IMPACT OF LIGNIFICATION OF CORN STOVER FRACTIONS ON CELL WALL DEGRADATION BY RUMEN MICROORGANISMS AND RESPONSE TO AMMONIA TREATMENT
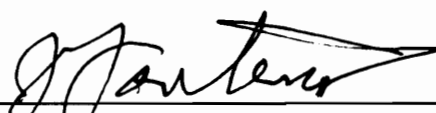
by

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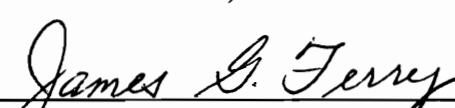
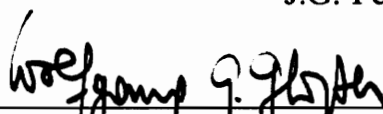## DOCTOR OF PHILOSOPHY

in

Animal Science

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IMPACT OF LIGNIFICATION OF CORN STOVER FRACTIONS ON CELL WALL DEGRADATION BY RUMEN MICROORGANISMS AND RESPONSE TO AMMONIA TREATMENT

by

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(Abstract)

Changes in cell wall composition and *in vitro* degradation of corn stover fractions (leaf, upper stem and lower stem) with advancing maturity and in response to NH_3 treatment were determined, and possible inhibitory mechanisms of lignin were evaluated. With advancing maturity, IVDMD decreased ($P < .001$), associated with decreases ($P < .001$) in CP and water soluble carbohydrates (WSC), and increases ($P < .001$) in NDF and ADF. The IVDMD of leaf was higher ($P < .001$) than of stems, associated with higher CP, hemicellulose:cellulose, and arabinan:xylan, and lower lignin methoxyl content.

A hypothesis of formation of reactive quinone methide intermediates from lignin during rumen fermentation was tested *in vitro* by incubating corn stover fractions with S-containing reducing agents. Sulphur incorporation into residual fiber occurred ($P < .05$), indicative of nucleophilic addition to quinone methide intermediates. Degradation of NDF was highly correlated with lignin methoxyl content.

The impact of lignin on cellulose degradation was studied using lignocellulosic hydrogels, in which hydroxypropylated or unmodified hardwood lignin was blended

with cellulose. *In vitro* cellulose degradation of lignocellulose blends was higher ($P < .01$) than of control. Addition of lignin at incubation depressed ($P < .01$) cellulose degradation. Hydroxypropylation enhanced ($P < .001$) the increase in cellulose degradation with lignin blending, and reduced ($P < .001$) the inhibitory effect of lignin addition at incubation.

Treatment of drought-stressed corn stover with 3% aqueous NH_3 decreased ($P < .05$) NDF, compared to isonitrogenous NH_3 addition and control, associated with solubilization of hemicellulose. Esterified phenolic acids were released ($P < .05$) by NH_3 treatment in upper stem. The IVDMD and NDF degradation increased ($P < .001$) after ammoniation, with higher ($P < .05$) values for NH_3 treatment than NH_3 added in leaf.

The *in vitro* response to ammoniation of fractions of drought-stressed and non-drought stressed corn stover harvested in subsequent years was compared, using N-sufficient and N-limiting buffers. Response was highest ($P < .001$) for non-drought stressed stover fractions, and in N-limiting medium. Response appeared to be affected by high concentration of WSC in lower stalks of drought-stressed stover.

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List of Abbreviations

ADF	acid detergent fiber
ADIN	acid detergent insoluble nitrogen
ADL	acid detergent lignin
ANOVA	analysis of variance
CP	crude protein
DM	dry matter
GLC	gas-liquid chromatography
GLM	General Linear Model
HPLC	high performance (pressure) liquid chromatography
IVDMD	*in vitro* dry matter disappearance
n	number of samples
NDF	neutral detergent fiber
OM	organic matter
r	correlation coefficient
R^2	multiple coefficient of determination
SE	standard error
UV	ultraviolet
VFA	volatile fatty acid

Introduction

Optimal utilization of forages and crop residues is becoming increasingly important in livestock production systems in many regions of the world. In densely populated areas of high soil fertility, emerging problems of overproduction and soil pollution have emphasized the necessity of paying attention to sustainability of production systems. An important aspect of maintaining sustainability is to refocus from maximum production to optimum efficiency in production as the primary goal. This can be achieved by improved utilization and recycling of available resources, whereby the use of external inputs can be limited to complement the local resources.

By virtue of rumen microorganisms, ruminants can utilize plant fibers undegradable to mammalian enzymes, as well as non-protein N (NPN). Therefore, the ruminant is the ideal animal to utilize resources that would otherwise be wasted. Ruminants can graze range forages on non-arable land, and can utilize fibrous agricultural by-products. In many developing countries, ruminant production depends on fibrous agricultural by-products such as corn stover, wheat straw and rice straw as feed resources, since most of the available cultivatable land is dedicated to food crop cultivation, leaving little opportunity for permanent pastures or forage crops.

The extent to which mature forages and crop residues can be utilized is limited due to low concentration of nutrients available to the animal. Forage quality declines rapidly with increasing maturity, associated with a change in the relative contribution of leaves and stems, towards more stems, and a declining concentration of easily digestible cell solubles and increasing cell wall content. Crop residues consist mainly of cell wall material, because the cell contents have been transformed into cell wall material or transported to the seed. Stems are generally less easily digested than leaves, due to lower CP, higher cell wall content, different cell wall carbohydrate composition and a higher proportion of lignified tissue. The differences in composition between stem and leaf are related to their respective functions, which are primarily support and transport functions for stem, and photosynthesis/respiration for the leaf blade, with a secondary support function for the leaf sheath.

The processes associated with the decline in overall nutritive quality of corn plants are of practical interest, because corn, unlike small grains, can be harvested at various maturity stages for different purposes. Whole corn plants for silage can be harvested from the hard dough to the mid dent stage, depending on overall DM concentration. Within this range, the grain to stover ratio still increases, but this seems unimportant in determining silage quality (Russell et al., 1992). If corn grain is harvested at a relative immature stage (moist grain), the stover could still be ensiled (Irlbeck et al., 1993), as opposed to mature, fully dried corn stalks, which are of lowest quality. Changes in overall corn forage composition with maturity are fairly well described, but the underlying processes within botanical fractions have not been extensively evaluated.

The carbohydrate components of mature cell walls are potentially digestible, but increasing lignification of cell walls with maturation is a major limiting factor.

The inhibitory role of lignin is not well understood, and forage digestibility can vary without major changes in lignin concentration, e.g. in corn stover (Russell, 1986). Although the changes with advanced maturity in overall composition of corn stover may seem sufficient to explain the low digestibility, it is likely that lignin does affect rate and extent of microbial degradation, similar to those forages for which a more narrow relation exists between lignin content and digestibility. Lignin is not an inert polymer. Its monomer composition varies among species, plant parts and with maturity, associated with changes in the interactions of lignin with other cell wall constituents.

Nutritive quality of mature forages and crop residues can be enhanced by improved management practices, selection, and various physical and chemical pretreatments, of which NH_3 treatment is most generally applicable. Urea-ammonia treatment of crop residues is most appropriate in developing countries, for reasons of availability, farmers' familiarity with urea, simplicity of the treatment, and its effectiveness due to high temperature. Other treatments, such as alkali treatment with NaOH, oxidative delignification with alkaline H_2O_2, ozone, or SO_2, and steam explosion treatment may be more effective in increasing biodegradability of fibrous materials, but have more limited application for socio-economic or environmental reasons. Although the overall improvements in digestibility and intake after NH_3 treatment are well described, the underlying mechanism(s) are not well identified.

The overall objective of the research contained in this dissertation was to identify the structural components of corn stover of advanced maturity that impose limitations to its utilization by ruminants. To achieve this objective, experiments were conducted, describing cell wall composition of different botanical fractions of corn stover as affected by maturity and NH_3 treatment, in relation to degradation by

rumen microorganisms. In addition to the work on corn stover, in which the role of lignin was emphasized, the impact of lignin on cellulose degradation was studied in a separate *in vitro* model degradation study.

Review of Literature

Composition and Digestibility of Corn Stover as Affected by Maturity

The digestibility of corn stover, as for most other forages, decreases with later harvest, both before and after physiological grain maturity (Johnson et al., 1966; Weaver et al., 1978; Berger et al., 1979; Phipps and Weller, 1979). The reduction in digestibility, unlike that for other forage species, is associated primarily with an increase in cell wall concentration due to the loss of cell solubles (Weaver et al., 1978; Berger et al., 1979; Russell, 1986), rather than with increased lignification of cell walls. Water soluble carbohydrates (WSC) are translocated to the ear and deposited as corn starch, hence, WSC concentration in the stalk decreases before physiological maturity (Phipps and Weller, 1979). Corn harvested 4 to 5 wk after physiological maturity had a higher grain-to stover-ratio, higher stover concentrations of NDF, ADF and ADL, lower stover concentrations of CP and total non-structural carbohydrates (TNC), and lower IVDMD than corn harvested at physiological maturity (Russell, 1986; Irlbeck et al., 1993). Russell (1986) found a moderate negative correlation between IVDMD of stover and grain-to-stover ratio. However,

grain-to-stover ratio was not a significant variable in multiple regressions predicting IVDMD of whole corn forage or silage (Russell et al., 1992) and stover forage or silage (Irlbeck et al., 1993). Concentrations of NDF, ADF, and ADL in stover were significant variables in these equations (Irlbeck et al., 1993). With later harvest, concentration of ADL in DM increases, but its concentration in NDF seems unaffected by maturity (Russell, 1986; Irlbeck et al., 1993).

Cell Wall Components

Plant cell walls are composed of three main types of compounds: homopolysaccharides (cellulose), heteropolysaccharides (pectins, hemicelluloses) and phenolic compounds (lignin, tannins, phenolic acids) (Bacic et al., 1988). Pectins are polymers that are solubilized in hot water, chelating agents or dilute acid. Hemicelluloses are polymers extractable under alkaline conditions and cellulose is the carbohydrate residue after such extraction. (Tucker and Mitchell, 1993). Lignin is the primary phenolic polymer in most forages. The structural properties of cellulose, hemicellulose, and lignin are very diverse.

Cellulose. Cellulose is a linear homopolysaccaride composed of $\beta(1->4)$-linked D-glucopyranosyl residues (Daniel, 1985). Each anhydroglucose unit is rotated at 180°C relative to the adjacent unit. On the basis of tacticity and enzyme specificity (cellobiase), the only repeat unit in cellulose is cellobiose. The glucan chain has a two-fold screw axis, which is stabilized by intramolecular hydrogen bonding. The linearity of the cellulose polymer, and the rigidity of the $\beta(1->4)$ linkage permit the formation of intermolecular hydrogen bonds with adjacent cellulose chains, resulting

in crystalline association and the formation of microfibrils. Within the cellulose polymer, both crystalline and amorphous regions occur. The degree of polymerization of cellulose has been well characterized for cotton (Marx-Figini and Schulz, 1966). Cellulose in primary walls displays a relatively low degree of polymerization, and is polydisperse (2000 to 6000 anhydroglucose units), whereas that of the secondary wall cellulose is more highly polymerized and essentially monodisperse (14,000 units). The stiffness of the joint cellulose fibrils results in high tensile strength, but also high brittleness. In plant cell walls, cellulose is plasticized by water, hemicellulose, and lignin.

Hemicellulose. Hemicelluloses and pectins are mainly heteropolysaccharides with a variety of units (mannose, galactose, glucose, xylose), substituent side groups (arabinose, glucuronic acid, acetyl groups), branching, and linkage types (Wilkie, 1979). Primary walls of grasses differ from those of legumes in that pectic substances form only a minor part of the wall. Immature tissues of grasses contain large amounts of ß-glucans (Kato and Nevins, 1986), consisting of ß-D-glucopyranosyl residues linked by (1->3) and (1->4)-glycosidic linkages. The interrupted (1->4), (1->3) linkage pattern gives ß-glucan its overall irregular shape, preventing extensive intermolecular association.

The predominant hemicelluloses in primary walls of grasses and secondary walls of all angiosperms are heteroxylans. They comprise a family of polysaccharides consisting of a linear (1->4)-linked ß-D-xylopyranosyl backbone. Arabinoxylans or arabinoglucuronoxylans consist of a backbone of 50 to 200 xylose units substituted with residues of glucuronic acid and/or arabinose (Wilkie, 1979). Xylans of grasses also contain acetyl groups and esterified phenolic acids, which can be released with alkali (Bacon et al., 1981). Although the conformation of hemicellulose is similar to

that of cellulose, the side chains and acetyl groups prevent tight association among arabinoglucuronoxylan chains. Due to its lower molecular weight and the substituent groups, hemicellulose can be extracted by alkali.

Hemicellulose can form various types of interlinkages with other cell wall components. With exception of linear xylans (Hatfield, 1989), hemicelluloses are rarely involved in hydrogen bonding, which is reflected in its morphological structure. Hemicelluloses are part of the amorphous cell wall matrix, surrounding the cellulose crystallites, to which they are associated superficially, probably by hydrogen bonds, and form interlinkages with lignin. In a sense, hemicelluloses serve as plasticizers ('shock absorbers') of cell walls and as coupling agent between cellulose and lignin.

Lignins. Lignins have been classified as "core lignin" and "non-core lignin" (Gordon and Griffith, 1973; Gordon, 1975; Jung, 1989). Core lignins are polyphenols formed by dehydrogenative polymerization of phenylpropanoid precursors into a three-dimensional network (Sarkanen and Ludwig, 1971), which may contain up to 20 different bond types (Nimz, 1974). Phenolic monomers are often referred to as non-core lignin (Jung, 1989). In this review, core lignin is referred to as simply lignin, as opposed to the monomeric phenolic acids. Lignin can be classified into guaiacyl and guaiacyl-syringyl lignin, based on the ratio of the aldehyde derivatives (vanillin and syringaldehyde) found in nitrobenzene oxidation products (Sarkanen and Ludwig, 1971). The first class (guaiacyl) comprises gymnosperm lignin, and the second class (guaiacyl-syringyl) comprises angiosperm lignin, including monocotyledons. Large amounts of *p*-hydroxybenzaldehyde (H) are found only in grasses, which has lead to the distinction of grass lignin (GSH-lignin) from other angiosperm lignin (Nimz et al., 1981). More recently, it has become apparent that with respect to monomer composition, variations exist within the lignin fractions derived from a single species.

In the case of corn, different monomer compositions were demonstrated by thioacidolysis for different varieties, but also within a single botanical fraction (upper versus lower stem) of the same plant (Gaudillere and Monties, 1989). Wallace et al. (1991) suggested similar differences even among the layers of a single cell wall.

The two major low molecular weight phenolic monomers present in forage cell walls are ferulic acid (FA) and p-coumaric acid (p-CA), which are actually precursors to the lignin polymer. Phenolic acid concentrations in the corn plant were found to be higher in stem than in leaf, with intermediate levels in the leaf sheath (Hartley and Haverkamp, 1984). Phenolic acids occur mainly in the cell wall as monomers, but dimers of FA (dehydrodiferulic acid) (Hartley and Jones, 1976; Markwalder and Neukom, 1976), and cyclodimers of p-CA and FA have been identified (Hartley et al., 1988; Ford and Hartley, 1988, 1989, 1990).

Ferulic acid is generally linked to hemicellulose, whereas p-CA is associated more with the core lignin (Atsushi et al., 1984), although a compound containing p-CA bound to hemicellulose has been isolated by Mueller-Harvey et al. (1986). Phenolic acids are generally ester-linked via their carboxyl group to lignin or hemicellulose. Scalbert et al. (1985) provided evidence for ether linkage of FA via its phenolic group to lignin. Therefore, FA could form alkali-labile bridging units between lignin and hemicellulose by being simultaneously esterified to hemicellulose (Smith and Hartley, 1983) and etherified to lignin. Another possibility for cross linkage would be through dimers of both FA and p-CA (Hartley and Ford, 1989). Generally, content of FA is stable or decreases slightly, while p-CA increases with increasing maturity (Chaves et al., 1982; Atsushi et al., 1984; Grabber et al., 1991; He and Terashima, 1991).

Silica. Silica is present in forages and crop residues in the opaline form ($SiO_2.nH_2O$) and is probably deposited in the wall together with the carbohydrates during the process of secondary wall thickening (Jones et al., 1963). Silification of cell walls is another mechanism by which plants obtain mechanical support, hence the silica in grass leaf cell walls helps to keep leaf blades erect. Silica is high in rice straw and retards cell wall degradation (Van Soest and Jones, 1968), although in temperate straws the level of silica normally encountered does not influence digestibility (Hartley, 1981).

Cell Wall Development

Function of Cell Walls. Plant cell walls determine the size and shape of cells. Cells are connected to each other by a common boundary, the middle lamella, consisting of pectinaceous material (Harris, 1990). After cell division, the primary wall is deposited inside the middle lamella. One of the functions of primary walls is to yield to a certain extent to turgor pressure resulting in cell expansion. During expansion, wall material is continuously deposited. Cellulose is polymerized at the cell surface by a complex enzyme system transported to the plasma membrane (Northcote, 1972). Hemicellulose and pectin, which make up the cell wall matrix, are polymerized in the endomembrane system and are secreted in a preformed condition to the outside of the wall. After deposition, further modification (such as acetylation) may occur in the wall. During the early stages of development of primary cell walls, elasticity and plasticity are maintained to enable controlled expansion of cell walls (Preston, 1979). Therefore, interactions among polysaccharides at this time are

different from those occurring at secondary cell wall formation, being mainly limited to ionic interactions (Ca bridges) between uronic acids of polygalacturonans (pectin) and possibly glucuronoxylans, and limited hydrogen bonding among linear matrix polysaccharides and at the interface between cellulose microfibrils and matrix polysaccharides (Carpita, 1983). The degree of hydrogen bonding depends on a linear ribbon-like conformation to bring polysaccharides in close proximity (Preston, 1979). Therefore, with decreased degree of branching or substitution of the polysaccharide backbone, the extent to which polysaccharide chains become associated by H-bonding will increase. Covalent linkages are present among pectinaceous polysaccharides and possibly between pectin and arabinoxylan (Hatfield, 1989).

At completion of expansion, changes in the mechanical properties of the wall are required so that it will no longer yield to turgor pressure, i.e. deposition of the secondary wall and subsequent lignification (Harris, 1990). Some plant tissues remain unlignified, such as the parenchyma cells in stem pith and cortex, and leaf mesophyll. Their walls are usually thin and unlignified. Parenchyma cells are usually vacuolated and have living protoplasts. The turgidity of parenchyma cells contributes much of the mechanical strength of some plant organs, and their walls have sufficient tensile strength to resist high turgor pressure (Carpita, 1985). Parenchyma walls of dicotyledons typically contain a high proportion of pectic polysaccharides, smaller amounts of xyloglucans, extensins and other proteins (Bacic et al., 1988). Parenchyma walls of the monocotyledon family *Poaceae* (grasses and cereals) contain heteroxylans (glucoronoarabinoxylans) as the major matrix component together with variable amounts of (1->3),(1->4)-ß-glucans (Bacic et al., 1988) and proteins. Ferulic acid and other phenolic acids occur bound to the walls (Harris and Hartley, 1980).

Cell Wall Maturation. During cell enlargement, the primary wall appears to be a disorganized mass. During cell differentiation and enlargement the plant undergoes various structural modifications, while maintaining its physical function of support. The ß-glucans, initially high in immature primary walls of corn are turned over rapidly after initial growth, and are low or absent in mature tissues (Luttenegger and Nevins, 1985). Along with changes in ß-glucans, there are changes in the types of xylans in primary walls. With increasing maturity, xylans become less substituted with arabinose residues (Carpita, 1983; Morrison, 1974a; Wilkie, 1979), but it is not clear whether this represents a shift in synthesis pattern or enzymatic cleavage of arabinose from the xylose backbone. The reduced arabinose substitution leads to a less pliable matrix due to increased potential for hydrogen bonding between the less branched xylans and cellulose microfibrils (Hatfield, 1989).

In some cell types (support tissues), wall material is deposited inside the primary wall after cell expansion has stopped (Harris, 1990). This is the secondary wall, which may be lignified or unlignified (Bacic et al., 1988). Unlignified walls have a high tensile strength and stiffness due to the mechanical properties of cellulose. Concurrent with secondary cell wall formation is the initiation of lignin formation. Lignin is transported in the cytosol in the form of its building units (or their glycosides), and polymerization of lignin precursors occurs in the cell wall. Lignification, as studied by microspectrophotometry, occurs first at the time of onset of secondary wall formation (Northcote, 1972). It is first deposited at the cell corners just inside the primary wall, then extends to the middle lamella and spreads into the primary wall, and finally into the secondary wall.

Secondary walls of sclerenchyma and xylem consist of three layers based on the orientation of cellulose microfibrils: a relatively thick middle layer (S_2), and

relatively thin outer (S_1) and inner (S_3) layers. The lignification of pine xylem was shown to proceed in three distinct stages, always preceded by deposition of polysaccharides (Terashima et al., 1988). In the first stage, after the start of formation of the outer S_1 layer of the secondary wall, lignification occurs at the cell corner and middle lamella. The second stage is a slow lignification occurring during polysaccharide deposition in the S_2 layer. The main lignification occurs in the third phase after the start of S_3 formation. The resulting hydrophobic lignin displaces the water in the matrix surrounding the microfibrils, which results in a rigid wall with high compressive strength and low porosity. Lignin deposition always occurs in the preformed carbohydrate matrix, which is a key factor in creating the unique architecture of plant cell walls. Covalent bonds are formed during the dehydrogenative polymerization of monolignols in the pectin or hemicellulose gel. One of the causes of lignin heterogeneity among cell wall layers may be the fact that the dehydrogenative polymerization of monolignols occurs in distinct, preformed layers of pectin in middle lamella and primary wall and hemicellulose in primary and secondary walls, resulting in a more condensed lignin in the middle lamella and primary wall (Terashima et al., 1988).

Plant cell types which at maturity have lignified secondary walls are sclerenchyma fibers and xylem tracheary elements (vessels) (Harris, 1990). Sclerenchyma fibers are long and narrow, have pointed tips, and have thick, lignified secondary walls. Sclerenchyma fibers have an important support role in the plant. Vincent (1982) calculated that sclerenchyma fibers accounted for more than 90% of the longitudinal stiffness of the ryegrass (*Lolium perenne*) leaf, even though they contribute only a minor fraction of the total cross-sectional area. A minor change in volume fraction of sclerenchyma had a large effect on leaf strength. Contrary to the

traditional view that cells with lignified secondary cell walls are dead, sclerenchyma fiber cells in ryegrass (*Lolium temulentum*) contained cytoplasm and continued to elongate even after laying down a thick, lignified secondary wall (Lawton et al., 1979a,b). The lignified walls of vascular tissue are impermeable to water and resist the tensile forces generated in the lumen of these cells during transpiration. Unlike sclerenchyma fibers, the secondary wall of tracheary elements usually is not deposited uniformly over the primary wall (Harris, 1990). Tracheary elements that are formed initially (protoxylem) in elongating organs have lignified, secondary wall thickenings deposited in the form of rings or helices, which allow the cells to stretch. In the later formed metaxylem, the pattern of lignified, secondary thickening is more extensive with different patterns, i.e. reticulate or pitted.

The major matrix polysaccharides in sclerenchyma fibers and xylem vessels from dicotyledons are heteroxylans (4-O-methylglucuronoxylans) (Bacic et al., 1988). Gordon et al. (1985) found arabinoglucuronoxylans in sclerenchyma cell walls of ryegrass (*L. perenne* and *L. multiflorum*) leaves, and FA and *p*-CA bound to these walls. Parenchyma cell walls isolated from plant parts of orchardgrass (*Dactylis glomerata* L.) and switchgrass (*Panicum virgatum* L.) at different maturities showed considerable variation in composition, whereas sclerenchyma walls were more uniform (Grabber et al., 1991). The ratios of xylose to arabinose and *p*-CA to FA were generally lower in parenchyma than in sclerenchyma, and in leaf blade or sheath compared with stem.

Molecular orientation, intermolecular associations and supermolecular structure of lignohemicellulose matrices are heavily subject to speculation. By Raman microprobe study of secondary cell walls, the aromatic ring of lignin monomers has been found to be arranged in the plane of the cell wall surface (Agarwal and Atalla,

1986), indicating a relation to the preferential orientation of cellulose microfibrils. However, no orientational order of lignin towards cellulose has been found in wood by solid state NMR spectroscopy (Hatfield et al., 1987). Lignin can be physically and chemically associated with other cell wall polymers, notably hemicellulose, protein, and cellulose (Fengel and Wegener, 1989), the type of association differing according to plant species. During and after lignin deposition, covalent bonds are formed between carbohydrates and lignin. Evidence exists for linkages through etherification of lignin to phenolic acids (particularly FA), previously esterified to arabinose residues of arabinoxylan (Scalbert et al., 1985; Lam et al., 1992b), by addition of the phenolic hydroxyl to lignin quinone methide intermediates. It has been proposed that ester-linked FA could serve as target points for lignification (Lam et al., 1992b). Direct ester linkage of lignin to hemicellulose exists in hardwoods (Obst, 1982; Eriksson et al., 1980) and jute fiber (Das et al., 1981). In beech wood lignin-carbohydrate complex, the glucuronic acid substituents of glucuronoxylan are esterified to the α-C of lignin by addition to quinone methide intermediates (Takahashi and Koshijima, 1988). Ester linkage of lignin to cellulose fractions has been suggested also (Eriksson et al., 1980).

During plant maturation, cell walls increase considerably as a proportion of the total biomass, while relative proportions of lignin and cellulose increase, pectin decreases (especially in legumes), and hemicellulose remains stable (Hatfield, 1989). Lignification begins at the completion of cell wall expansion. Concentration of lignin is highest in the middle lamella and primary wall, but these represent only a minor portion of the total lignin in the cell wall (Saka and Goring, 1985). The secondary wall is the area of highest lignin accumulation. As secondary cell wall formation begins, a shift from a hydrated matrix to a hydrophobic one occurs (Northcote, 1972).

It has been proposed that the composition of lignin is more important than its quantity in inhibiting cell wall degradation (Gordon, 1975; Reeves, 1985a,b), but locally high concentrations of lignin effectively protect middle lamella and primary walls (Engels and Schuurmans, 1992). Overall, composition of lignin changes with maturity towards a more syringyl-rich lignin (Buxton and Russell, 1988), which could change the type and extent of interactions between lignin and polysaccharides.

Grass xylans may be synthesized in a highly substituted configuration and modified, once they are within the wall (Carpita, 1983). In the secondary wall, a low degree of substitution upon the xylan backbone allows closer packing. This, along with the replacement of water by cellulose allows formation of hydrogen bonds between xylan chains and between xylan and cellulose microfibrils. Because of the tighter association of the matrix, the interactions between lignin and carbohydrates could have larger impact upon cell wall degradation. During maturation, xylans are decreasingly substituted with arabinose or glucuronic acid side chains (Brice and Morrison, 1982). This is associated with an increase in isolated hemicellulose degradability, which is not expressed in the presence of lignin (Brice and Morrison, 1982). Changes in the frequency or type of linkages between polysaccharides and lignin further enhance wall strength and resistance to degradation.

