nearly the same as the components of the essential oil of Hybridsorgo. With respect to the total amounts of carbonyl compounds and phenols, however, a remarkable difference was recognized between these essential oils. Accordingly, it was concluded that the odor difference between Sudangrass and Hybridsorgo does not depend upon the kinds of compounds present, but rather upon their relative amounts.

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Digestibility of Delignified Forage Cell Walls

Franklin E. Barton, II* and Danny E. Akin

The effect of lignin on the digestion of plant cell walls by rumen microorganisms was examined. Intact cell walls of Coastal bermudagrass (*Cynodon dactylon* (L.) Pers.) and Ky-31 Tall fescue (*Festuca arundenacea* Schreb.) were delignified with potassium permanganate in an acetic acid buffer. The delignified cell walls and untreated controls were incubated with rumen microorganisms and the relative ease and extent of digestion observed with the scanning electron microscope. Sclerenchyma tissue was more susceptible to the permanganate treatment than the inner bundle sheath. The rate of digestion of all tissues in the cell walls was increased in the treated samples. The vascular bundle of the delignified sample (as determined histochemically) was not digested. These data suggest that while lignin does impede the rate and extent of digestion, the plant cell wall polysaccharides still show marked differences in their rate and extent of digestion when lignin is not a barrier. The relationship of anatomical characteristics to digestibility should include considerations of the association of cell wall polysaccharides with lignin as well as of the type, site, and extent of lignification.

Lignin content has been correlated with decreased forage digestibility and more specifically with decreased digestibility of particular cell wall polysaccharides (Wilkins, 1969, 1972; Smith et al., 1972; Duble et al., 1971; Waldo et al., 1972; Akin et al., 1975; Barton et al., 1976). Kamstra et al. (1958) also showed that cellulose and hemicellulose isolated as holocellulose (Phillips et al., 1960) from orchardgrass and alfalfa were digested in vitro more completely than intact plant cellulose in situ. Addition of lignin to in vitro samples isolated by the procedure of Patton (1943) did not depress in vitro digestibility of these forages. Cross et al. (1974) showed that cellulose digestibility was increased from 66 to 91% by chemical removal of lignin with sodium chlorite. Although lignin has been related to decreased forage digestibility, no definitive evidence has been found to show the nature of its effect on the digestion of forage tissue by rumen microorganisms.

Morrison (1975) and Bailey and Pickmere (1975) showed that the amount and type of hemicellulose isolated from various plant species depended on whether the plant material is delignified before hemicellulose extraction. Lignin–carbohydrate complexes isolated from birchwood (Bolker and Wang, 1969) and ryegrass (Morrison, 1973; Hartley, 1972) yielded upon hydrolysis monosaccharides which correspond to the plant hemicellulose fraction. Therefore, the plant polysaccharides apparently are not simply encrusted by lignin, but are probably covalently bonded as suggested by Harkin (1973). The type and extent of lignin–polysaccharide bonding could affect digestion more than the amount of lignin per se.

All the above authors examined the relationship of lignin to cell walls and digestibility, but only Akin et al., (1975) did so by direct observation of intact cells using scanning electron microscopy. They observed differential rates of digestion for various plant tissues in fescue and bermudagrass and found that the lignified cells were indigestible.

In this study the scanning electron microscope (SEM) was used to observe intact cell walls digested in vitro by rumen microorganisms before and after chemical treatment to remove lignin. The objective of the study was to visualize the effect of lignin and lignified tissue on the digestion of intact cell walls, i.e., compared to a properly fixed cross section of a grass leaf blade the tissues in the treated sections appear to be undisturbed, swelled, or distorted. Any distortion noted in the treated samples must then be attributed to the treatment. The samples were treated as leaf sections and were not ground.

EXPERIMENTAL SECTION

Preparation of Grass Samples. Samples of Coastal (CBG) and Coast-cross-1 (CX-1) bermudagrass (*Cynodon dactylon* (L.) Pers.) and Kentucky-31 (Ky-31) tall fescue (*Festuca arundinacea* Schreb.) and its annual ryegrass hybrid Kenhy (KHY) were harvested after 4 weeks of summer regrowth, immediately frozen with dry ice, and maintained at −30 °C until used. Sections of leaf blades,

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3–5 mm long were cut with a sharp razor blade as previously described (Akin et al., 1975). Additional samples of each forage were freeze-dried and ground to pass a 20-mesh screen.

