in cattle and on the experimentally produced bloat in the ewe, of which severe distention of the rumen was not always present, would tend to suggest that death of the animal may in some instances be due to disturbances other than the physical effects of acute distention of the rumen."

In the experimentally produced bloat cases, the alfalfa saponin undoubtedly played a dual role in the collapse and death of the animal. First, all the reactions caused by intraruminal pressure resulted originally from the interference of eruc-

tation by the action of the alfalfa saponin. Second, the systemic effects of the alfalfa saponin on respiration, cardiovascular functions, and the central nervous system were undoubtedly additive to those resulting from the intraruminal pressure. It is also probable that an increase in intraruminal pressure resulted in increased absorption of the saponin and, in turn, to more systemic effects.

The animals that died after a prolonged interval of time did not appear to be in distress, following the subsidence of any bloat symptoms, until a few hours before death when the customary symptom was a rasping, labored respiration.

Intravenous administration of the saponin in quantities in excess of 1 gram resulted in the death of all sheep.

Subcutaneous and intraperitoneal administration of small quantities of alfalfa saponin (2 to 3 grams or less) to sheep produced extreme irritation at the sites of injection and led to fatal results within a few days, with symptoms similar to those caused by a 1-gram intravenous dose.

Toxicity experiments with other species have been limited to intravenous administration of the saponin to three mature goats, weighing 95 to 110 pounds, and to one calf. In all these cases, the administration of the saponin resulted in more marked nervous disturbances of the animals than the disturbances noticed with the sheep. One goat died immediately following an intravenous dose of 3 grams of the saponin, while the other two, receiving doses of 1 and 2 grams, respectively, displayed marked nervous reactions for 2 to 3 hours and then died in 2 to 3 days with symptoms similar to those displayed by the sheep. A steer calf, weighing 360 pounds, was given 3.6 grams of the saponin in two equal intravenous doses. The first dose of 1.8 grams had little effect on the animal other than to increase the respiratory rate and to slow the rate of eructation. The second dose given 54 minutes later resulted in marked nervous symptoms, incoordination, a complete failure of eructation with a rapid buildup of intraruminal pressure, and respiratory failure within 17 minutes.

## Gross Pathology Resulting From Alfalfa Saponin

The gross findings on autopsy of some animals that had died after receiving alfalfa saponin administration are given below. The autopsy findings are given only if the post mortem examination was conducted within 2 hours following the death of the animal. The following cases represent only a small portion of the autopsy reports, but are typical of the overall results.

SHEEP No. 61: *100 grams alfalfa saponin given intraruminally; severe bloat; died in 2 hours.*

Rumen: Walls were congested and reddish in color. Rumen contained frothy ingesta and free gas.
Reticulum: Walls were injected.
Omasum: Distended.
Abomasum: Edematous and reddish in color. Contained frothy ingesta.
Small intestine: Marked inflammation.
Lungs: Congested.
Heart: Hemorrhages in ventricles and muscles.
Kidneys: Very dark and edematous.
Spleen: Engorged and dark red.
Liver: Dark in color.
Trachea: Some hemorrhage.

SHEEP NO. 50: *50 grams of alfalfa saponin given intraruminally; severe bloat; died in 8 hours.*

Rumen: Hemorrhage in walls, edema in cardiac fold. Frothy ingesta and free gas.
Reticulum: Normal.
Omasum: Normal.
Abomasum: Hyperemic.
Small Intestine: Moderate amount of inflammation throughout.
Lungs: Small amount of free blood.
Heart: Normal.
Kidneys: Normal.
Spleen: Considerable hemorrhage.
Liver: Normal.
Trachea: Normal.

SHEEP NO. 20: *50 grams of alfalfa saponin given intraruminally; killed, in 27 hours, for examination.*

Rumen, reticulum, omasum, lungs, heart, spleen, liver, and trachea all appeared to be normal.
Abomasum: Diffuse physiological hyperemia.
Kidneys: Slight enlargement plus edema.

SHEEP NO. 64: *1 gram of alfalfa saponin given intravenously; died in 6 days.*

Rumen, reticulum, omasum, abomasum, and liver all appeared to be normal.
Small intestine: Small areas of inflammation throughout.
Lungs: Lower border of apical and cardiac lobes were consolidated.
Heart: Two hemorrhagic areas in ventricles.
Kidneys: Swollen and light in color.
Spleen: Slight darkening in color; enlarged and soft.

SHEEP NO. 72: *1 gram of alfalfa saponin given intravenously; died in 3 days.*

Rumen, reticulum, and omasum all appeared to be normal.
Abomasum: Moderate congestion with a few hemorrhages in the cardiac area.
Small intestine: Few hemorrhagic areas in duodenum.
Lungs: Extreme congestion; liverlike in appearance.
Heart: Ecchymotic hemorrhages over epicardium.
Kidneys: Pale in color and slightly edematous.
Spleen: Soft, swollen, and edematous.
Liver: Slight enlargement.
Trachea: Intensely inflamed and contained free blood.

STEER NO. 17: *3.6 grams alfalfa saponin given intravenously; moderate bloat; died in 1 hour and 17 minutes.*

Rumen: Hemorrhage in rib area.
Reticulum: Small area of hemorrhages near omasum junction.
Omasum: Serosal surface had gelatinous hemorrhagic appearance.
Abomasum: One-half of the surface area was light pink in color.
Small intestine: Dark colored and engorged.
Lungs: Apical and cardiac lobes were hyperemic.
Heart: Normal.
Kidneys: Normal.
Spleen: Engorged and darkened in color.
Liver: Dark colored and congested.
Trachea: Normal.

Congestion of lung tissue; inflammation, hemorrhage, and congestion of the small intestine: and hyperemia of the walls of the abomasum were typical findings regardless of the site of the saponin administration. Although gross pathology of the kidneys and liver was not so apparent and typical as the above findings, histological examination revealed consistent and severe damage to these organs. Heart abnormalities were found in less than half of the gross examinations. The spleen was found to be engorged or hemorrhagic in most of the cases where the animals collapsed with marked bloat. Hemorrhage of the walls of the rumen was found in all cases where the animals collapsed with marked bloat symptoms. The rumen, reticulum, and omasum were found to be essentially normal in animals that did not display marked bloat symptoms, except for one instance where erosion of the mucosal surface of the rumen was found following a 100-gram dose of saponin.

## Histopathology Resulting from Alfalfa Saponin

Histological data for certain animals are given below. The following cases again represent only a portion of the complete work and are given to illustrate typical findings.

SHEEP No. 61: *100 grams alfalfa saponin given intraruminally; severe bloat; died in 2 hours.*

Rumen: The mucosal vessels were congested, but there were no other marked changes.

Reticulum: Some congestion of the vessels in mucosa and tunica muscularis. Interfascicular edema in the muscularis. No marked changes in the muscle morphology.

Abomasum: Fundic region; slight to moderate congestion of vessels of mucosa.

Small intestine: Section from duodenum: vessels moderately to markedly congested in the villi and tunica propria. The tunica muscularis was normal with the exception of some edema in the outer longitudinal smooth muscle layer. Some of the Brunner's glands were greatly dilated and contained a stringy, lightly staining eosinophilic material. A few small hemorrhages were present in the submucosa.

Lungs: Marked congestion of interalveolar capillaries, with some extravasation of red blood cells into alveoli. One bronchiole contained red blood cells and mucus.

Liver: Moderate congestion of sinusoids and proliferation of Kupffer cells; many phagocytosing red blood cells.

Brain: No abnormalities.

Spleen: Marked hyperemia.

Kidney: Very marked congestion of peritubular vessels and glomeruli; many of the nuclei had disappeared in the glomeruli; the red blood cells almost completely replaced the endothelial and epithelial cells. Vacuolation of the tubular epithelium—these vacuoles were not positive for fat after appropriate staining. Hemosiderin could not be found in the kidney sections; hemoglobin casts were not seen in the lumens of the tubules.

Heart: Moderate subendocardial hemorrhages, moderate congestion of myocardial vessels with some red blood cell extravasation.

SHEEP No. 64: *1 gram alfalfa saponin given intravenously; died in 6 days.*

Abomasum: Congestion of tunica propria.

Reticulum: Congestion of submucosal vessels but not in mucosa. Mucosal cells showed vacuolar degeneration.

Small intestine: Moderate congestion in focal areas. Other areas showed diffuse marked infiltration by eosinophiles, these latter being the only cellular elements in the tunica propria in the involved areas.

SHEEP No. 64—Continued

Lungs: Moderate congestion with slight extravasation of red blood cells into alveoli; most of the latter were empty, however.
Liver: Moderate congestion of sinusoids.
Brain: Several small perivascular hemorrhages in the medulla; congestion of meningeal vessels in region of posterior colliculus.
Spleen: Slight hyperemia.
Kidney: Very severe vacuolar degeneration of both proximal and distal tubules. Hemoglobin casts in some of cortical tubules and in many of medullary tubules.
Heart: Moderate congestion of myocardial capillaries. Moderate diffuse subepicardial hemorrhages.

