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Received for review May 7, 1976. Accepted September 14, 1976. We are grateful to the Fats and Proteins Research Foundation, Inc., Des Plaines, Ill., for a grant which in part supported this research. Scientific Journal Series Paper No. 9524, Minnesota Agricultural Experiment Station. A portion of this paper was presented at the 58th Annual Meeting of the Federation of American Societies for Experimental Biology, April, 1974, in Atlantic City, N.J. (*Fed. Proc., Fed. Am. Soc. Exp. Biol.* **33**(3), 657 (1974)).

Some Properties of Soluble Proteins from Alfalfa (*Medicago sativa*) Herbage and Their Possible Relationship to Ruminant Bloat

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The soluble herbage proteins from alfalfa were analyzed by polyacrylamide gel electrophoresis and separate gels were stained with Coomassie Blue, Sudan Black, and periodic acid–Schiff reagent. Seventeen discrete protein bands were readily identified against heavy background staining. Four protein bands, in the fraction II group, were stained by Sudan Black. Possibly these proteins could make a greater than average contribution to the viscosity of rumen foams. Additional Sudan Black staining material was present in crude extracts but this material was removed by ion-exchange chromatography. Very faint staining was obtained with periodic acid–Schiff reagent. Fraction I protein stained most intensely with this reagent. The isoelectric point of fraction I protein was 5.6. Isoelectric points of fraction II proteins ranged from 3.3 to 6.2. Fraction II proteins present in highest concentrations had isoelectric points of 4.4, 4.8, 5.0, and 5.1. Both fraction I and the majority of fraction II proteins would be expected to produce viscous foams at the pH of rumen ingesta during bloat in cattle fed fresh alfalfa.

Several lines of evidence implicate soluble plant proteins as the foaming agents responsible for the frothy bloat which may occur when ruminant animals graze clover or alfalfa pastures (Clarke and Reid, 1974). For descriptive purposes the soluble herbage proteins are frequently classified into two fractions. Fraction I is a single protein, ribulosebiphosphate carboxylase, which comprises 30 to 50% of the total soluble protein. The other soluble proteins are known as fraction II proteins, a group which contains many proteins, each present in small amount.

Earlier studies on some foaming properties of alfalfa leaf proteins implicated fraction I protein as the primary foaming agent responsible for pasture bloat (McArthur et al., 1964; Stifel et al., 1968), but in these studies the fraction II proteins were apparently incorrectly identified and the experimental basis for excluding the fraction II proteins from consideration as foaming agents was invalid (Jones and Lyttleton, 1969; Howarth et al., 1973). We therefore wished to reexamine the question: do all soluble herbage proteins produce foams of equal persistence or do the various soluble herbage proteins differ in their abilities to produce persistent foams?

Jones and Lyttleton (1969, 1972) have compared some foaming properties of fraction I and II proteins from red and white clovers and found that both fractions gave persistent foams in the pH range which occurs in the rumen of bloated animals. On the basis of foam volume measurements, Rommann et al. (1971) suggested that alfalfa lipoproteins could play a role in the formation of persistent foam during ruminant bloat.

In our investigations we wished to compare the foaming properties of alfalfa fraction I and fraction II proteins and, in addition, to compare foaming properties of proteins within the fraction II group. However, the isolation of individual fraction II proteins in sufficient quantities for direct measurement of foaming properties would have been a difficult and time-consuming operation. Therefore, an indirect approach was selected. Since foaming and surface interfacial properties of proteins result from certain physical–chemical properties of proteins, we have characterized the soluble alfalfa proteins by measuring their isoelectric points and by their reactions with lipid and carbohydrate specific stains. From this information we have deduced probable foaming properties of specific alfalfa protein fractions.