Chemical Composition. The reagents used in the neutral (NDF) and acid (ADF) detergent fiber and permanganate lignin (PML) analyses were prepared, and analyses and fibrous preparations conducted, according to the procedures of Van Soest (1963; Van Soest and Wine, 1967, 1968), with modifications as described by Barton et al. (1976). NDF and delignified NDF were hydrolyzed by refluxing in 1.0 N sulfuric acid for 1 h, and the ADF washing procedure was used to produce fractions designated as ADF* and the delignified analogue of ADF*, respectively.

Preparation of Leaf Section Cell Walls. Intact leaf sections of each forage were placed in flasks with 100 mL of NDF solution and refluxed for 120 min. The sections were washed in a 250-mL beaker with 100 mL of hot (>98 °C) deionized, distilled water for 3–5 min, then washed for 5 min in 100 mL of acetone. The sections were blotted dry and divided into three groups. Group 1 was placed in vials containing 4% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.0 for 18–20 h, postfixed in buffered 1.5% OsO_4 for 4 h, dried at the critical point in liquid CO_2 without transitional solvents, adhered to aluminum stubs with conductive paint, coated in a vacuum evaporator with gold–palladium alloy (60:40) for conductive purposes, and observed in a field emission SEM at about 15 kV. Samples were run in triplicate. Group 2 was used as the NDF digestion control, and group 3 was treated to produce delignified cell walls that would be intact for observation by the SEM.

Preparation of Delignified Cell Walls. Group 3 NDF leaf sections and ground NDF residues were treated with potassium permanganate and demineralized according to the procedures of Van Soest and Wine (1968), modified because NDF instead of ADF was delignified and leaf sections, in addition to ground forage, were required. Leaf sections were placed in each reagent and each combination of reagents (i.e., the $KMnO_4$; the acetic acid buffer; the demineralizing solution; $KMnO_4$ plus acetic acid buffer; and $KMnO_4$, acetic acid buffer, and demineralizing solution) and prepared for SEM as described above. The effect of each reagent on the NDF leaf sections was monitored by SEM. Optimum times were established as follows: 30–40 min in permanganate plus acetic acid buffer, followed by 10 min in demineralizing solution. Some of the delignified NDF leaf sections were prepared for SEM as above. The other delignified NDF sections were used as substrates for in vitro digestion. Thin sections (cut 16 μm thick with a cryostat) and intact 3–5 mm sections of delignified and control NDF sections were evaluated histochemically for the extent of delignification. Acid phloroglucinol was used to stain the residual lignin (Jensen, 1962). The NDF residues of ground forage were treated as specified in the Van Soest and Wine (1968) procedures. The $KMnO_4$-treated NDF residue of ground samples was also checked for residual lignin using the usual Van Soest and Wine (1968) procedure.

Digestion Procedures. In vitro dry matter digestibility (IVDMD) was determined for ground forage samples by the Tilley and Terry (1963) two-stage procedure. The ground forage NDF samples (0.4 g) were incubated in 30 mL of buffer–inoculum mixture (buffer to rumen fluid, 2:1) in test tubes (with bunsen valves) at 39 °C and removed after 1, 2, 4, 6, 24, and 48 h incubation to evaluate the forages for rate of digestion. The NDF and permanganate extracted leaf sections were placed in a 500-mL Erlenmeyer flask with 300 mL of rumen fluid and McDougall's buffer (McDougall, 1948) and constantly bubbled with CO_2 at 39 °C. Five to six leaf sections from each flask were removed for observation at 1, 2, 4, 6, 24, and 48 h and prepared for SEM. Control leaf sections of NDF and $KMnO_4$-treated NDF were incubated in McDougall's buffer without inoculum for 20 h and prepared for SEM.