SHEEP No. 72: *1 gram alfalfa saponin given intravenously; died in 3 days.*

Rumen: Congestion of submucosal vessels.
Abomasum: Moderate to marked congestion of vessels in submucosa.
Large intestine: Slight congestion of mucosa.
Lungs: Severe hemorrhage and edema into alveoli with no polymorphonuclear leukocytes. Considerable red blood cell pigment in macrophages from red blood cell breakdown.
Liver: Moderate congestion, marked fatty changes.
Brain: Slight perivascular hemorrhages in floor of fourth ventricle. Slight to moderate perivascular hemorrhages in section of superior colliculus. Moderate hemorrhages in brachium pontis and in ventral part of this section. The medulla showed consistent hemorrhages.
Spleen: Moderate to severe hyperemia; considerable blood pigment in macrophages.
Kidney: Vacuolar degeneration, primarily of distal tubules, congestion of glomeruli and vessels. Moderate number of hemoglobin casts in medulla but little or none in cortex.
Heart: Congestion, focal and diffuse hemorrhages. Granular degeneration of myofibrils not so marked as in some cases. Cross striations still quite visible.
Lymph Node: Mediastinal: Diffuse hemorrhages and edema, considerable pigment in macrophages from red blood cell breakdown.

SHEEP No. 20: *50 grams alfalfa saponin given intraruminally. Killed after 27 hours for pathological examination.*

The kidney was the only organ that was sectioned for histological examination.

Kidney: Vessels slightly congested. Many of the distal convoluted tubules and straight tubules of the cortex and some of the collecting tubules of medulla contained solid pink staining casts that were probably of hemoglobin origin. A large number of these tubules were filled with small pink staining globules. The proximal tubular epithelium was vacuolated—these were not fat vacuoles. Several glomeruli were atrophied and filled with an albuminous precipitate.

GOAT 411: *2 grams alfalfa saponin given intravenously; died in 50 hours.*

Abomasum: Focal area congestion.
Omasum: Slight congestion.
Small intestine: Diffuse and focal hemorrhages, necrosis, heavy eosinophile, and monocytic infiltration.
Lungs: Pneumonia-acute. All alveoli of sections studied contained masses of polymorphonuclear leucocytes.
Liver: Severe diffuse fatty changes.
Brain: Number of focal hemorrhages in section of superior colliculus. Few small perivascular hemorrhages in region of floor of fourth ventricle.
Spleen: Moderate to marked hyperemia. Central areas of some follicles undergoing necrosis.
Kidney: Marked congestion; severe vacuolar degeneration proximal and distal tubules: hemoglobin casts in tubules in cortex and medulla.
Heart: Swelling, granulation of all myofibrils.
Adrenal: Normal.
Pancreas: Pancreatitis, focal and diffuse infiltrates of polymorphonuclear leucocytes along with macrophages.

STEER NO. 17: *3.6 grams alfalfa saponin given intravenously; moderate bloat; died in 1 hour and 17 minutes.*

Rumen: Marked congestion of the mucosal vessels; edema of submucosa and between fasciculi of smooth muscle tunic.
Reticulum: Marked congestion of mucosa and edema and congestion of tunica muscularis.
Abomasum: Fundus—may have been slightly edematous but no marked changes.
Omasum: Few scattered hemorrhages in papillae of mucosa.
Small intestine: Moderate to marked congestion of mucosal and submucosal vessels.
Lungs: Marked congestion. Alveolar hemorrhage and edema.
Liver: Slight congestion of central veins and very slight congestion in sinusoids. Moderate fatty changes.
Brain stem: No significant changes.
Spleen: Marked congestion with replacement of about one-half of the normal lymphoid tissue by red blood cells.
Kidney: Moderate congestion of glomeruli and vessels in cortex and medulla. Hemoglobin casts were seen in some of the cortical tubules.
Heart: Some diffuse myocardial hemorrhages with loss of muscle fibers.
Gastric mesenteric lymph node: Marked edema in the medullary part with a moderate increase in the number of polymorphonuclear and monocytic cells. The only relatively normal lymphoid tissue was at the periphery of the cortex and here it was mostly diffuse with only a few remaining germinal centers. In the edematous medullary part, the lymphoid cells were all perivascular (around arterioles), about 7 or 8 cells in depth, with edema and fine stromal tissue separating these small perivascular accumulations.
Mesenteric lymph node: Moderate edema but did not show the loss of lymphoid tissue that the gastic nodes did; however, many follicles contained large pools of red blood cells as though the follicular arteries had ruptured or undergone lysis. Many monocytes and macrophages were present in the edematous parts and there was marked erythrophagocytosis in these edematous areas.

The overall impression from the gross and histological examination of a number of animals was that the lesions resulting from administration of toxic quantities of alfalfa saponin represent an endotheliotoxic and endotheliolytic reaction. The most striking pathologic change was an almost total attack on the kidney parenchyma. No definite changes in the neurons in the brain could be detected, but no doubt those in the area of perivascular hemorrhage suffered some ischemic changes, and hemorrhages were detected in areas of the medulla affecting the vital centers. There was an apparent loss of lymphoid tissue both in the spleen—although this was not severe in all of the animals—and in the lymph nodes. All the pathological changes, with the exception of pneumonia, were without an inflammatory reaction, the most active cell apparently being the macrophage, in an attempt to handle the extravasated red blood cells. It appeared that the kidney and possibly liver alterations were severe enough in the animals examined to have caused eventual death, without either brain damage or the development of pneumonia.

## Toxicity of Saponins Other Than Alfalfa to Sheep

Intraruminal administration to sheep of up to 100 grams of two saponin preparations from the yucca plant resulted in no apparent toxic reactions. The intravenous administration to sheep of 1 gram of one of these saponins (labeled as toxic) did not result in any apparent toxic reactions in four different experiments. However, the intravenous administration to sheep of 1 gram of quillai saponin re-

sulted in the death of both animals, with gross pathology similar to that found from alfalfa saponin administration. Intraruminal administration of 25 grams of the quillai saponin also resulted in the death of a mature sheep.

The yucca saponins belong to the steroid class of saponins, while the alfalfa saponin and the quillai saponin belong to the triterpenoid class.

The different responses from intravenous administration of different saponins to sheep raises the question as to whether or not the rate of absorption from the gastrointestinal tract is the only factor in toxicity of saponins.

## Summary

The composite alfalfa saponin as used in these experiments was quite toxic. On a single-dose basis, the minimum lethal oral dose of the composite alfalfa saponin for mature sheep is approximately 50 to 60 grams. This saponin is 50 to 60 times more toxic when given intravenously than when given orally.

Results of gross and histological examination of animals that received a toxic level of alfalfa saponin are given.

# Action of Alfalfa Saponin in Experimental Bloat and Possible Relationships of Saponin to Clinical Bloat

By *Ivan L. Lindahl* and *R. E. Davis*

The studies reported in this bulletin illustrate that the isolated alfalfa saponins have pronounced physiological as well as surface tension activity. They also illustrate that the physiological as well as the surface tension activity of plant saponins can vary considerably.

During the course of the studies, experimental bloat symptoms in sheep were produced that resulted primarily from the physiological activity of the alfalfa saponins; in other cases, bloat symptoms were produced as a result primarily of the surface tension properties of the saponins. In still other cases, it appears that both physiological activity and surface tension properties of the saponins were instrumental in inducing the experimental bloat symptoms. Mild bloat symptoms were produced in animals displaying active ruminal motility, but severe bloat symptoms were produced only when ruminal motility appeared to be markedly reduced.

In summing up the studies on the production of experimental bloat symptoms in sheep by alfalfa saponin administration, two outstanding facts are evident: Bloat symptoms were induced more readily when the experimental animals were receiving a pretreatment diet of Ladino clover pasture than when they were receiving other dietary regimens; and that severe ruminal distention was produced only when the animals were also given amounts of the alfalfa saponin that were at or near the toxic levels.

The observation that bloat symptoms could be developed more readily in animals receiving a pretreatment diet of Ladino clover pasture cannot be adequately explained on a physiological basis alone. It appears, however, from the in vitro and the in vivo experiments on froth formation that the alfalfa saponin could have been additive to the factors existing in the rumens of the animals, resulting in enough froth to have interfered with eructation. The experimental data also indicate that froth formation was not sufficient to cause all the ruminal distention observed in the experiments.

Experimental work presently in progress at the Agricultural Research Center indicates that a complete blockage of eructation, even for extended periods of time, may not result in severe bloat when sheep are fed diets composed of No. 2 grade alfalfa hay or grass hay. More fundamental data on the rate of gas production on various dietary regimens and the importance of "physiological fill," etc., are definitely indicated before a complete understanding of why experimental bloat symptoms were produced more readily in animals pastured on Ladino clover pasture than in animals receiving other dietary regimens.

A reduction in ruminal motility in many cases was undoubtedly additive to the formation of froth in interfering with eructation. The cinefluorographic and intravenous experiments also illustrate that the larger doses of the alfalfa saponin were sufficient to have resulted in a considerable reduction in the efficiency of eructation from physiological actions alone. Also, in the advanced stages of the severe bloat symptoms the larger doses of saponin were undoubtedly sufficient to have resulted in a complete blockage of eructation.

It appears that the most logical explanation for the role of the alfalfa saponin in the acute experimental cases is that both the surface tension and physiological activities of the saponin were instrumental in producing the overall results. It also appears that in severe cases the pharmacological actions of the alfalfa saponin on the respiratory and cardiovascular systems contributed to the early collapse and death of the animals.

Although experimental bloat cases were produced in these experiments, it cannot be concluded that any of the cases were typical of clinical bloat. The most severe cases were produced by administering large amounts of saponin in a very short period of time. Some of the lesions observed on autopsy of these animals have not been observed by any of the authors of the bulletin or reported in the literature from examination of equally fresh specimens that have died of clinical bloat. Many of these lesions appeared to have been severe enough to have resulted in the eventual death of the animals without the effect of the intraruminal pressure. So far, severe bloat symptoms have not been produced with moderate levels of alfalfa saponin, either alone or in combination with other materials.