EXPERIMENTAL SECTION

Extraction of Soluble Alfalfa Proteins. Alfalfa herbage was harvested during the prebud growth stage from the apical 10–15 cm of plants in a field plot of Beaver alfalfa. It was stored at −20 °C and ground in a plate grinder cooled with liquid nitrogen (Hikichi and Miltimore, 1970). Weighed samples were homogenized in a Duall, ground-glass tissue grinder (Kontes Glass Co., Vineland, N.J.) in 0.1 M Tris-glycine buffer (pH 8.9) and centrifuged at 70 000*g* for 30 min. The supernatant solution was used for electrophoretic characterization of the soluble alfalfa

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proteins and is referred to herein as the crude extract.

In addition, three protein fractions of greater purity were prepared from crude extracts. These fractions were: (1) fraction I protein, (2) high molecular weight fraction II proteins (fraction IIA), and (3) low molecular weight fraction II proteins (fraction IIB). Fraction I protein was obtained by sodium sulfate precipitation and chromatography on Sepharose 6B. The fraction II proteins were obtained by ammonium sulfate precipitation and chromatography on Sephadex G-25, Sephadex G-150, and DEAE-Sephadex. The separation into high and low molecular weight groups was based upon the elution volume from the G-150 column. Details of these procedures have been reported previously (Sarkar et al., 1975).

Electrophoresis. Electrophoresis of the crude extract and protein fractions was conducted according to procedures described previously (Howarth et al., 1973) except that 5% polyacrylamide gels were used. Gels were stained with either: (1) a protein specific stain, Coomassie Blue R-250 (Howarth et al., 1973); (2) a carbohydrate specific stain, periodic acid–Schiff (PAS) reagent (Zacharius et al., 1969); or (3) a lipid specific stain, Sudan Black B. Gels stained in Sudan Black B were kept overnight in 0.1% Sudan Black B dissolved in 50% ethanol and they were destained in 40% ethanol.

Densitometric scans of the electropherograms were obtained using a Joyce-Loebl integrating densitometer equipped with a thin-layer chromatography attachment.

Isoelectric Focusing. Isoelectric points of the alfalfa proteins were determined by analytical isoelectric focusing in polyacrylamide gels (Finlayson and Chrambach, 1971; Righetti and Drysdale, 1971). Composition of the gels was 5.0% (w/v) acrylamide, 0.25% (w/v) *N,N'*-methylenebisacrylamide, 2.7% (w/v) carrier ampholytes (Ampholine, LKB-Produkter AB, Sweden), 0.1% (w/v) ammonium persulfate and 0.1% (v/v) *N,N,N',N'*-tetramethylethylenediamine. The gels were poured into 0.7 × 11.5 cm glass tubes. The lower and upper electrode chambers contained 0.1 N phosphoric acid and 0.75 M ethylenediamine, respectively. Sample solutions containing 1 to 4 mg of protein/ml and 50% sucrose were carefully layered onto the upper surfaces of the gels. Sample size was 150 to 200 μl. The anode and cathode were connected to the lower and upper electrode chambers, respectively. The electrical supply was 50 V for 1 h followed by 100 V for 15 h.

After focusing was complete the polyacrylamide gels were removed from the glass tubes and cut into halves along their longitudinal axes using a Stadie tissue slicer blade. One-half of each gel was stained in 0.5% (w/v) Fast Green FCF dissolved in acetic acid–methanol–water (7:25:68). It was destained in the same solvent and the location of each protein band was determined by measuring its distance from the anode end of the gel. Since the gels swelled during staining and destaining, locations of protein bands in unstained gels were calculated from the relative lengths of the gels before and after staining.

The second, unstained half of each gel was cut into 1-cm sections beginning at the anode end. Each section was placed into 2 ml of distilled water in a small test tube, and shaken for 3 h to elute the carrier ampholytes. A microcombination electrode was used to measure the pH (25 °C) of the ampholytes extracted from each gel section. The pH gradient along the gel was plotted, as a function of distance from the anode end of the gel, and the isoelectric point of each protein band was obtained from the graph.

