
ARTICLES

Scanning Electron Microscopy of Acid Detergent Fiber Digestion by Rumen Microorganisms

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Leaf sections of four grasses were extracted with acid detergent reagent and/or incubated with rumen microorganisms in vitro. The samples obtained from these treatments and controls were prepared for observation with a scanning electron microscope. The response of each grass to the acid detergent reagent varied. In all cases the combination of acid detergent extraction and in vitro digestibility caused greater destruction than either treatment alone. Treatment order also had an effect. Although tissue destruction was extensive in samples extracted with the acid detergent and followed with digestion by rumen microorganisms, the extent of disruption of lignified tissues was less severe than that in the reverse order of treatments. Gravimetric analysis of ground forage confirmed that the combination of treatments removed about 6-24% more dry matter than either treatment alone and showed that the difference in order of combined treatments was 5-12% units, depending on the grass species.

Recent studies by Akin et al. (1975) and Barton and Akin (1977) have identified specific fibrous tissues that make up the acid detergent fiber (ADF) and neutral detergent fiber (NDF) and have shown that delignification of the fibrous tissues of plant cell walls affects their subsequent degradation by rumen microorganisms. The values from gravimetric analyses of ADF have been correlated with in vitro digestibility (Van Soest, 1965), and values of ADF and NDF with in vitro dry matter disappearance (IVDMD) (Barton et al., 1976) and intake (Van Soest and Mertens, 1977). These two fibrous residues, ADF and NDF, have been proposed as the basis for predicting relative feeding value in a hay grading system (Rohweder et al., 1977) which has been applied to temperate forages. All of these studies suggest the use of percentage ADF for predicting forage digestibility. The studies of Akin et al. (1975) and Barton and Akin (1977) have shown that the fibrous fraction ADF is not the same for warm- and cool-season grasses. These differences could have a bearing on the use of ADF for predicting forage quality.

The ADF fraction is thought to be the most highly lignified and least digestible portion of the plant cell wall (Van Soest, 1963). The decomposition of lignocellulose is cited as one of the most important barriers to utilization of cellulose by microorganisms (Bryant et al., 1977; Belamy, 1976). The use of ADF values to denote the potential extent of digestion has also been proposed (Barnes and Marten, 1979).

Both the chemical analysis, ADF, and the bioassay, IVDMD, are gravimetric measurements. The dried residue after extraction (ADF) or fermentation (IVDMD) is weighed, and the numerical weight becomes the percentage ADF or IVDMD. Neither of these procedures considers the structure of the plant as an influence on the determination.

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In this study scanning electron microscopy (SEM) was used to observe the microbial digestion of ADF isolated from Bermuda grass, orchard grass, and fescue. The objectives were to identify the tissues that make up ADF and nondigestible residue from microbial digestion and to compare the degradation of forage by the chemical and microbiological methods using microscopy and gravimetric analysis. Because we imposed two treatments on the forage (in vitro rumen microbial fermentation and acid detergent extraction), it was also necessary to determine the effect of the order of treatments.

EXPERIMENTAL PROCEDURES

Preparation of Grass Samples. Coastal (CBG) and Coastcross-1 (CX-1) Bermuda grass [*Cynodon dactylon* (L.) Pers.], Kentucky-31 (Ky-31) tall fescue (*Festuca arundinacea* Schreb.) and its ryegrass hybrid Kenhy (KHY), and Boone orchard grass (*Dactylis glomerata* L.) (OG) were harvested after 4 weeks of summer regrowth. These samples came from experimental plots in subsequent years and received the same fertilization as those previously reported (Barton et al., 1976). The grass samples were prepared for ADF extraction as described by Akin et al. (1975).