These results, however, do not imply that saponins may not be one of the important factors in the pathogenesis of clinical pasture bloat, but rather that the saponin fractions used constitute only one of the factors and that the right combination of additional factors have not been used in the experiments to date.

Several more fundamental questions remain to be solved before the experimental results can be definitely correlated with the pathogenesis of clinical bloat.

Reports in the literature from many different areas, together with the observations of the authors, indicate that frothing of the ingesta of animals is an important factor in the pathogenesis of clinical bloat. Whether frothing of the ingesta alone is sufficient to explain all of the manifestations of clinical bloat may still be questioned at the present time.

The results of the in vitro tests with the alfalfa saponin indicate that rather small amounts of saponin could greatly aid in the formation of froth when certain other factors are present. The postulation that a saponin-protein-carbohydrate interaction may contribute

much of the plant sources for the formation of frothy ingesta needs confirmation. In addition to the plant factors, one or more animal factors also appear to be necessary for the development of large amounts of stable froth.

Not enough data on the physiological manifestations of clinical bloat are available at present, so that definite correlations of the physiological actions of the saponins can be made with clinical bloat. Certain questions concerning ruminal-reticular motility and clinical bloat still remain to be answered. Considerable evidence exists that a reduction in ruminal motility, by itself, is insufficient to result in ruminant bloat. It is logical, however, that a reduction in the strength of the contractions of the reticulum and reticulo-ruminal fold would contribute to difficulty in clearing the cardia of frothy ingesta. The observations that both legume press juices and alfalfa saponin can materially reduce ruminal motility indicate that legumes could possess marked physiological activity. Experimental data indicate that animals may vary considerably in their reaction to the physiological constituents in legume press juices or to alfalfa saponin. The experimental data also indicate that ruminal motility can be very active in some animals during the development of experimental frothy bloat symptoms and that ruminal motility can be materially reduced in others. The observations that, during an increase in intraruminal pressure after the administration of legume press juice and alfalfa saponin, some ruminal contractions may be reduced in amplitude while others may be increased in frequency and amplitude may also be important in correlating the physiological studies with clinical observations on ruminal motility during the development of frothy bloat arising from the ingestion of forage legumes.

It has been established in these studies that the isolated alfalfa saponins are quite toxic to ruminants, but whether the naturally occurring saponins can contribute to the large number of deaths occurring at times on some pastures cannot be answered until more clinical data are available.

Much work remains to be done before the six or more saponins of alfalfa are fractioned and tested for their physiological activity. It appears quite unlikely that all of these saponins will possess the same physiological activity or that the various fractions will always be found in the same ratio in growing legumes. Analytical work conducted at the Agricultural Research Center, using a preliminary method of analysis, indicates that the total saponin content of alfalfa may vary from approximately 0.5 to 2 percent or more. If it is also found that the ratio of individual saponins varies from sample to sample, it is conceivable that alfalfa forage could vary considerably in its physiological activity from time to time or from area to area.

It should also be pointed out again at this point that the composite alfalfa saponin fractions as used in the experimental animal work did not constitute all the saponin present in the original plant material. Relative proportions of the individual saponins in the recovered product were in some instances lower and in other instances higher than they were in the original plant material. The question as to whether this partial fractionation of saponins intensified or reduced the physiological activity and toxicity of the test fractions cannot be answered at the present time. The fact that a partial frac-

tionation of the natural saponins did occur must be considered in attempts to correlate the experimental data with clinical conditions.

Here are some of the important questions that remain to be answered before a complete understanding of the role of saponins in ruminant bloat is obtained. Were the isolated saponin fractions as used in the above experimental work more active or less active than the naturally occurring saponins? Do other plant substances counteract the physiological activity of the saponins? How rapidly are the saponins released from the forage during digestion by animals? And, how are the saponins detoxified, and how rapidly, under normal conditions existing in the gastro-intestinal tract of ruminants?

## Preparation and Chemistry of Legume Saponins

By *C. R. Thompson, G. R. Van Atta, E. M. Bickoff, E. D. Walter, A. L. Livingston,* and *Jack Guggolz*

Saponins are naturally occurring plant glycosides that have soaplike properties and also usually display considerable physiological activity. They may be broadly divided into two classes: in one the nuclei or aglycones are steroids, while those of the other are triterpenoids.

The saponins are surface-active (that is, they cause foaming in water or may act as wetting agents) because the aglycones, or more specifically the sapogenins, are fat-soluble while the carbohydrate portions of the intact saponin molecules are water-soluble.

Saponins have been employed in some countries as detergents and have limited use in this country as wetting agents in the textile industry. They are also used in foam-type fire extinguishers. Recent interest has been directed chiefly, however, to steroidal saponins because of their importance as drugs and precursors of mammalian hormones. Accordingly, considerable literature has accumulated concerning this class of saponins. Because comparable incentives have been absent, much less effort has been spent in the study of triterpenoid saponins, to which saponins in legume forages appear to belong. Only within recent years has the structure of triterpenoid sapogenins begun to attract the interest of several groups of organic chemists. At present, information about these compounds is far from being so complete as that concerning steroidal sapogenins. Very little indeed is known regarding the relationship between the structures of triterpenoid saponins and their physiological activities.

Methods that have been used for recovering saponins vary almost as widely as the plants from which they are obtained. Many reagents, such as various metal salts and tannins, are reported to precipitate these compounds from aqueous or alcoholic solution. However, this method of recovery may be far from complete and often results in alteration of the saponins. The glycosides are usually insoluble or nearly so in ethyl ether, acetone, petroleum ether, and benzene, but dissolve in such polar solvents as pyridine, alcohol, and water. Some of the legume saponins crystallize from alcohol-water solutions as calcium-magnesium salts. In water solution some also form sparingly soluble complexes when treated with an excess of cholesterol. Both of these properties have been utilized in the studies reported below on recovery of saponins from legumes.

## Recovery of Crude Saponins From Alfalfa

One hundred kg. of dehydrated alfalfa (*Medicago sativa*) was slurried with 800 liters of water, and 667 gm. $Ca(OH)_2$ was added. The heavy slurry (pH 6.9) was heated by free steam to about 95° C. and filtered hot in a horizontal centrifugal filter. The aqueous extract (525 to 550 liters) was cooled to 40°, and 335 ml. of acetic acid and 4 liters of freshly thawed egg white were added (pH 5.5). The mixture was heated to boiling in steam-jacketed kettles to coagulate the egg white, which acted as a defecant for colloidal material. The major portion of the coagulum was removed by straining through a wire basket. Residual coagulum was removed by Sharples centrifugation. The solution at this time was a reddish-amber color. A final polishing was obtained by filtration in a Sparkler filter with precoated filter papers.

The polished extract was stirred with 5.34 kg. of powdered cholesterol and boiled by use of closed steam coils for 10 minutes and then was allowed to cool to 30° C. by circulation of cold water through the coils. Four and forty-five hundredths kg. of filter aid was added and the cholesterol-saponin addition product was filtered off in a plate-and-frame press. The cake was washed with cold water until no more color was removed. Yield was about 16.7 kg. of cake at a moisture content of 35 percent.

To recover saponins from the cholesterol-saponin addition product, wet cake was slurried with 16.7 liters of water. Thirty and one-half kg. of 95 percent alcohol and 27.6 kg. of benzene were mixed with the slurry in a steam-jacketed digestion pot equipped with a circulating pump and a reflux condenser. The mixture was refluxed and continuously mixed for 1 hour, then filtered on Büchner funnels. The filtrate was transferred to a 240-liter funnel for phase separations. The upper phase was set aside for later reclamation of cholestrol and solvents. The lower phase was returned to the steam-jacketed pot and concentrated by distillation to about 42 liters, at which point foaming prevented further concentration. The remaining solution was cooled to 65° C. and 4.2 kg. of alcohol and 8.4 kg. of benzene were added. This mixture was refluxed 30 minutes and then transferred to the funnel for phase separation. The lower phase was taken back to the still for further concentration, cooled, and refluxed 30 minutes with 1.8 kg. of 95 percent alcohol and 4.2 kg. of benzene. After phase separation in the funnel and final concentration of the lower phase, the water-alcohol-saponin solution was evaporated to dryness in shallow stainless-steel pans in an explosionproof draft oven at 65° C. Yield was 118 grams (0.12 percent of alfalfa meal). Batch analyses of the product showed that it contained from 0.16 percent to 0.32 percent of cholesterol.

## Chemistry of Saponins From Alfalfa

Alfalfa saponin as isolated by the cholesterol-addition procedure was shown to be a mixture of several saponins (*48*). Partial resolution of the mixed saponins by organic solvent fractionation gave several fractions that differed materially in their optical rotation and mobility on paper and which by acid hydrolysis were also found to differ significantly from one another in both their sugar and aglycone components. Unfortunately, there is an unequal distribution of the various saponins of alfalfa (and probably the same is true of saponins from other legumes) when recovery is

effected by cholesterol addition. This is evident when paper chromatograms of the mixed sapogenins derived from this product are compared with chromatograms of the mixed sapogenins recovered by the analytical procedure that is to be described. This means that the relative proportions of the individual saponins in the recovered product are in some instances lower and in other instances higher than they are in the original plant material. The significance of this fact must not be overlooked in interpreting experiments with animals.