RESULTS AND DISCUSSION

Experimental Modifications. In the Van Soest and Wine (1968) procedure for the determination of lignin, ADF is treated with a saturated solution of $KMnO_4$ in acetic acid–*tert*-butyl buffer, then demineralized with an oxalic and hydrochloric acid 80% aqueous ethanol solution. The starting material for permanganate lignin determinations, i.e., ADF, differs for representative warm and cool season grasses (see Figure 9 and 10 in Akin et al., 1975). In fescue the extent of tissue removal by the ADF reagent was greater than by rumen microorganisms (in vitro) in 72 h. In bermudagrass some digestible tissues remained in ADF residue. The NDF, however, was essentially the cell walls in an undegraded form in both bermudagrass and tall fescue (see Figures 3 and 4 in Akin et al., 1975). For this reason the NDF of leaf sections was chosen as the material to delignify in this work. Each reagent, separately and in combination, was evaluated to determine its effect on the forage cell wall. Figure 1 (a–f) shows the results of these treatments on Coastal bermudagrass (CBG). When the CBG leaf sections were treated with the NDF reagent (Figure 1a), the cell walls were undisturbed and the cell contents were removed only from those cells ruptured during sectioning. The acetic acid buffer and demineralization with oxalic acid (Figure 1b and 1c, respectively) did not distort any of the tissues in the cell walls. Potassium permanganate, however, produced alterations in the cross sections compared with the NDF extracted control sections; these alterations (Figure 1d–f) were apparent only in the lignified tissues (i.e., sclerenchyma and vascular tissue). Figure 1d shows that alterations in the structure were affected by $KMnO_4$ per se. The sclerenchyma (s) was beginning to separate into individual cells, and inner bundle sheath cells (i) were separated from the nonlignified tissues, which were not distorted. Even the thin-walled mesophyll (Figure 1d (M)) and phloem (p) patch were undisturbed. The treatment with saturated $KMnO_4$ was sufficient for observation but the presence of manganese dioxide (MnO_2), a mild oxidizing agent, and a residue in $KMnO_4$ oxidations could interfere with the digestion of the cell walls by rumen microorganisms. The 3.0 M acetic acid buffer used by Van Soest and Wine (1968) when mixed with the $KMnO_4$ (Figure 1e) again distorted only in the lignified areas. Individual mesophyll (M) cells were left intact but were separated from adjacent vascular bundles. The effects of the full delignification and demineralization treatment are shown in Figure 1f. The leaf section showed separation of the lignified portions of the vascular bundle and sclerenchyma (S) while the nonlignified tissues (mesophyll (M), phloem (P), and outer bundle sheath (O)) were unaltered. Histochemical evaluation of treated sections with acid phoroglucinol detected no lignin for about 25% of the depth of the section, indicating that the surface of the section has been delignified; however, the vascular bundles of untreated leaf sections of CBG and Kentucky-31 tall fescue (Ky-31) were red stained with acid phoroglucinol.

In Vitro Digestion of Treated Sections. Figure 2a–d shows the control cross sections of CX-1-NDF and de-