RESULTS

Electrophoretic Patterns. A complex pattern of soluble proteins in the crude extract was obtained in polyacrylamide gel electrophoresis (PAGE) gels stained with Coomassie Blue. A simplified schematic illustration is shown in Figure 1.

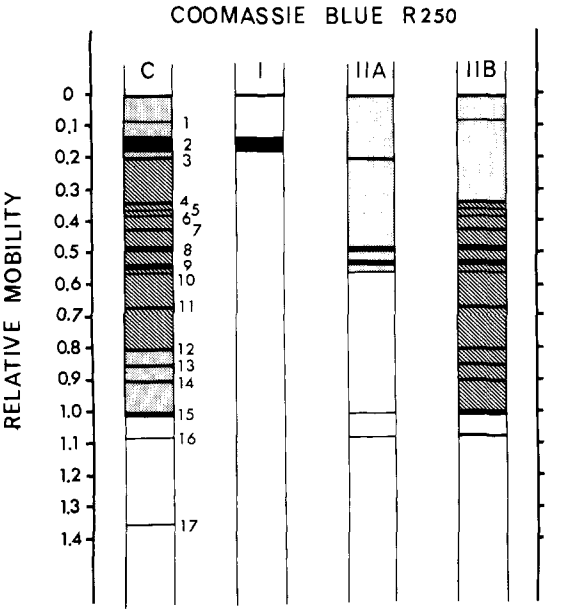

Figure 1. Schematic drawings of electropherograms stained with Coomassie Blue R-250, a protein specific stain. Typical patterns are illustrated for crude extract (C), fraction I protein (I), high molecular weight fraction II proteins (IIA), and low molecular weight fraction II proteins (IIB).

Seventeen discrete protein bands were easily identified but some of these probably consisted of closely spaced multiple bands. Resolution was always impaired by heavy background staining and in some gels additional bands were visible. Several different staining and destaining procedures failed to reduce background staining. A tracing from a densitometer scan (Figure 2) illustrates the incomplete resolution of protein bands caused by the background staining.

Band number 17 is the only one which was not retained in one of the three purified protein preparations while some proteins, particularly bands 8 and 9, were present in both fraction IIA and fraction IIB.

Discontinuous PAGE patterns for soluble alfalfa proteins have been reported by Rommann et al. (1971) and Hood (1973). The general pattern was similar except band number 17 is not resolved by discontinuous PAGE. Kleczkowska (1969) published starch gel electrophoresis patterns of soluble alfalfa proteins. Compared to PAGE, fewer bands were observed.

Figure 3 illustrates electropherograms stained with Sudan Black, a lipid specific stain. The crude extract contained a broad area of high intensity with two additional bands close to the origin. A typical densitometer scan is shown in Figure 2. Two broad bands in the fraction IIB group gave significant staining intensity with Sudan Black. These correspond to bands 4 to 6 and band 7, respectively, in gels stained with Coomassie Blue (Figure 1). The purified protein fractions contained less lipid than crude extracts. Lipid was removed during chromatography on DEAE-Sephadex. This suggests that much of the lipid-staining material in crude extracts was not true lipoprotein but was protein-bound, polar lipid.

When electrophoresis gels were stained by the PAS procedure only very faint background staining was observed (Figure 4). The most intense band in elec-

