# INHIBITION OF RUMEN CELLULOSE DIGESTION BY EXTRACTS OF SERICEA LESPEDEZA[1]

S. J. LYFORD, JR.,[2] W. W. G. SMART, JR.[3] AND T. A. BELL[4,5]
*North Carolina Agricultural Experiment Station, Raleigh*

SERICEA forage (*Lespedeza cuneata*) has gained wide acceptance in southern United States due to its adaptability to land conditions not suitable for the production of other legumes. However, Hawkins (1959) found this forage to be less productive for growth and milk production than alfalfa. He also found it to be less palatable and high in lignin.

Smart *et al.* (1961) have shown that sericea forage contained a material inhibitory to sodium carboxymethyl cellulose hydrolysis by bovine rumen fluid, while no detectable inhibition was found in extracts of alfalfa, soybean or ryegrass forages. Certain nonherbaceous plants have also been shown to contain an inhibitory material with similar properties (Bell *et al.*, 1962, 1965). Chemical studies of the sericea extract have shown that it is a polyphenolic leucoanthocyanin-like compound of high molecular weight, and not necessarily associated with the tannin portion of plants (Bell *et al.*, 1960, 1965; Porter *et al.*, 1961; Smart *et al.*, 1961; Lyford, 1963).

The extent of cellulose hydrolysis detected in soluble cellulose systems (carboxymethyl cellulose) is often not related to native cellulose in rumen digestion. Because the sericea inhibitor has been demonstrated only with soluble cellulose, the effect of this inhibitor on native cellulose was investigated with rumen cellulase extracts and intra-ruminal cellulose digestion trials.

## Experimental

The sericea forage used for the preparation of the inhibitor was obtained from the Patterson farm of the McNair Seed Company, Laurinburg, North Carolina. The material was cut, put in coarsely-woven bags, chilled and returned to the laboratory where it was cut into 10–15 cm. lengths, and then frozen in air-tight plastic bags until required for extraction. The extraction and purification procedure used in the preparation of the sericea inhibitor was that of Bell *et al.* (1965). The product was lyophilized and further purified by passing an aqueous solution through a Sephadex G-25 column. This purified sericea inhibitor (PSI) was again lyophilized and stored in a dry air-tight container.

To test for the possible use of other forms of the sericea plant for use in inhibitor extractions, samples were obtained from a single field area (1) just as the material left a direct-cut forage chopper (about a 1 cm. chop), (2) about 30 min. later as the truck load was dumped into a commercial, high-temperature dehydrator and (3) as this material left the dehydrator. In addition, whole plant samples cut about 8 cm. from the ground were obtained. These samples were collected in duplicate, placed in small air-tight plastic bags and kept cold with dry ice until arrival at the laboratory (about 4 hr. later) and were then frozen until analyzed.

Concurrently with the collection of samples for the chemical analysis, fresh whole plants of sericea and commercially dehydrated sericea (as prepared by the McNair Seed Company for commercial use) from the same field area were obtained in larger quantities for a sheep digestion trial. Pre-frozen "canned ice" was put into the bags of the fresh sericea for transportation to the laboratory where it was then frozen in air-tight plastic bags until fed. The dehydrated sericea was stored in paper bags until fed.

The chemical methods for leucoanthocyanins were the vanillin-HCl and 4% HCl tests of Bate-Smith (1954) with modifications as suggested by Burns (1963). Extracts of sericea were also tested for rumen cellulase inhibition employing the method of Bell *et al.* (1955), using bovine rumen fluid as the en-

[1] Contribution from the Departments of Animal Science and Food Science, North Carolina Agricultural Experiment Station, Raleigh, North Carolina. Published with the approval of the Director of Research as Paper No. 2263 of the Journal Series.

[2] Based on thesis data presented by the senior author to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Ph.D. degree. Present address: Veterinary and Animal Sciences, University of Massachusetts, Amherst, Massachusetts.

[3] Present address: Chemistry Department, Georgia Experiment Station, Experiment, Georgia.

[4] U. S. Food Fermentation Laboratory (one of the laboratories of the Southern Utilization Research and Development Division, U.S.D.A.).