ADF Dry Matter Determinations and Isolation. The ADF determinations were made according to the procedures of Van Soest as modified by Barton et al. (1976). The procedure is, essentially, the extraction of a 1.0-g dried, ground grass sample with boiling 2% hexadecyltrimethylammonium bromide in 1.0 N sulfuric acid. The isolation of ADF was also analogous to the procedures of Barton et al. (1976) except that an extra washing step was included. After each sample was washed with acetone, it was resuspended in boiling water and washed with boiling water until no detergent was visible (i.e., no foam could be seen at the base of the sintered glass disk). The sample was then washed with 3 volumes of acetone and dried at 65 °C in a forced draft oven.

Preparation of the Acid Detergent Extracted Leaf Sections. Leaf sections extracted with acid detergent were prepared for SEM in two ways. The first method was identical with that of Akin et al. (1975) in which there was no stirring of the sections in the heated flask. The second

procedure was analogous to the analytical procedure of Barton et al. (1976) in which the samples were stirred and filtered through a sintered glass crucible. The extracted leaf sections were prepared for viewing by SEM as previously reported (Barton and Akin, 1977), with the exception that they were not critical point dried (Anderson, 1951). The extracted leaf sections were fixed in buffered (cacodylate, pH 7.2) glutaraldehyde, postfixed with buffered osmium tetroxide mounted, and coated with a gold/palladium (60:40) alloy. With tissues this fragile, the wash steps and the pressure within the dryer could disrupt or destroy the samples if care was not used in their preparation. Critical point drying (Anderson, 1951) involves the exchanging of water with ethanol by steps and then exchanging the ethanol with liquid carbon dioxide (CO₂). This is done in a pressure vessel. After several exchanges, the temperature of the liquid CO₂ is raised above 88 °C, and the liquid and gas phases of CO₂ are in equilibrium. The pressure is slowly released and the dried sample removed.

Digestion Procedures. In vitro dry matter disappearance (IVDMD) was determined for samples (grasses and isolated ADF) ground through a Wiley mill to pass a 20-mesh screen by the Tilley and Terry (1963) two-stage procedure as reported by Barton et al. (1976) and Barton and Akin (1977). This procedure uses McDougall's buffer and strained rumen fluid (2:1) to digest 0.4 g of a sample incubated at 39 °C for 48 h. The digestion study was repeated 4 times. The ADF extraction of digested grass residue and the digestion, in vitro, of the leaf sections previously extracted with acid detergent were conducted as described above and constituted the combined treatments.

RESULTS AND DISCUSSION

Leaf sections from these seven treatments were evaluated by SEM: (1) leaf section controls, (2) leaf sections in buffer (McDougall's) controls, (3) acid detergent leaf sections, (4) acid detergent leaf sections in buffer controls, (5) leaf sections digested for 48 h, (6) acid detergent leaf sections digested for 48 h, and (7) leaf sections digested for 48 h and then extracted with acid detergent. Previous histochemical and SEM studies of leaf blades had identified the lignified tissues, which generally are not degraded by rumen microorganisms. Specifically, sclerenchyma cells gave a positive reaction for lignin with the chlorine-sulfite stain, whereas the xylem cells, including the inner bundle sheath of the vascular bundles, were positive for lignin when stained with acid phloroglucinol (Akin and Burdick, 1975). The terms *digestion*, *fermentation*, *extraction*, and *degradation* have slightly different meanings. However, the SEM cannot distinguish these differences nor quantitatively measure them but only reveal the presence or absence of a particular tissue type. Composite images can be made showing differential degradation.

Leaf Section Controls. Leaf blade sections of the five grasses served as the basic control. For all the grasses, the leaf blades showed normal intact plant tissues as previously observed (Akin and Burdick, 1975).