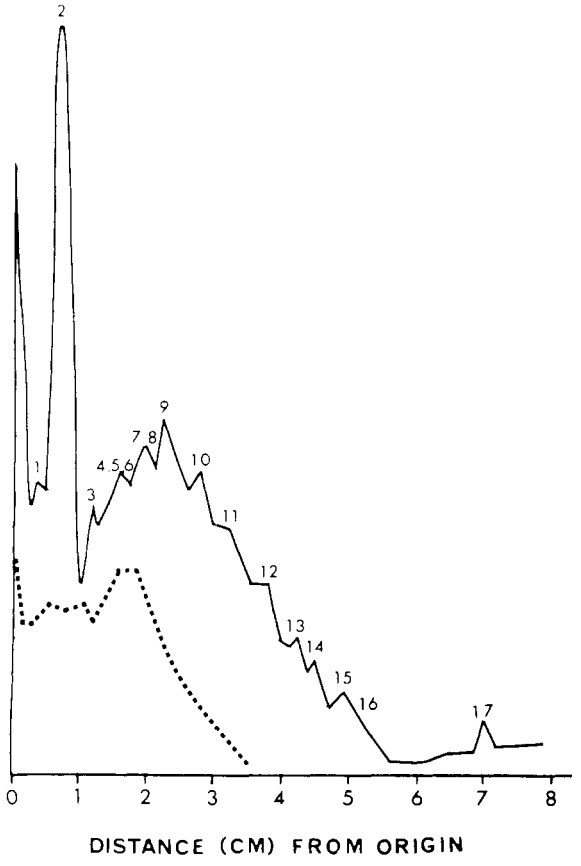

Figure 2. Densitometer scans of electropherograms for crude extracts after staining with Coomassie Blue R-250 (solid line) and Sudan Black B (broken line). The numbers identify the protein bands shown in Figure 1.

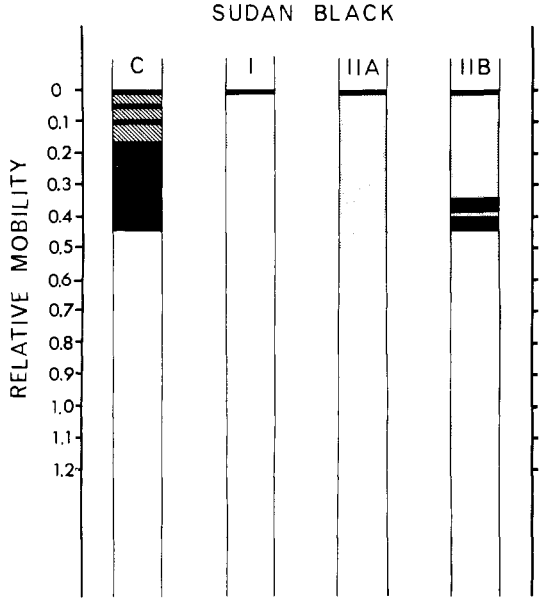

Figure 3. Schematic drawings of electropherograms stained with Sudan Black B, a lipid specific stain. Typical patterns are illustrated for crude extract (C), fraction I protein (I), high molecular weight fraction II proteins (IIA), and low molecular weight fraction II proteins (IIB).

tropherograms for crude extracts corresponded to fraction I protein. The purified fraction I protein also gave a weak stain with PAS. Highly purified tobacco fraction I protein contains no carbohydrate (Sakano et al., 1973), but fraction I from both rice and spinach contains carbohydrate

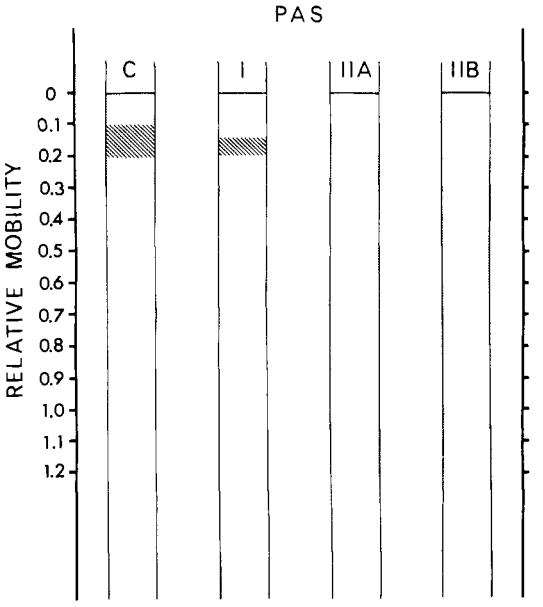

Figure 4. Schematic drawings of electropherograms stained by a periodic acid-Schiff (PAS) procedure. Typical patterns are illustrated for crude extract (C), fraction I protein (I), high molecular weight fraction II proteins (IIA), and low molecular weight fraction II proteins (IIB).