[5] The authors wish to express appreciation to Mr. Hervey Evans, Jr. and to Dr. John Green of the McNair Seed Company, Laurinburg, North Carolina, for the use of their facilities and materials furnished for this study.

zyme source with a 3-hr. incubation period at 30° C. The rumen fluid was prepared for use as described by Smart *et al.* (1961).

Intra-ruminal cellulose digestion studies were conducted according to the procedure of Pettyjohn *et al.* (1964). Ten cellophane bags (containing cellulose, inhibitor and rumen fluid) were suspended in a perforated plastic cylinder. The substrate was ground, 60-mesh, chromatographic grade filter paper, 0.5 gm. per cellophane sack. The PSI was then added, followed by 20 ml. of the rumen fluid preparation. Two cylinders constituting a single experiment were placed in the rumen of a fistulated steer for an incubation period of 48 hours. Determination of the remaining cellulose was according to the method of Halliwell (1957).

The *in vitro* cellulose digestion studies were conducted as follows: Substrate, 100 mg. of ground, 60-mesh, chromatographic grade filter paper and treatment effects (PSI and/or casein) were added to 50 ml. Erlenmeyer flasks. This was followed by 25 ml. of rumen fluid prepared as in the intra-ruminal studies. The contents of the flask were stirred and $CO_2$ bubbled vigorously through the mixture. The flask was stoppered immediately and connected in series with the other flasks of the given trial, and the system was thoroughly flushed with $CO_2$ every 12 hours. A closed system at atmospheric pressure was maintained through the use of 20% NaCl reservoir solution. The flasks were incubated at 37–38° C. for 48 hours. Determination of the residual cellulose was by the Crampton and Maynard (1938) procedure as modified by Matrone (1944).

The digestibility of cellulose from fresh frozen and dehydrated sericea forage was determined with four wether lambs (40–60 lb.). They were treated to remove parasites, placed in conventional digestion crates and randomly assigned to forage treatments. The sheep were offered either the fresh-frozen or dehydrated sericea *ad libitum* twice daily. The fresh-frozen material was removed from storage just before feeding and was partly frozen when fed. The preliminary period of the digestion trial was 7 days followed by a 4-day (limited due to shortage of material) collection period. The feces and orts were collected daily, dried in a forced-air oven at 60° C. for 48 hr. and weighed. Collected samples of dehydrated and fresh-frozen sericea, as well as the feces, were ground to pass a 40-mesh screen and thoroughly mixed, and aliquots were taken for analysis. Dry matter was determined according to A.O.A.C. (1950) procedure, and the cellulose determined according to Crampton and Maynard (1938) as modified by Matrone (1944). The data were analyzed statistically according to Steel and Torrie (1960).

## Results and Discussion

Extraction of 2.5 kg. of the fresh-frozen sericea forage yielded 35.95 gm. of lyophilized inhibitor, amounting to 1.44% or 3.43% inhibitor as calculated on a fresh or dry weight basis, respectively. Active inhibitory preparations appear to be water extractable only from the fresh or fresh frozen forage. Table 1 shows the results of the chemical properties of extracts of the forage in several stages of preparation. Chopping and wilting of the forage in the field significantly reduced the amount of extractable substances, as shown by the lower absorbence values for the leucoanthocyanin tests. This finding was closely associated with the biological response of rumen cellulase in-

TABLE 1. LEUCOANTHOCYANIN TESTS AND INHIBITION PROPERTIES OF SERICEA FORAGE AT VARIOUS PREPARATION STAGES

| Forage preparation | Vanillin-HCl test Absorbence at 520 mμ | 4% HCl test Absorbence at 520 mμ | Degree of inhibition of soluble cellulose hydrolysis by rumen cellulase[a] |
|---|---|---|---|
| 1. Entire plant | 0.236[b] | 0.354[b] | 4+ |
| 2. Field chopped, fresh | 0.063 | 0.168 | ... |
| 3. Field chopped, sampled at dehydrator | 0.088 | 0.216 | 1+ |
| 4. Dehydrated by the commercial process | 0.057 | 0.136 | ... |
| 5. Partly wilted, used for digestion trial | 0.054 | 0.128 | 2+ |

[a] Degree of cellulase inhibition (Bell *et al.*, 1962) is as follows: 0%–25% enzyme inhibition is considered doubtful to negative; 25%–60%=1+ (weak); 60%–80%=2+ (moderate); 80%–90%=3+ (strong); and greater than 90%=4+ (very strong).