Leaf Sections in Buffer Control. In all five grasses the mesophyll was disturbed and slightly collapsed (Figure 1, M). For Ky-31 (Figure 1b, E) the epidermis showed some distortion, and in OG the parenchyma bundle sheath (Figure 1d, B) was partially collapsed in some cases. In CX-1, (Figure 1c, P) the phloem was removed to some extent, but infrequently. These samples were not critical point dried during preparation for SEM. While critical point drying will preserve the intact sections, detrimental effects were noted with many of the treated samples and

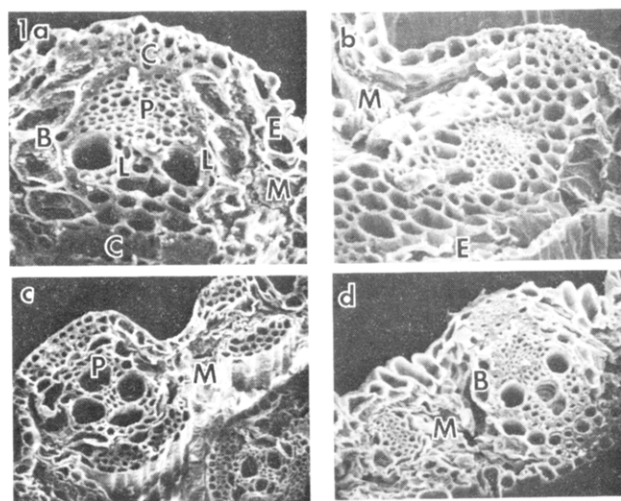


Figure 1. SEM of control leaf sections incubated in buffer. (a) is labeled to show the tissue found in the blades of all of these grasses. Tissues present are epidermis (E), parenchyma bundle sheath (B), vascular bundle including the lignified inner bundle sheath and xylem cells (L) and the unligified phloem (P), the lignified sclerenchyma cells (C), and mesophyll (M). (a) Coastal Bermuda grass. Tissues are intact but with collapsed mesophyll (M) cells. $\times 240$. (b) Ky-31 tall fescue. Tissues are intact but with collapsed mesophyll (M) and distorted epidermis (E). $\times 153.6$. (c) CX-1 Bermuda grass. Mesophyll (M) is somewhat distorted and the phloem (P) removed from some sections. $\times 153.6$. (d) Orchard grass. Mesophyll (M) tissues are distorted and parenchyma bundle sheath (B) partially collapsed. $\times 153.6$.

in particular with the acid detergent extracted leaf blades. These effects included disruption due to agitation during washing and dehydration and explosion of sections if the pressure of the critical point dryer was released too quickly. In some cases, the control leaf section in buffer for 48 h showed a loss in rigidity (although tissues were not removed) in many of the tissues when compared with the leaf section control where all tissues, except mesophyll, maintained structural integrity.

Acid Detergent Extracted Leaf Sections. Generally, the cell walls of the mesophyll, phloem, and other fragile and readily hydrolyzable cells were removed leaving a residue of lignified cells, i.e., sclerenchyma and lignified vascular bundles (Figure 2). Usually, the parenchyma bundle sheath was removed in the cool-season grasses but often remained in the warm-season grasses (Figure 2c, arrows). This residual parenchyma bundle sheath tissue (arrow) was generally found associated with the small vascular bundles. In earlier studies (Akin et al., 1975) with no stirring and washing and without vacuum filtration of samples, considerably more parenchyma bundle sheath tissue was seen associated with the large vascular bundles. In the present study, physical agitation incurred by stirring the sample during ADF extraction probably contributed to excessive tissue destruction and apparent removal. This physical treatment is, however, a part of the analytical procedure used on ground grass samples, and, therefore, physical disruption of tissue could occur in the normal preparation of ADF. In all the samples the sclerenchyma was separated from the vascular tissue and, in many cases, separated into individual fiber cells (Figure 2b, C). At low magnification, a rigid structure resembling a leaf still was observed in all grasses except Ky-31. Ky-31 ADF was shattered, showing extensive destruction and tissue removal and a residue of loose fiber with no resemblance to a leaf section.