Microbial Degradation of Different Plant Tissues and Cell Wall Constituents

Morphologically Distinct Tissues. Lower degradability of barley straw stems compared with leaf blade and sheath were closely related to the extent of secondary cell wall thickening and to the pattern of lignin distribution among the different cell

types (Goto et al., 1992). The general consensus on the order of cell wall digestibility of different leaf tissues is as follows: mesophyll = parenchyma > epidermis > parenchyma bundle sheath > sclerenchyma > vascular tissue (xylem) (Akin and Burdick, 1975; Wilson, 1990). Tissue structures that limit forage degradation are primarily the highly lignified support tissues, xylem and sclerenchyma. Temperate (C_3) and tropical (C_4) grasses are readily distinguished from each other by the lower proportion of vascular tissue and sclerenchyma and higher proportion of mesophyll in the temperate species (Akin and Burdick, 1975; Akin, 1989; Wilson et al., 1990). Tropical and temperate legumes do not show this difference (Minson and Wilson, 1980). The proportions of vascular tissue and sclerenchyma vary little with the age of an individual leaf (Akin et al., 1977) or with growth temperature (Akin et al., 1987). A marked increase in wall thickness and/or lignification may occur with age or high temperature, causing a decrease in digestibility. However, the proportions of these tissues can differ substantially between leaves on the same tiller, with less requirement of mechanical support for the earlier formed leaves and thus less vascular tissue and sclerenchyma (Wilson, 1976a,b).

Parenchyma bundle sheath cells, comprising up to 25% of the cross-sectional area of the C_4 leaf, are essential for photosynthesis in tropical grasses. They are highly resistant to bacterial degradation, presumably due to deposition of suberin in the middle lamella (Wilson and Hattersley, 1983). The higher proportion of these tissues, and the growth conditions, combine to limit breakdown of warm season grass leaves compared to cool season grass leaves. Leaflets of cool- and warm-season legume species are readily degraded due to a high proportion of mesophyll tissue. In grass stems, a higher proportion of cross sections is occupied by non-digestible epidermis, sclerenchyma ring and vascular tissue than in leaf, and poorly degradable

parenchyma tissue (Akin and Burdick, 1975; 1981). Parenchyma in legume stems is totally degraded, leaving a hollow cylinder of highly lignified material. Chemical treatments (strong alkali, oxidation) tend to affect the slowly degradable parenchyma of grass stems more than the 'undegradable' tissue, which is related to the relative content of low molecular weight phenolics and the different lignin composition in the respective tissues (Akin, 1989). With oxidative treatment, a decrease in S units is observed (Reeves, 1985b) along with a decrease in staining intensity of stem parenchyma cell walls by chlorine-sulfite (Akin et al., 1985), which stains syringyl units red (Sarkanen and Ludwig, 1971).

In the overall plant, the proportion of poorly degradable tissues increases considerably, which in terms of overall composition is expressed as an increase in NDF, and small increases in the proportion of cellulose to hemicellulose (due to the higher proportion of cellulose in the secondary wall), and an increase in lignin (Akin et al., 1977). The increase in lignin may be small, relative to the total cell wall increase, but may render a large proportion of these walls poorly digestible or undigestible.

Cell Wall Constituents. Plant cell walls are degraded in the rumen by cellulolytic bacteria, and to a smaller extent, protozoa (Coleman, 1978) and fungi (Bauchop, 1979). The most important fiber degrading bacteria, commonly found on cut edges of cell walls, are the gram negative *Bacteroides succinogenes*, and the gram positive *Ruminococcus albus* and *Ruminococcus flavefaciens* (Latham et al., 1978a,b; Akin, 1979). Bacteria, especially *B. succinogenes*, adhere closely to the cell wall, which may involve specific binding by adhesins or non-specific binding between the negatively charged glycocalix and cell wall polysaccharides.

Chesson (1983), in a holistic approach to cell wall degradation, postulated

that, since cell wall digestion is a superficial phenomenon, degradation of cell wall components is limited by the accessibility of digestible carbohydrates. Lignin accumulation at the inner plant cell wall surface during microbial digestion would be a general mechanism of retarding cell wall degradation (Chesson, 1986). This was based on the observation that barley straw and its indigestible residue have similar carbohydrate composition, (Gordon et al., 1983), implying no occurrence of selective degradation of one component. Lignin was found in slightly increased proportion in digested residue, but lignin accumulation at the surface apparently resulted in a protective layer.

Forages undergo substantial changes in composition during ruminal degradation, with a general increase in xylose residues and a decrease in arabinose and cellulose, possibly due to selective protection of hemicellulose by lignin, although this interpretation is under dispute (Chesson and Forsberg, 1986). For example, in early cut ryegrass and red clover (*Trifolium pratense* L), glucuronic acid and arabinose are indeed more readily digested than xylan and cellulose (Chesson et al., 1983; Gordon et al., 1983). The high proportionate loss of uronic acids and arabinose from immature plants was, however, related to selective degradation of easily digested mesophyll cells (Chesson et al., 1986). However, when isolated parenchyma and sclerenchyma cell types from leaf blades, leaf sheaths and stems of orchardgrass were subjected to *in vitro* digestion, removal of FA, arabinose and glucuronic acid was greater than that of other components across all cell types and plant parts (Grabber and Jung, 1991). Piwonka et al. (1991) reported xylan to be the least digestible polysaccharide constituent of mesophyll and non-mesophyll cell walls from Caucasian bluestem (*Bothriochloa caucasia* Trin) leaf tissue harvested at four maturity stages, with no differences in glucose, xylose or arabinose digestibility among tissue types,

and a decrease in xylan digestibility with increasing maturity. Xylan has consistently been the least fermentable neutral polysaccharide in alfalfa (*Medicago sativa* L) (Albrecht et al., 1987; Ben-Ghedelia and Miron, 1984; Titgemeyer, 1992), presumably due to linkage of glucuronic acid residues with lignin. The difference in disappearance among sugars indicates that lignin does not affect all cell wall components equally (Grabber and Jung, 1991).

When assessing the effect of cell wall components and their interactions on cell wall digestibility, it is imperative to distinguish between the characteristics of the individual fiber components and the characteristics of various types of composite cell walls as encountered in different plant tissues. Only then can the causes for different biodegradability, and the effects of maturity and chemical treatments be understood. For effective degradation of cellulose, a cellulolytic-enzyme complex is required, including exo- and endo- (1->4)β glucanases (Chesson and Forsberg, 1988). A restraint to cellulose degradation is the extensive hydrogen bonding giving the cellulose its crystalline nature. Cotton linters, which are highly crystalline, are known to be digested at a slower rate than samples of isolated wood cellulose (Baker et al., 1959). In studies with isolated cellulose, degradation may be limited by the degree of crystallinity: rapid degradation occurs in non-crystalline regions, which slows down greatly when the crystalline regions are attacked (Soltes, 1983). However, results obtained with X-ray diffraction by Beveridge and Richards (1975) indicated that the highly ordered and less ordered regions of cotton cellulose were equally rapidly degraded in the rumen. They also indicated that cellulose in spear grass had similar proportions of highly ordered cellulose before and after rumen digestion, suggesting that factors other than crystallinity are more important to the digestibility of cellulose from cell walls. Isolated holocelluloses from forages examined by X-ray diffraction

were more amorphous than isolated wood cellulose (Baker et al., 1959). Once cellulose is isolated from cell walls, the negative effect of maturity on digestibility diminishes, which was attributed to the presence of lignin (Kamstra et al., 1958).

Chemistry of Lignification

Increasing cell wall content and lignification of cell walls decrease forage quality during maturation (Allinson and Osbourn, 1970). The relationship between lignin content and forage digestibility was observed over 50 yr ago (Crampton and Maynard, 1938), but it was not until the 1960's that routine lignin assays were proposed as part of the detergent system for fiber analysis (Van Soest, 1963; Van Soest and Wine, 1968). A major problem in relating lignin content to digestibility is that the compounds comprising forage lignins are ill-defined. For example, Klason lignin of herbaceous material may contain cutins, residual carbohydrate, nitrogenous material, and ash. Furthermore, lignin itself is not one single compound; its composition and structure are heterogenous. The phenolic monomer composition of core lignin, as well as the type of intra-molecular linkages and interactions with other cell wall components contribute to the heterogeneity (Scalbert et al., 1985). Core lignin monomer composition (Reeves, 1985a,b), interlinkages between lignin and other cell wall components (Van Soest, 1981) and lignin distribution over the cell wall layers (Engels, 1989) are likely to affect cell wall degradability by rumen micro-organisms. When considering mechanisms of cell wall degradation, also the accessibility of the various wall types and layers to microbial enzymes should be taken into account. An overview of lignin biosynthesis is pertinent with regard to the

different types of lignin found in softwoods, hardwoods and grasses in relation to interactions with other cell wall polymers that may limit microbial degradation of plant cell walls.

Control of Biosynthesis of Lignin Precursors. Lignins are plant cell wall polymers derived from the p-hydroxycinnamyl alcohols or monolignols p-coumaryl (I), coniferyl (II) and sinapyl (III) alcohol shown in Figure 1 (Freudenberg, 1965; Freudenberg and Neish, 1968). The biogenesis and subsequent polymerization of lignin monomers have been studied in detail (Brown, 1961; Freudenberg, 1965; Freudenberg and Neish, 1968; Higuchi et al., 1967a, 1977; Adler, 1977; Gross, 1977; Leary, 1980) and recently reviewed (Grisebach, 1981; Glasser and Kelley, 1987; Higuchi, 1990; Lewis and Yamamoto, 1990).

Lignin is a three-dimensional network molecule formed by random, peroxide induced and radical propagated polymerization of *p*-coumaryl, coniferyl, and sinapyl alcohols (Freudenberg, 1965). Despite the random nature of the polymerization, lignification is under strict control by the plant. Lignin is synthesized from L-phenylalanine, L-tyrosine and cinnamic acids (Figure 2), which are produced from sugars through shikimate and cinnamic acid pathways (Higuchi, 1971). The synthesis of cinnamic lignin precursors (cinnamic acid and *p*-CA) from L-phenylalaline and L-tyrosine is under control by the activity of phenylalaline NH_3 lyase (PAL) and tyrosine NH_3 lyase (TAL) in the cell. Activity of the NH_3-lyases rises to a maximum at the time of differentiation when xylem vessels form. The distribution of TAL is limited to grasses (Higuchi et al., 1967a). L-tyrosine is not converted to lignins in gymnosperms (softwoods) and most angiosperms (hardwoods), but is transformed by grasses into lignin monomers and esterified *p*-CA (Higuchi et al., 1967a,b). The reactions catalyzed by the respective enzymes are practically irreversible, therefore

I

II

III

γ H₂COH

ß HC

α HC

p-Coumaryl
alcohol

Coniferyl
alcohol

Sinapyl
alcohol
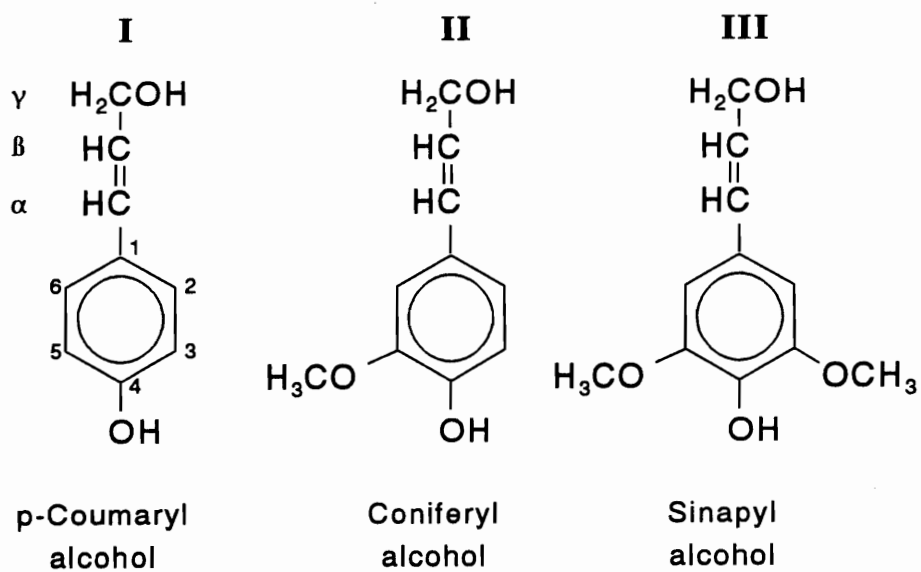
Figure 1. Chemical structure and nomenclature of the three major lignin precursors.
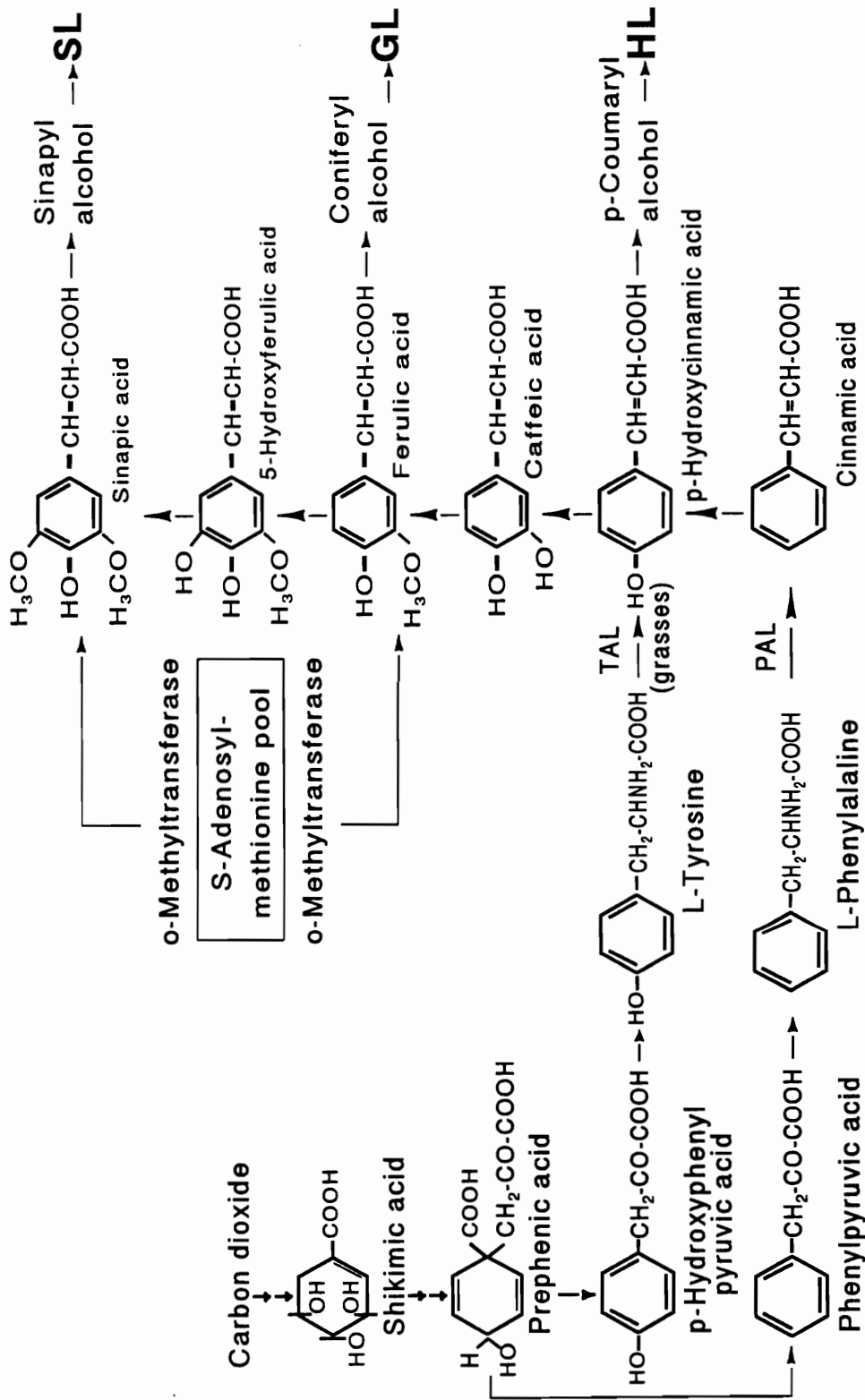
Figure 2. Cinnamic acid pathway of biosynthesis of lignin precursors (adapted from Higuchi, 1990). TAL = tyrosine ammonia lyase, PAL = phenylalaline ammonia lyase. Syringyl, guaiacyl, and *p*-hydroxyphenyl lignins are represented by SL, GL, and HL, respectively.

the appearance of NH$_3$-lyases in differentiating tissues is a definite signal to lignification (Higuchi et al., 1977). Several growth factors inducing PAL activity, as well as inhibitors of these growth factors, have been identified (Northcote, 1989).

The first direct lignin precursor, p-CA, is formed by hydroxylation of cinnamic acid (by cinnamic acid 4-hydroxylase), or by direct conversion from L-tyrosine induced by TAL. The next steps in the synthesis of the monolignols are two alternate hydroxylations of the aromatic ring and methylations of the hydroxyl groups, yielding the lignin precursors, FA and sinapic acid. The methylations are catalyzed by O-methyltransferases in the presence of S-adenosylmethionine as a methyl donor. The p-hydroxycinnamic acids are subsequently reduced to their respective alcohols, p-coumaryl, coniferyl and sinapyl alcohol. The alcohols are dehydrogenated by peroxidase enzymes into radicals, which triggers the polymerization into lignin.

The synthesis of predominantly guaiacyl lignin in gymnosperms or guaiacyl/syringyl lignins in angiosperms depends on the substrate specificity of the available O-methyltransferases (Higuchi et al., 1977). In grasses, the large pool of p-CA formed by TAL saturates the first hydroxylation step, so that parts of the acid are reduced to p-coumaryl alcohol, leading to p-hydroxyphenyl units, or converted to esterified p-CA, characteristic of grass lignins (Higuchi et al., 1977).

The conversion of the precursors p-CA, FA and sinapic acid into the respective alcohols occurs via a two-step reductive process, producing first the aldehydes, and subsequently the alcohols (Grisebach, 1981). After the monolignol formation in the cytosol, these intermediates are further metabolized into their respective glycosides, which are generally thought to be transported across the cell membrane into the cell wall, although they may only function in a storage capacity (Yamamoto et al., 1989).

Lignin Polymerization. In the wall, the dehydrogenative polymerization of the p-hydroxycinnamyl alcohols is initiated by various peroxidases and H_2O_2 (Figure 3). The reaction then proceeds by random coupling of free phenoxy radicals. This process produces a large number of different interunit linkages (Nimz, 1974). The frequency of particular linkages formed depends on the free π-electron spin density of the radicals involved, which are highest on the 1-, 3-and 5-positions, the phenolic oxygen, and the ß-carbon (Martensson and Karlsson, 1969).

However, in syringyl lignins, both ring positions 3 and 5 are occupied by methoxyl groups. In guaiacyl lignins, only the 3-position is occupied. The relative contribution of the phenolic oxygen (attached to the 4-position of the aromatic ring, thus termed O-4) to the linkage pattern is thus much larger for syringyl lignin than for guaiacyl lignins (see Table 1). Most linkages in guaiacyl lignin involving the 5-position are carbon-carbon bonds, which are much more stable than the ether linkages involving the phenolic oxygen. This makes softwoods more resistant to chemical delignification than hardwoods.

Every coupling of two phenoxy radicals through a ß-carbon position will produce a reactive quinone methide structure (Leary, 1980; Glasser and Kelley, 1987). Beta-coupling is estimated to be involved in up to 70% of the links between C_9-units in softwood lignins (Adler, 1977; Higuchi, 1990), and even more so in hardwood lignins, since syringyl structures cannot form linkages at the 5-position. Since quinone methides are numerically very important intermediates, they will have a significant effect on the way the lignin polymer is cross linked with itself and with other cell wall molecules.

The quinone methide intermediates formed by the coupling of two phenoxy radicals through the ß-carbon will react further to form stable bonds by nucleophilic
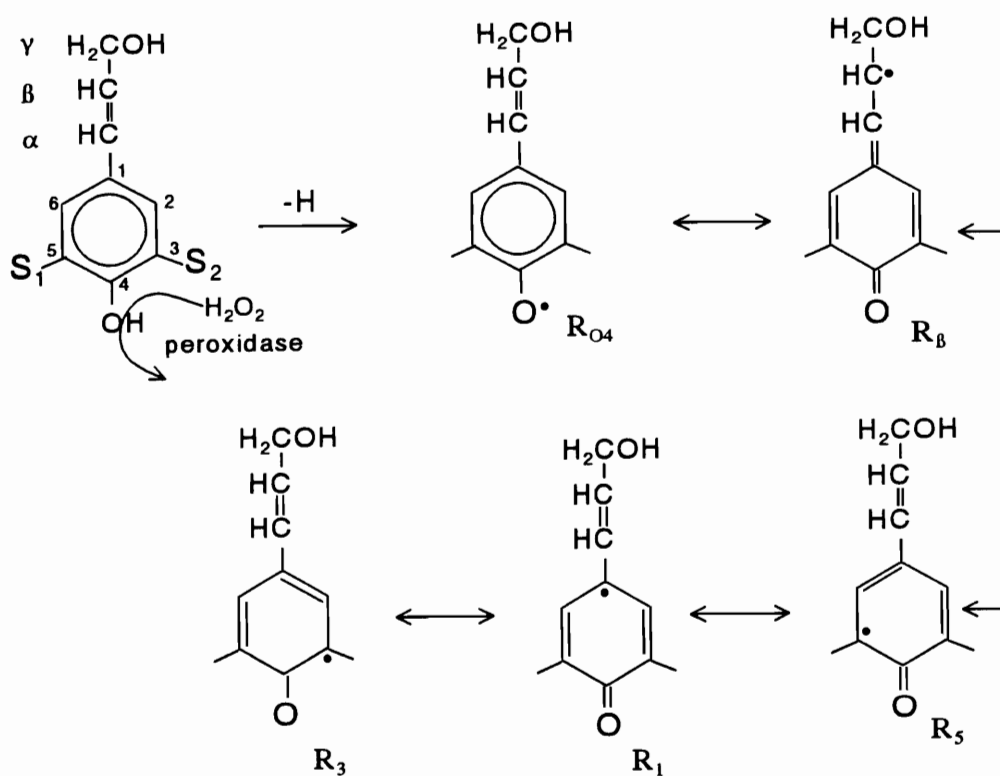
Figure 3. Formation of radical species from lignin precursors by peroxidase/H_2O_2. $S_1 = $ H in *p*-coumaryl alcohol, OCH_3 in coniferyl and sinapyl alcohol, $S_2 = $ H in *p*-coumaryl and coniferyl alcohol, OCH_3 in sinapyl alcohol. R_{O4}, R_β, R_5, R_1, and R_3 are isomers with free radicals on oxygen-4, ß-carbon and ring carbons in the 5, 1, and 3 positions, respectively.

Table 1. Intramolecular linkages formed by phenoxy radical coupling[a]

Radical	R_{O4}	R_β	R_5	R_1
R_{O4}	unstable	ß-O-4	4-O-5	1-O-4
R_β	ß-O-4	ß-ß	ß-5	ß-1
R_5	4-O-5	ß-5	5-5	1-5
R_1	1-O-4	ß-1	1-5	1-1

[a] Glasser and Kelley (1987)

addition, tail-to-tail dimerization or addition of olefins, of which the addition of nucleophiles is most likely to occur in plant tissues (Leary, 1980). Plant tissues are abundant in weak nucleophiles such as phenolic and aliphatic hydroxyls in lignin, hydroxyls and carboxyls in carbohydrates and water. Furthermore, dilignols not formed by ß-coupling are further dehydrogenated by the peroxidase-H_2O_2 system to their radicals, which are finally converted to lignin and lignin-carbohydrate complexes via radical couplings followed by nucleophilic attack on the benzyl carbons of the oligomeric quinone methides by water, hydroxyl groups or carboxyl groups (Higuchi, 1990).

Formation of Lignin-Carbohydrate Linkages. The reactions with quinone methides occur at the α-carbon, and typical products are alcohols (addition of water), ethers (addition of alcohols) and esters (addition of carboxylic acids) (Leary, 1980) (Figure 4). Steric considerations and pKa are factors determining the relative nucleophilicities of possible substituents. The steric environment of the hydroxy group is important as illustrated by the rates of disappearance of a quinone methide in the presence of simple alcohols, which follow the sequence methanol > ethanol > isopropanol > *t*-butanol (Leary et al., 1977). The ratio of products formed depends on the relative nucleophilicities of the reactive species at a given pH. It was shown in vitro that under mildly acidic conditions (pH of 4 to 6), the reaction with a carboxylic acid was favored, resulting in an ester linkage. Formation of a benzyl ether through addition of an alcohol also occurred. At higher pH values the formation of ethers and alcohols was favored. Thus, in neutral or weakly acidic plant tissues, formation of lignin-carbohydrate ester linkages may be favored, and lignin-carbohydrate ether bonds are likely to form also. The α-carbon of the quinone methide is preferentially attacked by the carboxyl group of D-glucuronic acid and the

QM intermediate

Addition product

$$+ HX \longrightarrow$$

HX=HOOC-,
HO-, HS-,
H_4N^+, H_2O
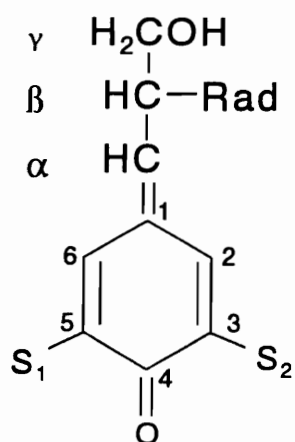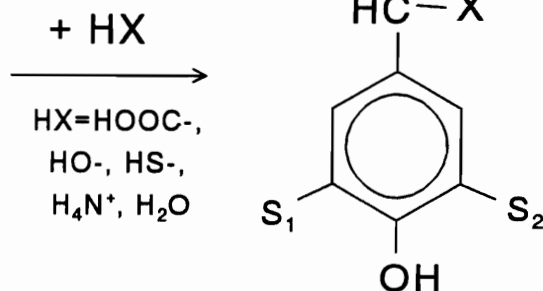
Figure 4. General reaction scheme of nucleophilic addition to quinone methide (QM) intermediate. S_1 and S_2 are H and(or) OCH_3 (see Figures 1 and 3). Rad = radical coupled to ß-carbon. HX = nucleophile, comprising carboxyl-, hydroxyl-, and sulfhydryl groups, ammonia, and water.

C-6 hydroxyl group of D-glucose (Tanaka et al., 1976,1979) to give ester and ether linkages, respectively, when these sugars were added to a solution of the quinone methide of guaiacylglycerol-ß-guaiacyl ether. In plant cell walls and middle lamellae, a considerable part of the lignin seems to be linked by the α-carbon to the primary alcohols and carboxyl groups of hemicelluloses and pectins. Eriksson et al. (1980) and Takahashi and Koshijima (1988) suggested that in both hardwoods and softwoods, lignins are ester linked to glucuronic acids of hemicelluloses (probably through benzyl esters), and ether linked to the other sugar units (probably by benzyl ethers).