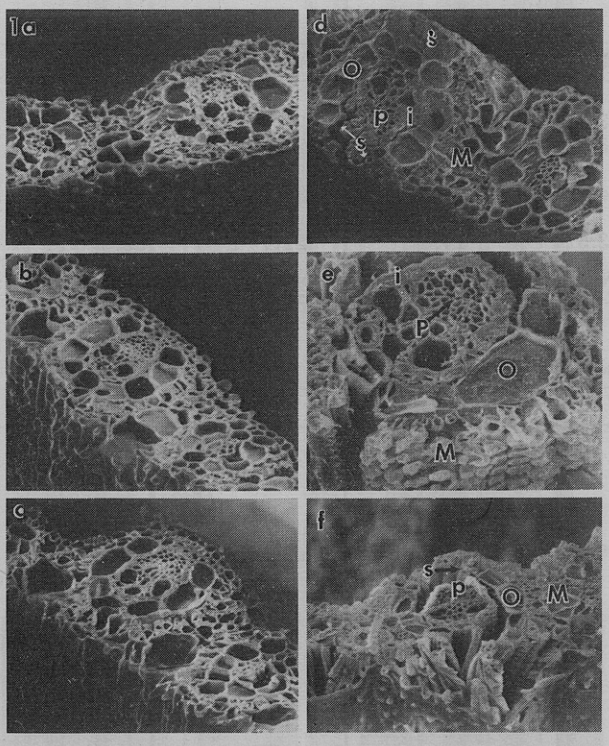

Figure 1. Effect of delignification on Coastal bermudagrass neutral detergent fiber (CBG-NDF) leaf sections. A. Control CBG leaf section treated for 120 min with NDF reagents. All tissues are intact and show no distortion: ×600. B. Cross section of CBG-NDF treated for 30 min with acetic acid buffer used in the $KMnO_4$ reagent. All tissues are intact and there is no evidence of cell wall hydrolysis by the buffer: ×600. C. Cross section of CBG leaf NDF treated for 15 min with demineralization reagent. All tissues are intact with no evidence of cell wall hydrolysis: ×600. D. Cross section of CBG-NDF treated with saturated $KMnO_4$ for 30 min and washed with water. Cells of the mesophyll (M), phloem (p), and outer bundle sheath (O) are intact but the inner bundle sheath (i) and sclerenchyma (s) are being separated from the outer bundle sheath and the sclerenchyma below the phloem patch is being separated into individual cells: ×600. E. Cross section of CBG-NDF treated for 30 min with buffered $KMnO_4$ reagent and washed with water. The same tissues are intact as in D above, but separation of inner bundle sheath (i) from the outer bundle sheath (O) is more pronounced: ×1200. F. Cross section of CBG-NDF treated for 30 min with buffered $KMnO_4$ reagent and demineralized for 10 min. The mesophyll (M) and phloem (p) are intact while the outer bundle sheath (O) is almost completely separated from the rest of the vascular bundle. The sclerenchyma has been divided into individual cells and separated from the vascular bundle: ×384.

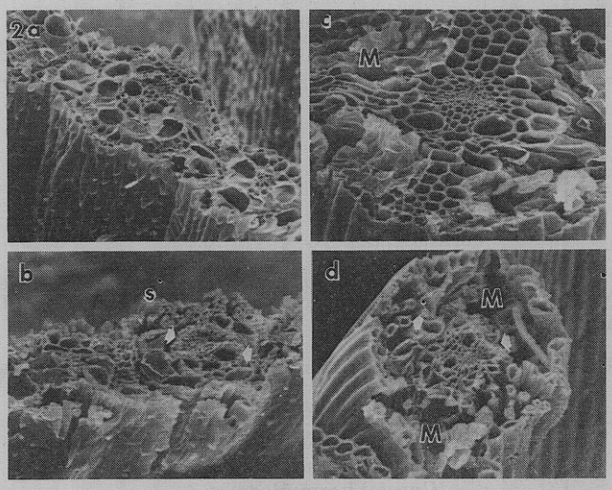

Figure 2. The NDF and delignified NDF controls of Coastcross-1 bermudagrass (CX-1) and Ky-31 shown for comparison of response to the treatment between cool- and warm-season species. A. Cross section of CX-1-NDF. All tissues are intact: X384. B. Corss-section of CX-1-NDF delignified with $KMnO_4$ reagent. The nonlignified tissues are not distorted, however, and the lignified tissues (arrows) and sclerenchyma (s) have been separated: X384. C. Cross section of Ky-31 tall fescue treated with NDF reagent. All tissues are present though some collapse in the mesophyll (M) is evident: X384. D. Cross section of Ky-31 leaf NDF delignified and demineralized. Lignified tissues (arrows) are distorted and separated and some mesophyll (M) has been removed: X384.

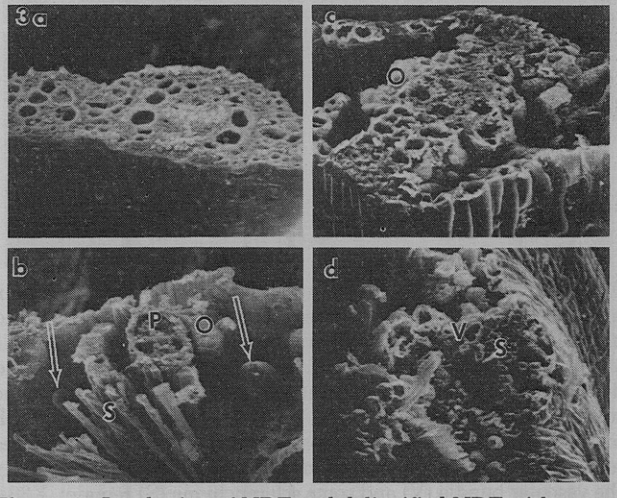

Figure 3. Incubation of NDF and delignified NDF with rumen microorganisms for 1 h. A. Cross section of CBG-NDF incubated with rumen microorganisms. No digestion is evident: ×384. B. Delignified CBG-NDF digested. The mesophyll is completely removed, while the phloem (P) and outer bundle sheath (O) are beginning to be removed. Sclerenchyma (S) is broken apart and rumen protozoa (arrows) are present between vascular bundles: ×384. C. Cross section of Ky-31-NDF incubated. Some mesophyll has been removed. Other tissues are intact: ×384. D. Cross section of incubated delignified Ky-31-NDF. Extensive digestion has removed mesophyll and phloem. Schlerenchyma (S) is separated from the vascular bundle (V): ×384.