Figure 3 shows that the effect of buffer on the ADF residue was to further remove tissue in the acid detergent

Table I. Percentage Composition and IVDMD^a of Grasses

grass	NDF	ADF	PML	CP	IVDMD	NDR
Coastal Bermuda grass	72.66	34.90	4.34	8.54	57.41	42.59
Coastcross-1 Bermuda grass	74.04	36.67	4.65	9.85	61.17	38.83
Ky-31 tall fescue	47.10	25.85	3.52	13.08	64.54	35.46
Kenhy fescue	47.37	26.42	3.07	13.51	66.87	33.13
orchard grass	53.64	30.78	3.61	11.84	64.53	35.47

^a NDF = neutral detergent fiber, ADF = acid detergent fiber, PML = permanganate lignin, CP = crude protein, IVDMD = in vitro dry matter disappearance, and NDR = nondigestible residue (100 - IVDMD).

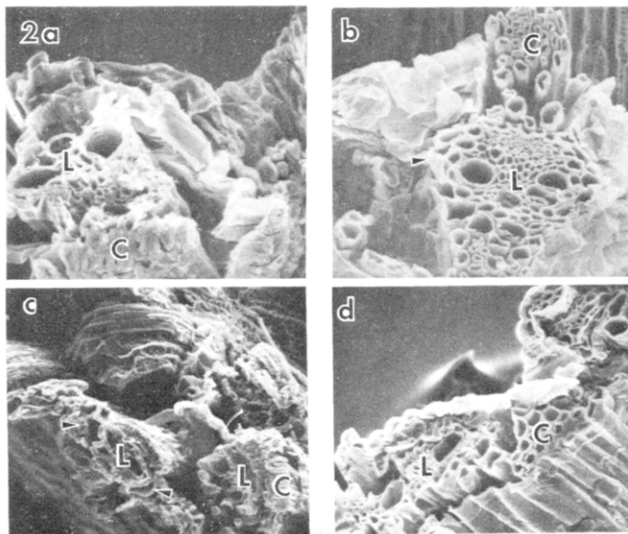


Figure 2. SEM of acid detergent extracted leaf blade sections. Generally, the more fragile tissues such as mesophyll and phloem have been removed (cf. Figure 1a) and the lignified tissues make up the residue. Tissues are extensively distorted and disrupted. (a) Coastal Bermuda grass. The residue consists of sclerenchyma cells (C) and lignified vascular tissue (L). $\times 480$. (b) Ky-31 tall fescue. The sclerenchyma has been separated into individual cells (C), but the lignified vascular tissue (L) is intact. Note that the parenchyma bundle sheath (arrow) occasionally resists extraction. $\times 153.6$. (c) Coastcross-1 Bermuda grass. In addition to sclerenchyma cells (C) and lignified vascular tissues (L), some parenchyma bundle sheath cells remain (arrow). $\times 153.6$. (d) Kenhy tall fescue. Sclerenchyma cells (C) and lignified vascular tissue (L) remain. $\times 153.6$.

extracted samples of OG. All samples showed some additional washout of tissues, with Ky-31 exhibiting the most tissue removal. Orchard grass ADF which was incubated in buffer consisted of essentially lignified vascular bundles with phloem and patches of sclerenchyma held together by cuticle. Acid detergent leaf sections of OG control (Figure 3a) and blades incubated in buffer (Figure 3b) were essentially identical, with a little more distortion of the blade and removal of sclerenchyma in sections of ADF in buffer. The CBG sample of ADF in buffer did not have any parenchyma bundle sheath associated with the large vascular bundle.