There has not been complete agreement concerning the nature of the ether compounds resulting from nucleophile addition to quinone methides (Leary, 1980; Nimz, 1981; Leary, 1982). Freudenberg and Werner (1964) had initially shown that addition reactions to quinone methide mainly involve phenols, leading to intramolecular α-O-4 bonds, which are stable in weak alkali. However, Hemmingson and Leary (1980) found that in aqueous systems only the product of alcohol addition, a benzyl alkyl ether could be detected. Recent findings (Brunow et al., 1989; Sipila and Brunow, 1992) indicate that the formation of non-cyclic benzyl alkyl ethers is important only in systems where the concentration of water is lower than alcohol. With advanced maturation and ongoing cell wall lignification, conditions in the cell wall matrix will presumably shift from hydrophylic to hydrophobic, so that both benzyl aryl ethers and benzyl alkyl ethers can be expected.

Role of Phenolic Acids in Interlinkages. In grasses, the phenolic acids, *p*-CA and FA, are present in appreciable amounts (Harris and Hartley, 1980), whereas sinapic acid usually is found in traces. These phenolic acids are the immediate precursors for the monolignols p-coumaryl (I), coniferyl (II), and sinapyl (III) alcohols. Ferulic acid is predominantly ester linked to hemicellulose, and *p*-CA is almost exclusively ester

linked to lignin, although a hemicellulose/p-CA complex was isolated by Mueller-Harvey et al. (1986).

Scalbert et al. (1985) reported occurrence of FA ether-linked to lignin fractions isolated from wheat straw. The ether linkage might be established after dehydrogenation of FA, similar to dehydrogenation of immediate lignin precursors, and the resulting phenoxy radical would combine with other lignin radicals to yield an aryl ether. p-Coumaric acid has been shown to copolymerize with coniferyl alcohol, forming an alkali-stable linkage (Shimada et al., 1971; Nakamura and Higuchi, 1978). A second mechanism, via addition to quinone methide intermediates, is also possible. Phenolic acids might add to quinone methides either through their phenolic or carboxylic group, yielding benzyl aryl ethers or benzyl esters (Scalbert et al., 1986b). When p-CA and FA were allowed to react with a model quinone methide, they yielded the benzyl esters (Scalbert et al., 1986b). Thus, when both the carboxyl and phenol groups are available, the carboxyl group adds preferentially to the quinone methide. This was in agreement with previous work (Freudenberg and Werner, 1964; Leary et al., 1977). However, methyl esters of p-CA and FA reacted with the quinone methide to give the respective benzyl aryl ethers. The authors suggested that similar reactions occur in grass cell walls, which would explain the existence of FA ethers and especially that of FA bridging units. The reaction sequence might be as follows: in young non-lignified cell walls, FA is esterified to arabinoglucuronoxylans (Smith and Hartley, 1983; Kato et al., 1983; Mueller-Harvey et al., 1986). During lignification, these FA esters may add through their free phenolic group to a quinone methide. As a result, FA would be simultaneously etherified to lignin and esterified to hemicellulose, thus forming an alkali-labile cross link between the two cell wall polymers. The finding of FA dimers ester-linked to

hemicellulose may be explained by a similar mechanism and could provide an additional way of cross linking hemicellulose and lignin (Hartley et al., 1992; Lam et al., 1992b).

Besides the presence of phenolic acids in cell walls of grasses and some legumes, forage lignins also differ from wood lignins in that they contain small amounts of nitrogenous compounds (Bondi and Meyer, 1948). These may be protein bound to lignin via peroxide induced radical coupling (Whitmore, 1978), presumably with aromatic amino acids or via nucleophilic addition to quinone methide intermediates.

Proposed Roles of Lignin in Limiting Cell Wall Degradation

Over 60 yr ago it was recognized that lignified plant tissues are not attacked by bacteria (Woodman and Stewart, 1932). It was postulated that the protective effect was due to a certain degree of antiseptic action of the lignin resulting from its phenolic nucleus (H. Hibbert, pers. comm., quoted in Crampton and Maynard, 1938). In these early studies it was already recognized that the manner of lignin deposition in the plant may affect its impact on cell wall digestibility. Polymeric lignin is assumed not a substrate for anaerobic microorganisms, but is not inhibitory to their growth (Jung et al., 1983a,b). Considerable solubilization of phenolics from ^{14}C-labelled lignocellulose of highly lignified forages by mixed rumen fungi from the rumen has been shown to occur without conversion into $^{14}CO_2$ (Akin and Benner, 1988). Fungi are presumed to play an important role in physically weakening lignified tissues, thereby increasing the accessibility to microbial enzymes (Borneman and

Akin, 1990).

Among the mechanisms proposed in the literature to explain the effect of core lignin on cell wall digestion are a physical 'encrustation' or shielding of the cell wall components by core lignin (Van Soest, 1981), the inability of microbial enzymes to recognize the carbohydrate substrate (hemicellulose) after establishment of covalent linkages with lignin (Jung, 1989), and the formation of an 'inert layer' of core lignin at the cell wall surface during microbial digestion (Chesson, 1988).

Encrustation. Encrustation of cellulose or cell walls by an impenetratable layer of lignin has been one of the earliest proposed mechanisms, and is attractive in the light of high local concentration of lignin in the outer regions of the wall, the primary wall/middle lamella (Saka and Goring, 1985; Terashima et al., 1988). However, mastication and rumination are effective in physically cracking cell walls, and microbial attack often begins from the cell lumen. Furthermore, encrustation or shielding cannot account for the existence of indigestible soluble lignin-carbohydrate complexes in the rumen (Gaillard and Richards, 1975), which are recovered in the feces after coagulating at the lower pH prevailing in subsequent gastro-intestinal compartments (Neilson and Richards, 1978). It cannot account for the increase in digestibility in mildly alkaline treated straws where lignin has not been removed from the product (Van Soest, 1981).

Lignin-Carbohydrate Linkages. It seems that attachment of lignin to the cell wall matrix is a prerequisite for its negative impact (Jung, 1990). Although substantial amounts of lignin are solubilized in the rumen, the concentration of low molecular weight compounds does not reach toxic levels (Kivaisi et al., 1990). Addition of isolated polymeric lignin does not influence *in vitro* digestion (Han et al., 1975; Jung, 1990). Contrary to addition of lignin to incubation media, artificial lignification (using

peroxidase, H_2O_2 and eugenol) of digestible cellulose resulted in a reduction of digestibility (Gressel et al., 1983; Jung, 1990).

The attachment of lignin to carbohydrates aids in maintaining a rigid cell wall matrix, in which penetrating of microbial enzymes is limited. The rigidity also limits its physical breakdown by mastication. Also, interlinkages will keep the lignin 'skeleton' in place during microbial degradation by preventing solubilization. The linkages themselves seem to have a limited direct effect on degradation of carbohydrates as evidenced by the relatively low carbohydrate content (2 to 8%) of soluble lignin-carbohydrate complexes isolated from ruminal fluid (Conchie et al., 1988; Neilson and Richards, 1982; Nordkvist et al., 1989). These complexes appear to consist mainly of polyphenolic material to which small 'stubs' of carbohydrates are attached. Inhibition of enzymatic substrate recognition by attachment of core- or non-core lignin to hemicellulose is a possibility, but has not been demonstrated.

Accumulation of Lignin during Microbial Digestion. Microorganisms selectively attach to cell walls with a low phenolic content (Akin, 1979). During rumen fermentation, microbial enzymes remove carbohydrates from the cell wall matrix, leaving the surface relatively high in undegradable lignin, which may form a layer that prevents further degradation of the matrix. Chesson (1984) showed that while the phenolic content of extensively degraded barley straw increased by 11% overall, the increase at the surface was about 45%. It is the changing chemistry of the upper surface which dictates the rate and extent of degradation (Chesson, 1988). In highly lignified materials the buildup is faster, which agrees with the indirect relationship between lignin content and digestibility. However, the extent to which phenolic compounds are linked to cell wall carbohydrates is likely to contribute to the retention of lignin at the cell wall surface. This is of particular significance when

considering chemical treatment of crop residues. Alkali treatment saponifies ester bonds between hemicellulose and lignin, and also releases esterified phenolic acids (Chesson, 1988). Cleavage of the lignin-hemicellulose linkages aids in the solubilization of exposed lignin and slows down the build up of the inert layer.

Lignin Heterogeneity and Cell Wall Degradation

Lignin Heterogeneity. The mechanisms discussed above all suffer from the lack of recognition of lignin heterogeneity and its possible effect on cell wall degradation. The phenolic monomer composition of core lignin, as well as the type of intra-molecular linkages and interactions with other cell wall components contribute to its heterogeneity (Scalbert et al., 1985). Lignin structure differs among plant species (Nimz et al., 1981), mutant varieties (Kuc and Nelson, 1964; Gee et al., 1968; Bucholtz et al., 1980), plant parts (Gaudillere and Monties, 1989), tissue types (He and Terashima, 1990; 1991), and cell wall layers (Terashima et al., 1988; Wallace et al., 1991).

Many researchers have tried to relate lignin content with digestibility by linear regression models (Allinson and Osbourn, 1970; Tomlin et al., 1965), but curvilinear models often fit the data better (Jung and Vogel, 1986). In the case of 'flattening' response, this may be due to a "law of diminishing returns" (Jung and Vogel, 1986). On the other hand, increasing ratios of indigestible residue to lignin content with increasing maturity (Quicke and Bentley, 1959; Grabber et al., 1992) may be due to a different type of lignin (with respect to monomer composition) being deposited in the cell wall at a later stage of maturity, or a difference in type and extent of

interlinkages established among core lignin, hemicellulose and phenolic acids at a later stage of maturity. An interacting factor that may contribute to the curvilinear nature of the regression of lignin on cell wall digestibility is the observation by Brice and Morrison (1982) that with increasing maturity the xylan backbone of hemicellulose becomes less substituted with arabinose side chains. On the one hand, this would allow for less cross-links between hemicellulose and lignin through arabinose side chains at higher maturity. On the other hand, the decreased substitution with arabinose would allow a more dense packing of xylan chains with the possible formation of intermolecular hydrogen bonds.

An observation first made decades ago is the fact that the most lignified forage is not always the least digestible (Kamstra et al., 1958). Jung and Russelle (1989) were able to influence fiber digestibility of birdsfoot trefoil (*Lotus corniculatus* L.) by manipulating light regime, NH_4^+/NO_3^- ratio and S content of nutrient solutions without affecting lignin concentration. Within species or varieties, changes in maturity produce changes in digestibility inversely related to lignin content (Tomlin et al., 1965; Allinson and Osbourn, 1970; Jung and Vogel, 1992; Cone and Engels, 1993). Indications are that for comparison of species, varieties, and plant parts within maturity stages, lignin composition is more valuable than lignin concentration (Jung and Vogel, 1992). Although lignin content is higher in legumes than in grasses, increased lignification with maturity depresses digestion in grasses more than in legumes (Tomlin et al., 1965; Buxton and Russell, 1988; Buxton, 1989). According to Allinson and Osbourn (1970), alfalfa and ryegrass lignin respond differently to maturity. Increasing maturity of ryegrass but not alfalfa was associated with a lignin fraction forming a UV difference peak at 350 nm, which indicates aromatic hydroxyls conjugated with a carbonyl in the *para*-position. Diploid ryegrass, having a lower

cellulose digestibility than tetraploid ryegrass, also showed a higher absorbance at 350 nm. Overall lignin content only increased slightly with maturity, indicating that the qualitative character of the lignin 'complex' varied independently from actual lignin content.

Gordon and coworkers initiated a systematical evaluation of lignin properties of different forage species (Gordon and Griffith, 1973; Gordon, 1975; Gordon and Neudoerffer, 1975). They found that a greater proportion of grass lignin dissolved in alkali (50% of ADL) than alfalfa lignin (30% of ADL). Corn lignin was even more alkali-soluble. Ruminal digestion increased the alkali-solubility of the lignins. Alkali-extracted alfalfa lignin had more total but fewer aromatic protons, fewer groups reacting with *N*-trichloro-*p*-benzoquinone imine, fewer ionizable hydroxyl groups and fewer methoxyl groups than grass lignin (Gordon, 1975). Ruminal digestion reduced the number of groups reacting with *N*-trichloro-*p*-benzoquinone imine in grass (Gordon, 1975) and corn (Gordon and Neudoerffer, 1973), but seemed to have the opposite effect for alfalfa (Gordon, 1975). Fecal lignin contained similar number of methoxyl groups as the hays and alfalfa.

Overall, these findings imply that alfalfa lignin is a more highly condensed lignin than grass lignin, leaving fewer reactive sites for combining with other molecules. This explains why legume lignins act more as an inert energy diluent than grass lignins, because grass lignins interact to a greater extent with other cell wall constituents.

Lignin Composition of Grasses and Legumes. The different properties of grasses and legumes described by Gordon and coworkers, as well as the differential response to maturity (Allinson and Osbourn, 1970) are related to the different phenolic monomer composition (including the presence of esterified *p*-CA), as well

as to the finding that the increase with maturity in syringyl/guaiacyl ratio is higher in grasses than in legumes (Buxton and Russell, 1988; Buxton, 1989). The vanillin proportion of nitrobenzene oxidation products (NBO) has also been found highly negatively correlated with IVDMD (Reeves, 1985a) when comparing grasses, legumes and hulls. However, within grasses, proportion of syringaldehyde was negatively correlated with IVDMD. In an assessment of the effect of various chemical treatments on lignin composition (Reeves, 1985b), it appeared that alkaline peroxide treatment effectively decreased syringyl content of lignin in corn stover, but the impact on peanut hull lignin (predominantly guaiacyl lignin) and digestibility was negligible. Buxton (1989) suggested that selecting plants for low NBO product yield or low syringaldehyde content of NBO products, (thus low syringyl content of core lignin), along with low p-CA concentration would reduce the inhibitory effect of lignin on rate and extent of forage cell walls.

Brown Midrib Mutants. Cell walls of the more digestible brown midrib mutant (*bmr*) of corn often contain less lignin (Hartley and Jones, 1978), but even at lignin content similar to normal corn varieties, digestibility has been higher for the mutant (Sommerfeldt et al., 1979). The higher digestibility of the *bmr* mutant of corn is associated with a change in lignin composition related to increased activity of catechol-O-methyltransferase (Grand et al., 1985). In the mutant corn, a lower syringyl/guaiacyl ratio is found (Cymbaluk et al., 1973; Gaudillere and Monties, 1989) as well as a decrease in concentration of p-CA (Cymbaluk et al., 1973; Hartley and Jones, 1978; Hartley and Haverkamp, 1984) and lower ratio of p-CA to FA (Gaudillere and Monties, 1989). The NMR spectra of *bmr* and normal corn lignin indicated a higher degree of intramolecular cross linking of the propane side chain of the lignin molecule, resulting in a more condensed *bmr* lignin than normal lignin

(Gordon and Griffith, 1973). In *bmr* mutants of sorghum, a lower contribution of syringyl units to the lignin structure has been reported (Akin et al., 1986). The amount of *p*-CA released by alkali from parenchyma tissue of *bmr* mutants of millet (Hartley et al., 1992) and sorghum (Akin et al., 1986) was considerably lower than that of the normal varieties, resulting in significant improvement in digestibility. The enhancement of fiber degradation for the *bmr* mutants is most marked in stem parenchyma tissue (Grenet and Barry, 1988; Akin et al., 1991; Hartley et al., 1992), associated with lower *p*-CA concentration and/or lower syringyl lignin content. Besides the differences between normal and mutant varieties, lignin composition in corn stover appeared to differ between lower and upper internodes from the same stalk (Gaudillere and Monties, 1989), with lower syringyl to guaiacyl and *p*-CA to FA ratios in the upper internode for both normal and mutant corn. Differences in overall lignin composition between corn leaf and stem have not been reported. Of interest with regard to leaf and stem differences are the findings by Cherney et al. (1991) that sorghum leaf blade and stem are equally digestible, even though sorghum stem contains about 50% more lignin than leaf and 2 to 3 times as much esterified *p*-CA.

Lignin Distribution in Cell Walls. Histological studies in combination with physicochemical measures of lignin distribution (microautoradiography, UV-microspectrophotometry) have proven valuable. Different concentrations of guaiacyl and syringyl lignins have been demonstrated in different cell types, in similar cell types of different internodes (maturity effect) and in different cell wall layers. Hartley et al. (1990a), using microspectrophotometry and histological staining, found that bermudagrass (*Cynodon dactylon* L Pers) parenchyma had lower UV absorbance than sclerenchyma, and tested negatively with acid phloroglucinol (absence of guaiacyl lignin) but positive tests with diazotized sulphanilic acid (indicating phenolic acids)

in upper and lower internodes and with chlorine-sulphite (indicating syringyl-rich lignin) in the lower internode. Sclerenchyma in lower internodes of bermudagrass was higher in UV absorbance (total phenolics) and of a higher syringyl content than sclerenchyma in lower internodes. Similar qualitative differences in syringyl and guaiacyl contents between secondary and primary cell wall layers of lignified cells have also been indicated (Akin, 1988), which are related to differential incorporation of lignin monomers at different stages of cell wall development.

Tissues with different reactions to the acid phloroglucinol and chlorine sulfite tests consistently show different responses to chemical treatment and biodegradation (Akin, 1989; Borneman and Akin, 1990). Xylem in leaf blades and stems, and epidermis and sclerenchyma ring in grass stems resist degradation almost completely, and are consistently positively stained with acid phloroglucinol. Rapidly digested tissues (phloem and mesophyll in grass leaf blades, and phloem and immature parenchyma in grass stems) do not give any positive reaction with light histological methods for lignin. Slowly and partially digested tissues, such as epidermis and parenchyma bundle sheath of C_4 grasses, leaf sclerenchyma, and mature parenchyma in stems of C_3 and C_4 grasses, are often stained by chlorine sulfite (Akin and Burdick, 1981). Digestion of tissues stained by chlorine sulfite (sclerenchyma in grass leaf and parenchyma in stems) is enhanced by alkali treatment to a larger extent than digestion of tissues stained by acid phloroglucinol (e.g., sclerenchyma ring in stems) (Spencer and Akin, 1980). Stem parenchyma is more completely 'delignified' by potassium permanganate than other lignified stem tissues (Akin et al., 1985). The effects of alkali and oxidative treatments on parenchyma tissue point to a large contribution of etherified syringyl units to the lignin in these secondary wall.

Of interest with regard to the degradability of maturing stem parenchyma are

the recent findings by Grabber et al. (1992) that the indigestible residue from parenchyma walls was linearly correlated to the p-CA/FA ratio, but exponentially to the lignin content, indicating a larger inhibitory effect per unit of lignin deposited with increasing maturity. This may be related to the chemical properties of the lignin in the secondary wall (predominantly syringyl lignin) or to a change in parenchyma cell wall architecture. In barley straw, a barrier covering the lumen side of lignified parenchyma cell walls was found ('warty layer'), restricting access by rumen microorganism (Engels and Brice, 1985).

Primary cell walls of corn stem sclerenchyma become resistant to microbial degradation as soon as formation of the secondary wall is started (Engels and Schuurmans, 1992). However, secondary layers of lignified cell walls in thin sections have been shown to be more extensively degraded in the rumen than primary layers (Chesson et al., 1986; Engels, 1989; Engels and Schuurmans, 1992; Mulder et al., 1992), the residual lignin showing a highly positive acid phloroglucinol reaction (Engels, 1989; Engels and Schuurmans, 1992). Treatment of these cell walls with $KMnO_4$ resulted in a colorless secondary wall (indicating complete delignification) and a soft orange stained middle lamella/primary wall identical to the cell wall residue after digestion (Engels and Schuurmans, 1989). However, from two adjacent cells separated by middle lamella/primary wall, only the secondary wall in a mechanically opened cell lumen could be digested, indicating that lignin 'encrustation' turned the primary wall into an indigestible layer protecting the secondary wall. Although the bulk of the lignin in cell walls is present in the secondary wall, lignin concentration in primary walls and middle lamella is much higher than in the secondary wall.

In agreement with the qualitative staining with acid phloroglucinol and the

relative resistance of the primary wall lignin to $KMnO_4$ delignification are growth and lignification studies using microautoradiography and ultraviolet microscopic spectroscopy, indicating that lignin in primary walls/middle lamella is essentially a condensed guaiacyl lignin with *p*-hydroxyphenyl lignin in the cell corners (Terashima et al., 1988; He and Terashima, 1990; Wallace et al., 1991). Due to the high concentration of lignin in the primary wall and its more condensed character, it probably serves more as a physical barrier to microbial attack, which renders the carbohydrates in the primary wall inaccessible. On the other hand, the association of lignification of the secondary wall with reduced degradability is more likely due to interactions of syringyl lignin with other cell wall components. This reduced degradability can be partially overcome by chemical treatments targeting alkali-labile bonds or etherified syringyl units. The lignification of secondary wall layers with increased maturity causes an increase in the proportion of syringyl to guaiacyl units in the overall forage, which explains the negative correlation of syringyl content with forage digestibility. However, even after lignification is completed, cell wall digestibility may continue to decrease, associated with regeneration of quinone methide structures from lignin, referred to as the "lignin aging" effect (Leary, 1980). Therefore, the larger the contribution of syringyl units is to the overall lignin structure, the larger the decrease in digestibility is with increasing maturity.

Lignin-Carbohydrate Complexes. Morrison (1974b) was the first to address the composition and nature of linkages of lignin-carbohydrate complexes (LCC) isolated from forages. Using LCC extracted from *Lolium perenne* with dimethyl sulphoxide (DMSO), he demonstrated the presence of three different bond types between lignin and carbohydrate: one cleaved on borohydride reduction, one alkali labile, and one alkali stable. In LCC isolated from wood by DMSO extraction, a number of different

linkages have been proposed. Definite proof of the nature of chemical cross links are difficult to obtain due to the drastic conditions employed for the isolation of lignin-carbohydrate complexes. Among all the different possibilities, glycosidic linkages (Koshijima, 1972), ester linkages (Higuchi and Nakamura, 1978) and ether bonds (Leary et al., 1983) have been demonstrated, using model compounds.

Alkali Solubility. Lapierre et al. (1989b) explained the alkali solubility of wheat straw lignin by the high content of ionizable phenolic hydroxyl groups after they found that through methylation of the phenolic hydroxyl, the dissolution of wheat straw lignin in alkali was drastically reduced. Ionization of acidic groups (including the phenolic hydroxyl) by alkali promotes lignin solubilization, either by increasing the solubility of lignin fragments, or by inducing swelling of the cell wall matrix (Tarkow and Feist, 1969), allowing diffusion of lignin fragments.

Scalbert and Monties (1986) explained the high alkaline solubility of wheat straw lignin by the high content of alkali-labile linkages within the lignin network or among lignin and polysaccharides. Rupture of alkali-labile linkages increases the content of acidic groups in lignin (phenolic and carboxylic groups from ether and ester linkages), thereby enhancing solubilization (Scalbert and Monties, 1986). The overall result would be increased access of the cell wall matrix to microbial enzymes.

Delignification. Various researchers have tried to use chemical delignification (oxidation treatments) or biological delignification (lignolytic fungus *Phanerochaete chrysosporium*) as a tool to study the impact of lignin on cell wall degradation (Jung et al., 1992; Miron and Ben-Ghedelia, 1993). This approach has generally been unfruitful in establishing close relationships between the extent of delignification and the resulting cell wall degradability (Jung et al., 1992), attributable to a more general disruption of the cell wall matrix structure beyond simple removal of lignin (Jung et

al., 1992), and a certain extent of uncontrolled condensation and(or) cross-linking reactions (Ford, 1986).

Interactions of Quinone Methide Intermediates with Cell Wall and Ruminal Digesta Components

Quinone methides, intermediates in lignin polymerization, can be regenerated from lignin under mild conditions (Leary, 1980). It seems therefore likely that the bonding in lignin will change during lignin aging, with the progressive conversion of benzyl alcohols to more stable benzyl ether structures. The new bonds may be lignin-carbohydrate linkages or lignin-lignin bonds of the α-O-y type. Continuing formation of cross linkages with carbohydrates would provide explanation of the reduction in cell wall digestibility with increased maturity, with relatively little additional deposition of lignin.

The observation that quinone methides can be regenerated from lignin under mild conditions (Leary, 1980) raises the possibility of their formation in the rumen. Although strongly reducing conditions prevail in the rumen, micro-environments may exist in the fiber matrix where oxidative degradation of fiber components, notably lignin, can take place as suggested by Nordkvist et al. (1989). They found a large proportion of stable radicals, probably of the quinone type, in residual LCC from wheat straw digested in rumen fluid. Residual LCC from wheat straw and red clover in that study also contained considerably more ash than the original fibers, indicating that LCC are capable of binding non-cell wall components from rumen fluid.

Quinone methides, if formed in the rumen, would immediately undergo

nucleophilic addition reactions, whereby lignin would repolymerize, or, more likely, cross-link with carbohydrates, nitrogenous compounds, or mineral complexes. The soluble lignin-carbohydrate complexes in the rumen were found not to be affected by further digestion (Neilson and Richards, 1978), suggesting that they may not have been formed by simple solubilization, but by formation of linkages. Nitrogen content of LCC isolated from rumen fluid has been conspicuously high (Wallace et al., 1991). This N may be of plant origin, as it has been suggested that lignin becomes linked to extensin during lignification (Whitmore, 1978). However, N could become incorporated into lignin carbohydrate complex by nucleophilic addition of rumen-NH_3 or amino acids to quinone methide intermediates. Lignin has also been shown to act as a binding agent of minerals (Thompson and Weber, 1982) and possible scavenger of nitrates and carcinogens (Rubio et al., 1979). Polyphenoloxidase treatment of straw reportedly inhibits fiber digestion *in vitro* (Han et al., 1975), presumably by producing quinone derivatives. The formation of quinone methides, whether it occurs in the maturing cell wall or during cell wall degradation, is a factor that has been overlooked. An increased syringyl content of lignin is directly related to the likelyhood of formation of quinone methide intermediates.