[b] Value significantly ($P<.05$) different from all other values within column.

hibition. Extractions from both the fresh and partly wilted sericea gave levels of inhibition significantly greater than the other preparations. The inhibitory substance in the partly wilted material was more active than the color reaction would indicate (table 1). The field-chopped sericea obtained as a truck load and dumped for dehydration showed a non-significant increase in color that was associated with a low level of inhibition. The fresh sericea gave consistently the highest levels of inhibition. Similar observations have been recently reported by Bell *et al.* (1965).

The inability to extract the sericea inhibitor after the plant cells become mascerated and changed by field chopping or drying may be explained on the basis that the inhibitory substance was stored in special plant structures. In the discussion of possible roles of natural inhibitors of cellulase in the interrelationships between fungi and plants, Mandels and Reese (1963) suggest that the plant may use an inhibitor to protect itself from invasion by fungal enzymes. These authors suggest that, since the inhibitory materials are also toxic to the host tissues, they may be stored in special structures or in inactive complexes with proteins or polysaccharides until released by the attacking fungus.

*Intra-ruminal Cellulose Digestion.* Results from four experiments employing a constant substrate level with varying amounts of inhibitor are presented in figure 1. While considerable variability in level of cellulose hydrolysis between trials was apparent, a consistent pattern of inhibition was observed. A marked drop in rate of hydrolysis occurred when 10 mg. of PSI were added to the reaction mixture. The "negative" hydrolysis noted was believed due to the cellulose determination

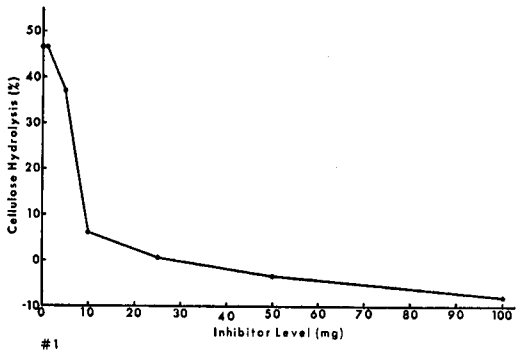


Figure 1. Intra-ruminal digestion of a constant cellulose level with increasing levels of inhibitor (PSI).

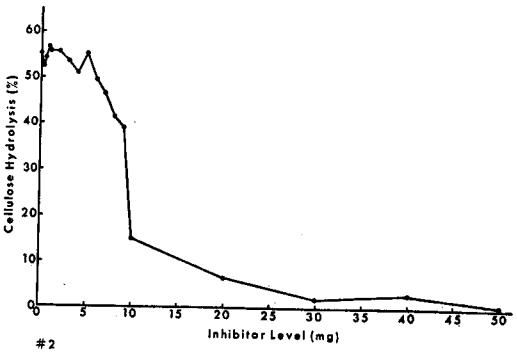


Figure 2. Cellulose hydrolysis with increasing levels of inhibitor (PSI), in vitro.

procedure in which the weak acid and weak alkali treatment used was not sufficiently strong to completely remove the rather large amounts of non-cellulosic material present. However, definite inhibition of cellulose hydrolysis under normal conditions was produced by the PSI.

Because of the limited amounts of the inhibitor available, studies were not undertaken to determine directly the influence on cellulose hydrolysis by digestion trial. However, placing the reactants in a dialysis bag with subsequent incubation in the rumen of a fistulated animal provided the normal rumen pH, temperature, digestion and end products removal (Pettyjohn *et al.*, 1964). Intact cellulosic material (ground filter paper) was used in these trials in conjunction with the intra-ruminal technique. The relative results of inhibition obtained by this procedure further substantiates the belief that the inhibitory material extracted from sericea forage is responsible in part for the reduced digestibility of sericea forage.