Leaf Sections Digested for 48 Hours. All untreated grasses exhibited extensive digestion by rumen microorganisms. In CBG, however, some parenchyma bundle sheath remained attached to the lignified vascular bundle (Figure 4a, arrow). The sclerenchyma was difficult to distinguish from pieces of fiber which had broken loose but was obviously present in the mat of fiber obtained in Ky-31. The CX-1 and OG (parts b and d of Figure 4) samples were virtually identical; the only tissue that remained was lignified vascular bundles held together by the cuticle. However, all of the samples retained a rigid enough structure to resemble a leaf. These leaf section samples compared favorably with the samples from earlier studies

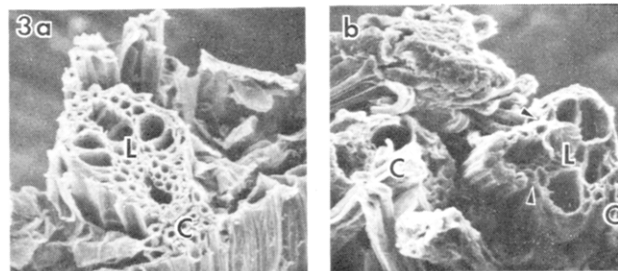


Figure 3. SEM of acid detergent extracted leaf sections of orchard grass. (a) Control (i.e.) sample has not been incubated in buffer. Sclerenchyma is being separated into cells (C) but the lignified vascular tissue (L) is generally intact. $\times 240$. (b) Incubated in buffer for 48 h. Blades lack the rigidity of controls and sclerenchyma (C) and lignified vascular tissues (L) show extreme disruption and distortion (arrows). $\times 240$.

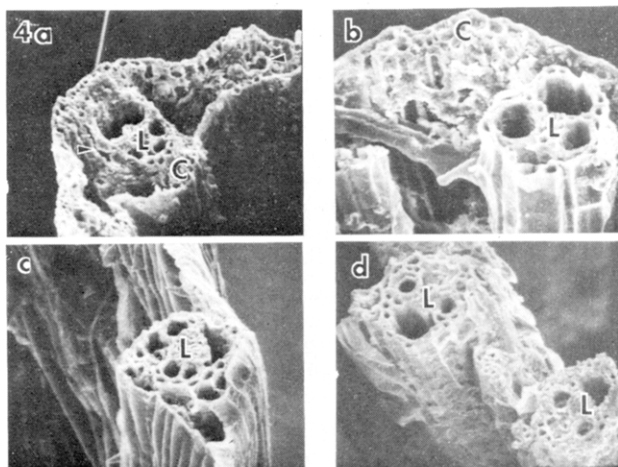


Figure 4. SEM of leaf blades incubated with rumen microorganisms for 48 h. Generally, only sclerenchyma cells (C) and lignified vascular tissue (L) resist microbial digestion. (a) Coastal Bermuda grass. Note the remnants of parenchyma bundle sheaths (arrows) that resist digestion. $\times 153.6$. (b) Coastcross-1 Bermuda grass. $\times 240$. (c) Kenhy tall fescue. $\times 153.6$. (d) Orchard grass. $\times 153.6$.

(Akin et al., 1975), even though the IVDMD of the present grass samples was lower [Table I vs. Barton et al. (1976)]. In Ky-31 there was some evidence of breakdown of the outer portion of the inner bundle sheath cells.

Acid Detergent Extracted Leaf Sections, Digested in Vitro for 48 Hours. The acid detergent extracted residue from in vitro digestion from all the grasses showed degradation in excess of the acid detergent extracted leaf sections incubated in buffer controls, but the response was variable (Figure 5) for different species. In CBG and CX-1 some of the vascular bundles were broken, and the sclerenchyma was separated into individual cells and, for the most part, were not observed. Figure 5a,b shows the isolation of lignified vascular bundles of CX-1. Some parenchyma bundle sheath tissue was infrequently attached to lignified portions of large vascular bundles in CBG, but