Determination of Lignin Content and Composition

Lignin Content. Unambiguous quantification of lignin content or determination of its structure in forages is impossible. The various methods available to quantify lignin; Klason lignin (TAPPI, 1985), ADL (Van Soest, 1963), $KMnO_4$ lignin (Van Soest and Wine, 1968), chlorite lignin (Collins et al., 1978), acetyl bromide lignin

(Iiyama and Wallis, 1988; 1990), and triethylene glycol lignin (Edwards, 1973) all seem to measure different lignin fractions (Reeves, 1988; 1993). Oxidation with sodium chlorite or dissolution in triethylene glycol does not remove all the lignin, and acid insoluble (Klason lignin, ADL) and $KMnO_4$ lignin include some nitrogenous substances in the lignin value. The sulfuric acid lignins involve two acid hydrolysis steps, one with approx. 1 M H_2SO_4 (giving ADF), and a second with 72% H_2SO_4 (giving ADL); and vice versa for Klason lignin. Klason lignin gives higher values than ADL, which cannot be completely accounted for by their respective N contaminations (Jung et al., 1991). Dilute (1 M) H_2SO_4 hydrolyzes glycosidic linkages but in doing so also produces degradation products (furfurals), which condense with lignin, and complexes of lignin with undegraded carbohydrate complexes. These lignin fractions are insoluble in acid, and are therefore included in Klason lignin. The presence of a detergent in preparing ADF facilitates dissolution of the lignin-carbohydrate fractions by trapping them in detergent micelles, thus reducing the lignin content measured as ADL and $KMnO_4$ lignin (Stewart and Morrison, 1992). Permanganate lignin and ADL can therefore be regarded as estimates of condensed lignin, whereas Klason lignin includes non-condensed lignin structures and several contaminants. Finally, acetyl bromide lignin is determined by measuring the UV absorbance at 280 nm after lignin dissolution, thus including both polymeric lignin and phenolic acids. Furthermore, no suitable calibration standards exist.

Lignin Composition. Insight into structural aspects of lignin, especially its monomer composition, is important for understanding the limitations lignin imposes on cell wall degradation by rumen microorganisms. Guaiacyl lignin is more resistant to degradation than syringyl lignin (Glasser et al., 1983b), due to the larger contribution of carbon-carbon bonds, which are more stable than the ether linkages

involving the phenolic oxygen (e.g. ß-O-4), which are more predominant in syringyl lignin (Glasser and Kelley, 1987). This explains why crop residues with syringyl-rich lignin, e.g. corn stover, are more prone to degradation during oxidative treatments than materials with guaiacyl-rich lignin, e.g. peanut hulls (Reeves, 1985b). For similar reasons (larger involvement of the ß-position in polymerization), syringyl lignin is more likely to form intermolecular linkages with cell wall carbohydrates via quinone methide intermediates than guaiacyl lignin, which may be the major reason why forage cell wall degradation decreases with increasing maturity (increasing syringyl content). Structural features of interest with respect to lignin reactivity are the guaiacyl/syringyl ratio or, alternatively, methoxyl content, the content of free phenolic hydroxyl groups, and the contribution of ether bonds to the total linkage pattern. These features can be determined with degradative and non-degradative techniques.

Since the 1930's, the classical nitrobenzene oxidation is the degradative technique that has been most widely used by wood (Higuchi et al., 1967b) and forage (Jung et al., 1983a; Reeves, 1985a,b, 1987) scientists . Unfortunately, yield of degradation products is rather low (20 to 30%). When alkaline nitrobenzene oxidation or the similar CuO degradation is followed by a $KMnO_4$ oxidation step, yields of degradation products are somewhat higher (Morohoshi and Glasser, 1979). The major problem with oxidative degradation techniques is that the reactivities of guaiacyl and syringyl lignin units towards oxidation are different (Sultanov and Wallis, 1991). In degradative analysis of lignin composition, the targets of degradation are essentially the uncondensed alkyl aryl ether bonds between the lignin monomers. p-Hydroxyphenyl (H) and guaiacyl (G) units are less likely to form ß-O-4 linkages than syringyl (S) units, and are more frequently involved in often multiple carbon-carbon linkages in the more condensed moieties of the lignin macromolecule,

whereas S units are predominantly linked through ether bonds in the uncondensed moieties. For this reason the monomer ratios (H/G, S/G, or H:G:S) obtained with degradative techniques do not represent the entire lignin macromolecule. However, intermolecular linkages are established mainly through the uncondensed lignin moieties (Gordon, 1975), and therefore the S/G ratio in the non-condensed lignin may actually be more relevant to the digestibility of cell wall carbohydrates than the characteristics of the entire lignin macromolecule. Thus, degradative analysis of lignin composition may give information more pertinent to the association with carbohydrates than non-degradative techniques. The degradative technique that most exclusively attacks ether bonds (non-oxidative degradation by thioacidolysis) would then be preferable.

Another drawback of nitrobenzene oxidation is its inability to distinguish phenolic acids present in grasses from their respective counterparts in lignin (Lapierre et al., 1989a). Phenolic acids can be distinguished from lignin monomers in non-oxidative procedures, e.g. thioacidolysis, which are also less laborious than CuO/KMnO$_4$ oxidation. Thioacidolysis is preferred over acidolysis because side reactions are more controlled (Lapierre et al., 1985b). Experiments with thioacidolysis have shown that wheat lignins are more condensed than hardwood lignins and that the relative importance of p-hydroxyphenyl units in their uncondensed moiety is negligible (Lapierre et al., 1989a). A drawback of thioacidolysis is, again, a low overall monomer yield (Morohoshi and Glasser, 1979). If thioacidolysis is preceded by methylation, the frequency of free phenolic hydroxyl groups in the H, G and S moieties linked by ß-aryl ether bonds can be estimated (Lapierre and Rolando, 1988; Lapierre et al., 1988). By this technique it appeared that in wheat straw, 43% of the lignin monomers had free phenolic groups, compared

to 20 to 30% in poplar lignin. When the phenolic hydroxyls were methylated, the high alkali solubility of wheat straw was drastically reduced (Lapierre et al., 1989b).

For qualitative determination of lignin monomer ratios in total wood lignin (uncondensed and condensed), spectroscopic analysis involving ^{13}C nuclear magnetic resonance (NMR), in solution or solid state, is preferable. Solution ^{13}C NMR has the highest resolution, but is limited by the relatively low solubility of lignin. Solution ^{13}C NMR spectra of grasses show more complexity than those of softwoods or hardwoods (Himmelsbach and Barton, 1980; Nimz et al., 1981; Scalbert et al., 1985, 1986a; Jung and Himmelsbach, 1988). However, application of ^{13}C NMR spectroscopy for quantitative lignin analysis (Landucci, 1985; Lapierre et al., 1985a) is time consuming, and not straightforward.

Accurate S/G determination in the presence of significant amounts of H is very difficult. Direct spectral analysis by peak integration suffers from spectral overlap of H with G peaks (Lapierre et al., 1985a). Therefore, spectroscopic analysis of grass lignin structure seems to be limited to qualitative aspects due to the presence of p-hydroxyphenyl units. More specific quantitative information on lignin composition can be obtained by ^{13}C labeling. High resolution ^{13}C spectra can be obtained by photosynthetic ^{13}C-labeling (Lapierre et al., 1984) or administering ^{13}C-labeled lignin precursors (e.g. FA) to intact growing plants (Lewis et al., 1989). By solid-state ^{13}C NMR of specifically labeled intact plants, these authors showed that the predominant bonding pattern for *Leucaena leucocephala*, a hardwood, was through ß-O-4 linkages, whereas for young, growing wheat plants no significant contribution of ß-O-4 bonding was evident. These findings are in agreement with those of Nimz et al. (1981), who concluded that grass lignins are more different from hardwoods than from softwoods with respect to formation of ß-O-4 linkages, even

though grasses are still often regarded as a special kind of hardwoods.

Methoxyl Content. Much simpler than determination of S/G ratios in lignin is the determination of the methoxyl group content in lignin with standard methods involving treatment of isolated lignin with hydriodic acid (TAPPI, 1972). The resulting methyl iodide is converted to iodic acid, which is titrated with standard thiosulfate solution. Relatively large numbers of samples can be subjected to this procedure. For gramineae lignins containing appreciable *p*-hydroxyphenyl units, the methoxyl content is actually more easily interpretable than the S/G ratio or H/G/S ratio, since it is through the presence or absence of methoxyl groups on the aromatic ring that S, G and H units become more or less involved in ether or carbon-carbon bonds. Changes in relative methoxyl content due to maturation, treatments or digestion can also be determined by infrared (IR) or NMR spectroscopy.

Phenolic Acids and Cell Wall Degradation

Wall-bound Phenolic Acids. Whether simple phenolic acids depress cell wall digestibility in a manner similar to core lignin, is still subject to speculation. Concentration of phenolic acids generally increases with maturity, which has been associated with decreased digestibility (Hartley, 1972). The increase in *p*-CA usually occurs simultaneously with lignin deposition in the secondary wall, whereas FA accumulates in earlier stages and is more concentrated in the primary wall, associated with hemicellulose. Hydroxycinnamic acids are known as precursors of core lignin in plant metabolism (El-Basyouni et al, 1964). *p*-Coumaric acid is esterified mainly to the terminal hydroxyl groups of some of the propane side chains of lignin, thereby

contributing 5 to 10 % of the lignin (Higuchi, 1990). The formation of p-hydroxyphenyl lignin and occurrence of esterified *p*-CA in grass lignin seems to be derived from a high concentration of *p*-CA directly supplied from L-tyrosine by tyrosine-ammonia lyase.

In light of lignin biosynthesis, *p*-CA esterified to lignin should not be regarded a separate factor with regard to cell wall degradation, but its effect should be seen as part of the lignin polymer. Similar to lignin, *p*-CA progressively increases during the course of ruminal digestion, while FA decreases (Graham and Aman, 1984). However, Mueller-Harvey et al. (1986) have isolated a *p*-CA ester-linked to the arabinosoyl residue in arabinoxylan, and work by Ford (1986) suggests that *p*-coumaric esters form bridging units between core lignin and cell wall polysaccharides. A similar role has been postulated for FA, since it can be both esterified to arabinoxylan and etherified to lignin (Lam et al.,1992b). Most of the FA is ester-linked to polysaccharides during primary wall formation.

Etherification of FA was shown to be an early event in maturation (Iiyama et al., 1990). Ferulic acid, once esterified to arabinose residue of arabinoxylans, could serve as target points for lignification by reacting with lignin quinone methide intermediates to form benzyl ether linkages (Scalbert et al., 1986a). Cell walls of both grasses and legumes also contain small amounts of dehydrodiferulic acid (Hartley and Jones, 1976; Markwalder and Neukom, 1976; Eraso and Hartley, 1990), thought to arise from radical coupling between two polysaccharide-bound FA molecules in the presence of peroxidase and H_2O_2 (Fry, 1982, 1986). Recently, Lam et al. (1992a) showed dehydrodiferulic acid to be involved in both ester and ether linkages in wheat (*Triticum aestivum*) and Phalaris (*Phalaris aquatica*) internodes, and proposed that this dehydrodiferulic acid, in the form of di-ester bridges between polysaccharide

chains, is also etherified to lignin. In addition to dehydrodiferulic acid, cyclobutane dimers are present in tropical and temperate grasses (Ford and Hartley, 1988, 1989, 1990) in small amounts formed by photodimerization in sunlight involving p-coumaroyl and feruloyl groups, probably linked to cell wall polysaccharides (Eraso and Hartley, 1990). Photodimerization of p-CA and FA ester-linked to carbohydrate fractions has been shown *in vitro*, whereby the carbohydrate fragments become cross-linked (Hartley et al., 1990b).

The structure and composition of the fraction to which phenolic acids are esterified will influence the degree to which they affect digestibility. Bohn and Fales (1991) demonstrated that neutral detergent solution extracts 9 to 27 times as much phenolic acids (p-CA and FA, respectively) from alfalfa than 95% ethanol, whereas in reed canarygrass (*Phalaris arundinacea* L) and switchgrass similar amounts of phenolic acids were extracted with neutral detergent and 95% ethanol, respectively. Apparently, both p-CA and FA in alfalfa are linked to a water soluble component, presumably WSC. Most of the ester-linked FA and cyclobutanes in cell walls of tall fescue (*Festuca arundinacea* Schreb) and coastal bermudagrass could be released with 0.1 M NaOH (Hartley and Morrison, 1991), whereas one third to half of the saponifiable p-CA required treatment with at least 1 M NaOH for effective release from tall fescue and coastal bermudagrass cell walls.

The facile release of FA and the cyclobutanes appears indicative of linkage to the 5-*O* position of arabinosoyl residues in arabinoxylan (Hartley and Morrison, 1991) rather than linkage to lignin. However, treatment of bermudagrass sections with 0.1 M NaOH (Akin et al., 1992) not only released the major portion of the phenolic acids, but also partially disrupted the more refractory tissues (sclerenchyma, xylem) and decreased the response to histochemical staining for lignin, whereas 1 M

NaOH actually reduced lignified tissues to single-cell fibers. Sclerenchyma walls are affected by alkali treatment, particularly in the middle lamella (Spencer and Akin, 1984). When sections saponified with 0.1 M NaOH were subjected to *in vitro* digestion, degradation of mesophyll and phloem were increased, indicating that phenolic acids limit the degradation of relatively easily digested tissues. Saponification with 1 M NaOH resulted in increased digestion of lignified tissues.

Reduced *in vitro* fiber degradation was demonstrated in model systems containing *p*-CA and FA esterified to neutral detergent fibers and celluloses (Sawai et al., 1983; Jung and Sahlu, 1986; Bohn and Fales, 1989). Jung et al. (1991) demonstrated that the effect of phenolic acids esterified to oatspelts xylans was limited to reducing the degradability of the side chain sugars of the hemicellulose.

Toxicity. The potential toxicity of free and esterified phenolic acids to rumen micro-organisms has been studied extensively under *in vitro* conditions. Phenolic acids are released from plant cell walls by alkali (Hartley, 1972), commercial cellulases (Hartley et al., 1976), and plant hydrolases released from vacuoles during ingestion and mastication (Woodhead and Cooper-Driver, 1979). Cellulolytic bacteria and their enzymes may therefore be exposed to high local concentrations of phenolic acids, since they are closely associated with the cell wall surface (Chesson et al., 1982). Addition of phenolic acids to *in vitro* fermentations reduces cellulose degradation and suppresses microbial growth (Akin, 1982; Chesson et al., 1982; Jung and Fahey, 1983; Akin and Rigsby, 1985; Jung 1985). Ferulic acid and *p*-CA inhibit ß-glucosidase of *Bacteroides succinogenes* but not of *Bacteroides ruminicola* (Martin, 1990). However, growth of both organisms on cellobiose is inhibited by the phenolic acids, suggesting that other metabolic steps involved in cellobiose catabolism are affected. *p*-Coumaric but not FA inhibits extracellular carboxymethylcellulase and xylanase from *B.*

succinoges (Martin, 1990). Attachment of bacteria to plant cell walls is also inhibited, particularly by *p*-CA (Akin et al., 1988). Also, phenolic acids may interfere with sugar uptake by ruminal bacteria (Martin, 1990). Generally, *p*-CA appears more inhibitory to rumen microorganisms than FA (Martin, 1990).

Hartley and Akin (1989) showed that methyl and *t*-butyl esters of *p*-CA were as effective as *p*-CA in inhibiting growth of pure cultures of *B. ruminicola* and *Ruminococcus flavefaciens* and reducing IVDMD of bermudagrass, but the photodimer of *p*-CA was only marginally inhibitory, possibly related to the loss of the unsaturated $C=C$ bond in the side chain of the acid. Soluble phenolic-carbohydrate complexes released during previous *in vitro* fermentations have depressed degradability of cellulose and hemicellulose (Jung, 1988), but the most inhibitory phenolic esters appeared to be the benzoic acids and aldehydes rather than the cinnamic acids normally associated with reduced cell wall digestion (Burritt et al., 1984; Hartley, 1972). Inhibition of cellulolysis by addition of phenolic acid-carbohydrate esters at relatively high concentration appeared mainly due to the sugar and not to the phenolic moiety (Akin et al., 1992). Inhibition of *in vitro* digestion by phenolic-carbohydrate complexes released from sorghum cell walls appeared to be higher for those complexes with low molar *p*-CA/FA ratios and high xylose/arabinose ratios (Cherney et al., 1992).

In spite of the potential antimicrobial activity of phenolic acids, it is evident that phenolic compounds are extensively degraded in the rumen (Martin, 1982; Jung et al., 1983b). Certain bacterial strains (*Pseudomonas spp.*) are able to metabolize phenolic acids as their sole carbon source (Perez et al., 1990). When *p*-CA and FA were added to a hay diet for sheep at 20 g/kg diet DM, or infused intraruminally at 20 g/day, intake and digestibility were not affected (Lowry, 1990), indicating that free

phenolic acids never reached levels sufficient to depress microbial activity. Presumably a chemical reduction of the phenolic functions occurred, as there were no phenolic conjugates present in the urine, and benzoic acid was excreted as hippurate. Hydrogenation of the double C-C bond in the side chain, followed by reductive demethylation and dehydroxylation leads to the production in the rumen of 3-phenylpropanoic acid (3-PPA) (Martin, 1982), which is actually a growth factor for the cellulolytic bacterium, *Ruminococcus albus* (Hungate and Stack, 1982). Subsequent degradation of 3-PPA by the animal would occur in the liver by ß-oxidation, yielding benzoic acid, which is excreted as the glycine conjugate hippuric acid (Lowry, 1990). The loss of N by the excretion of hippurate may be an important aspect of phenolic acid metabolism, since most mature grasses and crop residues are low in N. Post-ruminal starch digestion was also shown not to be inhibited by oral administration or abomasal infusion of *p*-CA and FA (Mahmoudzadeh et al., 1989).

Alkali and Ammonia Treatment of Mature Forages and Crop Residues

Fibrous agricultural by-products such as corn stover and wheat and rice straws are abundantly available for ruminant feeding in both developed and developing countries (Kossila, 1984). However, feed resources such as straws and stovers are generally inadequate as sole sources of nutrients (O'Donovan, 1983; Givens, 1987). Crop residues are characterized by a low CP, low rate and extent of digestion, low intake and imbalanced mineral content (Schiere and Ibrahim, 1989). Crop residues consist mainly of cell wall material, because the cell contents have been transported to the seed or transformed into cell wall material.

A large variability in nutritive quality of straws and stovers exists due to differences among varieties, growth conditions and harvest procedures. Varietal differences are often greater than differences between straw species and are mainly related to different proportions of stems and leaves (Wales et al. 1990; Doyle and Champongsang, 1990).

Ammonia can be applied to fibrous feedstuffs with two distinct purposes, i.e. upgrading of low-quality residues, and preservation of moist hay or silage of relatively high quality (Tetlow, 1983). Ammonia treatment of crop residues or mature hay results in improved fiber digestibility and(or) intake (Horton and Stacey, 1979), whereas application of NH_3 to wet-baled hay reduces heating and molding (Weiss et al., 1982; Thorlacius and Robertson, 1984). Addition of NH_3 to alfalfa forage preserves plant protein of alfalfa through ensiling (Kung et al., 1984; Glenn, 1990).

Chemical Effects of Alkali Treatment. A major effect of alkali treatment is the saponification of ester linkages between cell wall components (Tarkow and Feist, 1969). As a result of cleavage of links between lignin and hemicellulose, the hemicellulose and cellulose become more accessible to microbial enzymes, increasing the potential digestibility of both cellulose and hemicellulose. Associated phenomena are partial solubilization of hemicellulose (Evans, 1979; Chesson, 1981; Hartley, 1987) and morphological swelling of the cellulose structure (Tarkow and Feist, 1969; Evans, 1979). The loss of acetic and phenolic acid esters, as well as the partial solubilization of hemicellulose and silica appear to be associated phenomena not directly related to enhancement of digestibility (Chesson, 1981).

In treatment with strong alkali at elevated temperature, lignin also undergoes cleavage of ether linkages between phenylpropane units (Iiyama et al., 1990; Lam et al., 1990). The increase in biodegradability has been shown to be linearly related to

the UV absorbance at 280 nm by phenolic material solubilized by alkali treatment (Lau and Van Soest, 1981), or, more specifically, by the amount of p-CA and FA released by alkali treatment (Hartley, 1987). The negative impact of lignin upon digestion of forage cell walls apparently depends on covalent linkage between lignin and carbohydrates. Due to the relatively unaltered composition of alkali-treated cell walls, the use of lignin content as a negative index of quality is a problem with unwashed, alkali-treated straws.

Treatment with Ammonia. Ammonia treatment is a relatively simple alkali treatment. Van Soest et al. (1984) demonstrated that NaOH and NH_3 do not differ in their basic effects upon cell walls of graminaceous plants. Infrared spectral characteristics of untreated and ammoniated tall fescue hay indicated a decrease in ester bond absorbance and an increase in amide bond absorbance (Buettner et al., 1982). Ammonia is a slow-reacting alkali that requires several days for effective treatment (Waiss et al., 1972), which has misled some researchers to believe that NH_3 is not an effective alkali. However, NH_3 treatment is only slightly less (Wanapat et al., 1985) or equally (Oji et al., 1977; Borhami et al., 1983; Deschard et al., 1988) effective as NaOH in terms of increasing digestibility, and it is equally (Oji et al., 1977) effective as NaOH in increasing voluntary intake. The slightly lower effectiveness of NH_3 as a saponifying agent seems to be offset by the N it supplies to the diet. Borhami et al. (1983) reported that total N, NH_3-N and microbial N reaching the duodenum of sheep were higher for NH_3 treated straw than for NaOH treated straw supplemented with urea, which indicates an additional benefit of NH_3 treatment in that part of the NH_3-N becomes bound to the fiber, where it is better utilized by cellulolytic micro-organisms. Performance for animals fed crop residues treated with NaOH or NH_3 is similar (Sundstol et al., 1978).

Urea is a readily available source of NH_3 for farmers in developing countries. The solid form of urea makes it very easy to handle and transport without the precautions necessary for gaseous or aqueous NH_3. The increase in digestibility due to urea and aqueous NH_3 treatments are generally in the same range (Kiangi et al. 1981; Llorente, 1986; Ali, 1991), although at low ambient temperature anhydrous NH_3 has shown a slight advantage over urea (Wanapat et al., 1985, 1986). With adequate moisture content and suitable temperature conditions, microbial and/or plant urease is capable of extensively hydrolysing urea (Oji and Mowat, 1977; Tetlow, 1983; Williams et al. 1984a,b) with the formation of ammonium compounds. Primarily NH_4OH, but also ammonium carbonate and -bicarbonate are formed, which then permeate through the straw.

Similar to NaOH treatment, a major effect of ammoniation is the saponification of ester linkages between cell wall components. A common observation in NH_3 treatment, irrespective of the form in which it is applied, is a reduction in NDF content (Kiangi et al., 1981), whereas ADF is unaffected, resulting in a lower values for hemicellulose (Kiangi et al., 1981; Givens et al., 1988; Mason et al., 1988, 1990; Schneider and Flachowsky, 1990; Kondo et al., 1992). The decrease in hemicellulose is thought to be caused by alkali-induced 'peeling' reactions (Mason et al., 1988) in which degradation of sugar moieties occurs at the reducing end of hemicellulose chains (Wilkie, 1979), or by partial solubilization after rupture of bonds between lignin and hemicellulose, since unbound hemicellulose is partially water-soluble (Wilkie, 1979). Among the hemicellulose components, arabinose seems to be released from hemicellulose more extensively than xylose (Morrison and Brice, 1984). Reduction in hemicellulose, particularly in pentose residues carrying alkali-labile substituents, can be regarded as evidence of effective NH_3 treatment. Graham and

Aman (1984) reported an increase in digestibility of barley straw by 23 percentage units for NH_3 treatment, which was due mainly to the solubilization of straw constituents during treatment, rather than enhanced degradation of residually bound fiber components. However, improvements of up 10 percentage units in digestibility have been reported with only minor reduction in the proportion of arabinose residues (Goto et al., 1991).

The extent to which FA and p-CA are released from cereal straws by treatment with increasing concentrations of alkali has been directly related to the observed improvement in digestibility (Chesson, 1981). Proportionately more FA than p-CA is released by treatment with NH_3 (Mason et al., 1988) and NaOH (Besle et al., 1988). A reduced acetyl content was observed in NH_3-treated barley straw (Morrison and Brice, 1984).

Laboratory Evaluation of Treatment Effectiveness. Organic matter digestibility of treated straws is poorly correlated with overall cell wall (Mason et al., 1988; Goto et al., 1991) and cell wall polysaccharide (Goto et al., 1991) composition. The relation between lignin content and digestibility is obscured by NH_3 treatment, e.g., within untreated or treated straw samples, regressions between lignin and digestibility are significant, but the difference in digestibility between untreated and treated straws does not allow lignin content to be a meaningful measure of treatment (Van Soest et al., 1984).

For alkali-treated roughages, *in vitro* digestibility values often overestimate digestibility *in vivo* (Berger et al., 1979). Van Soest et al. (1984) postulated that *in vitro* digestibility evaluation overvalues treated straws since the cleaved phenolic compounds are soluble but probably indigestible. *In vivo* digestibility values may not be improved by ammoniation due to higher passage rate and shorter retention time

in the rumen associated with a higher voluntary dry matter intake. The higher dry matter intake is however, a major benefit of NH_3 treatment, and leads, even without substantial increase in OMD, in an increase in DOM intake. However, when evaluating NH_3 treatment under restricted feeding conditions, the increase in *vitro* digestibility is reflected in higher *in vivo* digestibility (Kiangi et al., 1981). Overall, the *in vitro* digestibility seems to be an adequate means of measuring effectiveness of NH_3 treatment, as the method can also be adapted to estimate differences in rate of degradation, which are related to differences in voluntary intake.

Ammonia treatment, unlike treatment with NaOH, rarely produces the extensive solubilization of cell wall phenolics and carbohydrate (Chesson, 1981). Effectiveness of NH_3 treatment can however be measured by monitoring changes in optical density in the ultraviolet region. Mason et al. (1988) observed increased soluble phenolics, which mainly consist of esterified hydroxycinnamic acids released by saponification. Kondo et al. (1992), in a systematic characterization of soluble lignins from untreated and NH_3-treated wheat straw, provided evidence of cleavage of FA bridges by NH_3 treatment and the subsequent solubilization of a FA-rich lignin fraction.

There is a close relation between optical density at 280 nm (OD_{280}) of buffer or neutral detergent extracts and in vitro digestibility for untreated and treated samples (Van Soest et al., 1984). An exception to this close relation are straws exposed to heat during treatment (Mason et al., 1990; Garrido et al., 1992), which have a higher UV absorbance at 280 nm due to generation of soluble phenolic compounds by conversion of sugars to phenols. This reaction (Maillard reaction) is promoted by amino acids, and, apparently, NH_3 can substitute for amino acids in this reaction (Mason et al., 1990). The most important carbohydrate sources are

hemicelluloses and sucrose, and the products are artifact lignin polymers of high N content (Van Soest, 1965) and soluble low molecular weight phenolics (Hodge, 1953). Soluble phenolics have also been associated with the occurrence of toxicity problems (Garrido et al., 1992) producing hyperexcitability (Perdok and Leng, 1987).

Contrary to the mechanism of mild alkali treatment, oxidative treatment methods such as treatments with H_2O_2, O_3 or SO_2 are aimed at delignification of the fiber, after which the remaining cellulose and hemicellulose are more easily degraded. Ammonia and oxidative treatment appear to have additive effects, as shown by Fahmy and Klopfenstein (1992), who subjected maize stalks to urea-NH_3 treatment, SO_2 treatment, and urea-NH_3 treatment followed by SO_2 treatment, which resulted in improvements of *in vitro* OM digestibility of 22, 19, and 47%, respectively.