From the mixture of saponins, one sapogenin and its diacetate and its diacetate dimethyl ester were prepared in crystalline form. Its dimethyl ester and monobromolactone were obtained as noncrystalline products. Properties of this sapogenin and its derivatives indicate that it is a monounsaturated dihydroxy dicarboxylic acid, having the molecular formula $C_{30}H_{46}O_6$. These properties together with the specific rotation of the sapogenin, +111°, and of its diacetate, +87°, suggests a triterpenoid, since the steroid-sapogenin side chain usually confers pronounced levorotation.

The acidic character of the sapogenin and the fact that it contains 30 carbon atoms further support the idea that it belongs to the triterpenoid rather than the steroid class.

A search of the literature disclosed no description of a sapogenin coinciding in all respects with that of the present substance. Castanogenin, a dihydroxy dicarboxylic acid sapogenin obtained by Sines (*43*) from the wood of *Castanospermum australe*, apparently has the same molecular formula ($C_{30}H_{46}O_6$) as sapogenin derived from alfalfa. However, melting points and specific rotations reported for the diacetate and the diacetate dimethyl ester differ from those of the corresponding substances prepared from alfalfa.

Further studies with the total sapogenin fraction obtained by acid hydrolysis of alfalfa saponins showed that the sapogenin acetates could be separated by column chromatography on Special Filtrol[7] into 5 fractions. One of these was very different from the high-rotating material described above. The rotation of this crystalline acetate was +7.7° and its melting point was 297° to 299° C. Two carboxyl groups were present, but the number of hydroxyl groups remains to be determined. Qualitative color tests indicate that it is also a triterpenoid. Two other crystalline acetates were obtained. One melted at 154° to 157° C. and had a specific rotation of 76°, while the other melted at 274° to 278° C. and had a rotation of 49°. The remaining fractions did not crystallize and purification was incomplete.

In other experiments with the saponin mixture it was found that a crystalline saponin could be obtained from a dilute alcohol solution with some preparations. This material was shown to be identical with a crystalline product obtained from Ladino clover. This crystalline substance was hydrolyzed with acid and was shown to contain galactose, glucose, and rhamnose as predominant sugars with arabinose and xylose in trace amounts. Glucuronic acid was also found. Chromatography of the sapogenin part of the hydrolysate on silica gel resolved it into soyasapogenols B, C, and a trace of A.

Some further fractionation of the saponins themselves has been accomplished, but much more work is necessary to resolve their mixture into its separate components.

[7] Manufactured by Filtrol Corp. Mention of this product and others does not imply recommendation by the U. S. Department of Agriculture.

TABLE 10.—*Constants of sapogenins obtained from alfalfa saponin*

| Fraction | Melting point | | Specific rotation | |
|---|---|---|---|---|
| | Sapogenin | Sapogenin acetate | Sapogenin | Sapogenin acetate |
| | ° C. | ° C. | *Degrees* | *Degrees* |
| 1 | 349–350 | 206 | 111 | 87 |
| 2 | | 297–299 | | 7. 7 |
| 3 | | 154–157 | | 76 |
| 4 | | 274–278 | | 49 |
| Soyasapogenol A | 318–320 | | | |
| Soyasapogenol B | 260 | 180–181 | 91 | 78 |
| Soyasapogenol C | 240–241 | 200–203 | 63 | 58 |

Present information is summarized in table 10, which shows that at least six saponins, and possibly several more, occur in alfalfa.

## Isolation and Chemistry of Saponins From Ladino Clover

Ladino white clover (*Trifolium repens*) is reported to be as capable of causing bloat in ruminants as alfalfa. Experiments with water extracts of Ladino clover designed to isolate saponin as the cholesterol addition product as had been done with alfalfa (*35*) yielded only small amounts of cholesteride. However, direct isolation of crystalline saponin from the alcohol extract of Ladino clover, without the use of cholesterol, proved to be possible. This product is the same as the crystalline fraction of alfalfa saponin which, as previously stated, yields soyasapogenols B and C and a trace of soyasapogenol A.

Preliminary small-scale laboratory experiments led to the following procedure: 150 kg. of freshly cut Ladino clover (30 kg. dry weight) was immersed in 360 liters of 95 percent alcohol for 2 days. Then the extract was separated and filtered. The filtered extract was concentrated in an open stainless-steel pot to about 30 liters. This solution along with some dark-green lipid fraction was transferred to separatory funnels. The residue in the pot was transferred with diethyl ether, followed by a small quantity of water. Sufficient ether was added until two layers were obtained. The funnels were gently inverted several times (vigorous shaking gave emulsions) until most of the chlorophyll and lipid material were in solution. After about 30 minutes the aqueous layer was drained and allowed to stand until the saponin crystallized in shimmering micro plates. The ether layer was then repeatedly washed with small quantities of water; the water solutions were combined and allowed to stand for an additional crop of crystalline saponin. The aqueous phase was centrifuged and the crystalline saponin was washed in the centrifuge tubes several times with small quantities of water. The saponin was finally washed with acetone until no more colored material was removed. This yielded white, crystalline saponin, which was dried in vacuum at 60° C.; yield was 70 grams, or about 0.23 percent of the dry weight of Ladino clover.

The saponin as isolated contained 6.7 percent ash and proved to be the calcium-magnesium salts of three closely related saponins (*47*).

Upon hydrolysis with acid this material gave galactose, glucose, rhamnose, and glucuronic acid plus traces of arabinose and xylose. Chromatography of the sapogenins on silica gel yielded three compounds that had been characterized previously by Ochiai and coworkers (*38*) and Meyer and others (*35*) as soyasapogenol B, soyasapogenol C, and traces of soyasapogenol A.

## Comparative Measurement of Saponins in Forage Legumes

A method whereby the saponin contents of different lots of forage can be quantitatively compared is important in the study of the relationship of saponin content to ruminant bloat and to inhibition of chick growth (*22*).

The problem presents many formidable difficulties, and its solution has seldom been attempted. Instead, workers usually content themselves with estimates of the sapogenins that the saponins yield by acid hydrolysis. Even this alternative is frequently far from easy and often the information gained is qualitative rather than quantitative. For those inquiries that are concerned primarily with the chemistry of the sapogenins rather than their intact glycosides, these partial findings suffice. However, the present purpose in the study of ruminant bloat requires more information. Accordingly, the Western Utilization Research Branch has undertaken development of quantitative analytical procedures for individual saponins in alfalfa and other forage legumes. As one of the first steps a tentative method for assaying the total sapogenins of alfalfa has been developed.

With this method in its present form it is possible to obtain close agreement between analyses performed upon duplicate samples of alfalfa. The method, however, has not been conclusively proved to be free of systematic error. Further work will be required. Results obtained may not be true measures of the total sapogenins present in the samples. Although the results are in terms of total sapogenins rather than individual saponins or sapogenins, the procedure can be useful in making approximate comparisons of alfalfa samples. The suitability of the method for analysis of other forage legumes such as the clovers and vetches has not yet been fully tested.

### PREPARATION OF SAMPLE

The plant material should be dried and ground to a meal before analysis. Extremely fine grinding is unnecessary; the fineness of ordinary dehydrated alfalfa meal is satisfactory. Drying is important, since the presence of moisture in the sample leads to low analytical results. Air-dry meal should be further dried by maintenance at 70° C. in a vacuum oven for 40 hours.

### PREPARATION OF PLANT EXTRACT

Weigh 10.0 grams of dry meal into a 25- x 80-mm. paper extraction thimble and cover with a tuft of cotton. Place the thimble in a small Soxhlet extraction apparatus and extract 48 hours with benzene-absolute ethanol azeotrope (67.6: 32.4 parts by volume). For the extraction use 200 ml. of the solvent in a 300-ml. short-neck flat-bottom boiling flask.

Dry the thimble and its contents, using either an explosionproof mechanical convection oven or a vacuum oven to remove the last of the benzene-ethanol.

Allow the benzene-ethanol extract solution to stand until it has cooled and any suspended matter in it has settled on the wall of the flask. Pour the cooled solution

from the flask and gently rinse the inside of the flask with several small portions of benzene, taking care not to dislodge any powdery deposit clinging to the inside surface. Draw air through the flask to evaporate residual benzene.

Return the dried thimble and its contents to the extractor, put 200 ml. of 95 percent ethanol into the dried flask, connect the flask to the extractor, and extract the contents of the thimble 48 hours. If the first runnings of ethanol extract solution are green, the period of extraction with benzene-ethanol should have been longer.

Filter the ethanol extract solution through Whatman's No. 1 paper. Rinse the flask with a small portion of water and transfer the washings to the filter with 95 percent ethanol. Use a dropping pipette to wash the paper with 80 percent ethanol.

At this point in the procedure the ethanol solution always contains small but significant quantities of fat and fat-soluble pigments that must be removed by liquid-liquid extraction. To do this, first evaporate the ethanol solution nearly to dryness. Now transfer the extract to a 125-ml. Squibb separatory funnel with sufficient $H_2O$ to bring the volume to 30 ml. Add 10 ml. of 95 percent ethanol and mix the contents of the funnel by shaking. Now add 40 ml. of U. S. P. diethyl ether, stopper the funnel, and again mix thoroughly by shaking. Let the funnel stand until its contents separate into two layers. Drain all the bottom layer into a second 125-ml. separatory funnel. Inclusion of several drops of the upper phase in the liquid drawn into the second funnel will do no harm. Discard the remaining upper liquid phase. Add 5 ml. of 95 percent ethanol to the contents of the second funnel and mix by shaking. Next add 40 ml. of U. S. P. ether and shake vigorously again. If clean separation of two liquid phases does not occur promptly on standing, make further small additions of ethanol until separation does quickly follow shaking. Draw all of the bottom liquid phase into a glass evaporating dish and evaporate it to substantial dryness over steam.