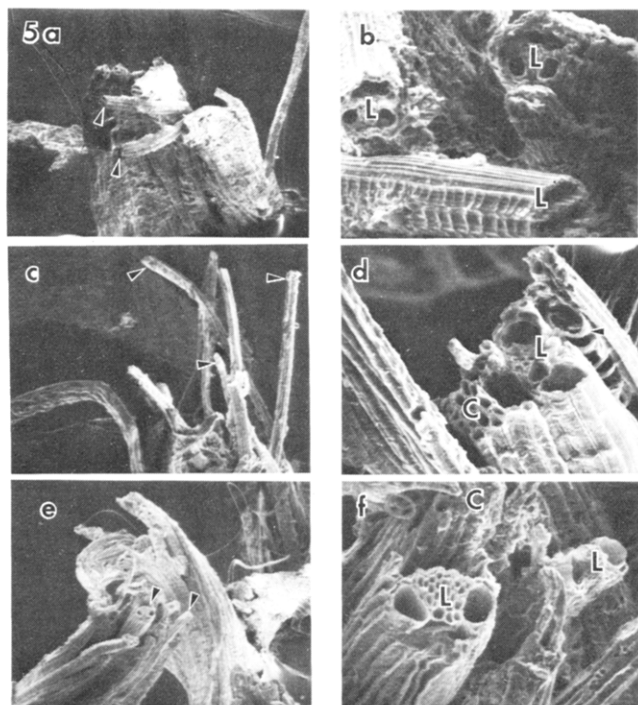


Figure 5. SEM of acid detergent extracted leaf blades that were then incubated with rumen microorganisms for 48 h. (a) Coastcross-1 Bermuda grass. The residue of the leaf and isolated vascular bundle (arrows) are recognizable. $\times 24$. (b) Coastcross-1 Bermuda grass. Isolated lignified vascular bundles (L) are present. $\times 153.6$. (c) Orchard grass. Breakup of the blade is advanced and cuticle (C) and isolated vascular bundles (arrows) are present. $\times 24$. (d) Orchard grass. The lignified vascular (L), cuticle (C), and sclerenchyma cells are generally intact, but the inner bundle sheath is broken at the metaxylem vessel (arrow). $\times 153.6$. (e) Kenhy tall fescue. Breakup of the blade is advanced and vascular bundles (arrows) are present. $\times 24$. (f) Kenhy tall fescue. Breakup of the lignified vascular bundles (L) occurred and sclerenchyma cells (C) are distorted. $\times 153.6$.

none was seen for CX-1. The breakup of OG leaf sections was extensive (Figure 5c,d), and although the sclerenchyma cells were still attached to the vascular bundle, all the larger vascular bundles were broken at the metaxylem vessels. The spiral structure of the xylem cells apparent in Figure 5d (arrow) indicates that the lignified tissues were partially degraded. The exposed xylem spirals were not apparent when only one treatment, i.e., microbial digestion or acid detergent extraction, was used. Kenhy was the least degraded of the cool-season grasses (Figure 5e,f), but generally the large vascular bundles were still partially degraded (Figure 5f, L). The sclerenchyma cells (Figure 5f, C) remained in patches but had broken away from the vascular bundle. There was little residue remaining for the Ky-31 sample and no tissue which could be identified with the SEM. These findings appear to differ from the gravimetric results reported earlier (Barton et al., 1976); however, SEM affords no way to assess the actual amount of dry matter removed, but only that it appears to be extensive; SEM will reveal nothing about the mechanism of tissue removal.

Leaf Sections Digested for 48 Hours and Then Extracted with Acid Detergent. For all grasses except Ky-31 this order of treatments, i.e., microbial digestion followed by ADF, showed destruction of tissue in excess of either treatment alone (Figure 6). Even CBG, the grass most resistant to both digestion and acid detergent extraction, showed cleavage of the large vascular bundle, with most of the sclerenchyma distorted and separated from