Table I. Isoelectric Values of Reference Compounds

Ref compds	Isoelectric points	
	Obsd	Lit. values
Fast green FCF	3.06[a]	3.05[b]
Methyl red	3.78	3.75[c]
Ovalbumin	4.61	4.6[d]
Human serum albumin	4.60	4.8[d]
Catalase	5.55	5.6[d]
Myoglobin	6.55	7.0[d]
Hemoglobin	6.93	6.9[d]

[a] Standard errors of means were 0.1 unit; n = 3 to 5. [b] Conway-Jacobs and Lewin, 1971. [c] Svensson, 1962. [d] White et al., 1968.

(Akazawa et al., 1965; Ridley et al., 1967).

Isoelectric Points. Isoelectric points of some reference proteins and dyes were measured to evaluate the accuracy of the isoelectric focusing method. Table I shows our observed isoelectric values for these compounds along with values reported by other investigators. There was excellent agreement except in the cases of human serum albumin and myoglobins for which we obtained isoelectric values that were 4.2 and 6.4%, respectively, lower than those cited by White et al. (1968). We considered the technique to be sufficiently accurate for our purposes. Low observed values for human serum albumin and myoglobin may have resulted from destruction of amide groups during commercial preparation of these samples.

Isoelectric points for the alfalfa protein fractions are shown in Figure 5 along with schematic diagrams illustrating relative intensities of the stained protein bands and their locations in the polyacrylamide gels. The fraction I protein preparation contained a trace of a contaminating protein with pI = 5.4. Eleven protein bands appeared consistently in the fraction IIA group. There were two major protein bands with pI values near 5.0 and there was background staining from pI = 4.8 to 5.4, probably caused by the presence of many indiscrete protein bands. Fifteen protein bands appeared consistently in the low molecular weight fraction II group and there were four major protein

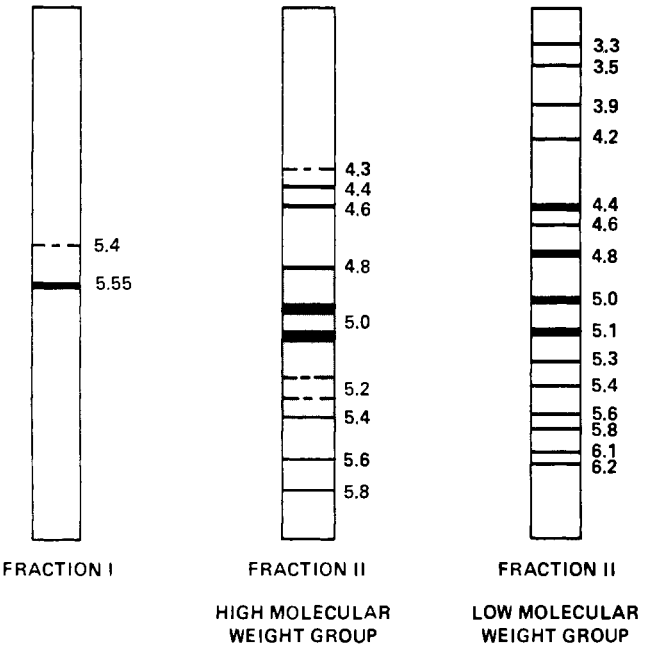

Figure 5. Isoelectric points of soluble alfalfa herbage proteins. The isoelectric points are means of three or more determinations. Standard errors of the means are 0.1 unit.

bands with isoelectric values of 4.4, 4.8, 5.0, and 5.1.