*In Vitro Cellulose Hydrolysis.* To further study the effects of the PSI on cellulose hydrolysis, a series of *in vitro* trials was carried out. The effect of PSI on cellulose hydrolysis under *in vitro* conditions (shown in figure 2) essentially duplicated the results of the inhibition observed with the intra-ruminal trials. Under the conditions of both trials, 10 mg. of inhibitor were sufficient to give nearly complete inhibition of the cellulose hydrolysis. Further decreases in activity, however, required rather large additional amounts of the inhibitory material.

To consider possible relationships between the level of PSI and substrate, several trials were carried out in which the level of both PSI and the cellulose substrate were varied

(figure 3). Through use of controls, the data of each trial was adjusted to a common level of enzyme activity before presentation in figure 3. On this basis, given increases in sericea inhibitor gave definite added increments of inhibition of cellulose hydrolysis. The adjusted average cellulose hydrolyzed for the 0, 10, 20 and 30 mg. levels of inhibitor were 78.4, 62.5, 51.0 and 41.8 mg. of cellulose, respectively, with each mean being significantly ($P<.05$) different from each other mean. Statistical analysis of the data, however, revealed no interaction between level of substrate and degree of inhibition.

Halliwell (1957) found that two or more enzymes are required for complete cellulose hydrolysis. One enzyme must be capable of attacking the more complex insoluble cellulose, with a second to further hydrolyze the soluble cellulose. He found that many organisms are capable of hydrolyzing soluble cellulose while only a few are truly cellulolytic. Cell-free enzymatic preparations that were active in hydrolyzing soluble cellulose were found to be