## HYDROLYSIS OF ETHANOL EXTRACT

Measure 5 ml. of 85 to 90 percent formic acid into a beaker, and with a dropping pipette use it to moisten the extract in the evaporating dish. Cover the dish with a watchglass and warm it gently over steam. When all the extract has dissolved, pour the solution into a 125-ml. Erlenmeyer flask, using a thin stirring rod to prevent spread of the solution to the outside of the dish. Complete the transfer with an additional 5 ml. of formic acid delivered in several portions from a slender dropping pipette. Finally, use 10 ml. of water in several portions to rinse the last of the acid into the flask. Insert a cold-finger condenser into the neck of the flask and heat the contents over steam for 40 hours.

Dialysis of hydrolysis mixture: Cut off about 35 cm. of cellulose sausage casing (circumference 7 cm.), and after soaking it in water tie a knot about 2 cm. from one end. Slip the other end of the casing over the stem of an 80-mm. powder funnel until only about 13 cm. of casing above the knot hangs free. Support the funnel so that the casing is immersed up to within about 2 cm. of the lower end of the funnel stem.

Pour the cooled hydrolysis mixture into the funnel and complete the transfer with not more than 15 ml. of water. Knot the casing as close above the top of the liquid in the bag as possible. For conven-

jence in handling, the casing can be knotted again near its open end after first drawing it between the thumb and forefinger to expel air. Dialyze 16 hours in running distilled water.

## EXTRACTION OF SAPOGENINS FROM DIALYZED HYDROLYSIS MIXTURE

Using a pair of shears, cut off the "pigtail" below the knot at the bottom of the dialysis bag. Now with one hand hold the bag so that its bottom end hangs well down in an 80-mm. powder funnel that is supported by the mouth of a 100-ml. graduated cylinder. Use a chisel, which may be ground from a small triangular file, to pierce the bag just above the bottom knot, thus allowing the contents of the bag to drain through the funnel into the graduated cylinder. When no more liquid will drain from the bag, cut the top of the bag off just below the knot at the top. Using a wash bottle, flush the last of the contents of the bag into the cylinder with a small volume of water. Rinsing the inside of the bag with a minimum of water is facilitated by snipping off the bottom of the bag just above the bottom knot. Note the volume of liquid in the cylinder before emptying it into a 275-ml. Squibb separatory funnel. Measure one-third volume of 95 percent ethanol into the cylinder, swirl it to rinse the cylinder, and pour it into the separatory funnel. Stopper the separatory funnel and shake it vigorously to break up as completely as possible any agglomerates of solid particles that may be present. Now measure in the same graduated cylinder a volume of U. S. P. diethyl ether equal to $1\frac{1}{3}$ times that of the dialyzed hydrolysis mixture and add it to the contents of the funnel. Stopper the funnel and shake it again. Let the mixture stand in the funnel until the two liquid phases separate, then make a mark on the funnel at the level of the boundary of the two liquid phases. Draw the lower phase containing suspended brown solids into a second 275-ml. separatory funnel. Drain the clear upper phase onto a 15-cm. Whatman's No. 1 filter paper and catch the filtrate in a 500-ml. Erlenmeyer flask.

Wash the turbid aqueous phase 5 more times with ethanol and ether, using in each wash the same volume of ether but only half as much ethanol as was used in the first wash. Keep the volume of the lower phase constant by adding water as needed. The volume of water to be added can be determined only after each mixture of aqueous phase, ethanol, and ether, has been shaken together. After the requisite quantity of water has been added to each wash mixture, it is necessary to mix by shaking again. In performing each wash always add the ethanol first, mix by shaking before adding the ether. When all of the successive ether-extract solutions have been filtered into the Erlenmeyer flask, wash the filter with 95 percent ethanol. Evaporate the combined filtrate and washings to dryness by transferring successive portions to an 80- × 45-mm. glass evaporating dish heated over steam. Finish drying the residue in an oven at 80° C.

## CHROMATOGRAPHIC PURIFICATION OF SAPOGENIN EXTRACT

Prepare a chromatographic column as follows: Ram a small cotton plug to the bottom of column tube having an inside diameter of 7 mm. Tamp about 1 cm. of powdered cellulose (Whatman's Standard Grade for Chromatography) on top of the cotton plug. Now without packing, fill the tube with Special Filtrol to a depth of 3 cm. above the cellulose. Tap the tube while applying suction to pack the Filtrol. When packed in this manner the

Filtrol column will be 2 cm. long. It will contain about 0.65 gram of Filtrol. Before use, pass methanol through the column until the effluent becomes free of turbidity and discard the washings.

Dissolve the residue from the ether extract in a small volume of methanol and transfer the solution to the Filtrol column, using a further small quantity of methanol to complete the transfer. A slender dropping pipette for dispensing methanol will assist in keeping to a minimum the volume of solvent required. Not more than about 8 ml. of methanol is needed in making and transferring the solution.

After all the methanol solution has passed into the column, elute the column with 25 ml. of methanol. Use only moderate suction and thus avoid boiling the solvent in the column. Receive the eluate in a 50-ml. beaker, the wall of which has been marked at a height corresponding to a capacity of 9.5 ml. Evaporate the eluate until its residual volume is between 6 and 9 ml.

Prepare a second chromatographic column 7 mm. in diameter as follows: Plug the bottom of the tube with cotton and cellulose powder as described before. Above the cellulose powder put 0.7 gram of 1:1 (w/w) mixture of Darco G–60 activated carbon and cellulose powder. Tap the tube while applying suction to pack the column.

Prepare a stock quantity of 1:19 (v/v) mixture of 85 to 90 percent formic acid and methanol for use as solvent and column eluent.

Wash the column with the formic acid-methanol solution until the effluent is free of turbidity and discard the washings.

Make the volume of the concentrated eluate from the Filtrol column to the 9.5-ml. mark on the beaker by adding methanol; then add 0.5 ml. of 85 to 90 percent formic acid. Mix the contents of the beaker by swirling. Transfer the liquid to the carbon column as in the previous instance, using formic acid-methanol solution to assist in the transfer.

When all of the sample solution has passed into the packing, elute the column with 45 ml. of formic acid-methanol solution. Apply only very moderate suction during elution. Receive the eluate in a 100-ml. tared beaker. Evaporate the eluate.

Before weighing it, dry the residue in an oven at 80° C. Record the weight of this residue as "Total Sapogenin."

## Some Pharmacological Effects of Alfalfa Saponin on Nonruminants and on Isolated Muscle Strips

By *Robert H. Wilson, Martin B. Sidcman,* and *Floyd DeEds*

Saponins have long been known to modify normal physiological reactions and activities. Sollmann (*46*) has reviewed briefly the various pharmacological and toxic reactions attributed to saponins. He has indicated that not all saponins are equally toxic, that degree of absorption varies with the particular saponin, and that pharmacological actions differ either qualitatively or quantitatively. In view of the present increased interest in the saponins of bloat-producing forages, it seemed desirable to conduct a more extensive study on the pharmacological properties and toxicity of alfalfa saponin. This study was made possible because of the isolation and purification of alfalfa saponins by the Field Crops Utilization Section of the Western Utilization Research Branch.

## Experimental Data

The investigations were along three major lines: Acute and subacute toxicity, effects on isolated muscle strips, and hemolytic activity. The saponins studied were (1) a water-soluble composite from alfalfa; (2) a crystalline calcium-magnesium saponin, poorly soluble in water; and (3) two commercially available saponins, presumably extracted from yucca, one obtained from Eastman Kodak Co., the other from J. T. Baker Chemical Co. The crystalline calcium-magnesium saponin came from two sources—alfalfa and Ladino clover. Chemically, according to the Field Crops Utilization Section, the two are very similar and may be identical; pharmacologically, both produced the same reactions and will be considered in this section as one substance. To increase water solubility and thus make testing more easy and dosage more certain, the calcium and magnesium were removed by filtering an alcoholic solution of the crystals through an ion-exchange column of Dow-X50 and neutralizing the acid saponin with NaOH. For convenience, this preparation will be called crystalline saponin throughout this section.

## Methods and Results

### ACUTE TOXICITY

Mice were used to determine acute toxicities. The materials were administered by intraperitoneal injection, by stomach tube, and, in a few instances, intravenously. The data, where extensive enough to warrant it, were analyzed by the method of Litchfield and Wilcoxon (*33*) to obtain the $LD_{50}$ (the dosage lethal to 50 percent of the animals) and the 19 20 confidence limits, i. e., the statistical spread within which the $LD_{50}$ could be found in 19 out of 20 trials. The data are given in tables 11 and 12.

Water-soluble alfalfa had an $LD_{50}$ of 8.5 mg./kg. of body weight, with confidence limits of 6.3 to 11.4 mg./kg., when given intraperitoneally. Animals showing signs of toxic reaction became progressively more quiet, felt cold to the touch, and died in a state of shock. Death occurred in 8 hours to 2 days after injection. Autopsy revealed no gross abnormalities, except that there was a partial hemolysis of the red blood cells in those animals receiving the larger doses and dying in the shorter times.