DISCUSSION

A number of previous investigations into the nature of the foaming agents responsible for pasture bloat have been based on the assumption that viscosity of foams is the physical property primarily responsible for the persistence of rumen foams during frothy bloat (Mangan, 1959; McArthur and Miltimore, 1966; Jones and Lyttleton, 1972). In this report our interpretation is based upon the same assumption. The viscosity of protein foams results from the surface viscosity of the protein film at the air–liquid interfaces within the foam. Surface viscosities of protein films vary over a wide range depending upon the nature of the protein (Langmuir and Schaefer, 1939). Viscous films are produced by proteins which form a concentrated or thick surface film and, conversely, dilute or thin surface films have little or no measurable viscosity (Boyd et al., 1973). Evans et al. (1970) showed that film concentration is related to protein structure. Proteins of high hydrophobicity and having a random coil configuration give dilute surface films while globular proteins, with many intramolecular constraints and low hydrophobicity, give concentrated surface films. It follows that globular proteins should produce more viscous foams than proteins which have a random coil configuration. As an initial approach to our study of the leaf proteins we have identified protein fractions which are conjugated with lipid and with carbohydrate. We reasoned that lipoproteins have a globular structure and hence should form viscous foams. Conversely, proteins conjugated with carbohydrate would be likely to have less globular structure and more random coil configuration. These proteins would be expected to produce foams of lower viscosity. Using Sudan Black, Rommann et al. (1971) noted significant variation in staining intensity among alfalfa clones and suggested that alfalfa lipoproteins could have a role in the formation of persistent foam during ruminant bloat. Our reasoning that lipoproteins should stabilize more viscous foams than glycoproteins is consistent with their proposal. However, the present results show that much of the lipid is reversibly bound to the proteins and is polar lipid. Since the polar, leaf lipids have antifoaming properties (Clarke and Reid, 1974), much of the material in crude extracts which stains with Sudan Black may not be an important causative agent in pasture bloat. Protein bands 4 to 7, inclusive (Figure 1), may be true lipoproteins and may produce more viscous foams compared to other soluble proteins.

In view of the very faint intensity of staining obtained with the PAS stain, there appears to be very little carbohydrate associated with the soluble leaf proteins and this approach provides no basis for distinction between the foaming properties of the soluble proteins.

Isoelectric points of the alfalfa proteins were measured because the foaminess of proteins is affected by pH (Bikerman, 1973). The viscosity of a protein foam is greatest at a pH just alkaline to the isoelectric pH of the protein (Jones and Lyttleton, 1972). Maximum viscosity for clover fraction I protein occurred at pH 5.7, and for fraction II proteins over the pH range 4.0 to 6.0 (Jones and Lyttleton, 1972). The isoelectric point for alfalfa fraction I was 5.6 while the greatest proportion of alfalfa fraction II proteins had isoelectric points in the range 4.4 to 5.4. Therefore, one can predict that soluble alfalfa proteins would yield foams with viscosity properties essentially identical with clover proteins. The pH for maximum viscosity of alfalfa fraction I protein foam and the majority of alfalfa fraction II protein foams would be expected to coincide with the pH of rumen ingesta. Rumen pH ranged from 5.2 to 6.5 in bloated cattle fed fresh alfalfa herbage (McArthur and Miltimore, 1969).

The alfalfa fraction II proteins with p*I* less than 4.0 probably contribute little to foam viscosity at the pH of rumen contents and, on the basis of stain intensity, they constitute a minor proportion of the fraction II group. Therefore, we conclude that both fraction I protein and the majority of the fraction II proteins will produce foams with maximum viscosity at the pH of rumen ingesta.