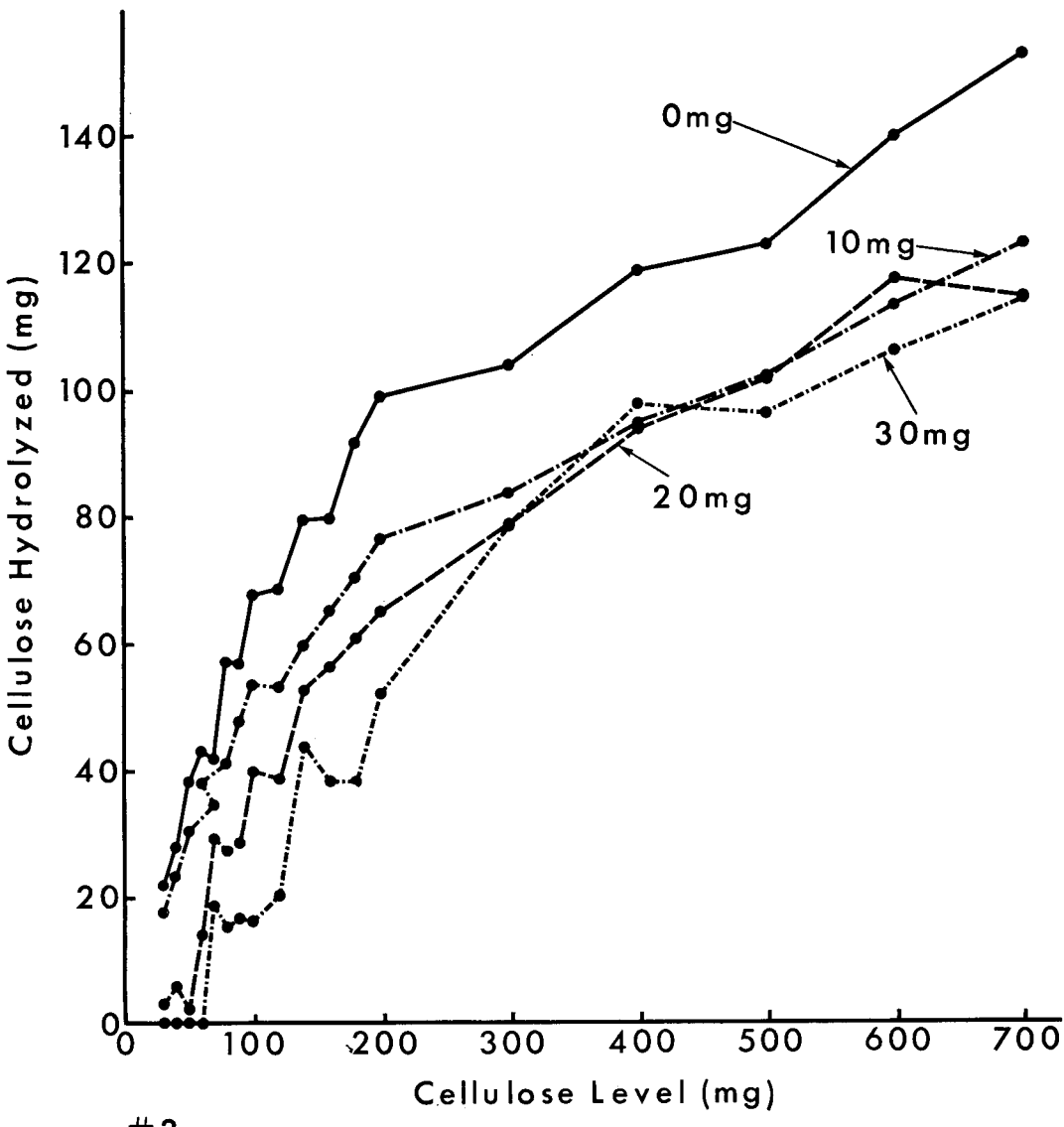


Figure 3. Hydrolysis of increasing levels of cellulose with the inhibitor (PSI), in vitro.

TABLE 2. COMBINED EFFECTS OF PROTEIN (MILK CASEIN) AND PURIFIED SERICEA INHIBITOR ON CELLULOSE DIGESTION *IN VITRO*[a]

| Milk casein | Sericea inhibitor | Av. cellulose remaining | Digestion |
|---|---|---|---|
| mg. | mg. | mg. | % |
| 0 | 0 | 41 | 59.0[b] |
| 0 | 10 | 90 | 10.1 |
| 0 | 50 | 100 | 0.4[d] |
| 0 | 100 | 98 | 1.9[d] |
| 10 | 0 | 38 | 62.0[b] |
| 10 | 10 | 85 | 15.3 |
| 10 | 50 | 101 | 0.0[d] |
| 10 | 100 | 104 | 0.0[d] |
| 30 | 0 | 30 | 70.5[b] |
| 30 | 10 | 70 | 30.0[c] |
| 30 | 50 | 102 | 0.0[d] |
| 30 | 100 | 104 | 0.0[d] |
| 100 | 0 | 32 | 68.4[b] |
| 100 | 10 | 47 | 52.8[b] |
| 100 | 50 | 103 | 0.0[d] |
| 100 | 100 | 104 | 0.0[d] |

[a] 36 hr. incubation with three replications per treatment and 100 mg. cellulose per incubation flask.
[b] Individual means with letter "b" show digestion significantly ($P<.05$) greater than all other means, while the one with letter "c" shows digestion significantly ($P<.05$) greater than other means with letter "d".

inactive on the insoluble substrate. He found that the presence of the cellulolytic organism was needed to hydrolyze insoluble cellulose, suggesting that the initial attack on the cellulose is by a very labile enzyme. Soluble cellulose hydrolysis is not necessarily indicative of a corresponding property of hydrolysis of insoluble cellulose, such as found in plant constituents. Halliwell (1957) and Gill and King (1957) have shown that soluble cellulose systems that employ viscometric procedures measure very small changes in total substrate. The PSI has been found to be an effective inhibitor of the cellulolytic enzyme system of the rumen with either soluble or filter paper cellulose as substrate.

While the mechanism of action of cellulases is poorly understood, extensive studies of various chemical inhibitors of certain plant cellulase systems by Basu and Whitaker (1953) and Sison *et al.* (1958) suggested that thio groups were necessary for enzymatic activity. Evidence for the interaction of the PSI with sulfhydryl groups has also been observed (Lyford, 1963). Certain proteins, cysteine, glutathione and 2,3-dimercapto-1-propanol were found to be effective in protection of the activity of several carbohydrate-associated enzyme systems. To extend these observations to cellulose digestion by rumen contents, milk casein was added to *in vitro* incubations.