Treatment of cereal straw with strong alkali also releases a substantial portion of the core lignin in cell walls (Chesson, 1981), which suggests linkages between core lignin and arabinoxylan similar to the linkages between phenolic acids and cell wall components. Ammonia treatment, however, reduces lignin content only slightly, due to the generation of ADIN, especially in treatments involving heating, i.e. thermo-ammoniation (Van Soest and Mason, 1991). Increased lignin content by heating may occur irrespective of ammoniation (Schneider and Flachowski, 1990; Van Soest and Mason, 1991).

Nitrogen Utilization. An advantage of NH_3 treatment is the supply of NPN. The N required by rumen microbes is related to the potentially available fermentable energy, which is so low in straw and mature stover that the nitrogen present may well be adequate. When increasing the digestibility with alkali and other chemicals, supplemental N will however result in a more optimal rumen fermentation. Orskov and Grubb (1978) demonstrated that the response in voluntary intake and

digestibility to either NaOH treatment of straw or urea supplementation was limited in the absence of the other. The utilization of NH_3 depends on the total N concentration in the straw, the speed at which N is released in the rumen, the amount of available energy in the rumen, and the degradability of dietary protein. Although NaOH-treated roughages may require supplemental N for optimal rumen fermentation, NH_3 treated materials may require a supplemental energy source (Herrera-Saldana, 1982) depending on available energy in the straw.

Gordon and Chesson (1983) divided the N in NH_3 treated straw into water-soluble NH_3-N, water-soluble non-NH_3-N, and water insoluble non-NH_3-N. During storage, the N concentration of ammoniated straw decreases slightly, which is mainly due loss of water-soluble NH_3-N. Ammonia treatment increases microbial protein synthesis (Borhami et al., 1983; Hvelplund, 1989). However, a proportion of the NH_3-N appears to be tightly bound to the straw and unavailable to the microorganisms (Borhami et al., 1983). Solaiman et al. (1979) reported 12.6% of the NH_3-N to be bound to ADF, and Mason et al. (1988) reported a high concentration of N in indigestible cell walls.

Whether the effect of NH_3 treatment is mainly due to N supplementation or to alkali treatment of the straw, is unclear. Schneider and Flachowski (1990) found that the increase in DM digestibility of wheat straw resulting from ammoniation at increasing levels was poorly correlated with the increase in N-content (r = 0.58). Digestibility of DM and cell wall components of NH_3-treated timothy (*Phleum pratense* L) hay was higher than of untreated timothy with urea addition at feeding (Chiquette et al., 1992). Additional increases in rate of rumen degradation (Djajanegara and Doyle, 1989b; Ibrahim et al. 1989), intake and digestibility (Wanapat et al., 1985a; Djajanegara and Doyle, 1989a,b) of urea treated rice straw

versus isonitrogenous urea supplementation have been reported, part of which may still be explained by a more even N distribution and NH_3 adsorption to the straw substrate, and by increased susceptibility of the straw to physical breakdown. Of interest is the approach by Manyuchi et al. (1992), who were able to increase the rate of *in sacco* degradation of untreated straw by supplementing sheep with small amounts of NH_3-treated straw. The increased rate of degradation was attributable to the stimulatory effect in the rumen of the increased supply of fermentable energy provided by the NH_3 treated straw.

Digestibility of CP is sometimes slightly decreased with NH_3 treatment (Oji et al., 1977; Morris and Mowat, 1980). However, when Hvelplund (1989) increased the N-content of barley straw from .48 to 1.32% by ammoniation, 70% of the N was degraded in the rumen, versus 41% for untreated barley straw. Of the respective residual N entering the small intestine, an additional 36% was degraded, versus 32%, indicating the low value of rumen undegradable N in both treated and untreated straw. Hassen and Chenost (1992) found that the fecal nitrogen was mainly water soluble N, with only minor quantities of N retained by indigestible cell walls, contrary to the results of Mason et al. (1989a).

Initial Quality. The effectiveness of NH_3 treatment depends on the initial straw quality and straw variety (Ibrahim et al., 1989), the urea/NH_3 concentration, the moisture content of the straw and the duration of treatment. Many interactive effects exist among these factors (Bae and Jung, 1988; Mason et al., 1990).

The response in digestibility to NH_3 treatment has been higher for substrates with lower inherent degradability when comparing cereal species (Horton and Steacy, 1979; Kiangi et al., 1981; Givens et al., 1988), varieties (Tuah et al., 1986; Walli et al., 1988; Ibrahim et al., 1989; Nakashima and Orskov, 1990; Goto et al., 1991), and

plant parts (Walli et al., 1988; Nakashima and Orskov, 1990). In spite of the larger effect on poorer substrates, the substrates of higher initial quality generally still rank higher after treatment.

Maturity and alkali treatment may show complex interactions. Berger et al. (1979) reported a higher efficiency of NaOH treatment for late versus early harvested corn stalks in reducing NDF and increasing IVDMD. This may be, in part, due to differences in overall composition (e.g. cell soluble content), intrinsic characteristics of the fiber fraction (e.g. extent of interlinkages), or DM distribution between botanical fractions. Overall, the sites for alkali action appear to be more numerous in straws of poor quality.

The extent and nature of the bonding between lignin and hemicellulose is probably a key factor in determining initial cell wall degradability as well as potential improvement in degradability by ammoniation. Goto et al. (1991) reported that approximately one half of the difference in initial digestibility of different barley cultivars could be attributed to the variation in DM distribution among botanical fractions, with the remaining half due to inherent differences in the degradability of each fraction among varieties. However, the response to ammoniation for the leaf (8.2 percentage units) was similar to that for the stem fraction (8.8 percentage units). Although morphology had little effect on the overall response to NH_3 treatment, degradability of leaf tissue was independent of cultivar, whereas the response of stem tissue was highest for those cultivars with the lowest initial digestibility.

In a study by Kernan et al. (1979) on the effect of wheat variety, environment (location and year), and ammoniation, the effects of initial quality and ammoniation tended to be additive. Dias-da-Silva and Guedes (1990) did not observe a significant relation between initial DM digestibility of wheat, rye and triticale straws and the

increase in DM digestibility with urea-NH_3 treatment, but found a high negative correlation between buffering capacity and response to urea-NH_3 treatment. Cation-exchange capacities and buffering capacities of plant fibers are known to vary widely (McBurney et al., 1983). Knowledge of buffering capacity may be important to predict the optimum level of NH_3 application. For alkaline saponification of ester linkages, the minimum pH required is 9. In the case of urea treatment, the pH may not rise above 9, especially if a major part of the urea is converted into $(NH_4)_2CO_3$ or NH_4HCO_3. Saponification of ester bonds consumes a certain amount of alkali and releases acetic acid and phenolic acids, by which the alkaline environment is slowly neutralized. The rate of neutralization depends on the buffering capacity of the straw, which varies with different growing conditions.

Treatment Conditions. Generally, the different factors in NH_3 treatments show interactive responses, such as NH_3 level x moisture, NH_3 level x temperature, moisture x temperature, moisture x duration, and temperature x duration (Waiss et al., 1972; Borhami and Sundstol, 1982; Bae and Jung, 1988; Mason et al., 1990; Schneider and Flachowsky, 1990). Differences between straw species in required minimum urea/NH_3 level are not well described in the literature, but are possible on the basis of their differing buffering capacity. Individual treatment studies (Bae and Jung, 1988; Mason et al., 1990) have shown a curvilinear response to increased concentrations of urea or NH_3. For effective urea treatment, the quantity of urea should be at least 40 g/kg air dry straw (Schiere and Sewalt, 1988). Evidently, urea levels over 60 g/kg are too high and may depress intake, and increasing the urea level from 40 g/kg to 60 g/kg is usually not cost-effective. Response to increasing NH_3 concentrations in thermo-ammoniation of wheat straw were small once the application rate exceeded 20 g/kg DM (Mason et al., 1990). In aqueous or anhydrous

NH_3 treatment, generally 30 to 35 g/kg DM are optimal.

Increasing moisture content of urea treated rice straw has lead to both increasing (Bae and Jung, 1988) and decreasing (Tinnimit, 1988) digestibility. A minimum amount of water is needed as a vehicle for distribution and, in the case of urea, for the dissociation into NH_3. Too much (>100 L/100 kg straw) or too little water (<30 L/ 100 kg straw) may cause mold growth. Moisture content seems to affect the extent to which NH_3 remains bound to the straw (Schneider and Flachowsky, 1990). Airtight sealing helps to keep the NH_3 in the stack and avoids drying and mold formation, especially in small heaps of uncompacted straw. Aeration before feeding results in considerable loss of N. Spraying of NH_3 treated straw with organic acids (Borhami et al., 1982) can effectively reduce loss of NH_3 and increase N content of straw with no negative effect on DM digestibility and increased N digestibility.

Duration of treatment depends on ambient temperature, farmer attitude (whether small batches are treated for direct utilization or large batches are treated for upgrading and preservation) and compactibility of straw (mold formation). Generally, at high ambient temperature, a duration of 1 wk suffices for both NH_3 and urea treatments, provided a urease source is present (Ibrahim and Schiere, 1989). In two studies, 70% of the added urea was dissociated into NH_3 after 2 d, at room temperature (Oji and Mowat, 1977) or even at 12 °C (Tetlow ,1983). External urease sources such as soybean meal, legume seeds and leaves, rumen contents and cattle- and poultry feces have been investigated in laboratory trials to shorten the reaction time (Jayasuriya and Pearce, 1983; Ibrahim et al, 1985; Dias-da-Silva et al., 1988; Sahnoune et al., 1991), but extremely short treatment times of 3 d have been sufficient even without the addition of an external urease source (Ibrahim, 1985). At

low ambient temperature, a duration of 30 d is usually adequate (Sundstol et al., 1978). Effective thermo-ammoniation (55 to 90 °C) is achieved within 12 h (Mason et al., 1988).

Ruminal Fermentation. Total ruminal VFA production has been reported to be higher for animals fed NH$_3$-treated straws compared with untreated straws (Oji et al. 1979; Minato et al., 1989). Reports on the effect of feeding NH$_3$-treated straws on molar proportions of VFA's are conflicting. Oji et al. (1979) found increased molar proportions of proprionate and decreased butyrate, whereas Minato et al. (1989) reported increased proportion of butyrate. Density of protozoa is affected only slightly by feeding ammoniated versus untreated rice straw (Minato et al., 1989), although the percentage of *Entodinium spp.* increased with ammoniation. Increasing predominance of *Entodinia spp.* were observed also when increasing amounts of urea were supplemented, at the expense of cottonseed cake, to goats, sheep, cattle and buffaloe on a wheat straw diet. In the study of Minato et al. (1989), viable counts of cellulolytic bacteria, *Eubacterium spp.*, *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Succinovibrio spp.* were increased with ammoniation of rice straw, but *Bacteroides spp.* and *Butyrivibrio spp.* were dominant when untreated rice straw was fed. *In vitro* batch culture (Kolankaya et al., 1985) showed that anhydrous NH$_3$ treatment enhanced the rate of colonization of straw particles, increased straw solubilization, and increased microbial protein synthesis by *Bacteroides succinogenes*, *Ruminococcus albus*, and *R. flavefaciens*.

Nutritive Value. Although intake and digestibility of straws are increased by NH$_3$ treatment, the final product can still be considered as low-quality feed, allowing for maintenance or low production levels (Doyle et al. 1986; Schiere et al. 1989). In a review of feeding trials and *in vivo* digestibility trials, Sundstol et al. (1978)

concluded that the energy value of low-quality residues can be raised to that of medium quality hay. They observed a considerable variation in the degree of response to ammoniation, which may have been due to suboptimal conditions and to variation in initial quality.

For medium production levels, part of the straw will have to be replaced by concentrates or good quality forage. Increases in intake and weight gain by urea/NH_3 treatment of straw and concurrent supplementation with concentrates or green feed at low levels seem to be additive (Ghebrehiwet et al. 1988). At higher levels of concentrate supplementation, Creek et al. (1983) also obtained linear but converging responses to supplementation of untreated and NH_3 treated straw. Although urea treatment improves N and energy uptake by animals, mineral content remains unchanged. Mineral composition of straws is generally unbalanced, e.g. rice straw has an excess of K and Zn, contains adequate amounts of Ca, Mg and Na, but is deficient in P, S and Cu (Sewalt, 1989). By urea treatment, the added N will require even more S to maintain an adequate N:S ratio.

Ammoniation of Corn Stover. Several experiments have been reported concerning NH_3 treatment of corn stover (Oji et al., 1977, 1979; Oji and Mowat, 1979; Morris and Mowat, 1980; Kiangi et al., 1981; Saenger et al., 1982; Sankat and Bilanski, 1982). Oji et al. (1977) reported treatment of corn stover with aqueous NH_3 to be equally effective in increasing intake and digestibility of organic matter to treatment with $NaOH/Ca(OH)_2$, with no differences between 3% and 5% NH_3. At either level of NH_3, approximately 50% of the added N remained in the stover at feeding time, most of which was NH_3-N. 'True' protein (crude protein minus NH_3-N) was also increased with ammoniation, either by decreased proteolysis or by the formation of artifact protein due to the Maillard reaction.

Morris and Mowat (1980) reported that stack treatment of corn stover with anhydrous NH_3 increased intake of digestible dry matter by increasing both intake and digestibility of dry matter. In the same experiment, fine grinding of corn stover resulted in increased intake but decreased digestibility. The effects of ammoniation and grinding tended to be additive, implying that ammoniation results in chemical but not physical alterations in the fiber. Ammoniation of corn stover at elevated temperature for short periods ('thermo-ammoniation') has been superior to aqueous NH_3 treatment in increasing IVDMD and *in vivo* dry matter digestibility (Oji and Mowat, 1979), but the two treatments were similarly effective in increasing organic matter intake. Molar proportions of proprionate to total VFA in the rumen was higher in animals fed ammoniated stover than control (Oji et al., 1979).

Objectives

The overall objective of the research was to identify the structural components in corn stover that impose limitations to degradation by ruminal microorganisms. Changes in composition and digestibility of corn stover were determined in response to increasing maturity, and several hypotheses concerning inhibition of cell wall carbohydrate degradation by lignin were tested. Also, the response to NH_3 treatment of corn stover was evaluated. Specific objectives of individual experiments, corresponding to the respective Journal Papers, were as follows:

Experiment 1

- To determine the changes in overall composition, cell wall composition, lignin distribution, and *in vitro* digestibility of three corn stover fractions (leaf, upper stem and lower stem) in response to increasing maturity.

Experiment 2

- To test a hypothesis of occurrence of quinone methide formation and subsequent cross-linking reactions by nucleophilic addition during rumen fermentation of corn stover fractions.
- To relate overall cell wall composition, as affected by plant fraction, maturity, and conditions during maturation, to *in vitro* cell wall and DM digestibility.

Experiment 3

- To evaluate possible mechanisms by which lignin may affect microbial degradation of cellulose, i.e. close physical association with lignin, establishment of interlinkages after quinone methide formation, or hydrophobic enzyme adsorption to lignin, using a cellulosic hydrogel as model.

Experiment 4

- To determine the response of different corn stover fractions (leaf, upper stem, lower stem) to ammoniation.

- To separate the response to ammoniation into effects of non-protein N (NPN) addition and chemical treatment per se.

- To relate the response to NH_3 treatment to changes in linkages of phenolic acids with other cell wall components (ester and ether linkages).

Experiment 5

- To compare the *in vitro* response in cell wall degradation to NH_3 treatment of different corn stover fractions harvested in different years,

- To evaluate the relation between treatment response and initial quality,

- To separate the overall response to NH_3 treatment into NPN supplementation and chemical treatment effects, interrelated with rumen environment as simulated by different buffer systems.

Journal Paper 1. Cell Wall Composition and Digestibility of Corn Plant Fractions as Affected by Maturity

ABSTRACT: Composition and IVDMD of corn stover fractions (leaf, upper stem, lower stem) from plants harvested at six maturity stages, ranging from soft dough to full maturity, were evaluated. Lignin distribution in tissues and cell walls of corn fractions of advanced maturity was studied using Scanning Electron Microscopy (SEM) and Energy Dispersive Analysis of X-rays (EDAX). At all maturity stages, stem fractions were lower (P<.001) in DM and CP, and higher (P<.001) in water soluble carbohydrates (WSC), than leaf. Lower stem remained higher (P<.05) in WSC than upper stem with advanced maturity. Neutral detergent fiber, ADF, and KMnO$_4$ lignin increased (P<.001) with maturity. Klason lignin in NDF was higher (P<.001) in stem fractions than in leaf, and increased (P<.01) with maturity. Glucan content of NDF was highest (P<.001) for lower stem and lowest (P<.001) for leaf, but was not affected (P>.05) by maturity. Xylan, arabinan, and galactan in NDF and methoxyl content of Klason lignin were higher (P<.001) in leaf than stems. Concentrations of arabinan, galactan, and lignin methoxyl groups in NDF increased (P<.001) with maturity. Leaf was higher (P<.05) in IVDMD than stem fractions at

all stages except full maturity. Changes in IVDMD with maturity were highly correlated with CP, NDF, and ADF. Within maturity stages, IVDMD was highly correlated to cell wall composition (lignin methoxyl content, xylan:arabinan ratio, glucan:xylan ratio) and CP. No firm conclusions on variation in lignin distribution could be drawn.

Key Words: *Zea mays*, botanical fractions, maturity, lignin methoxyl groups, electron microscopy.

Introduction

Botanical fractions of forages differ in composition, leaves generally being of higher quality than stems due to higher protein and lower lignin content (Morrison, 1980). Maturity of corn stover is associated with an increase in NDF concentration due to the loss of cell solubles (Weaver et al., 1978; Berger et al., 1979) after conversion of sugars into grain starch (Phipps and Weller, 1979). In corn stover, the effect of maturity on digestibility seems to be associated more with NDF than with lignin concentration (Russell, 1986). Factors other than lignin content per se may be more important in explaining differences in digestibility, e.g. lignin composition (Reeves, 1985) and distribution within cell walls (Wallace et al., 1991). The methoxyl content of lignin affects the formation of intermolecular linkages, e.g., between lignin and hemicellulose (Leary, 1980), and is, therefore, likely to affect digestibility of plant cell walls. The objectives of the research were: to determine the changes in composition, lignin distribution, and digestibility of three corn stover fractions (leaf, upper stem and lower stem) in response to increasing maturity.

Experimental Procedures

Plant Material. Six harvests of corn (*Zea mays*) plants were made from August 20 to October 18, 1991, at stages ranging from soft dough to full maturity (grain harvest). The corn (cv. SS 728, Southern States Cooperative, Richmond, Virginia) was mildly drought-stressed, after a dry summer and early fall (monthly precipitation, recorded approximately 8 km from experimental site, June to October: 32, 54, 67, 12, and 7 mm, respectively, average 34 mm). Each harvest was from six replicate field plots, and included six plants per plot. The plants were separated into leaf blades and stems (including leaf sheaths). Stems were further divided in upper (above ear) and lower (below ear) fractions. Replicate fractions were chopped through a 1-cm screen, and frozen at -20 °C. Subsamples were freeze-dried and ground in a Wiley mill to pass a 1-mm screen.

Chemical Analyses and In Vitro Dry Matter Digestibility. Dry matter, CP, and WSC (Dubois et al., 1956, as modified by Johnson et al., 1966) were determined after thawing. Detergent fiber components (Robertson and Van Soest, 1980), and IVDMD (Tilley and Terry, 1963) were determined on freeze-dried samples. Fractions harvested on August 20 (soft dough), September 20 (early dent), and October 18 (full maturity) were also analyzed for Klason lignin, acid soluble lignin, neutral cell wall polysaccharides. Cell walls were subjected to a two-stage acid hydrolysis (TAPPI, 1985, modified by Kaar et al., 1991) by suspending NDF ($\pm$ 100 mg) in 1 mL of 72% H_2SO_4 in uncovered 50-mL reaction tubes kept in a water bath at 30°C for 1 h. The initial hydrolysis was followed by dilution to 4% H_2SO_4 and autoclaving of the sealed tubes at 121 °C for 1 h. The hydrolysis mixture was passed through a glass fiber filter (Whatman grade 934 AH, particle retention 1.5 μm) in 30-mL gooch crucibles of fine

porosity (pore size 4-5.5 μm). The residue (Klason lignin) and filter were oven-dried (105 °C, overnight), weighed, and retained for further analysis (ash, N and methoxyl groups).

Neutral polysaccharides (glucan, xylan, arabinan, and galactan) were analyzed by HPLC (Kaar et al., 1991) after dilution of the filtrate to 100 mL, and buffering of a 10-mL aliquot to pH 5.3 with $Ba(OH)_2$ and addition of an appropriate amount of erythritol as internal standard. The buffered solution was centrifuged at 1175 g for 10 min., the supernatant evaporated under vacuum to about 2 mL and passed through an anion exchange column before analysis using an HPLC ion partition column. Furfural degradation products of glucose (hydroxymethyl furfural) and xylose (2-furaldehyde) were determined in non-buffered filtrate and used for correction of glucan and xylan contents. Acid soluble lignin (ASL) was quantified spectroscopically by UV absorption at 205 nm (TAPPI, 1989) using non-buffered filtrate. Lignin methoxyl groups were determined by reaction with 57% hydriodic acid at 145 to 150 °C in a methoxyl apparatus, and titration of the resulting iodine with sodium thiosulphate (TAPPI, 1972).

Statistical Analysis. Data were analyzed by repeated measures analysis of variance using the GLM procedures of SAS (1989) with plant fractions as main effects and repeated measures at the different maturity stages. Field plots served as experimental units (n = 6). Orthogonal contrasts tested were: leaf vs stems (upper and lower); upper vs lower stems; linear and quadratic effects with time (maturity) for all components and IVDMD; and cubic effects with time for proximate components and IVDMD; interactions between main effects and between fraction and maturity contrasts. Correlation coefficients of components with IVDMD were calculated using regression procedures of SAS (1989).

Electron Microscopy. Stovers harvested at early dent and full maturity were used for morphological comparison of leaf, upper stem and lower stem by scanning electron microscopy (SEM). Lignin distribution was determined by bromination of samples followed by energy dispersive analysis of X-rays (EDAX) coupled to the SEM (Saka et al., 1978). Specimens (approximately 1 x 5 x 5 mm) were cut from the first leaf above the ear and from the fourth and ninth internodes, above ground level. Sections were made with a razor blade to expose cross-sectional and longitudinal surfaces. Specimens preparation was modified after Saka et al. (1978). Specimens were extracted with benzene/ethanol (2:1, vol/vol) for 2 d, washed three times with ethanol, which was replaced with chloroform before bromination. The specimens were treated 10 min with dilute bromine solution prepared by mixing 0.3 ml reagent grade bromine in 90 ml chloroform (Saka et al., 1978). After bromination, the specimens were rinsed with chloroform to remove excess bromine, washed twice (15 min), and extracted overnight with chloroform. Chloroform was replaced with ethanol, and specimens were freeze-dried overnight, using a benchtop Virtis 3 L freeze-drier. Freeze-dried specimens were mounted on carbon specimen holders, and sputter coated under vacuum with 15 nm gold-palladium, using an Hummer X sputter coater (Anatech Ltd.). The specimens were examined in a Philips Scanning Electron Microscope (SEM 505) at an accelerating voltage of 20 kV, and micrographs were taken using polaroid equipment. Bromine distribution was determined by EDAX, using the EDAX PV 9900 system (EDAX International, Mahwah, NJ) at 0° tilting angle of the specimen holder, and generating dot maps for bromine.

Results and Discussion

Composition and In Vitro Dry Matter Digestibility. Dry matter content (Figure 5) increased (P<.001) with maturity, but at different rates for leaf vs stems (P<.001) and upper vs lower stem (P<.001) (fraction x maturity interaction, P<.001). At all maturities, stem fractions were lower (P<.001) in DM than leaf. Ash and silica were higher (P<.001) in leaf than stems (Figure 5). Upper stem was higher (P<.05) in ash, and tended to be higher (P=.052) in silica than lower stem (Table 2). Leaf protein was higher (P<.001) than stem protein (Figure 6), and decreased (P<.001) from 16% in August (soft dough) to 7.7% in September (early dent) (Table 2), after which further decrease was small (linear and quadratic effects, P<.001). Leaf was lower (P<.001) in WSC than stems (Figure 6, Table 2). Lower stem maintained more WSC (P<.05) than upper stem with advanced maturity. The mild drought stress apparently resulted in a relatively high residual concentration of WSC in the stalks. A similar observation was made by Russell (1986) in a season of inadequate precipitation.

In all fractions, NDF increased (P<.001) with maturity (Figure 7), but at different rates for leaf vs stems (P<.05) and for upper vs lower stem (P<.01). Initially, leaf NDF was higher than stem NDF, but this difference decreased with increasing maturity (fraction x maturity interaction, P<.001). Acid detergent fiber was lower (P<.001) in leaf than in stem (Figure 7), and increased (P>.001) with maturity. Hemicellulose (NDF minus ADF) decreased (P<.05) with maturity in leaf (Table 2), but increased (P<.05) in upper stem (fraction x period interaction, P<.001).