At pH 5.8 the solubility of clover fraction I protein decreased sharply to a very low value while the solubility of clover fraction II proteins decreased gradually from pH 6.5 to 3.5 (Jones and Lyttleton, 1972). Since proteins have minimum solubility at their isoelectric points one can predict that soluble alfalfa proteins have solubility curves essentially similar to clover proteins. The solubility of these plant proteins in rumen fluid may be an important point to be considered in elucidating their exact role as foaming agents in pasture bloat, especially in the case of fraction I protein. The pH of rumen fluid decreases after ingestion of feed (McArthur and Miltimore, 1969). Fraction I protein, with a p*I* of 5.6, would be the first of the major protein bands which is subject to precipitation. In some animals, which have low rumen pH, there may be insufficient fraction I protein in solution to make a significant contribution as a foaming agent.

The purpose of this research was to investigate whether or not the various soluble alfalfa proteins differ in their abilities to produce viscous foams. One group of fraction II proteins, bands 4, 5, 6, and 7, may be true lipoproteins and may therefore make a greater than average contribution to foam viscosity. A minor group of fraction II proteins has low isoelectric points and may contribute very little to foam viscosity at rumen pH. Finally, solubility of the proteins, especially fraction I protein, at the pH of rumen fluid may limit their contribution as foaming agents in ruminant bloat. These physical–chemical differences among the soluble leaf proteins provide a basis for further investigation. We have experiments in progress, to determine whether variations in concentrations of specific

soluble herbage proteins are associated with the occurrence of bloat in cattle.

ACKNOWLEDGMENT

Part of this research was conducted at the Agriculture Canada Research Station, Summerland, British Columbia. Skilled technical assistance was provided by M. Hikichi and R. Underwood.

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Received for review July 13, 1976. Accepted September 20, 1976. Contribution No. 651 of the Saskatoon Research Station.

Changes with Maturity in Anatomy, Histochemistry, Chemistry, and Tissue Digestibility of Bermudagrass Plant Parts

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Coastal bermudagrass (*Cynodon dactylon* (L.) Pers.) was harvested after 5.5 months of greenhouse regrowth and separated into leaf blades, sheaths, and stems of top, middle, and bottom portions to reflect differences in maturity of each plant part. The parts were evaluated for differences associated with maturity in the percentages of tissue types, histochemistry of sections, and compositional analyses, and these data were related to changes in the in vitro dry matter digestibility (IVDMD) and in the degradation of specific tissue types observed by scanning electron microscopy. In blades, no significant differences with maturity (as related to digestibility) were found among the parameters. In sheaths and stems, acid detergent fiber (ADF) and lignin increased with maturity. Histochemical studies indicated the presence of chlorine sulfite positive lignin in the mesophyll of sheaths and parenchyma of stems of middle and bottom portions only. These tissues were less degraded in the bottom sheath and stem portions, and their IVDMD decreased with maturity. Decreases in digestibility with maturity of bermudagrass appeared to be at least partially the result of chlorine sulfite positive lignin in thin-walled cells of bottom portions of sheaths and stems.

The effects of maturity on forage grasses have been studied on the basis of several criteria: chemical composition (Burton et al., 1964; Danley and Vetter, 1973; Deinum and Dirven, 1971; Moore et al., 1970), morphology (Johnston and Waite, 1965; Prine and Burton, 1956), and animal performance (Utley et al., 1971; Weston and Hogan, 1968). Additionally, decreases in forage digestibility with maturity have been shown in both warm-season grasses (Danley and Vetter, 1973; Burton et al., 1964; Moore et al., 1970; Ventura et al., 1975; Wilkinson et al., 1970) and cool-season grasses (Mowat et al., 1965; Pritchard et al., 1963; Terry and Tilley, 1964). More specifically, Coastal bermudagrass, pelleted at 4 and 8 weeks of age, showed a decrease in dry matter digestibility of about 10 percentage units in the older vs. the younger forage (Utley et al., 1971). Wilkinson et al. (1970) reported that the bottom portion of Coastal bermudagrass was 17 to 20 percentage units lower in dry matter digestibility than the top portion.

Reported decreases in the digestibility among the plant parts due to maturity have been consistent. Leaf blades usually decrease in digestibility, but not as much nor as rapidly as the stems (Pritchard et al., 1963; Mowat et al.,

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