Four levels of protein (milk casein) in conjunction with four levels of inhibitor were incubated with ground filter paper and rumen fluid. The results are presented in table 2. The analysis of the group means showed, as in the above experiments, the effectiveness of the sericea inhibitor ($P<.01$). The effect of protein on cellulose digestion was also significant ($P<.01$) with the 30 and 100 mg. levels promoting a 15% increase in cellulose digestion over the control and the 10 mg. level of the added casein. A significant ($P<.01$) interaction between the effects of protein and inhibitor was observed. Analysis of the individual means showed that the low level of inhibition was significantly affected by the 30 mg. and 100 mg. levels of casein, reflecting the results obtained with certain semi-purified enzyme systems (Lyford, 1963). The added casein was not effective in increasing cellulose hydrolysis at the higher levels of PSI.

*Sheep Digestion Trial.* To test for the presence of an unextractable form of the inhibitor in the forage material, a sheep digestion trial was undertaken with fresh-frozen and dehydrated sericea forages, sampled from the same field and harvested at the same time. The results, shown in table 3, reveal a marked difference in the cellulose and dry matter digestibilities of the two products. The cellulose digestibility and dry matter of the fresh frozen sericea was higher, even though it contained the extractable inhibitor. The mean digestion coefficients for cellulose were 53.7% and 43.6% for the fresh-frozen and dehydrated products, respectively, ($P<.05$), while those for dry matter were 51.7% and 42.8%, respectively, ($P<.01$).

Whether the decrease in cellulose digestibility observed can be ascribed to changes in either the inhibitor or cellulose components of the plant is questionable. Field drying or the high temperatures of the dehydration process

TABLE 3. THE DRY MATTER AND CELLULOSE DIGESTIBILITY OF FRESH-FROZEN AND DEHYDRATED SERICEA FORAGE

| Sheep no. | Sericea form | Digestion coefficient | |
|---|---|---|---|
| | | Dry matter | Cellulose |
| | | % | % |
| 1 | Fresh-frozen | 51.76[a] | 54.32[c] |
| 2 | Fresh-frozen | 51.60[a] | 53.05[c] |
| 3 | Dehydrated | 42.43[b] | 42.58[d] |
| 4 | Dehydrated | 43.16[b] | 44.62[d] |

[a] Coefficients with letter "a" are significantly different from those with letter "b" ($P<.01$), and those with letter "c" from letter "d" ($P<.05$).

could result in decreased digestibility of the cellulose. Honeyman (1959) has shown that cotton and wood celluloses regain more moisture after harvesting if they have never been dried than those that have been dried. The greater hydroscopicity of undried materials generally indicates a lower degree of crystallinity and increased susceptibility to enzymatic degradation. Walseth (1952) found that phosphoric acid treated cotton fibers having a high moisture regain value showed a marked increase in the extent of cellulose hydrolysis. He also reported the degree of hydrolysis of different cellulose preparations by cellulase to be associated with the extent of moisture-regain capacity. Cowling (1963) has observed that the native undried cotton fibers removed directly from the cotton boll and maintained constantly in water are susceptible to extensive hydrolysis in contrast to dried fibers. Apparently, drying of cellulose results in a collapse of the capillary structure normally present in high moisture sample. This could explain the difference found in cellulose digestibility between the fresh and dehydrated sources of the sericea forage. The low levels of inhibitor extractable from the chopped or dried sericea forage do not appear to be related to any improvement in over-all forage and cellulose digestibility, suggesting the continued presence and influence of the inhibitory material.

## Summary

Cellulose hydrolysis was found to be inhibited by a purified preparation of a water-extractable inhibitor from fresh sericea forage. This inhibition was obtained under rumen conditions as well as in *in vitro* trials. When casein was added to the cellulose-rumen fluid mixture containing PSI, considerable protection of activity was afforded to the cellulose degrading system; but at high levels of inhibitor, casein had no effect. While fine chopping of the fresh material or drying of the forage reduced the amount of extractable inhibitor to a low level, the digestibility of the cellulose of the dry form was not higher than the fresh forage.