The water-soluble alfalfa saponin was given to a few mice intravenously. Death was almost instantaneous with doses of 250 mg./kg. or more, and the blood was completely laked. At the dosage level of 50 mg. kg., death occurred in 1 or 2 days with no apparent hemolysis. However, animals receiving this dosage and sacrificed within a few hours of injection had partially hemolyzed blood, but no gross hemorrhages or other evidence of damage. The two animals receiving 10 mg./kg. developed no symptoms and were quite normal during 2 weeks of observation.

Administration of the water-soluble saponin by stomach tube indicated that the $LD_{50}$ would be somewhere between 200 and 250 mg. kg. No symptoms other than depression in those mice receiving the higher dosages were seen, and there was no hemolysis, hemorrhage, or other damage apparent at autopsy.

The crystalline saponin has a quite different toxicity. By intraperitoneal injection, the $LD_{50}$ was found to be 265 mg./kg., with 19/20 confidence limits of 180 to 390. Symptoms of poisoning were similar to those observed in mice given the water-soluble saponin, but no hemolysis was observed at autopsy. When given by stomach tube, the

TABLE 11.—*Acute toxicity of water-soluble saponin in mice*

| Route of administration | Dose | Mortality | |
|---|---|---|---|
| | | Number | Percent |
| | *Mg./kg.* | | |
| Intraperitoneal | 25 to 1,000 | 21/21 | 100 |
| | 20 | 7/7 | 100 |
| | 17 | 10/10 | 100 |
| | 15 | 10/15 | 67 |
| | 12 | 4/5 | 80 |
| | 10 | 10/17 | 59 |
| | 7 | 3/10 | 30 |
| | 5 | 3/17 | 18 |
| | 3 | 1/10 | 10 |
| | 2 | 1/5 | 20 |
| Oral | 1,000 | 2/2 | 100 |
| | 500 | 2/2 | 100 |
| | 250 | 5/5 | 100 |
| | 200 | 1/6 | 17 |
| | 150 | 0/5 | 0 |
| | 100 | 0/3 | 0 |
| | 80 | 0/3 | 0 |
| | 75 | 0/7 | 0 |
| Intravenous | 1,000 | 2/2 | 100 |
| | 250 | 2/2 | 100 |
| | 50 | 4/4 | 100 |
| | 10 | 0/2 | 0 |

TABLE 12.—*Acute toxicity of crystalline saponin (sodium salt from Ladino clover) in mice*

| Route of administration | Dose | Mortality | |
|---|---|---|---|
| | | Number | Percent |
| | *Mg./kg.* | | |
| Intraperitoneal | 1,000 | 2/2 | 100 |
| | 500 | 4/5 | 80 |
| | 300 | 5/6 | 84 |
| | 250 | 3/7 | 43 |
| | 200 | 1/8 | 13 |
| | 150 | 0/2 | 0 |
| | 100 | 0/5 | 0 |
| | 25 to 80 | 0/22 | 0 |
| Oral | 1,000 | 1/3 | 33.3 |
| | 750 | 1/3 | 33.3 |
| | 500 | 1/5 | 20 |
| | 400 | 0/3 | 0 |
| | 25 to 300 | 0/35 | 0 |

toxicity is very low; 1,000 mg./kg. causes the death of only one of three mice.

## SUBACUTE ORAL TOXICITY

Young, rapidly growing male rats, weighing about 90 grams, were presented an adequate basal diet to which had been added 1 percent of water-soluble alfalfa saponin. For 3 to 5 days the animals did not eat adequately and lost weight slightly. Following this, they grew nearly as rapidly as their controls. The rats were active and looked well during the 10 days of observation. That they did not like the diet was shown by the great food wastage. That there was no great intestinal irritation was apparent from the absence of diarrhea: on the contrary, the feces were scanty, small, and firm.

Two groups of guinea pigs were fed diets containing alfalfa saponin. The first was a group of adults, male and female, seven of which finally adapted themselves to ground rabbit diet supplemented daily with cabbage. Water-soluble alfalfa saponin was mixed with the ground diet and fed to the animals at a 0.5-percent level for 7 days and at 1 percent for the next 8 days. The animals maintained, or nearly maintained, their weight during the experimental period. There were no indications of intestinal upset. In the next experiment 300-gram rapidly growing guinea pigs were treated in the same manner. They were fed on a 1-percent saponin diet for 8 days, followed by an 8-day feeding at a 2-percent saponin level, with daily cabbage supplements. One of the three animals refused the saponin-containing diet and died before the end of the experiment. The other two continued to grow and appeared healthy and normally active. Three other young guinea pigs were given ground dehydrated alfalfa supplemented by cabbage in place of the saponin-containing diet. The alfalfa probably contained about 1 percent saponin. These animals grew and in other respects appeared well during the 15 days of observation.

A final feeding test was made, using young rabbits that weighed 2.15 to 2.35 kg. at the start of the experiment. The diets were the same as those given the young guinea pigs, except that no cabbage supplement was given. All animals, both those on the alfalfa diet and those on the saponin-containing diet, grew and appeared healthy throughout the 15 days of the experiment.

## ISOLATED INTESTINAL AND RUMINAL STRIPS

These studies were made in an isolated organ bath at 38° C., using well-aerated Tyrode's solution as the balanced physiological salt solution in which the tissues were suspended. In a test such as this, one end of the muscle strip (in this case, intestine or rumen) is anchored, and the other is attached to a lever that records contractions on a slowly moving paper. An intestinal strip can show two types of movement: One is a rhythmic contraction and relaxation, corresponding to peristalsis in the intact animal, and the other a more prolonged contraction or relaxation of the muscle, designated as tonus. Addition of certain drugs to the bath surrounding these isolated segments will cause a change in the amplitude or frequency of peristaltic contraction, or a change in tonus, or both. In the present experiments, the bath contained 60 ml. of saline to which was added the designated amounts of saponin.

Not all saponins affect isolated intestinal segments in the same way. For example, a commercial saponin (probably from yucca) decreased the tonus without altering the peristalsis. This is not the same reac-

tion as that seen with alfalfa saponin. Since it was not the purpose of this study to compare saponins from difference sources, this phase of investigation was not pursued further.

Using strips of rabbit ileum, it was found that the crystalline saponin and the water-soluble alfalfa saponin modified the contractions of intestine in the same way, but the saponins differed considerably in the dosage required to produce a reaction. With most strips, 10 mg. of crystalline saponin added to the 60 ml. bath would produce a clear response, whereas nearly 50 mg. of the water-soluble saponin was required. These saponins produced a regular sequence of responses (fig. 52). The first addition of saponin immediately decreased the amplitude of peristalsis, quickly followed by a considerable increase in tonus. The peristaltic movement continued to decrease to a point and might completely disappear. Within 5 to 10 minutes the strip began to relax and, if the reaction were allowed to continue, the tonus would drop to well below the initial level. Allowing the tissue to stay in contact with the saponin caused irreparable damage; within a few minutes the strip lost all spontaneous activity and failed to respond to high doses of usually effective drugs. If the strip were washed with fresh Tyrode's solution before this permanent damage occurred and rewashed two or three times with intervals of several minutes between washings, the strip usually regained its former tonus and peristaltic activity and responded normally to drugs such as epinephrine, histamine, pilocarpine, nicotine, and barium.

However, in spite of this apparent recovery, the first saponin exposure produced lasting changes that were revealed by subsequent treatments of the tissue with saponin. Successive exposures to saponin produced progressively increasing drops in tonus concomitant with the early decrease in

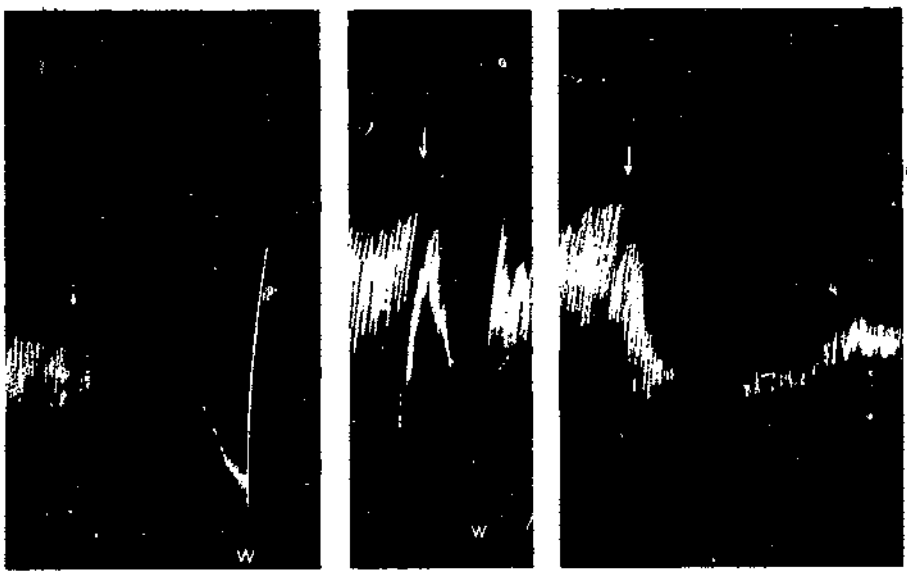



FIGURE 52.—Response of segment of rabbit ileum to first, second, and fourth additions of 50 mg. water-soluble alfalfa saponin. Arrows indicate time of additions; W indicates washing.

peristaltic amplitude. The following increase in tonus became progressively less and, after three or four exposures to saponin, disappeared entirely. At this stage the tissue might still respond to other drugs, but usually not so strongly, and sometimes not at all.