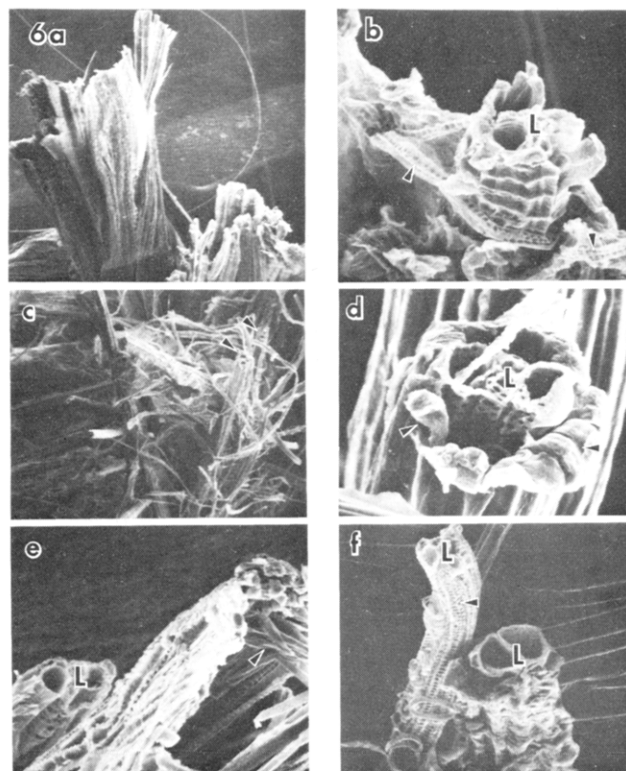


Figure 6. SEM of leaf blades incubated with rumen microorganisms for 48 h and then extracted with acid detergent reagent. (a) Coastcross-1 Bermuda grass. The blade structure is recognizable, but breakup is advanced. $\times 24$. (b) Coastcross-1 Bermuda grass. Lignified vascular tissue (L) are broken apart, and the pitted nature of the fibers is evident (arrows). $\times 240$. (c) Orchard grass. The blade structure has been totally reduced to fiber cells and some intact vascular bundles (arrows). $\times 24$. (d) Orchard grass. The lignified vascular tissues (L) are broken apart (arrows). $\times 480$. (e) Kenhy tall fescue. Remnants of vascular bundles (L) and fibers (arrow) are present. $\times 153.6$. (f) Kenhy tall fescue. Extreme degradation of lignified vascular tissue (L) showing the pitted (arrow) and other lignified cells. $\times 240$.

the vascular tissue. Many of the tissues took on a "filmy", as opposed to fibrous, nature. The small vascular bundles were removed. For all grasses, it appeared that the large vascular bundles (i.e., metaxylem vessels with connective tissue) made up the residue. The leaf integrity was destroyed, but enough tissue was left between the cuticle layers to hold them together (Figure 6a). The pitted nature of the lignified vascular cells (Figure 6b,d,f, arrows) indicated the extent of destruction resulting from the combination of these two treatments.

Gravimetric Analysis of Digestion. The chemical composition of the five grasses used in this study (Table I) were typical of those for grasses used in other studies (Barton et al., 1976). Although these grasses were harvested in 1978 from the same experimental plots as those in Barton et al. (1976), the Bermuda grasses in this study were lower in IVDMD by 5–8% units, and the NDF values were 12–14% units higher than those reported by Barton et al. (1976).

The electron micrographs in Figures 2 and 4 show the relative amount and type of tissue remaining after acid detergent extraction and 48-h incubation with *in vitro* rumen microorganisms, respectively. The residues that remained (Table I) were ADF (i.e., percentage ADF = residue) and the nondigested residue (NDR) from the *in vitro* fermentation (100 - IVDMD). In all cases, the percent ADF was smaller than the NDR, which substantiates the findings derived from the micrographs in Figures

Table II. Percentage Digestibility of the Five Grasses, ADF's,^a and the Combined Dry Matter Disappearance (CDMD)^b

grass	% IVDMD	% IADFD	% ADER	CDMD-1	CDMD-2
Coastal Bermuda grass	57.41	5.66	46.56	77.24	67.08
Coastcross-1 Bermuda grass	61.17	5.91	41.17	77.16	68.53
Ky-31 tall fescue	64.54	5.76	44.73	80.40	74.93
Kenhy tall fescue	66.87	7.18	47.15	82.49	75.50
orchard grass	64.53	6.98	53.86	83.63	71.35