Permanganate lignin increased (P<.001) with maturity (Figure 7, Table 2), with the largest increase in lower stem (fraction x period interaction, P<.001).
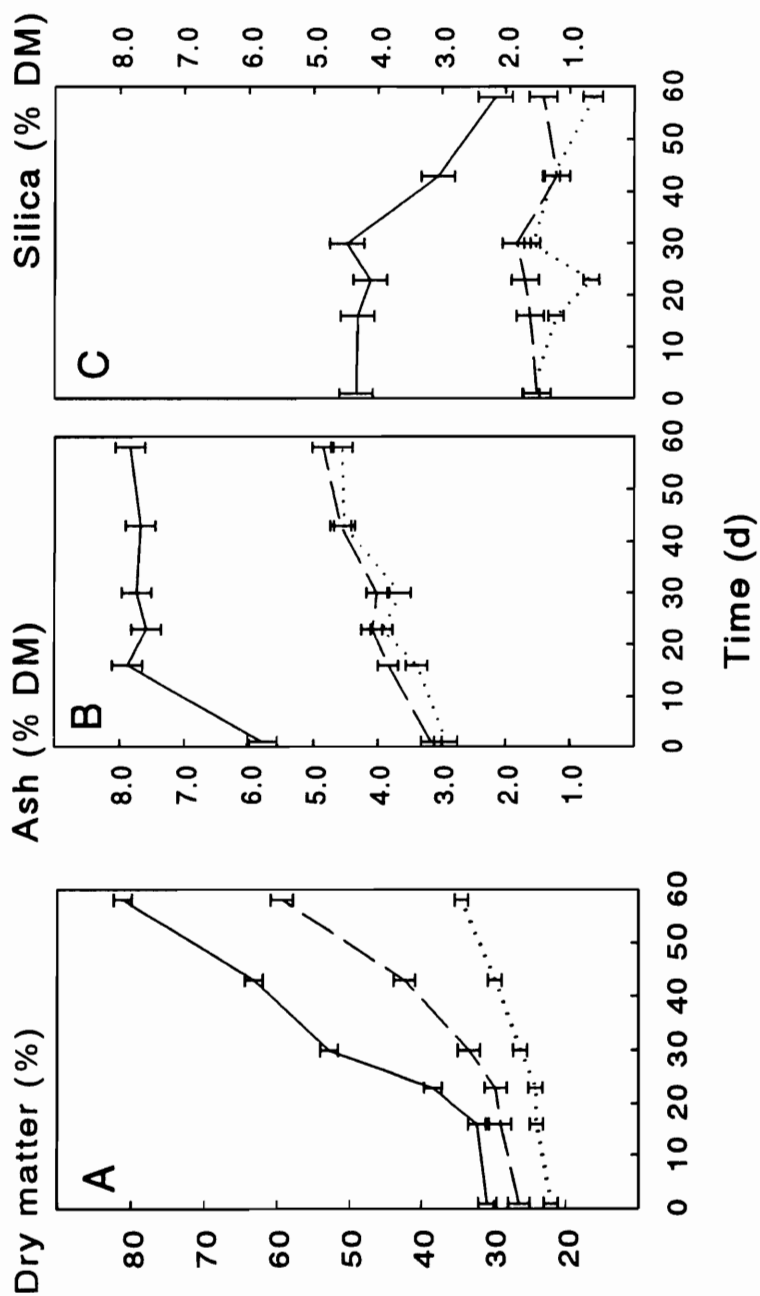
Figure 5. Dry matter (DM), ash, and silica in corn leaf (———), upper stem (— — —), and lower stem (. . . . .) as affected by maturity. Starting date (d 1) was August 20, 1991 (soft dough). Leaf differs (P<.001) from stems, and upper stem differs (P<.001) from lower stem for all components. Fraction x maturity interactions (P<.001); maturity effects different between leaf and stems (P<.001), and between upper and lower stem (P<.05) for all components. (A) DM. Linear (P<.001) and quadratic (P<.01) time effects for all fractions. (B) Ash. Linear and quadratic (P<.001), and cubic (P<.01) time effects for leaf. Linear time effects for upper (P<.001) and lower (P<.01) stem. (C). Linear and quadratic (P<.001) time effects for leaf. Linear and cubic (P<.01) effects for lower stem.

Table 2. Dry matter content, composition and *in vitro* dry matter disappearance of corn plant fractions harvested at three maturity stages

	Plant fraction by maturity[a]									SE
	Leaf			Upper stem			Lower stem			
	Soft dough	Early dent	Mature	Soft dough	Early dent	Mature	Soft dough	Early dent	Mature	
DM, %[bcdef]	30.9	52.8	81.1	26.5	33.4	59.3	22.1	26.4	34.5	1.2
Ash[bdeg]	5.80	7.74	7.84	3.16	4.02	4.86	2.93	3.65	4.56	.17
Silica[bdeg]	4.34	4.48	2.17	1.52	1.84	1.42	1.61	1.59	.64	.22
Cp[bdefg]	16.0	7.7	6.4	4.5	2.8	4.2	4.0	4.0	4.7	.23
WSC[bcdefg]	11.4	7.0	3.9	32.7	20.6	13.2	37.0	26.3	23.5	.98
NDF[bcdefg]	63.1	66.1	68.7	54.9	67.2	71.0	54.9	61.6	61.7	1.3
ADF[bdefg]	27.8	34.9	40.0	33.2	36.6	38.1	28.8	38.8	38.0	.80
Lignin										
KMnO$_4$[bcdefg]	1.64	2.88	4.35	3.73	3.69	4.53	2.18	5.68	5.81	.20
Klason[bdgh]	6.8	8.0	8.0	6.9	8.5	9.5	7.0	8.2	8.5	.29
IVDMD, %[bcdef]	71.2	59.3	52.4	60.6	45.4	46.6	57.4	51.2	52.5	1.3

[a] Harvest dates, August 20, September 20, and October 18, 1991, respectively.
[b] Leaf differs (P<.05) from stems.
[c] Upper stem differs (P<.05) from lower stem.
[d] Maturity effect (P<.05).
[e] Fraction x maturity interaction (P<.05); maturity effect differs (P<.05) for leaf vs stems
[f] Fraction x maturity interaction (P<.05); maturity effect differs (P<.05) for upper vs lower
[g] Percentage of DM.
[h] Linear maturity effect (P<.001); linearity differs (P<.05) for leaf vs stems and upper vs lower.
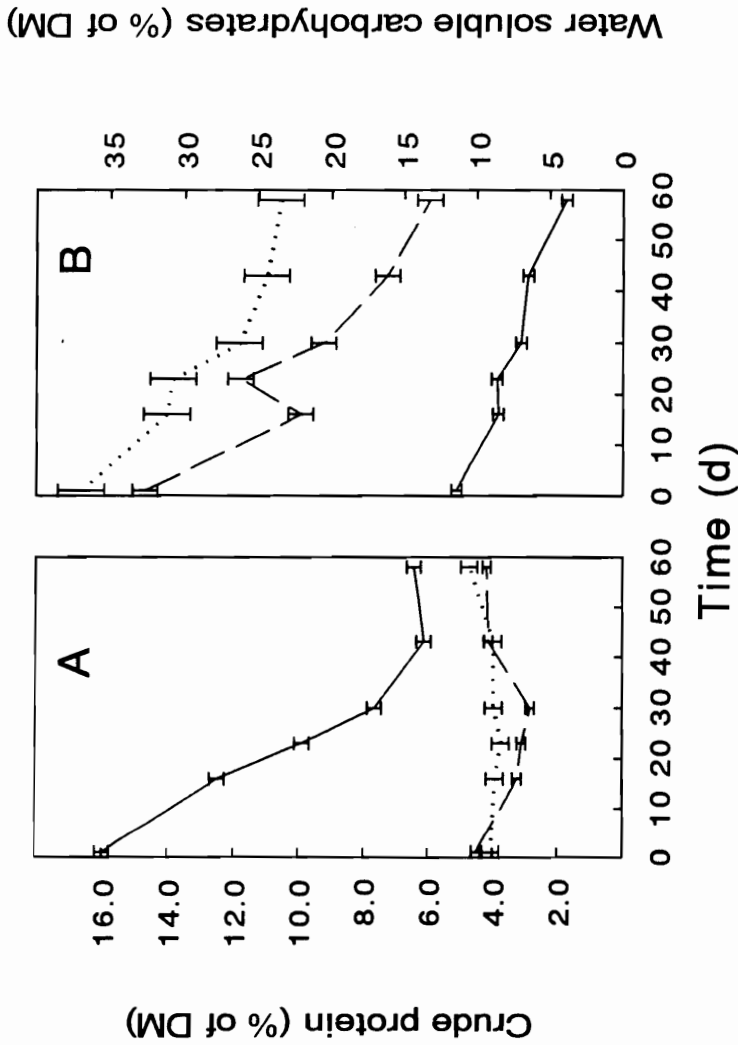
Figure 6. Crude protein and water soluble carbohydrates in corn stover leaf (————), upper stem (– – – –), and lower stem (. . . . .) as affected by maturity. Starting date (d 1) was August 20, 1991 (soft dough stage). Leaf differs (P<.001) from stems, and upper stem differs (P<.001) from lower stem; fraction x maturity interactions (P<.001); maturity effects different between leaf and stems (P<.001), and between upper and lower stem (P<.05) for both components. (A) CP. Linear (P<.001), quadratic (P<.001), and cubic (P=.047) time effects for leaf, quadratic (P<.001) and cubic (P<.001) time effects for upper stem, and quadratic (P<.001) and cubic (P<.001) time effects for lower stem. (B) WSC. Linear (P<.001) time effects for all fractions. and quadratic (P=.045) time effect for lower stem.
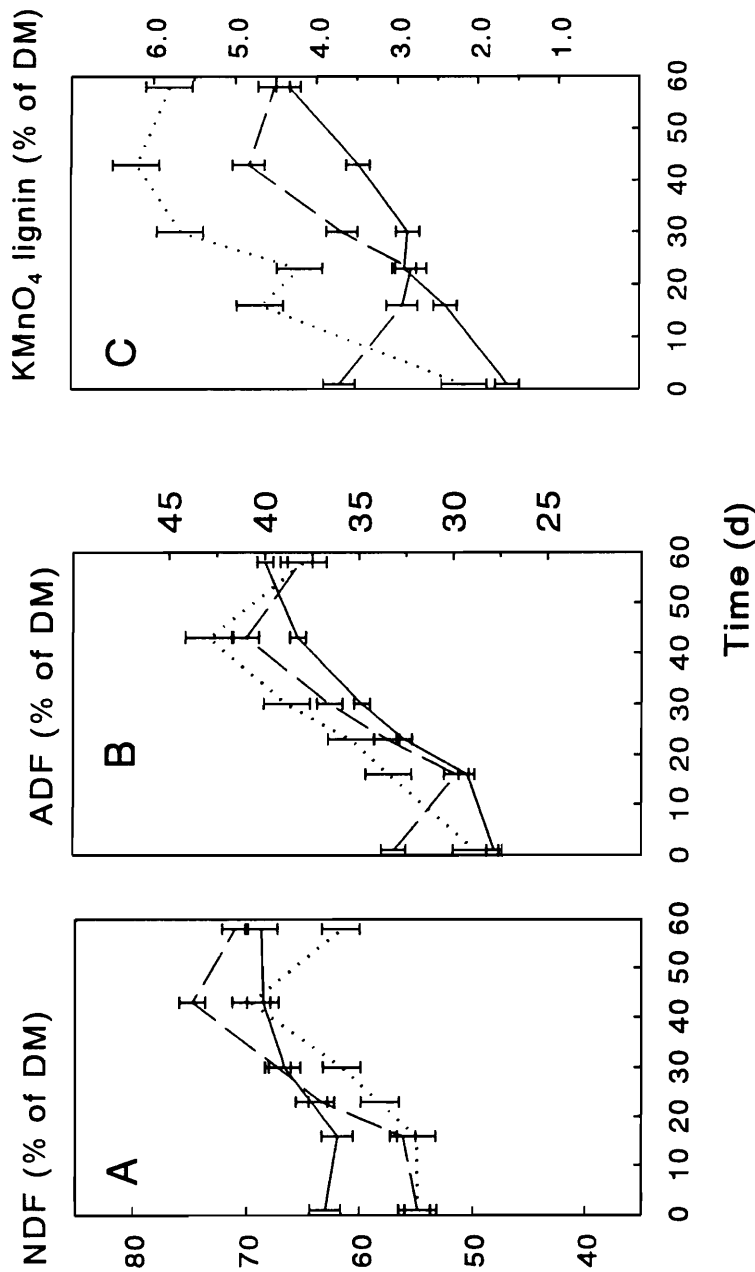
Figure 7. Detergent fiber components of corn stover leaf (———), upper stem (— — —), and lower stem (. . . . .) as affected by maturity. Starting date (d 1) was August 20, 1991 (soft dough). Leaf differs ($P < .001$) from stems; maturity effects differ between leaf and stems ($P < .01$) and between upper and lower stem ($P < .05$) for all components. (A) Neutral detergent fiber (NDF). Upper stem differs ($P < .001$) from lower stem. Linear ($P < .001$) effect of time for leaf. Linear ($P < .001$), quadratic ($P < .05$), and cubic ($P < .05$) time effects in upper stem. Linear ($P < .001$) and cubic ($P < .01$) effects in lower stem. (B) Acid detergent fiber (ADF). Linear ($P < .001$) effect of time for leaf. Linear ($P < .001$) and cubic ($P < .01$) effects in upper stem. Linear ($P < .001$), quadratic ($P < .05$), and cubic ($P < .05$) time effects for lower stem. (C) Permanganate (KMnO$_4$) lignin. Upper stem differs ($P < .001$) from lower stem. Linear ($P < .001$) and cubic ($P < .01$) time effects for leaf. Linear ($P < .001$), quadratic ($P < .05$), and cubic ($P < .05$) time effects for upper stem. Linear ($P < .001$) and quadratic ($P < .001$) time effects for lower stem.

Klason lignin was higher (P<.001) in stem fractions than in leaf, both in DM (Table 2) and in NDF (Table 3). Klason lignin in NDF increased linearly (P<.05) with maturity. Total lignin (Klason plus acid insoluble lignin) did not change (P>.05) with maturity. Methoxyl content of lignin was lower (P<.001) in leaf than stems, but was not affected (P>.05) by maturity. However, as percentage of NDF, lignin methoxyl groups increased (P<.001) with maturity due to the increase in Klason lignin content.

Glucan in NDF was higher (P<.001) in stems than leaf (Table 3), with lower stem higher (P<.001) than upper stem. Hemicellulosic polysaccharides (xylan, arabinan and galactan) in NDF increased linearly (P<.01) with maturity. Xylan in NDF was higher (P<.001) in leaf than in stem fractions. Both arabinan and galactan in NDF were different (P<.05) among all three fractions, with highest values for leaf and lowest values for lower stem.

Leaf was higher (P<.05) in IVDMD (Figure 8) than stem fractions at all stages except full maturity, at which time IVDMD of leaf was similar to the value for lower stem. Initially (August), IVDMD for upper stem was higher (P<.05) than for lower stem, but the relative values reversed with advanced maturity (maturity x fraction interaction, P<.001), probably due to the larger increase in NDF for upper stem associated with the higher retention of WSC in lower stem.

Due to the observed fraction x maturity interactions, IVDMD was only moderately correlated with a few components, notably CP and negatively with ADF (Table 4). The fraction of lowest IVDMD (lower stem) was lowest in NDF, which resulted in a poor overall correlation of NDF with IVDMD. Within fractions, changes in IVDMD with maturity were highly correlated with overall composition: CP of leaf, and WSC of leaf and upper stem were positively correlated with IVDMD; and ADF, lignin methoxyl groups (% NDF), and NDF content were negatively

Table 3. Cell wall composition of corn plant fractions harvested at three maturity stages

Component	Plant fraction by maturity[a]									SE
	Leaf			Upper stem			Lower stem			
	Soft dough	Early dent	Mature	Soft dough	Early dent	Mature	Soft dough	Early dent	Mature	
Acid sol. lignin[bcdef]	4.55	2.90	2.18	2.45	2.24	2.11	1.98	1.81	1.74	.061
Klason lignin (KL)[bcg]	10.8	12.0	11.6	12.6	12.7	13.4	12.7	13.3	13.8	.35
Glucan[bcdf]	38.2	42.3	42.8	44.4	42.4	43.2	47.2	45.8	46.4	.57
Xylan[bcfgh]	22.2	24.8	24.2	21.2	20.7	23.1	20.8	21.4	21.9	.34
Arabinan[bcdfg]	3.21	3.72	3.46	2.75	2.97	3.39	1.90	2.02	2.48	.105
Galactan[bcdg]	1.05	1.25	1.49	0.88	0.94	1.21	0.69	0.75	0.99	.100
Lignin methoxyl groups[i]										
(% of KL)[c]	10.6	10.1	11.9	14.6	14.8	14.9	15.5	14.8	15.2	.40
(% of NDF)[cdgj]	1.04	1.34	1.36	1.61	1.95	2.02	2.05	2.09	2.15	.065

[a] Harvest dates, August 20, September 20, and October 18, 1991, respectively
[b] Percent of NDF
[c] Leaf differs (P<.05) from stems
[d] Upper stem differs (P<.05) from lower stem
[e] Linear and quadratic maturity effects (P<.05)
[f] Fraction x maturity interaction (P<.05); effect of maturity differs for leaf vs stems
[g] Linear maturity effect (P<.01)
[h] Fraction x maturity interaction (P<.05); effect of maturity differs for upper vs lower stem
[i] Determined in duplicate for each fraction at each maturity (n=2)
[j] Linearity of maturity effect differs (P<.05) between upper and lower stem.
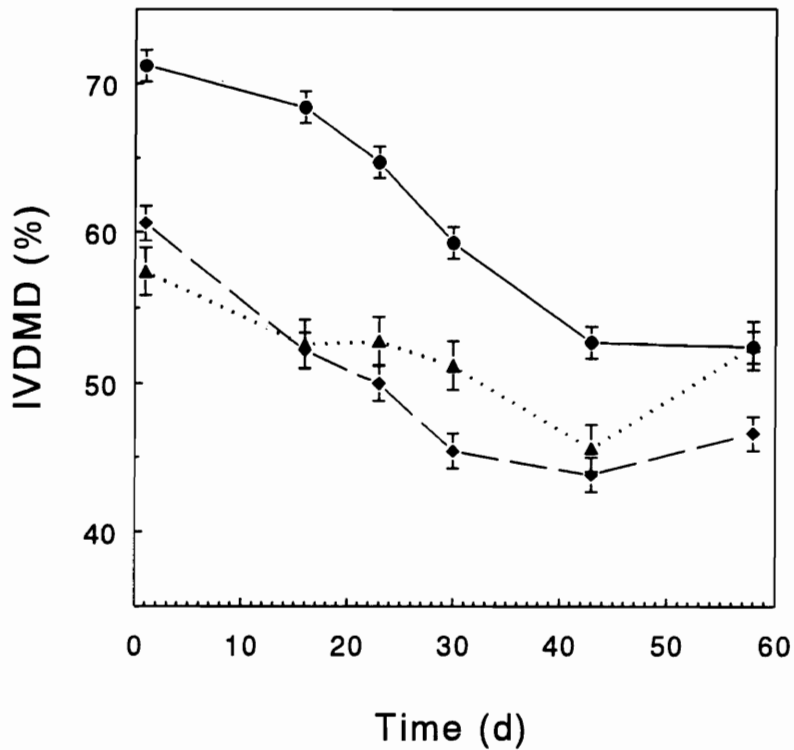
Figure 8. *In vitro* dry matter disappearance of corn plant leaf (————), upper stem (– – – –), and lower stem (. . . . .) as affected by maturity. Starting date (d 1) was August 20, 1991 (soft dough). Leaf differs (P<.001) from stems. Upper stem differs (P<.01) from lower stem. Fraction x maturity interaction (P<.001). Maturity effect different (P<.001) between leaf and stems and between upper and lower stem. Linear (P<.001) and cubic (P<.05) effects of time on leaf IVDMD. Linear (P<.01) and quadratic (P<.05) effects of time on IVDMD of stems.

Table 4. Correlation Coefficients of components with IVDMD

Component	Overall	Within stages			Within fractions		
		Soft dough	Early dent	Full maturity	leaf	Upper stem	Lower stem
Ash[a]	NS	.90	.74	NS	-.80	-.70	-.49
Silica[a]	.60	.86	.74	NS	.68	NS	NS
CP[a]	.79	.94	.89	.62	.95	.71	NS
WSC[a]	NS	-.94	-.68	NS	.95	.84	NS
NDF[a]	-.48	.82	NS	-.61	-.63	-.92	-.93
ADF[a]	-.78	-.45	-.64	-.40	-.96	-.89	-.87
KMnO$_4$ lignin[a]	-.63	-.45	NS	NS	-.92	NS	-.70
Klason lignin[b]	-.54	-.62	-.42	NS	-.49	NS	-.45
Acid sol. lignin[b]	.76	.96	.61	NS	.97	.75	.41
Glucan[b]	-.40	-.92	NS	NS	-.91	.68	NS
Xylan[b]	NS	.61	.78	NS	-.71	NS	NS
Arabinan[b]	NS	.84	.46	NS	-.42	NS	NS
Glucan/xylan ratio	-.30	-.91	-.68	NS	NS	.50	.42
Xylan/arabinan ratio	NS	-.76	NS	NS	NS	NS	NS
Methoxyl content							
% of KL	-.59	-.92	-.89	NS	NS	NS	NS
% of NDF	-.72	-.96	-.77	NS	-.90	-.97	NS

[a] Percent of DM
[b] Percent of NDF

correlated with IVDMD. The major decline in nutritive quality occurred before the early dent stage, associated with the effects of physiological maturation. After the early dent stage, further quality loss is mainly due to weathering.

Differences in IVDMD among corn plant fractions were highly correlated with CP (all stages) and cell wall composition (soft dough and early dent). The correlations with IVDMD were generally lower at advanced maturity, related to the lower variability in composition. At the soft dough stage, IVDMD was negatively correlated with the glucan:xylan and xylan:arabinan ratio's. This is in agreement with the general observation that in relatively immature forages, hemicellulose is more easily degraded than cellulose (Gordon et al., 1983). At higher maturity, xylan is often the least degradable carbohydrate (Piwonka et al., 1991; Titgemeyer et al., 1992), due to increasing interactions with lignin (Brice and Morrison, 1982) or larger extent of hydrogen bonding when xylan becomes less substituted (Hatfield, 1989).

Overall, concentrations of $KMnO_4$ lignin in DM and Klason lignin in NDF were moderately correlated with IVDMD, but $KMnO_4$ lignin was highly correlated with IVDMD within leaf and lower stem (Table 4). Although it is widely accepted that lignin is a major factor limiting fiber degradation, lignin content is often poorly correlated with digestibility (Jung and Vogel, 1986; Russell, 1986). This is in part related to limited variability in lignin content in forages, and to the difficulty of determining lignin (Reeves, 1993) as exemplified by the difference in lignin values obtained by the permanganate oxidation and acid hydrolysis.

For lignin to be important in limiting fiber degradation, lignin content and IVDMD need not necessarily be highly correlated. The decline in digestibility with maturity may be associated with increased lignin content, but also with changes in lignin composition (Buxton and Russell, 1988) and interlinkages (Brice and Morrison,

1982). The major route of establishment of interlinkages between lignin and carbohydrates during lignification is through nucleophilic addition reactions to quinone methide intermediates (Leary, 1980). The likelihood of formation of quinone methide intermediates is higher for syringyl lignin (Glasser and Kelley, 1987). Insight into the lignin structure (e.g. methoxyl content) is therefore a valuable tool in explaining differences in digestibility among plant fractions and maturity stages. The impact of functional groups in lignin (methoxyls and phenolic hydroxyls) on fiber degradation by rumen microorganisms is further evaluated in the accompanying Journal Papers 2 and 3.

Lignins of monocotyledons are generally considered to be of the H-G-S type, containing substantial amounts of p-hydroxyphenyl (no methoxyl groups), guaiacyl (monomethoxylated), and syringyl (dimethoxylated) units, but p-hydroxybenzaldehyde in traditional nitrobenzene oxidation mixtures is largely derived from p-CA (Higuchi et al., 1967). At first, lignin methoxyl content of the stover in our experiment may seem rather low (.75 to 1.0 OCH_3 group in each monomeric unit), possibly due to impurities. However, Klason lignin had been corrected for N and ash, and the only further contamination could be due to carbohydrate. As measured by the methoxyl content of ash-and N-corrected Klason lignin, it seems that the corn stover lignin in this experiment contained a considerable amount of p-hydroxyphenyl units, or esterified p-CA, in agreement with ^{13}C NMR spectra of grasses (Nimz et al., 1981). The lower methoxyl content of leaf lignin (.75 group per monomeric unit) vs stem lignin (1 group per monomeric unit) is either due to a larger contribution of p-hydroxyphenyl units or a lower content of syringyl units.

Morphology and Lignin Distribution. Micrographs of typical cross-sectional and longitudinal surfaces of leaf blade, and upper and lower stem internodes are

presented in Figures 9 and 10. Overall, tissue morphology of the three fractions did not substantially differ between early dent and full maturity (direct comparison not shown). The number of vascular bundles in stems containing thick-walled sclerenchyma and xylem was similar between upper and lower stem internodes (Figure 9), but the cross-sectional area occupied by vascular and support tissues appeared larger in the lower than in the higher internode (Figure 10). The high content of WSC in stems was confirmed by the observation of crystalline substances in the lower stem (Figure 9), even after the extensive extraction and washing procedures.

At both stages, lignin was present in all tissues, including stem and leaf parenchyma, as evidenced by bromine distribution patterns given in Appendix Figures 1 to 14. However, the method used resulted in rather non-specific bromine patterns. Lignin in corn leaf appeared somewhat more concentrated in epidermis than in phloem and mesophyll (Appendix Figures 5 and 6). However, no apparent differential lignin distribution was observed among vascular tissue, sclerenchyma, epidermis and parenchyma in the leaf midrib (Appendix Figures 1 to 4) and stem internodes (Appendix Figures 7 to 10, 13 and 14).