lignified CX-1-NDF and Ky-31-NDF and delignified Ky-31-NDF, respectively. The cell walls of the CX-1-NDF (Figure 2a) were completely intact while the delignified section (Figure 2b) showed changes in the lignified tissues of the inner and outer bundle sheath (arrows) and the sclerenchyma (S) as noted above. The mesophyll, phloem, and outer bundle sheath cells maintained their structural integrity. In Ky-31-NDF (Figure 2c) the mesophyll (M) was distorted, but all tissues were present. In the $KMnO_4$-treated Ky-31-NDF sections (Figure 2d) the lignified areas were affected as in CX-1, but in addition some mesophyll (M) was removed. Results were identical with analogous samples of CBG-NDF and KHY-NDF and the respective $KMnO_4$-treated NDF.

After 1 h of incubation, the NDF of CBG (Figure 3a) was undigested, although covered with rumen microorganisms while the NDF of Ky-31 (Figure 3c) showed digestion of mesophyll. The phloem of Ky-31-NDF was being removed as was part of the outer bundle sheath (O), a part of CBG which is not usually digested until after 24–48 h. Digestion of the delignified NDF samples was quite extensive after 1 h (Figure 3b and 3d). The mesophyll was digested and the phloem (P) was being digested, as was part of the outer bundle sheath (O) in the delignified CBG-NDF. The sclerenchyma (S) was broken apart and some of it had been removed. Rumen protozoa were also present (arrows in Figure 3b). The extent of digestion becomes increasingly greater with time for the delignified sections as shown by the depth to which de-

Table I. Percent Compositional Analysis

Grass	NDF[a]	ADF[a]	PML[a,b]	NDFPML[c]	DADF*[d]	DADF* + PML[e]
Kentucky-31 tall fescue	58.4	31.4	3.4	17.5	26.9	30.3
Kenhy fescue	58.2	33.6	3.7	17.2	23.5	27.2
Coastal bermudagrass	61.0	29.1	4.1	17.3	30.0	34.1
Coastcross-1 bermudagrass	60.0	31.9	3.5	17.4	29.7	33.2

[a] From Barton et al. (1976). [b] PML is permanganate lignin. [c] NDF PML % dry matter removed by the $KMnO_4$-treated NDF. [d] DADF* % residue from 1 N H_2SO_4 hydrolysis of $KMnO_4$-treated NDF. [e] DADF* + PML is the sum of DADF* and PML.

Table II. Percent in Vitro Dry Matter Digestibility of Grass and Isolated Fibrous Fractions

Sample	Grass[a]	NDF[b]	NDFPMLT[c]
Kentucky-31 tall fescue	64.2	63.1	77.0
Kenhy fescue	67.7	64.5	77.8
Coastal bermudagrass	66.8	63.5	79.8
Coastcross-1 bermudagrass	66.1	65.0	80.6

[a] From Barton et al. (1976). [b] NDF is neutral detergent fiber. [c] NDFPMLT is the permanganate-treated neutral detergent fiber residue.