A series of exposures to small amounts of alfalfa saponin—amounts too small to produce a noticeable reaction—will modify the tissue so that a larger dose no longer gives the reaction described above for a first, adequate dose. Instead, this adequate dose will now produce a picture resembling one of the subsequent responses previously described.

One experiment with unfractionated alfalfa juices was made, using strips of rabbit ileum as the test material. The four samples of alfalfa obtained on different days were received from the University of California at Davis. Bloating had been observed in cattle eating this alfalfa. The samples were kept frozen until time for testing. After thawing, juice was obtained by squeezing in a hydraulic press. Action of all four juices on the intestinal strip was comparable and is illustrated in figure 53. Three ml. of juice added to the 60-ml. bath decreased tonus slightly and also the amplitude of peristaltic contraction. Five ml. had a like effect on tonus, but decreased peristalsis rapidly to zero.

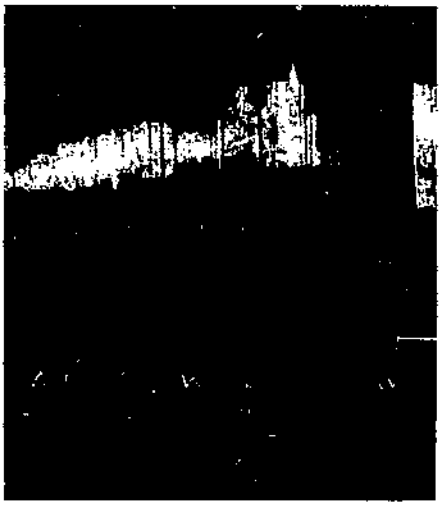


FIGURE 53.—Response of segment of rabbit ileum and alfalfa juice: *Af* signifies addition of alfalfa juice; *W* indicates where the drum was stopped while the juice was washed off the tissue.

Parsons and workers (*41*) observed similar changes in some of the forage juices they studied. These juices definitely had pharmacological activity, but the responses were not the same as those produced by alfalfa saponin. Saponin may have contributed to, but was not entirely responsible for, these changes in tissue activity.

## RUMEN STRIPS

The pharmacological actions reported above for rabbit intestine are not necessarily the same as those which would be obtained with ruminal strips. Even strips from cattle or sheep intestine, although coming from ruminants, would not necessarily act as does rumen. There are a few reports of experiments using strips of rumen in isolated-organ baths (*1*, *30*). These reports indicate that strips of rumen can be used satisfactorily. However, it is our experience that strips of ruminal musculature are extremely unreliable. The tissue must be obtained very shortly after slaughter, washed, and kept in a physiological saline. For use in an isolated-organ bath, the thick mucosal layer must be removed and the underlying musculature divided in small enough strips so that they are easily permeable to the constituents in the surrounding bath. Under these conditions, some of the strips will be active. Having found a reactive strip, what is more important, perhaps, is the variant action displayed toward certain standard drugs.

Strips were obtained from two recognizable regions of beef rumen.[5] The more reliable strips (those most apt to have spontaneous rhythm and to respond to drugs) were obtained from a muscle fold constituting the edge of the esophageal groove. The other area from which strips were taken was the wall of the rumen, a few inches lateral to the esophageal groove. Since direction of muscle fibers in this region was not apparent, strips were taken both parallel and perpendicular to the groove; direction of cutting did not greatly modify response. Aside from the greater possibility of activity in tissue from the muscular fold, strips from the two areas reacted similarly.

Active ruminal strips contracted when treated with pilocarpine, acetylcholine, barium, and histamine. Atropine did not always cause a response; when it did, the tissues relaxed. These findings are in general agreement with the results reported by Lienert (*30*), and are similar to those obtained from intestinal segments of laboratory animals. Our results differed from those of Lienert and from the usual reaction of intestinal strips in that in most instances (70 percent) we obtained a contraction after addition of epinephrine. A response of this nature is illustrated in figure 54. In this case, the muscle responded as might a sphincter muscle. Occasionally, epinephrine caused relaxation. All strips from a given animal reacted in the same direction. The contraction of ruminal strips to histamine is the response usually obtained on strips of smooth muscle. It differs, however, from the observation of

[5] Portions of rumen were kindly supplied by Lewis and McDermott Wholesale Butchers, Berkeley, Calif.

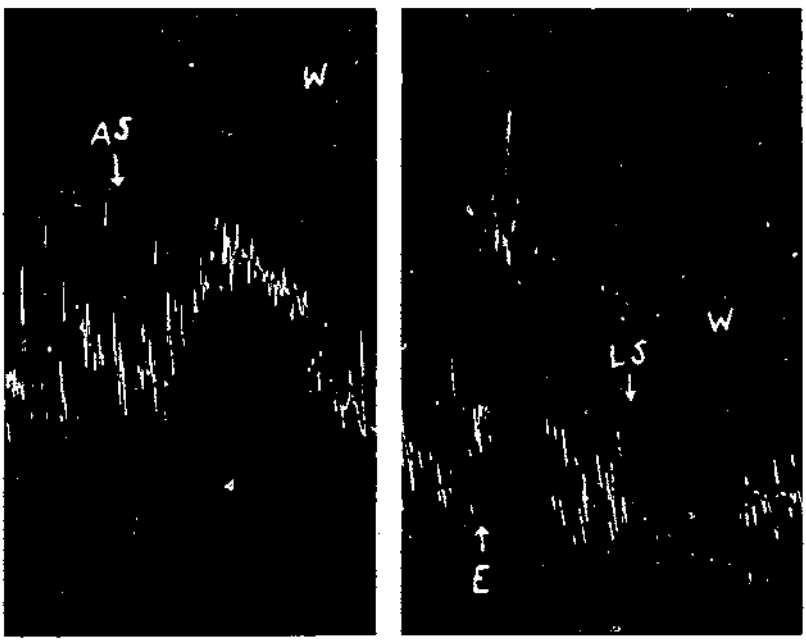



FIGURE 54.—Response of rumen strip to saponin and epinephrine; *AS* indicates addition of 20 mg. of water-soluble alfalfa saponin; *LS* indicates addition of 20 mg. of crystalline Ladino clover saponin; *E* indicates addition of 20 μg. of epinephrine; *W* is wash.

Doughterty (*11*) in the intact animal, where intravenous injection of histamine decreased the activity of the rumen. It seems apparent that responses of isolated ruminal strips to drugs and legume extracts can be applied only with reservation to responses in the living animal and that there are differences between animals, as shown by the diametrically opposite response of ruminal strips to epinephrine.

With this in mind we can consider the action of alfalfa saponin on strips of rumen. Usually, addition of saponin to the strip produced a relaxation such as that shown in figure 55. Sometimes there was a contraction, and in at least one instance, as illustrated in figure 54, a strip contracted after one addition of saponin and relaxed after another. Both crystalline and water-soluble alfalfa saponins seemed comparable in their actions, although quantitatively the crystalline saponin was more potent.

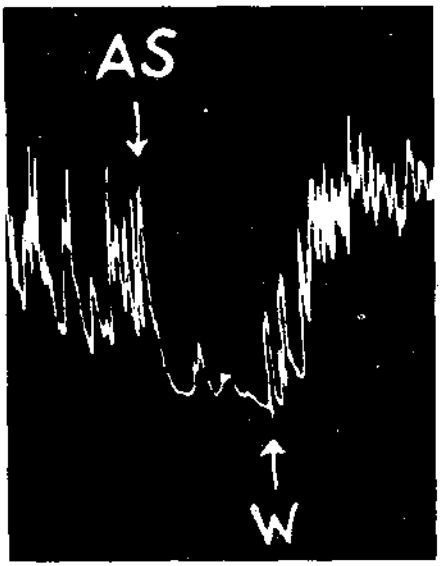



FIGURE 55.—Response of rumen strip to alfalfa saponin: AS indicates addition of 50 mg. of water-soluble alfalfa saponin; W is wash.

## HEMOLYSIS

A brief study of hemolysis was made, not to explain bloating properties, but as a possible guide to aid the Field Crops Utilization Section in its purification and fractionation of alfalfa saponins. A review of the hemolytic action of saponins by Sollmann (*46*) mentions reports with which our brief studies agree. Not only is concentration of saponin an important factor, but so also is the number of red cells on which the saponin may react. This would verify the earlier statements (*46*) that hemolysis occurs after combination of the saponin with the cell wall (possibly cholesterol). Time of reaction is likewise important, and the reaction will vary somewhat with cells from different animals. The hemolytic procedure finally evolved was as follows: Red cells from the rat were washed several times with saline and suspended in 0.9 percent NaCl in such concentration that 5 ml. of cell suspension mixed with 1 ml. of 0.9 percent NaCl would give a reading of 300 on a Klett-Summerson colorimeter equipped with a red No. 66 filter. Using this standardized cell-suspension and substituting varying concentrations of saponin in saline for the 1 ml. of saline previously mentioned, we found the concentration causing half-hemolysis in 15 minutes.

Hemolysis under these conditions was measured with the following concentrations of saponins (the concentrations mentioned are those in saline before mixing with the cell suspension):

1. A composite of water-soluble alfalfa saponins, 0.045 to 0.076 percent.
2. Various fractions of the composite water-soluble alfalfa saponin, 0.052 to 0.079 percent.
3. Crystalline saponin, half-hemolysis not obtained with a 1-percent solution.
4. Commercial saponin (yucca 71), 0.005 percent.