Possible differences in bromine concentration among tissues may have been obscured by differential penetration of X-rays in the different tissues (Saka et al., 1978). Due to penetration of X-rays in walls of underlying cells, bromine sometimes appeared to be as concentrated in the cell lumen as in the wall (Appendix Figures 3 and 10). No apparent differential lignin distribution was observed between xylem and sclrenchyma (Appendix Figure 11) or within a thick sclerenchyma wall (Appendix Figure 12), the latter being attributable to the limit in resolution obtainable with the SEM.
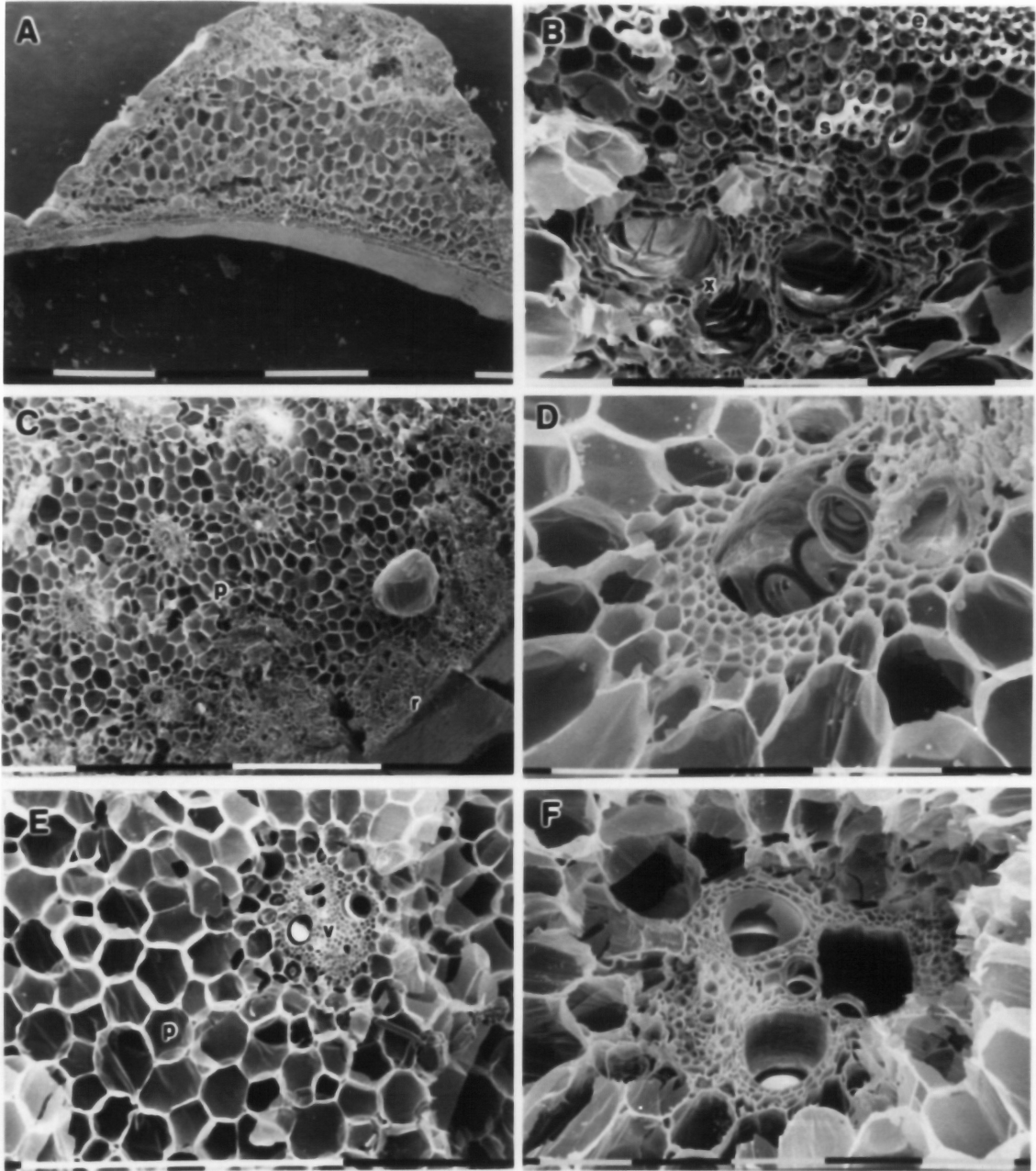
Figure 9. SEM micrographs of corn plant fractions. (A) Leaf midrib, full maturity (x 20, bar = 1 mm). (B) Magnification of (A), with, epidermis (e), sclerenchyma (s), xylem (x) (x 212, bar = .1 mm). (C) Upper stem internode, full maturity. Rind (r) and pith (p) with vascular bundles (v) (x 25, bar = 1 mm). (D) Upper stem internode, early dent. Vascular bundle (x 252, bar = .1 mm). (E) Lower stem internode, early dent. Pith with vascular bundle (v) in parenchyma (p) (x 55, bar = 1 mm). (F) Lower stem internode, full maturity. Vascular bundle (x 186, bar = .1 mm).
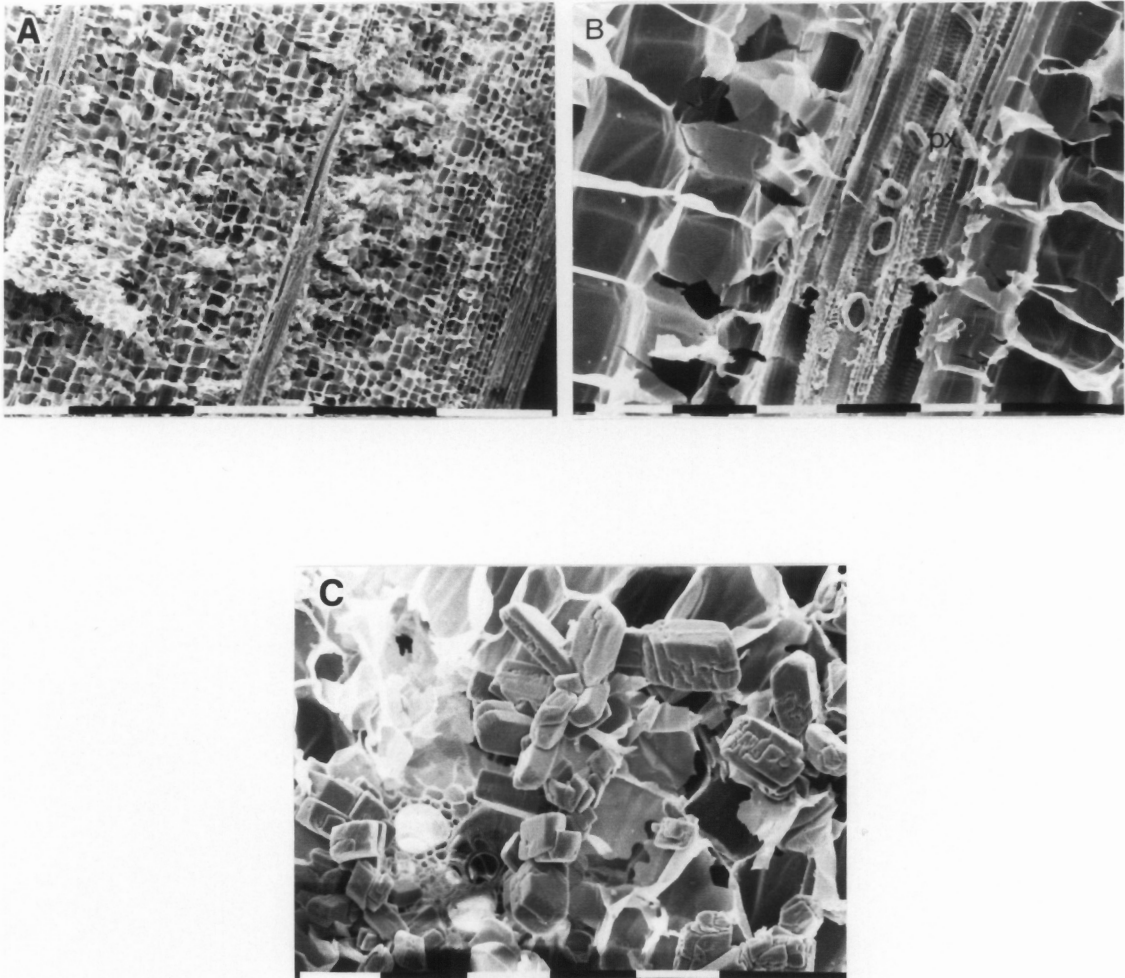
Figure 10. SEM micrographs of upper and lower corn internodes. (A) Upper stem internode, early dent, longitudinal (x 20, bar = 1 mm). (B) Upper stem, early dent, longitudinal. Tracheids embedded in parenchyma. Note protoxylem (px) rings (x 131, bar = .1 mm). (C) Lower stem internode, full maturity, with abundant crystalline structures (x 137, bar = .1 mm).

To overcome the limitations experienced in this study, thin specimens could be subjected to EDAX coupled to a transmission electron microscope (Saka et al., 1982), which would allow higher resolution and elimination of interference by underlying cells. However, even with SEM, the use of thin sections would prevent X-ray penetration in underlying cells, and embedding in an epoxy resin (Spurr, 1969) would limit possible differential X-ray penetration in tissue or cell wall layers of different density.

Implications

Generally, leaves are of higher nutritive quality than stems, but this research has shown that the difference between leaves and stems depends on stage of maturity and is apparently influenced by drought stress. The decrease in quality occurs mainly before early dent. Within each stage of maturity, digestibility was positively correlated with protein, and negatively correlated with cell wall carbohydrate composition and lignin methoxyl content. Results with regard to plant tissue distribution of bromine-labeled lignin obtained with SEM-EDAX were inconclusive. The high negative correlation between lignin methoxyl content and IVDMD suggests lignin methoxyl content can be a useful parameter in predicting digestibility and animal performance.

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Journal Paper 2. Formation of Quinone Methide Intermediates During Ruminal Fermentation of Corn Stover Fractions

ABSTRACT: The objective of this experiment was to test whether formation of quinone methide intermediates occurs during ruminal fermentation of corn stover, as evidenced by nucleophilic reaction with S-containing reducing agents. Corn stover fractions harvested at full maturity were incubated in buffered ruminal fluid without reducing agents, or with cysteine-HCl, Na_2S, cysteine-HCl plus Na_2S, or $(NH_4)_2SO_4$ (as S-control); and in only buffer with or without cysteine-HCl plus Na_2S. Sulphur content was determined of the residual fiber. *In vitro* NDF degradation after 48 h was not affected by adding reducing agents, but solubilization of NDF ($P = .070$) and DM ($P = .055$) in buffer alone tended to be enhanced, and S content of residual NDF was elevated ($P < .001$).

In a subsequent experiment corn stover fractions of varying lignin compositions harvested at two maturities (early dent and full maturity) in two subsequent years, were incubated in buffered ruminal fluid under addition of mixed reducing agents (cysteine-HCl and Na_2S), and incorporation of S into the undegraded

fiber was determined. Degradation of NDF was correlated with extent of S-incorporation into the fiber, and was highly correlated with lignin methoxyl content, especially in yr 1.

Key Words: *Zea mays*, rumen fermentation, lignin, quinone methides, sulphur.

Introduction

Utilization of mature forages and crop residues by livestock is limited by the extent to which they can be degraded in the rumen. Lignification inhibits or retards microbial cell wall degradation. Factors contributing to lignin heterogeneity, such as its monomer composition and type and extent of interlinkages, are likely to be related to its negative impact on cell wall degradation (Buxton and Russell, 1988; Jung, 1989).

Lignins are plant cell wall polymers composed of three different phenolic monomeric units varying in extent of methoxyl substitution of the aromatic ring. These components, termed p-hydroxyphenyl (not methoxylated), guaiacyl (monomethoxylated), and syringyl (dimethoxylated) units, are derived from the p-hydroxycinnamyl alcohols; p-coumaryl, coniferyl and sinapyl alcohol by dehydrogenative polymerization (Freudenberg, 1965), a random coupling of free phenoxy radicals initiated by peroxidase and H_2O_2. Coupling of two phenoxy radicals through a ß-carbon position produces reactive quinone methide structures, which further react to form stable bonds. The likelihood of quinone methide formation is highest for syringyl units, due to the relatively high contribution of ß-coupling to the linkage pattern for syringyl units. Nucleophilic addition of water, alcohols, phenols,

or carboxylic acids to quinone methide occurs at the α-carbon, producing benzyl alcohols, ethers, and esters respectively. By this mechanism, cross-linkages with carbohydrates are established, e.g. ester linkages with glucuronoxylans (Tanaka et al., 1976), and ether linkages with wall-bound hydroxycinnamic acids, as shown for ferulic acid (FA) (Scalbert et al., 1986; Lam et al., 1992). Quinone methides can be regenerated from lignin under mild conditions (Leary, 1980), resulting in a change in the bonding pattern of lignin during lignin aging towards more internal benzyl ether structures and lignin-carbohydrate bonds (Leary, 1980). In wood pulping, regeneration of quinone methides and subsequent nucleophilic addition reactions occur (Glasser, 1981), which are thought to limit the degradation rate.

The regeneration of quinone methide under mild conditions also raises the possibility of their formation during microbial fermentation in the rumen. Quinone methide intermediates, if formed during ruminal fermentation, would readily react with nucleophilic components present in ruminal fluid similar to those present in the intact cell wall. This mechanism would explain the existence of soluble lignin-carbohydrate complexes found in the rumen of cattle (Neilson and Richards, 1982) and sheep (Conchie et al., 1988).

The major objective of this experiment was to test the hypothesis of the occurrence of quinone methide formation and subsequent cross-linking reactions by nucleophilic addition during rumen fermentation. A secondary objective was to relate overall cell wall composition, as affected by conditions during maturation, plant fraction and maturity, to *in vitro* cell wall and DM digestibility.

Experimental Procedures

Plant Material. Corn (*Zea mays*) stalks were collected from six replicate field plots at early dent (silage harvest) and full maturity (grain harvest) in 1991 and 1992. In yr 1, the corn (cv. SS 728, Southern States Cooperative, Richmond, VA) was mildly drought-stressed, as evidenced by a red discoloration of the stalks, after inadequate precipitation during the summer and early fall (monthly precipitation, recorded approximately 8 km from experimental site, June, July, August, September, and October was 32, 54, 67, 12, and 7 mm, respectively). The corn in yr 2 (cv. SS 844) was harvested after a season of abundant precipitation (monthly precipitation was 155, 236, 128, 43, and 54 mm, respectively). For each harvest in each year, six plants per replicate plot were separated into the following fractions: leaf blade, upper stem (above ear), and lower stem (below ear). Stem fractions included leaf sheath, and were separated at the base of the ear. Replicate fractions were chopped, frozen at -20 °C, and freeze-dried.

Chemical Analyses. Dry matter, CP (AOAC, 1990), and water soluble carbohydrates (WSC) (Dubois et al., 1956, as modified by Johnson et al., 1966) were determined after thawing. Freeze-dried samples were ground to pass a 1-mm sieve. Detergent fiber components were determined according to sequential procedures of Robertson and Van Soest (1981). Lignin was determined in acid detergent fiber as $KMnO_4$ lignin and acid detergent lignin (ADL). Klason lignin in NDF was determined as the residue after treatment in 72% H_2SO_4 at 30 °C for 1 h followed by autoclaving at 121 °C in 3% H_2SO_4 for 1 h. In filtering, glass fiber filters (Whatman grade 934 AH, particle retention 1.5 μm) were used in 30-mL gooch crucibles of fine porosity (pore size 4-5.5 μm). No correction was made for ash or N

contamination of Klason lignin. Methoxyl content of Klason lignin was determined according to the standard TAPPI (1972) procedure.

In Vitro Experiments. To test the hypothesis of quinone methide formation and subsequent nucleophilic addition reactions, corn stover fractions, varying in lignin methoxyl content, were incubated in buffered ruminal fluid in two experiments. Strongly nucleophilic S compounds were added to the digestion media, and the S incorporation into the fiber fraction was determined. Ruminal fluid was taken from a rumen fistulated steer after an overnight fasting. The steer had been a diet of 50% corn stover and 50% corn silage (wt/wt, DM basis), supplemented with soybean meal (.9 kg), salt and $Ca_3(PO_4)_2$. The ruminal fluid was strained through four layers of cheesecloth, and mixed with reduced buffer of pH 6.8 (20% ruminal fluid, vol/vol). The buffer used was a mixed phosphate/bicarbonate (NaH_2PO_4/$NaHCO_3$/NH_4HCO_3) buffer, adapted from Goering and Van Soest (1970). The adaptation consisted of a higher contribution of NH_4HCO_3 to the bicarbonate portion of the buffer to provide 50 mg N/100 mL medium as NH_3-N. Tryptone was not added as protein source, but microminerals were added as originally described. During the handling of ruminal fluid, strict anaerobic conditions were not maintained.

In Exp. 1, the corn stover fractions from fully mature, drought-stressed stover (yr 1) were incubated for 48 h in buffered ruminal fluid. The following S containing reagents were added to different incubations in quantities recommended for mixed reducing agents (Goering and Van Soest, 1970): (1) cysteine-hydrochloric acid (cys-HCl) and disodium sulphide ($Na_2S.9H_2O$), (2) cys-HCl, (3) $Na_2S.9H_2O$, (4) ammonium sulphate (($NH_4)_2SO_4$, or (5) none. Additionally, samples were incubated in buffer only (no ruminal fluid added), either with (6) or without (7) addition of the cys-HCl/Na_2S mixture.

In Exp. 2, corn plant fractions of stover harvested at both maturities in both years were incubated for 48 h in buffered ruminal fluid containing both cys-HCl and Na_2S as reducing agents as recommended by Grant and Mertens (1992).

In each experiment, approximately .35 g sample were incubated in 50 mL-tubes fitted with bunsen valves, containing 35 mL medium (80% buffer, 20% ruminal fluid, vol/vol). At incubation, the tubes were purged with CO_2. The residues after *in vitro* incubation were washed into 600-mL Berzelius beakers with neutral detergent solution to a total volume of 150 mL, extracted for 1 h, and filtered using glass fibers filters (particle retention 1.5 μm) in 50-mL gooch crucibles of coarse porosity (pore size, 40 to 60 μm). The residue was rinsed thoroughly with hot water and 80% ethanol, and dried overnight at 70 °C.

Sulphur in the NDF residues after *in vitro* digestion, as well as in NDF preparations of original samples, was determined after wet-ashing using nitric/perchloric acid (Sandel, 1959). Sulphur content was determined by inductively coupled plasma spectrometry using a Jarrell Ash ICAP 9000, simultaneous spectrometer. Net S flux was calculated as follows:

$$\text{Net S flux} = \frac{S_{residue} \text{ (mg)} - S_{initial\ sample} \text{ (mg)}}{\text{initial NDF (g)}}$$

Sulphur incorporation into the fiber was calculated, taking the extent of cell wall degradation into account, and assuming that S disappeared at a rate similar to total NDF:

$$\text{S incorp. (mg/g NDF)} = \frac{S_{residue} \text{ (mg)} - (S_{initial} \text{ (mg)} \times \text{NDF degradation (\%/100))}}{\text{initial NDF (g)}}$$

Statistical analysis. In vitro degradation (incubation in buffered ruminal fluid) and solubilization (incubation in buffer only) results from Exp. 1 were analyzed by two-way ANOVA using the GLM procedures of SAS (1989) with plant fraction and S addition as factors. The general model was as follows:

$$A_{ijk} = \mu + fraction_i + sulphur_j + (fraction \times sulphur)_{ij} + error_{ijk},$$

in which

A_{ijk} =   the measured dry matter or cell wall disappearance;

μ =   the overall mean;

$fraction_i$ =   the effect of the ith fraction ($i = 1, 2, 3$);

$sulphur_j$ =   the effect of the jth sulphur subclass.

For the degradation data, the model comprised five sulphur subclasses, whereas for the solubilization data, the model comprised only two sulphur subclasses (none and mixed S agents). The following contrasts were tested: leaf versus stems, and upper stem versus lower stem, plus, for degradation results, control versus S added.

Composition and *in vitro* data from exp. 2 were analyzed as a three-way factorial by three-way analysis of variance with factors, years, maturities, and plant fractions. All two- and three-way interactions were included in the model, and the following contrasts were tested: leaf versus stems, and upper stem versus lower stem. Because of two- or three-way interactions consistently involving fractions, data were re-analyzed within each fraction by two-way analysis of variance using the following model:

$$A_{ijkl} = \mu_i + year_{ij} + maturity_{ik} + (year \times maturity)_{ijk} + error_{ijkl},$$

in which

$A_{ijkl} =$		the measured component or extent of cell wall degradation for the ith fraction subclass;
$\mu_i =$		the overall mean for the ith fraction subclass $(i = 1, 2, 3)$;
$year_{ij} =$		the effect of the jth year within the ith fraction subclass $(j = 1, 2)$.
$maturity_{ik} =$		the effect of the kth maturity stage method within the ith fraction subclass $(k = 1, 2)$;

Results and Discussion

Experiment 1. Detailed chemical composition of the fractions collected in yr 1 was reported in Journal Paper 1. Sulphur content of residual NDF (Table 5) was elevated ($P < .001$) by addition of a S-source, both for incubations with buffered ruminal fluid and with buffer alone. The extent of S incorporation appeared not to be affected by plant fraction as evidenced by the absence of fraction x S interaction ($P > .05$), even when the S contents in the residual NDF were corrected for differences in cell wall disappearance (data not shown). The results do indicate a certain degree of S binding during *in vitro* fermentation, with numerically higher values for the cys-HCl/Na$_2$S mixture than for the individual compounds.

Cell wall disappearance of the mature stover fractions was higher ($P < .001$) for leaf than stems, and higher ($P < .001$) for upper than lower stem (Table 6). Overall, cell wall disappearance was not affected ($P > .05$) by adding a S-source, whether in reduced (cysteine-HCl, Na$_2$S) or oxidized form ($(NH_4)SO_4$). However, the combination of cys-HCl and Na$_2$S tended to enhance cell wall disappearance. When incubated in buffer only, addition of a cys-HCl/Na$_2$S mixture tended to increase cell

Table 5. Sulphur content of cell wall residues of mature corn stover fractions (yr 1) after 48 h *in vitro* incubation in ruminal fluid/buffer or buffer alone with and without different sulphur sources

	Incubation medium by sulphur source								
	Ruminal fluid/buffer[abc]						Buffer alone[c]		
Fraction	Control	Cys-HCl /Na$_2$S	Cys-HCl	Na$_2$S	(NH$_4$)$_2$SO$_4$	SE	control	Cys-HCl /Na$_2$S	SE
	------------ mg/g NDF ------------						- mg/g NDF -		
Leaf	1.86	2.69	2.56	2.46	2.49		1.38	1.58	
Upper stem	1.65	2.06	1.84	1.98	1.75		1.19	1.52	
Lower stem	1.21	1.82	1.75	1.71	1.87		1.23	1.52	
						.116			.091

[a]Leaf different from stems (P<.001)
[b]Upper stem different from lower stem (P<.05)
[c]Control different from sulphur addition (P<.001)

Table 6. Disappearance of NDF from mature corn stover fractions (yr 1) after 48 h *in vitro* incubation in ruminal fluid/buffer or buffer alone with and without different sulphur sources

	Incubation medium by sulphur source							
	Buffered ruminal fluid[a,b]					Buffer alone		
Fraction	Control	Cys-HCL /Na$_2$S	Cys-HCl Na$_2$S	(NH$_4$)$_2$SO$_4$	SE	control	Cys-HCL /Na$_2$S	SE
	------------------------------ % ------------------------------					------ % ------		
Leaf	61.8	66.4	62.9	60.1	62.1	2.9	18.0	
Upper stem	52.9	54.6	50.0	46.7	50.3	10.0	15.4	
Lower stem	42.2	44.2	38.6	37.7	38.9	6.9	9.9	
					1.41			5.03

[a]Leaf different from stems (P<.001)
[b]Upper stem different from lower stem (P<.001)

wall solubilization (P = .070; Table 5) and dry matter solubilization (P = .055; data not shown). The hypothetical fate of the added S compounds is their addition to quinone methides, and the bonds established between sulfhydryl groups and quinone methides are irreversible (Glasser, 1981). As such, S addition interferes with the formation of crosslinkages among fiber components or between fiber components and cell solubles, which are reversible and may be degradable by microbial enzymes.

Experiment 2. The major differences in composition between years (Table 7) were a higher (P<.001) CP content of leaves in yr 2 (12.6 vs 6.9%), a higher (P<.001) concentration of WSC in the stem fractions in yr 1 (13 to 25% vs 6 to 7%), associated with a lower (P<.001) cell wall content of stems (63 to 71 % vs 76 to 81%). Cell wall composition was similar between years (Table 8), with exception of the content of Klason lignin, which was higher (P<.001) in yr 2, especially at full maturity (year x maturity interaction, P<.001). Lignin estimates varied considerably among procedures, with higher values obtained by the Klason method (12 to 19% of NDF), but only minor differences between $KMnO_4$ lignin (4 to 9% of NDF) and ADL (4 to 10% of NDF). The large discrepancy between Klason lignin and the other methods is probably due to dissolution of lignin fragments in the acid detergent used in the preparatory step for $KMnO_4$ lignin and ADL (Stewart and Morrison, 1992). Cell wall components were subject to three-way interactions (year x maturity x fraction, P<.05).

Disappearance of NDF and IVDMD were lower (P<.001) in yr 2 (Table 9), associated, in part, with the lower WSC and higher NDF in stems, and higher Klason lignin and methoxyl content in all fractions. Admittedly, different varieties were used, so that the higher IVDMD in yr 1 cannot be firmly attributed to drought stress. Digestibility of NDF decreased (P<.001) slightly with maturity, but the decrease was

Table 7. Crude protein, water soluble carbohydrates, and NDF in corn stover fractions harvested at two maturities in two years

Year	Maturity	Fraction	CP[abcde]	WSC[abcdfgh]	NDF[acdefg]
			----------- % of DM ---------		
1991	Early dent	Leaf	7.22	6.59	68.0
		Upper stem	2.69	19.44	69.2
		Lower stem	3.77	24.98	63.4
	Full maturity	Leaf	6.59	3.71	72.2
		Upper stem	4.16	12.70	70.6
		Lower Stem	4.74	22.65	63.1
1992	Early dent	Leaf	12.73	5.91	68.7
		Upper stem	3.52	6.54	77.9
		Lower stem	3.16	5.76	81.2
	Full maturity	Leaf	12.50	3.54	69.5
		Upper stem	4.74	7.12	76.3
		Lower Stem	3.95	7.12	79.1
SE			.208	.880	1.08

[a]Year effect ($P < .05$).
[b]Maturity effect ($P < .05$).
[c]Leaf different from stems ($P < .05$).
[d]Year x fraction interaction ($P < .05$).
[e]Maturity x fraction interaction ($P < .05$).
[f]Upper stem different from lower stem ($P < .05$).
[g]Year x maturity interaction ($P < .05$).
[h]Year x maturity x fraction interaction ($P < .05$).

Table 8. Cell Wall Composition of corn stover fractions harvested at two maturities in two years

Year	Maturity	Fraction	Hemi-cellulose[abcdefg]	Cellulose[bcdefgh]	KMnO₄ lignin[bcdefgi]	ADL[cdfi]	Klason lignin[bdeij]
			--------------------- % of NDF -----------------------				
1991	Early dent	Leaf	40.2	53.5	4.24	6.30	13.3
		Upper stem	44.2	46.9	5.34	8.91	13.7
		Lower stem	43.7	48.1	8.72	8.23	14.9
	Full maturity	Leaf	53.4	42.2	6.03	4.35	12.4
		Upper stem	48.0	44.3	6.43	7.73	14.0
		Lower Stem	36.9	52.7	9.02	10.3	15.0
1992	Early dent	Leaf	51.7	41.2	6.20	7.95	15.5
		Upper stem	46.7	47.8	5.29	5.77	14.2
		Lower stem	37.2	53.6	9.33	9.44	15.5
	Full maturity	Leaf	52.2	41.0	6.11	7.67	18.6
		Upper stem	46.7	47.7	6.03	5.07	16.5
		Lower Stem	40.3	52.5	7.76	6.59	18.8
SE			1.37	1.33	.240	.771	.42

[a](100 - ADF)
[b]Maturity effect ($P < .05$).
[c]Leaf different from stems ($P < .05$).
[d]Upper stem different from lower stem ($P < .05$).
[e]Year x fraction interaction ($P < .05$).
[f]Maturity x fraction interaction ($P < .05$).
[g]Year x maturity x fraction interaction ($P < .05$).
[h](ADF - ADL)
[i]Year x maturity interaction ($P < .05$).
[j]Year effect ($P < .05$).