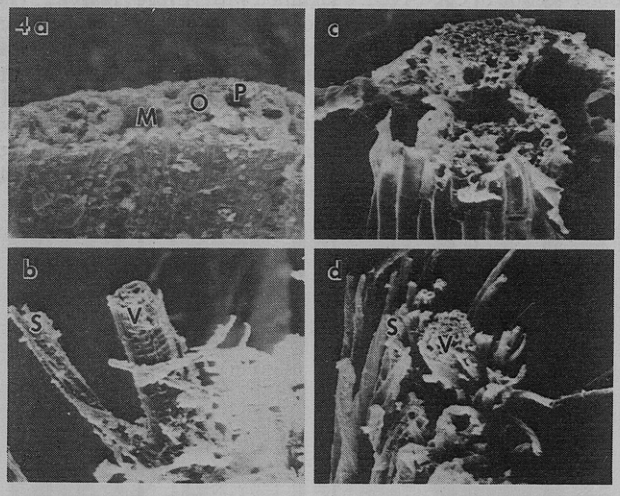

Figure 4. Incubation of NDF and delignified NDF with rumen microorganisms for 20 h. A. Cross section of CBG-NDF incubated mesophyll (M) and phloem (P) are digested while the outer bundle sheath (O) is still intact: X384. B. Cross section of incubated delignified CBG-NDF. Only the vascular bundle (V) and some sclerenchyma (S) remain: X384. C. Cross section of Ky-31-NDF incubated. Extent of digestion parallels 4a., but with more tissues removed: X384. D. Cross section of delignified Ky-31-NDF. Only the vascular bundle (V) and some sclerenchyma (S) remain: X384.

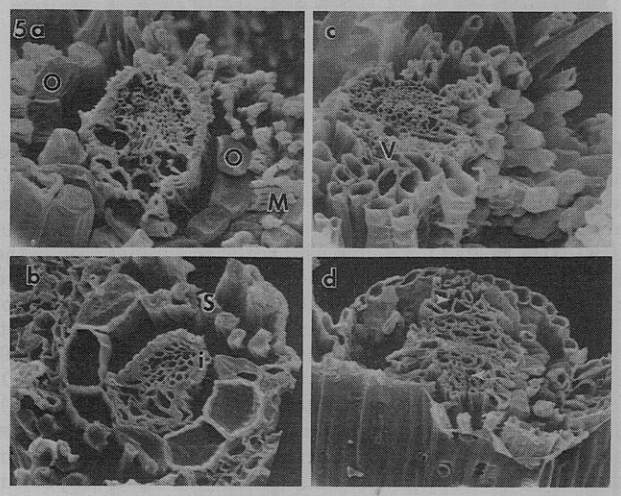

Figure 5. Controls of delignified CBG-NDF and KHY-NDF compared to the same samples incubated in McDougall's buffer for 20 h. A. Cross section of CBG-NDF and delignified CBG-NDF. While no digestion could have occurred, some tissues have been washed away; particularly mesophyll (M) and outer bundle (O): X600. B. Cross section of delignified CBG-NDF. All tissues are present and intact except that the sclerenchyma (S) and lignified inner bundle sheath (i) have been affected by the treatment: X1200. C. Cross section of delignified KHY-NDF incubated in buffer alone. No tissues have been digested but some tissues have been washed away from the vascular bundle (V): X384. D. Cross section of delignified KHY-NDF showing that the lignified tissues (arrows) are the ones affected by the permanganate treatment: X384.

lignified sections of both the CBG and Ky-31 are degraded. After 20 h of incubation (Figure 4), the delignified cell walls were degraded to the extent that the tissues were difficult to mount on the SEM stubs (Figure 4b and 4d). Only isolated, large vascular bundles (V) and some sclerenchyma (S) cells remained. The digestion of the delignified section was more extensive at 20 h than digestion of the grasses themselves at 72 h (Akin and Burdick, 1975). The extent of digestion of the NDF sections of CBG and Ky-31 (Figure 4a and 4c) was similar to the digestion of sections of these grasses incubated for a similar period of time (Figures 4 and 12 in Akin and Burdick, 1975). The SEM results obtained for the delignified sections of CBG-NDF sections incubated for 20 h only in McDougall's buffer (Figure 5a and 5c) show some further separation and slight removal of tissues which appeared to be a removal of loose cells, compared to the delignified controls of CBG-NDF and KHY-NDF (Figure 5b and 5d and Figure 1c–e).