^a Isolated ADF disappearance (IADFD) is the dry matter removed from isolated ADF by rumen microorganisms; acid detergent extracted residue (ADER) is the percentage dry matter removed by the extraction with acid detergent reagent of the in vitro residue. ^b CDMD-1 = (NDR × ADER/100) + IVDMD; CDMD-2 = (% ADF × IADFD/100) + (100 - ADF).

2 and 4 that the acid detergent extraction removed more dry matter than did the rumen microorganisms. However, there were certain discrepancies between the treatments in tissue types remaining. For CBG, some digestible tissue (parenchyma bundle sheath) resisted the ADF treatment but was removed by the rumen microorganisms in vitro. For Ky-31 the amount of tissue removed (hydrolyzed) by the ADF reagents far exceeded the dry matter removed by rumen microorganisms. The results of Barton et al. (1976) showed a larger difference than found in this study between the in vitro digestibility of isolated ADF from warm- and cool-season grasses. However, the warm-season grasses in that study were of higher quality (66.1 vs. 57.4% IVDMD) than those in this study, and the ADF might be expected to also have a higher digestibility.

The process of isolating the ADF could modify the tissues to the extent that they would be subject to increased tissue removal by a subsequent treatment, i.e., the action of rumen microorganisms during the determination of IVDMD. It is also possible that the preparation of ADF leaves traces of hexadecyltrimethylammonium bromide in the fiber that can inhibit digestion (Cross et al., 1974). Reversal of these treatments, i.e., in vitro digestion followed by treatment with acid detergent reagents, would remove more tissue than either alone. The electron micrographs in Figure 3, 5, and 6 show the effect on the grasses of combined (i.e., digestion and acid detergent extraction) treatments. The gravimetric results of those combined dry matter disappearance (CDMD) values are given in Table II. In all cases the effect of combined treatments (Table II; Figures 3, 5, and 6) was that more tissue was removed than was removed by either treatment alone. The order of the treatment also was important. More tissue was removed by treating digested leaf sections with acid detergent reagent than the reverse (Figures 5f and 6e).

Gravimetrically, the difference between the CDMD values was 5–12% units, depending on the grass (Table II). The methods of calculating CDMD reflected the treatment order (CDMD-1 and -2), i.e., for the effect of acid detergent reagents on the NDR (CDMD-1) and the effect of digestion (in vitro rumen microorganism fermentation) on isolated acid detergent extracted leaf sections (CDMD-2). These values (CDMD-1 and -2) are, essentially, material balances for the combined treatments.

These results support the conclusions of previous studies and show that the tissues in the leaf sections of the grass species examined, representing temperate and tropical

grasses, respond differently to acid detergent extraction (Akin et al., 1975), digestion (Akin and Burdick, 1975), and digestion of ADF (Barton et al., 1976). Further, the combination and digestion and ADF treatment removes more tissues, both qualitatively and quantitatively, than either treatment alone. Also, the order of treatments is important, for more tissue was removed by treatment of in vitro residues with acid detergent reagent than were removed by the in vitro digestion of acid detergent extracted leaf sections. Finally, Figures 2 and 4 show that ADF approximates the extent of digestion in vitro. The differences noted by Akin et al. (1975) and those reported herein for warm- and cool-season grasses should be used to interpret the use of ADF values in a quantitative prediction equation. The ADF value for warm- and cool-season grasses would overestimate, but to a lesser extent with the warm-season grasses. Therefore, ADF, as a predictor of the extent of digestion, is not adequate when used for both warm- and cool-season grasses. However, the use of qualitative data on the structure of the forage cell wall and the response of its respective tissues to extraction with ADF reagent can add additional information on the factors influencing forage digestion.

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