While markedly different hemolytic activities were found, which would easily particularize certain saponins, it was found to be impossible to differentiate the various fractions of water-soluble alfalfa saponin from each other or from the original composite by this method.

Incidental to the above, surface tension measurements were made on several saponin solutions.[9] Solutions of 0.125 percent in 0.225 percent NaCl were used. The results are:

| | *Dynes/cm.* |
|---|---|
| Crystalline saponin (alfalfa) | 60.5 |
| Crystalline saponin (Ladino clover) | 58.9 |
| Water-soluble alfalfa saponin | 60.0 |
| Commercial saponin | 43.4 |

Clearly there is no correlation between the surface tension of these saponins and their ability to cause hemolysis. This is in agreement with the findings of Woodward and Alsberg (*52*) in 1916.

## Discussion

Since the studies described above on the toxicities and on hemolytic activities of saponins are not clearly related to the problem of bloat and since our findings with alfalfa saponins are in general agreement with those reported and reviewed (*46*) for other saponins, no lengthy discussion of this phase seems necessary. Acute toxicities with alfalfa saponins can vary considerably from one saponin to another. Furthermore, toxicity by mouth is much less than by parenteral administration. Under conditions of the experiments, no subacute oral toxicity was noticed other than a dislike of the treated food by young rats during the first few days. Rabbits and guinea pigs did not appear to object to saponin-containing food. The problem with these animals, especially with guinea pigs, was to persuade them to eat a ground diet. Once they started eating such a diet, addition of alfalfa saponin did not modify food intake.

Hemolytic activity of crystalline saponin, both from alfalfa and from Ladino clover, was nearly nonexistent, whereas water-soluble alfalfa saponin has considerable hemolytic activity; and a commercial saponin, presumably prepared from yucca, is very potent. These facts would argue against hemolytic activity being correlated in any way with bloat-producing propensities. The crystalline Ladino clover saponin has been shown recently to be an experimentally ineffective bloat-producer.[10] This bulletin illustrates the bloat-producing effectiveness of water-soluble alfalfa saponin. And the saponin from yucca, which has great hemolytic activity, is reported to be ineffective in the production of bloat (*31*). Likewise, it would appear that ability to reduce surface tension by these saponins does not correlate well with bloating activity.

Saponins could cause bloat either through absorption and subsequent action on the central nervous system, or from direct action on the organs involved in bloat. If there is such a direct action, it might well be demonstrated in strips of muscle completely isolated from all body controls. The first step was to find if saponin affected isolated muscle strip. It does this but not in a simple manner. In the first place, the action of the saponin depends upon its origin, yucca saponin affecting the strip in a manner that differs from effects of alfalfa saponin. Secondly, the action of the strip is dependent on the previous history of saponin exposures. The reaction of the strips change pro-

[9] We wish to thank F. B. Stitt and Y. Tomimatsu, Analytical, Physical Chemical, and Physics Section, Western Utilization Research Branch, for these determinations.

[10] Lindahl, I. L., personal communication.

gressively with successive contacts with saponin. Even when the previous amounts of saponin have been too small to produce a noticeable response, the tissue has been modified as shown by its subsequent responses.

Reactions of intestinal and ruminal strips to saponin occur with little time lag. It was likewise shown in sheep (see section on ruminal motility) that intestinal and ruminal movements were modified very quickly in certain animals after administration of saponin. Little is known concerning the rate of absorption of saponin from rumen or intestine. Assuming a very rapid absorption, it seems doubtful that absorption into the blood stream and subsequent response of the central nervous system can be so fast as to cause the almost instantaneous reactions found in fistulated sheep. Even the early ruminal changes after intestinal administration of saponin (section on ruminal motility) could be more easily explained, on a time basis, on reflex responses originating from the intestine rather than central nervous control caused by absorbed saponin. However, some of the later effects seen in certain animals almost undoubtedly are caused by absorbed saponin.

In a previous section of this bulletin, Lindahl, Dougherty, and Davis observed a marked stimulation of the intestine immediately after intestinal administration of alfalfa saponin. This is very similar to the early tonic increase seen in intestinal strips subjected for the first time to adequate saponin dosage. It is tempting to speculate whether or not these two observations may not be slightly different manifestations of the same thing.

Certainly alfalfa saponin has toxic actions other than the bloat-producing action. These have been noted in the earlier sections of this bulletin, in particular after acute bloat has subsided. The prostration and shock seen in mice receiving toxic levels of saponin cannot be the result of bloat, since these animals are not ruminants; these reactions are probably similar to some of the later manifestations seen in ruminants.

A comparison of alfalfa saponins, of saponin from *Ladino* clover, and a commercial saponin was made on the basis of acute toxicity in mice, hemolytic activity, and, in particular, effects on intestinal and ruminal muscle strips. While saponins vary greatly in toxicity and in hemolytic activity, there appears to be no correlation between acute toxicity with small laboratory animals, hemolytic action, and the ability to cause experimental bloat in ruminants.

## Summary

Strips of rumen are not satisfactory as test materials, but they do respond to various drugs and to alfalfa saponin. Their response to drugs is usually the same as that shown by other smooth muscle strips, but they differ in the majority of cases with epinephrine, which usually produces a contraction of the ruminal strip rather than a relaxation. Rabbit ileum furnishes a more satisfactory muscle strip. A sequence of responses follows application of alfalfa saponin. There is a progressive diminution of peristalsis. Tonus is, at first, greatly increased, followed quickly by marked relaxation. After previous exposure to saponin, the increase in tonus is not found, and only the relaxation remains. These findings have been considered in their relationship to experimental bloat.

## Summary

The results of rather extensive studies on the chemical, physiological, pharmacological, physical, and toxocological properties of alfalfa saponins in relation to ruminant bloat are described in this bulletin.

Experimental bloat symptoms have been produced in sheep by oral and by intravenous administration of a composite mixture of saponins isolated from alfalfa.

Data are presented which indicate that alfalfa saponins can interfere with the mechanism of eructation through their physiological actions alone. Data are also presented which indicate that alfalfa saponins can contribute to the formation of froth, and thus interfere with eructation in an indirect manner. Evidence is also presented which shows that the pathogenesis of ruminant bloat is complex and cannot be attributed to a single factor. It appears that while alfalfa saponins may contribute to ruminant bloat, it cannot be concluded that saponins, per se, are the cause of naturally occurring bloat.

It has been demonstrated that the composite alfalfa saponins have pronounced pharmacological activity. No system is singled out by these drugs. Their actions do not permit classification under any known group. In some respects the saponins are parasympathomimetic in action; in other respects they are not.

The actions of the saponins on the digestive system are not limited to the rumen alone, but also include the reticulum, esophagus, and intestines. Different animals vary considerably in their reaction to the alfalfa saponins, some being very sensitive while others appear to be quite resistant. Although some actions of the saponins may be caused by a direct action on the ruminal musculature, all the observations cannot be explained on this basis.

The intraruminal administration of moderate amounts of the saponin or intravenous administration of smaller doses usually results in an increase in the respiratory rate and then in an irregular respiratory pattern. In all experiments in which the saponin was slowly injected intravenously until the animal collapsed, respiratory failure was proceeded by cardiac failure. Detailed data on an animal revealed that there was little or no change in oxygen consumption following the intravenous injection of the saponins, although there was a marked alteration in the respiratory rate and rhythm, indicating that the saponins may have a direct action on the respiratory centers.

The action of the saponins on the cardio-vascular system is rather complex and affects the cell counts, cardiac action, and blood pressure. Although saponins are well known for their ability to hemolyze blood, no definite correlation was found between blood hemolysis and other physiological actions.

The toxic levels of the composite alfalfa saponins for mature sheep appear to be approximately 50 to 60 grams when given orally or 1 gram when given intravenously. The typical findings on gross examination of the animals that died as a result of saponin administration have been congestion of lung tissue, inflammation, hemorrhage, and congestion and hyperemia of the small intestine and the walls of the abomasum, regardless of the site or mode of the saponin administration. Although gross pathology of the kidneys and liver was not so apparent and typical as the symptoms listed, histological examination revealed consistent and severe damage to these organs.

While saponins vary greatly in toxicity and in hemolytic activity,

there appears to be no correlation between acute toxicity with small laboratory animals, hemolytic action, and the ability to cause experimental bloat in ruminants. Studies were conducted to determine the action of saponins on isolated muscle strips. Strips of rumen did not prove completely satisfactory as test materials, but they did respond to various drugs and to alfalfa saponins. Their response to drugs is usually the same as that shown by other smooth muscle strips, but they differ in the majority of cases with epinephrine, which usually produces a contraction of the ruminal strip rather than a relaxation. Rabbit ileum furnishes a more satisfactory muscle strip. These findings were discussed in their relationship to experimental bloat in ruminants.

Methods for the isolation and determination of alfalfa saponins are presented in detail. Composite alfalfa saponin as isolated by the cholesterol-addition procedure was shown to be a mixture of several saponins. Partial resolution of the mixed saponins by organic solvent fractionation gave several fractions that differed materially in their optical rotation and mobility on paper and which by acid hydrolysis were also found to differ significantly from one another in both their sugar and aglycone components. Information is presented which shows that at least six saponins, and possibly several more, occur in alfalfa. Saponins in legume forage appear to belong to the triterpenoid class, while many plant saponins are of the steroid class.

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