Composition and in Vitro Dry Matter Determinations. The analysis of CBG, Ky-31, and two hybrids, Coastcross-1 bermudagrass (CX-1) and Kenhy fescue (KHY), is shown in Table I. When the NDF residues of the grasses were delignified, approximately 17% dry matter was removed. Gas chromatographic analysis of the alditol acetates from hydrolyzed NDF residues and delignified NDF indicated that a portion of the plant hemicellulose, particularly that containing arabinose, was removed from the NDF with the lignin (Barton, 1977). Sullivan et al. (1960) showed that hemicellulose was partially removed following delignification. The linking of lignin to the hemicellulose side chains through xylans and arabinoxylans had been reported (Morrison, 1973; Harkin, 1973). The delignified NDF that had been hydrolyzed in 1 N sulfuric acid (H_2SO_4) (i.e., DADF*, where ADF* is 1 N H_2SO_4 hydrolyzed NDF) is also given in Table I. For the temperate grasses, if the percent PML are added to the percent DADF*, the sums are less than their ADF

values. These values agreed with the SEM observation that $KMnO_4$ removed digestible tissue from the temperate grasses. The in vitro dry matter digestibilities (IVDMD) of the grasses and the isolated fibrous fractions are shown in Table II. The digestibilities were similar for the grasses and NDF but were higher for delignified NDF residues; the differences were about 15 percentage units for CBG and CX-1. Digestibility of delignified NDF was slightly higher for bermudagrass than for fescues.

Digestion appeared to be faster for the delignified NDF than for the corresponding grass NDF residues (Figures 3 and 4). Comparisons of the digestion with time for NDF and delignified NDF in ground samples are less conclusive. Grinding tended to level the ease of digestion of NDF and delignified NDF. Linear and exponential plots of IVDMD vs. time did not indicate a definite reaction order, so rate constants were not calculated. The half-lives of the delignified NDF digestion were all about 18 h. The half-lives of the NDF ranged from 22 h for CBG to 37 h for KY-31. Earlier work on the rate of digestion of leaf sections shows a marked difference in half-lives of forage digestion for CBG leaf sections (147 h) and Ky-31 leaf sections (53 h) (Barton, 1974).

Effect of Delignification. Several aspects of the $KMnO_4$ treatment and digestion of delignified cell walls were noted in this study. First, there was differential tissue response to the treatment between the warm- and cool-season cultivars; some mesophyll tissues were removed in the fescue NDF by $KMnO_4$ treatment. This could account in part for the lower digestibility of the delignified fescue NDF. Apparently less of the rapidly digestible tissues were available as substrate for the bacteria in the delignified fescue NDF than in the delignified bermudagrass NDF. The lignified portions of the sections were the most affected in all samples. Second, about 17% dry matter was removed from all the NDFs. Gas chromatography of alditol acetates from tissue hydrolyzates indicated that the tissue or dry matter removed with the lignin by the $KMnO_4$ would have been considered part of the plant hemicellulose (Barton, 1977). The data in Table I, where the DADF* values are similar to ADF values, indicated that the 17% dry matter (lignin and cell wall polysaccharides) removed came from the hydrolyzable polysaccharide fraction. Third, the relative ease and extent of digestion of all tissues, except the vascular bundles, were increased. In particular, digestibility of tissues that were not lignified to begin with (mesophyll, phloem, outer bundle sheath) was increased. During in vitro digestion the tissues in the $KMnO_4$-treated sections were removed by the bacteria at earlier time than in comparable digestions of grass sections (Akin and Burdick, 1975) and more tissues in the delignified sections were degraded by 24 h. Outer bundle sheath was digested as fast in $KMnO_4$-treated samples as mesophyll in the untreated samples. Also, tissues not usually digested in grasses (sclerenchyma) were degraded in the $KMnO_4$-treated cell wall. Fourth, the large vascular bundles were delignified by treatment with $KMnO_4$ but were not degraded (Figure 4b), indicating that the indigestibility of this tissue in grasses was not totally dependent on lignification.

A second possibility is that lignin aldehyde end groups that would be oxidized to carboxylic acid end groups (House, 1965) which are not reactive to acid phloroglucinol may still be present and inhibiting digestion. However, subsequent determinations of the residual lignin in permanganate-treated NDF indicate no lignin to be present.

Akin et al. (1977) showed histochemically (with a chlorine–sulfite stain) that, for CBG, maturity involves a laying down in the stem of one type of lignin analogous to that found in the sclerenchyma of blades. This tissue in leaves that was positive to chlorine–sulfite appeared to be more susceptible to the $KMnO_4$ treatment and to be degraded when incubated with rumen microorganisms. The relationship of anatomical characteristics to digestibility should include considerations of the association of cell wall polysaccharides with lignin as well as of the type, site, and extent of lignification.

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