## Literature Cited

A.O.A.C. 1950. Official Methods of Analysis (7th ed.). Association of Official Agricultural Chemists. Washington, D. C.

Basu, S. N. and D. R. Whitaker. 1953. Inhibition and Stimulation of Cellulase of *Myrothecium verrucaria*. Arch. Biochem. and Biophys. 42:12.

Bate-Smith, E. C. 1954. Leucoanthocyanins. I. Detection and identification of anthocyanins formed from leuco-anthocyanins in plant tissue. Biochem. J. 58:122.

Bell, T. A., J. L. Etchells and I. D. Jones. 1955. A method for testing cucumber salt stock brine for softening activity. U. S. Dept. Agr. Bul. ARS-72-5.

Bell, T. A., L. W. Aurand and J. L. Etchells. 1960. Cellulase inhibitor in grape leaves. Botan. Gaz. 122:143.

Bell, T. A., J. L. Etchells, C. F. Williams and W. L. Porter. 1962. Inhibition of pectinase and cellulase by certain plants. Botan. Gaz. 123:220.

Bell, T. A., J. L. Etchells and W. W. G. Smart, Jr. 1965. Pectinase and cellulase enzyme inhibitor from sericea and certain other plants. Botan. Gaz. 126:40.

Burns, R. E. 1963. Methods of tannin analysis for forage crop evaluation. Georgia Agr. Exp. Sta. Tech. Bul. N. S. 32.

Cowling, E. B. 1963. Structural features of cellulose that influence its susceptibility to enzymatic hydrolysis. p. 1–32. *In* "Advances in Enzymic Hydrolysis of Cellulose and Related Materials." (Edited by E. T. Reese). Pergamon Press Inc., New York.

Crampton, E. W. and L. A. Maynard. 1938. Relation of cellulose and lignin content to the nutritive value of animal feeds. J. Nutr. 15:383.

Gill, J. W. and K. W. King. 1957. Characteristics of fresh rumen cellulases. J. Agr. Food Chem. 5:363.

Halliwell, G. 1957. Cellulolysis by rumen microorganisms. J. Gen. Microbiol. 17:153.

Hawkins, G. E. 1959. Nutritive qualities of sericea forage. Alabama Polytech. Inst. Bul. 318:1.

Honeyman, J. 1959. Recent Advances in the Chemistry of Cellulose and Starch. Interscience Publishers, Inc., New York.

Lyford, S. J. 1963. Biological and Chemical Characterization of an Enzymatic Inhibitor Isolated from *Lespedeza Cuneata*. Ph.D. Thesis. North Carolina State University of the University of North Carolina, Raleigh, North Carolina.

Mandels, M. and E. T. Reese. 1963. Inhibition of Cellulases and Beta-Glucosidases. p. 115–157. *In* "Advances in Enzymic Hydrolysis of Cellulose and Related Materials." (Edited by E. T. Reese). Pergamon Press Inc., New York.

Matrone, G. 1944. A study of lignin and cellulose methods for the chemical evaluation of feeds. Master's Thesis. Cornell University, Ithaca, New York.

Pettyjohn, J. D., J. M. Leatherwood and R. D. Mochrie. 1964. Simplified technique for *in vitro* comparison of cellulose and dry matter digestibilities of forages. J. Dairy Sci. 47:1102.

Porter, W. L., J. H. Schwartz, T. A. Bell and J. L. Etchells. 1961. Probable identity of the pectinase inhibitor in grape leaves. J. Food Sci. 26:600.

Sison, B. C., Jr., W. J. Schubert and F. F. Nord. 1958. On the mechanism of enzyme action. LXV. A cellulolytic enzyme from the mold *Poria vaillantii*. Arch. Biochem. Biophys. 42:12.

Smart, W. W. G., Jr., T. A. Bell, N. W. Stanley and W. A. Cope. 1961. Inhibition of rumen cellulase by an extract from sericea forage. J. Dairy Sci. 44:145.

Steel, R. G. D. and J. H. Torrie. 1960. Principles and Procedures of Statistics. McGraw-Hill Book Company, Inc., New York.

Walseth, C. S. 1952. Occurrence of cellulases in enzyme preparations from microorganisms. Tappi 35:228.