Table 9. Cell wall degradation, IVDMD and S incorporation into NDF of corn stover fractions harvested at two maturities in two years after 48 h *in vitro* incubation with reducing agents (cysteine-HCl and Na_2S) and lignin methoxyl (OCH_3) groups

Year	Maturity	Fraction		Cell wall degr.[abcdef]	IVDMD[abcdfg]	Sulphur in NDF		Sulphur flux		Lignin OCH_3 groups	
						Initial	Residual	Net[bcdefg]	Incorp.[cdf]	% of Klason lignin[acdfghi]	% of NDF[abcdefgh]
				------ % ------		------ mg S / g NDF ------					
1991	Early dent	Leaf		68.4	78.9	1.67	2.86	-.776	.371	10.1	1.34
		Upper stem		56.8	70.9	1.04	2.26	-.064	.526	14.2	1.95
		Lower stem		45.3	66.4	.71	1.83	.290	.613	14.6	2.17
	Full maturity	Leaf		67.2	77.5	1.72	2.69	-.819	.326	10.9	1.36
		Upper stem		54.7	67.9	.71	2.06	.226	.613	14.4	2.02
		Lower stem		44.9	66.1	.49	1.82	.528	.744	14.3	2.15
1992	Early dent	Leaf		55.9	69.7	1.75	2.96	-.436	.540	12.2	1.90
		Upper stem		54.9	64.9	.98	1.99	-.083	.453	16.2	2.30
		Lower stem		44.0	54.5	.75	1.66	.173	.508	18.5	2.86
	Full maturity	Leaf		49.2	64.7	1.81	2.81	-.385	.506	10.9	2.02
		Upper stem		52.1	63.5	1.05	1.87	-.162	.390	17.3	2.84
		Lower stem		39.0	52.8	.81	1.67	.220	.531	17.7	3.32
SE				.89	.70	.10	.10	.051	.046	.26	.054

[a]Year effect (P<.05).
[b]Maturity effect (P<.05).
[c]Leaf differs from stems (P<.001).
[d]Upper stem differs from lower stem (P<.001)
[e]Year x maturity interaction (P<.05).
[f]Year x fraction interaction (P<.05).
[g]Year x maturity x fraction interaction (P<.05).
[h]Maturity x fraction interaction (P<.05).
[i]Determined in triplicate for each fraction at each maturity for each year (n=3).

larger in yr 2 than in yr 1 (year x maturity interaction, $P<.001$). Leaf components were more ($P<.001$) digestible than stems, and upper stem were higher ($P<.001$) than lower stem, irrespective of maturity. The differences in digestibility among fractions were larger in yr 1 than in yr 2 (year x fraction interaction, $P<.001$).

The differences among fractions in cell wall disappearance were associated with a higher ($P<.001$) S content of residual leaf NDF than of stem NDF, with upper stem higher ($P<.01$) than lower stem (Table 9). The net S flux was generally negative for leaf, indicating net loss of S. The net S flux was generally positive for lower stem, and negligible for upper stem. The calculated S incorporation was larger ($P<.001$) in stems than in leaf, although this was more apparent in yr 1 than in yr 2 (year x fraction interaction, $P<.001$). Sulphur incorporation into lower stem was higher ($P<.01$) than in upper stem. Extent of S incorporation followed closely the pattern of lignin methoxyl content in yr 1 (Table 9), but the relation was less obvious in yr 2, possibly due to contamination of Klason lignin with non-phenolic compounds.

The finding of S incorporation into fiber during ruminal fermentation, whereby establishment of crosslinkages between lignin and other cell wall or cell soluble components is inhibited, provides a partial explanation for the higher *in vitro* digestibility values obtained by Grant and Mertens (1992) with these S-containing compounds (cysteine-HCl, Na_2S). The main reason for adding these compounds *in vitro* has been to establish sufficiently reducing conditions, providing an optimal environment for the rumen microorganisms (Grant and Mertens, 1992). However, in exp. 1, no difference was observed in IVDMD between incubations with reducing agents (cys-HCl, Na_2S) or non-reducing SO_4^{2-}. It is known from studies into S dynamics in the rumen of sheep, that dietary sulphate (SO_4^{2-}) is rapidly converted

into sulphide (S^{2-}) (Kandylis and Bray, 1992) due to S-reducing bacteria in ruminal fluid.

The higher digestibility of leaf than stems in yr 1 seems attributable mainly to a lower lignin content, as well as the different lignin composition (less methoxyl groups), resulting in less quinone methide formation (Glasser and Kelley, 1987). This was confirmed by the higher net S loss from the fiber, or alternatively, the lower incorporation of S from the added reducing agents. In yr 2, the higher digestibility of leaf, compared to stems, could be due to its higher CP, lower NDF, higher hemicellulose/cellulose ratio and(or) the lignin composition, but not lignin content. The higher digestibility of leaf in yr 2, compared to stems, was not clearly related to the calculated S incorporation, although net S loss was higher from leaf than stems. It is possible that the assumption under which the S incorporation was calculated (equal loss of S and total NDF) was not valid for the leaf fraction in yr 2.

Cell wall digestibility of upper stem was considerably higher than of lower stem, irrespective of year or maturity, which may be attributable to a higher hemicellulose/cellulose ratio, lower $KMnO_4$ lignin, and lower lignin methoxyl content in NDF. Again, the likelihood of quinone methide formation is lower for lignin of a low methoxyl content (Glasser and Kelley, 1987), resulting in the lower S incorporation into the fiber of upper than lower stem.

Overall, lignin methoxyl content (% of NDF) was highly correlated ($r = -.94$) with IVDMD (Figure 11). Components well correlated with IVDMD were methoxyl content (% of Klason lignin) ($r = -.84$), Klason lignin ($r = -.68$), ADF ($r = -.64$), $KMnO_4$ lignin ($r = -.59$), and NDF ($r = -.58$), as well as the net S-flux ($r = -.65$). Degradation of NDF was also highly correlated with lignin methoxyl content in NDF ($r = -.84$) and as percentage of Klason lignin ($r = -.73$), $KMnO_4$ lignin ($r = -.74$), net S flux ($r = -.81$),
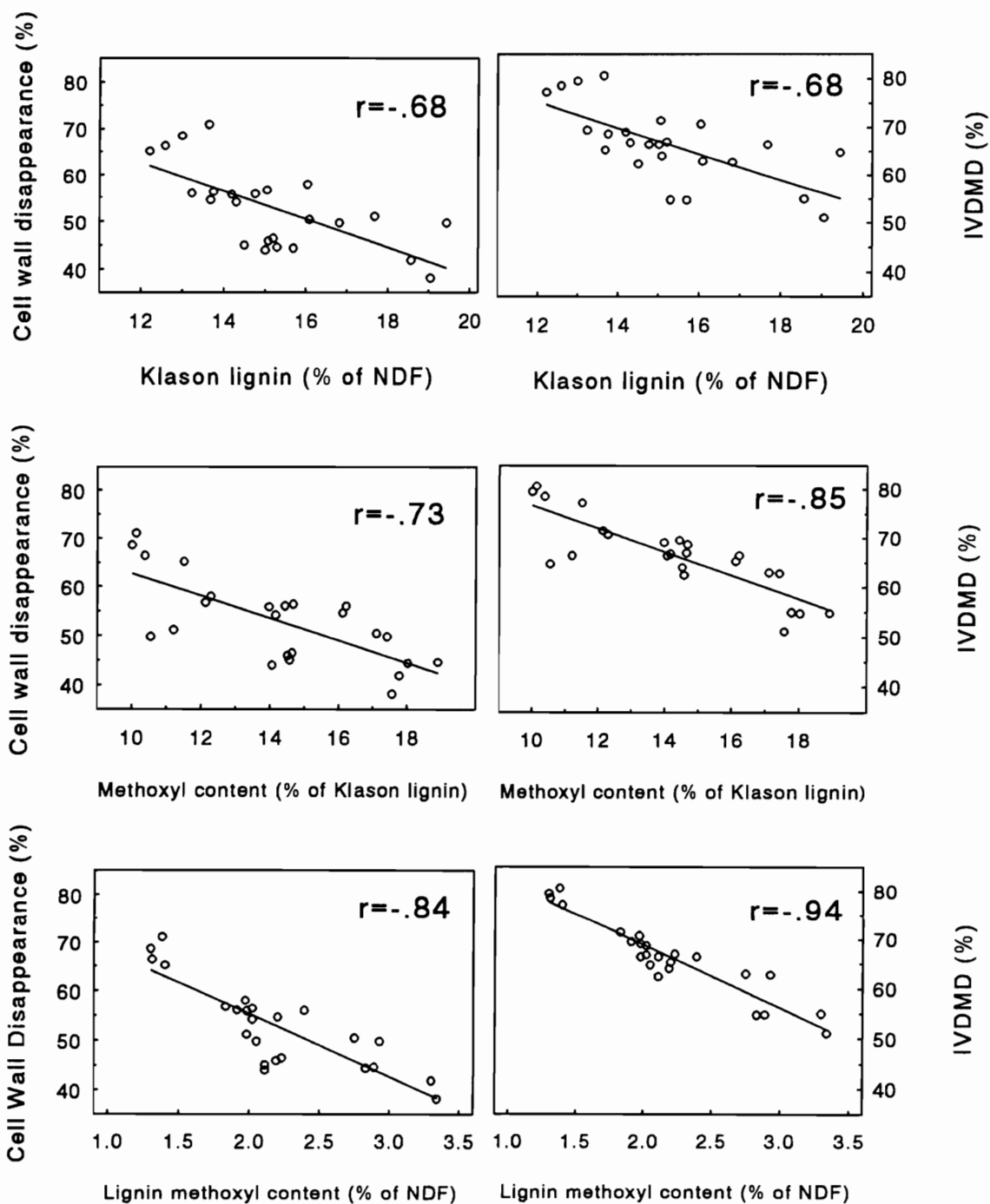
Figure 11. Relations between content and composition of lignin and *in vitro* disappearance of cell walls and DM. Values for lignin methoxyl content (% of NDF) were recalculated based upon Klason lignin (% of NDF) and methoxyl content (% of Klason lignin).

and to a lesser extent, with the calculated S incorporation ($r=-.54$).

Stepwise multiple linear regression for NDF degradation included ($P<.15$) S flux, $KMnO_4$ lignin, and WSC (overall $R^2=.76$). Within leaf, stepwise regression for NDF degradation included the net S flux ($R^2=.73$), followed by the calculated S-incorporation, resulting in a final model with $R^2=.99$. Within stems, IVDMD was highly correlated with NDF ($r=-.83$ and -.96 for upper and lower), Klason lignin ($r=-.85$ and $r=-.71$, respectively), and lignin methoxyl content ($r=-.91$ and $r=-.92$, respectively).

The formation of quinone methides in the rumen may at first seem unlikely, since these intermediates are normally formed in the plant under oxidative conditions in the presence of peroxide and H_2O_2. Although reducing conditions prevail in the rumen, micro-environments within the fiber matrix may allow oxidative degradation, as evidenced by the presence of stable radicals in lignin-carbohydrate complexes isolated from ruminal fluid (Nordkvist et al., 1989). The results from this experiment, however, indicate that nucleophilic addition to quinone methide intermediates does occur. In absence of a strong external nucleophile such as reducing HS^- or S^{2-}, this would cause a repolymerization of lignin and cross-linking with carbohydrates and other digesta components. However, new crosslinkages established by addition reactions to quinone methide may still be degradable. Therefore, quinone methide intermediates may affect rate of cell wall degradation, but not necessarily potential degradability.

Implications

Incorporation of S into corn stover fiber after addition of highly nucleophilic S-containing compounds to the *in vitro* incubation media supports the hypothesis that quinone methide intermediates are formed during ruminal fermentation. The close relation of S incorporation to the overall lignin methoxyl content indicates that quinone methides are formed at a higher rate during fermentation of fiber containing more lignin methoxyl groups. The formation of quinone methide intermediates at different rates from plant fractions, containing lignins of different methoxyl content, is a factor that needs to be taken into account in addition to overall and cell wall composition when comparing relative degradability of forages and their botanical fractions at varying maturity stages.

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Journal Paper 3. The Impact of Lignin on Cellulose Degradation, an In Vitro Model Study

ABSTRACT: The impact of lignin on cellulose degradation was studied in an *in vitro* degradation study using lignocellulosic hydrogels. Hardwood lignin and cellulose were blended in solution in two proportions (10:90 and 30:70, wt/wt) into lignocellulosic beads before or after modification of the phenolic hydroxyl group in (by hydroxypropylation of lignin). The *in vitro* cellulose degradation from freeze-dried beads after 24 h and 72 h incubation was compared to that of control (cellulose) beads and to that of cellulose beads to which lignin was added at time of incubation, in similar ratios. Rate of cellulose degradation from the hydrogels was low (12 to 16 % degradation after 24 h). At 72 h, 21 to 50% of the cellulose was degraded, and a clear impact of lignin on cellulose degradation was apparent. Blending lignin with cellulose enhanced (P<.01) cellulose degradation, whereas addition of lignin at time of incubation depressed (P<.01) cellulose degradation. The effect of lignin increased (P<.001) with lignin concentration. Hydroxypropylation of lignin enhanced (P<.001) the increase in cellulose degradation with lignin blending, and reduced (P<.001) the inhibition of cellulose degradation when

lignin was added at time of incubation. The interactive effects of lignin with cellulose degradation in this model study are related to forage cell wall degradability.

Key Words: Lignin, cellulose degradation, lignocellulosic hydrogels, model study.

Introduction

The mechanism by which lignin limits forage fiber degradation is not well understood. The complexity in interrelationships of lignin with other cell wall components usually does not allow for a linear relationship between lignin content and cell wall digestibility (Allinson and Osbourn, 1970; Jung and Vogel, 1986). Lignin is heterogenous with regard to its monomer composition and type and extent of interlinkages (Scalbert et al., 1986). Furthermore, lignin is not an inert polymer (Leary, 1980), implying that its relationships with other cell wall components may change with increasing maturity. The intrinsic variability in structure of the matrix polysaccharides is a factor contributing to the complexity of lignin-carbohydrate interactions (Hatfield, 1989).

Several mechanisms have been proposed by which lignin would limit degradation of carbohydrates. On a cellular or cell wall level, constitution of a physical barrier (highly lignified primary wall) (Engels and Schuurmans, 1992), and accumulation of an inert surface layer of lignin during microbial digestion (Chesson, 1988) are the most prominent. Both theories do not, however, account for the existence of indigestible soluble lignin-carbohydrate complexes in the rumen (Neilson and Richards, 1982). The protective barrier or 'encrustation' can neither account for the increase in digestibility of alkaline treated straws where lignin has not been removed from the product (Van

Soest, 1981), and the highly lignified primary wall is only effective as a physical barrier in mechanically undamaged cell walls (Engels and Schuurmans, 1992). Mastication and rumination cause extensive physical damage to cell walls, so that microorganisms are able to attack from within the cell lumen, resulting in extensive degradation of secondary cell walls (Chesson et al., 1986; Mulder et al., 1992). When secondary cell walls are attacked, the aspect of lignin build-up at the inner cell wall surface can provide a mechanistic model for retardation of cell wall degradation rather than a direct inhibitory mechanism of lignin.

At a molecular level, the extent to which phenolic compounds are associated with cell wall carbohydrates may contribute substantially to the retention of lignin at the cell wall surface (Chesson, 1988). In addition to enhancing lignin retention at the surface, interlinkages may affect the ability of microbial enzymes to recognize the carbohydrate substrate or may limit the enzyme accessible space (Jung, 1989). Alternatively, substrate accessibility might be limited simply due to the tight physical association of carbohydrates and lignin. Formation of lignin-carbohydrate linkages in the process of ruminal fermentation, in which quinone methide intermediates are involved, would be an additional restraint to carbohydrate utilization (Journal Paper 2).

Lignin is known as a potent adsorbent of carcinogens (Rubio et al., 1979) and bile acids (Kay et al., 1979). The binding of bile salts and acids is maximal at acidic pH and reduced at alkaline pH, and methylation of fiber results in enhanced adsorption (Eastwood, 1983), suggesting that suppression of ionization of hydroxyl and carboxyl groups is necessary for adsorption. In ruminants, the lignin component of cell walls may actually adsorb to microbial enzymes by similar processes, so that the enzymes would never reach their actual substrates.

In order to gain a more detailed understanding into the role of lignin in ruminal carbohydrate digestion, a simple but unique *in vitro* model study was conducted, in which the impact of lignin on microbial degradation of cellulose was studied. The model consists of "beads" of essentially non-porous, amorphous cellulose, in which lignin is blended. Although it is a simplified model of the plant cell wall, polymer morphology and thermodynamics of lignocellulose blends resemble the biocomposite (Rials and Glasser, 1989), and can be manipulated during the preparation (Rials and Glasser, 1990). The objective of this experiment was to evaluate if cellulose degradation by rumen microbes is affected by close physical association with lignin, establishment of interlinkages after quinone methide formation, or hydrophobic enzyme adsorption to lignin.

Experimental Procedures

Cellulose or lignocellulose substrates were prepared as hydrogels in the form of beads according to a method developed for chromatographic support systems (Glasser et al., 1993). Based on X-ray diffraction analysis, the product can be regarded a non-crystalline cellulosic hydrogel (Garnier, 1993). For the lignocellulose beads, an organosolv lignin from mixed hardwood (Aldrich Chem. Co., Milwaukee, WI) was blended in two concentrations (10 and 30% lignin) with the cellulose without modification or after modification of lignin by thorough hydroxypropylation (replacing the phenolic hydroxyl). This organosolv lignin has been previously characterized (Glasser et al., 1983a). For confirmatory purpose, the methoxyl content was determined (TAPPI, 1972). Control and lignocellulose beads were freeze-dried and stored over desiccant at

6 to 8 °C until the *in vitro* experiment (approximately 10 wk). *In vitro* degradation of control cellulose beads after 24 and 72 h incubation was compared with that of the following lignin 'treatments': (1) lignocellulose beads, physically blended in a cellulose:lignin ratio of 9:1; (2) as 1, but in 7:3 ratio; (3) as 1, but with hydroxypropylated lignin (HPL); (4) as 3, but in 7:3 ratio; (5) cellulose beads + lignin added at time of incubation in 9:1 ratio; (6) as 5, but in 7:3 ratio; (7) as 5, but with HPL; (8) as 7, but in 7:3 ratio.

Ruminal fluid was taken from a rumen fistulated steer after an overnight fasting. The steer had been fed a diet of 50% corn stover and 50% corn silage (wt/wt, DM basis), supplemented with soybean meal (.9 kg), salt and $Ca_3(PO_4)_2$. The ruminal fluid was strained through four layers of cheesecloth, and mixed with reduced buffer of pH 6.8 (20% ruminal fluid, vol/vol). The buffer used was a mixed phosphate/bicarbonate ($NaH_2PO_4/NaHCO_3/NH_4HCO_3$) buffer, adapted from Goering and Van Soest (1970). The adaptation consisted of a higher contribution of NH_4HCO_3 to the bicarbonate portion of the buffer to provide 50 mg N/100 mL medium. Tryptone was not added as protein source, but microminerals were added as originally described by Goering and Van Soest (1970). A mixture of cysteine-HCl and $Na_2S.9H_2O$ was added to the buffer as reducing agents, followed by CO_2 bubbling through the buffer until sufficiently reduced as indicated by change in color of added resazurin indicator from purple to colorless. Incubation media were kept at 38 to 39 °C at all times during the procedure, but strict anaerobic conditions were not maintained.

At inoculation, 20 mL of buffered ruminal fluid were added to 30 mL-tubes containing approximately .2 g of sample. The tubes were purged with CO_2 and closed immediately with screw caps. Sample size varied slightly by balancing for the varying cellulose content of the beads, to obtain incubation of equal amounts of cellulose (180

mg) in each tube. All incubations were replicated six times. After 24 or 72 h, the tube contents were extracted with neutral detergent solution as described in Journal Paper 2. Since KMnO$_4$, acetyl bromide, and Klason lignin methods were ineffective in determining lignin content of the beads, correction for lignin content before and after *in vitro* digestion was obtained by the difference in acid soluble lignin, as measured by UV absorbance at 280 nm after two-step acid hydrolysis of original and residual material.

In vitro results for control cellulose and lignin treatments were statistically treated by analyses of variance using the GLM procedures of SAS (1989). The initial analysis was by one-way ANOVA, testing the non-orthogonal (Bonferroni) contrasts (Lowry, 1992) 'control vs lignin blending (lignin treatments 1 to 4)' and 'control vs lignin addition (lignin treatments 5 to 8)'. All lignin treatments, arranged in a 2 x 2 x 2 factorial, were subsequently included in three-way analysis of variance, with factors (1) type of lignin inclusion (blending vs addition), (2) modification, and (3) concentration. Two-way and three-way interactions were included in this model. Effects of lignin modification and concentration were also analyzed within each type of lignin inclusion by two-way analysis of variance.

Results and Discussion

Extent of cellulose degradation after 24 h (Table 10) was rather low (10 to 16 %), with only minor differences among treatments. Apparently, rate of degradation of single-substrate material, such as the cellulose beads evaluated in this study, is lower than of whole forages containing a multitude of different substrates for the mixed rumen

Table 10. *In vitro* cellulose disappearance from cellulose beads after
24 h incubation as affected by blending or mixing
unmodified or hydroxypropylated lignin at two ratios

Ratio lignin: cellulose	Type of lignin inclusion[a]				SE
	Blending[b]		Addition[b]		
	Unmodified	HPL[d]	Unmodified	HPL	
	---------------------------- % ----------------------------				
Control[c]	12.5				.40
Lignin					
10:90	12.2	15.7	12.8	10.8	
30:70	12.6	13.6	11.6	12.0	
SE					.42

[a]Inclusion x modification interaction (P<.001)
[b]Blending different from addition at incubation (P<.001)
[c]Control different from lignin blending (P<.05)
[d]Hydroxypropylated lignin.

microorganisms. Also, the lack of microporosity will restrict hydration (Buleon and Bertrand, 1982) and enzyme accessibility (Focher et al., 1981). After 24 h incubation, cellulose disappearance from the beads was slightly increased (P < .05) by blending with lignin, but was not affected (P > .10) by addition at incubation. Lignin blending also resulted in higher (P < .001) cellulose disappearance than lignin addition, especially for HPL (lignin inclusion x modification interaction, P < .001).

In contrast to the minor differences after 24 h, the respective lignin treatments resulted in larger differences in cellulose disappearance after 72 h, at which time 21 to 50% of the cellulose had been degraded (Table 11). Lignin blending enhanced (P < .01) cellulose disappearance, whereas adding lignin at time of incubation partially inhibited (P < .01) degradation of cellulose, compared to the control value (31.5 %), irrespective of level of lignin or modification. However, the high lignin concentration (30%) was more effective (P < .001) than the low lignin concentration in enhancing cellulose disappearance from the lignocellulose blends, and in inhibiting cellulose disappearance when added at incubation.

Lignin blending apparently increased the accessibility of the cellulose substrate to the rumen microorganisms and their enzymes, either by limiting the opportunity of hydrogen bonding among cellulose molecules, or by influencing microporosity, unrelated to crystallinity. Earlier work had shown cellulose beads prepared by the same method to be amorphous (Garnier, 1993), and various workers have indicated the importance of microporosity to solvent and enzyme accessibility (Buleon and Bertrand, 1982; Excoffier et al., 1991; Samaranayake et al., 1993).

At both levels of lignin inclusion, blending of HPL with cellulose resulted in a larger (P < .001) increase in cellulose disappearance than blending of unmodified lignin. The positive effect of the presence of lignin between the cellulose chains is probably

Table 11. *In vitro* cellulose disappearance from cellulose beads after 72 h incubation as affected by blending or mixing unmodified or hydroxypropylated lignin at two ratios

Ratio lignin: cellulose	Type of lignin inclusion[a][b]				SE
	Blending[c]		Addition[c]		
	Unmodified[d]	HPL[de]	Unmodified[d]	HPL[d]	
	---------------------------- % ----------------------------				
Control[f]	31.5				.56
Lignin[g]					
10:90	36.3	44.2	25.8	29.9	
30:70	43.2	50.0	21.4	26.4	
SE					.52

[a]Inclusion x modification interaction ($P < .001$).
[b]Inclusion x quantity interaction ($P < .001$).
[c]Blending different from addition at incubation ($P < .001$).
[d]Unmodified different from HPL ($P < .001$).
[e]Hydroxypropylated lignin.
[f]Control different from blending ($P < .01$) and mixing ($P < .01$).
[g]10:90 different from 30:70 ($P < .001$).

counteracted by the formation of quinone methide intermediates from unmodified lignin and subsequent establishment of covalent linkages. Hydroxypropylated lignin is not converted to quinone methide structures, since it does not have any phenolic hydroxyl groups available.

The partial inhibition of cellulose degradation after addition of lignin at time of incubation is in disagreement with previous reports of no inhibition (Han et al., 1975; Jung, 1990). Jung (1990) concluded that covalent linkage, such as occurs during lignin biosynthesis, is a prerequisite for lignin to have a negative impact on carbohydrate digestion, based on the finding that artificial lignification (using peroxidase, H_2O_2 and eugenol, thereby promoting the formation of quinone methide structures) of digestible cellulose results in a reduced *in vitro* digestibility (Gressel et al., 1983; Jung, 1990).

The generally accepted assumption that covalent linkages between lignin and carbohydrate, established during lignification, are required for lignin to inhibit carbohydrate degradation, is contradicted by the results of this study in two ways. Formation of interlinkages apparently also occurs to a certain extent after blending of two pre-existing polymers or, alternatively, during rumen fermentation. The negative impact of lignin addition at incubation on cellulose disappearance in this study may have resulted from the higher lignin concentrations than in previous work (Han et al., 1975). However, the impact is more likely due to the fact that in this study a highly methoxylated hardwood lignin was used, in contrast to polyeugenol (Jung, 1990). Polyeugenol is non-methoxylated, and constitutes a poor model for lignin (Glasser, pers. comm.). The mixed hardwood lignin in this experiment had a methoxyl content of 23.1 %, or an OCH_3/monomer ratio of about 1.4:1. The likelihood of regeneration of quinone methide intermediates from hardwood lignin is relatively high, since the contribution of linkages involving the ß-carbon to the overall linkage pattern is higher

for lignin monomers in which the 3- and 5-carbon positions of the aromatic ring are methoxylated than for monomers that are mono- or non-methoxylated (Glasser and Kelley, 1987). These quinone methide intermediates may have been present in the lignin before the blending process, or may have been formed during preparation or regenerated during storage, or, alternatively, during *in vitro* fermentation (Journal Paper 2). The negative impact of quinone methide formation from lignin added at incubation is demonstrated by the larger (P < .001) decrease in cellulose degradation for unmodified lignin than for HPL (lignin inclusion x modification interaction, P < .001).

Toxicity of polymeric lignin towards rumen microorganisms has never been shown to occur (Jung et al., 1983), in contrast to phenolic monomers (Hartley and Akin, 1989; Jung, 1985). The organosolv lignin used in this experiment is an 'oligo-lignol' (molecular wt < 1000). If one can rule out the possibility of 'lignin toxicity' towards rumen microorganisms, unbound lignin in the medium could still affect cellulose degradation by the formation of quinone methide structures, forming interlinkages with the carbohydrate substrate and(or) binding extracellular microbial enzymes, or by directly adsorbing to microbial enzymes. The negative influence of HPL on cellulose degradation must be due to hydrophobic adsorption of enzymes, since HPL has no ionizable phenolic hydroxyl groups, and is thus unable to form quinone methide structures. Hydrophobic enzyme adsorption is likely to occur, since adsorption of bile acids to lignin is due to hydrophobic attraction, also (Eastwood and Hamilton, 1968; Eastwood, 1983). The additional negative impact of unmodified lignin implies that the phenolic hydroxyl group is also involved, either directly, by enhancing the adsorption of enzymes, or indirectly, by allowing the formation of quinone methide intermediates, and subsequent establishment of cross-linkages.

Although this simple *in vitro* model degradation study has clearly demonstrated the impact of lignin inclusion, modification, and concentration on cellulose degradation, the extent to which each mechanism contributes to the impact of lignin on forage cell wall degradation, is interdependent with structural differences among plant tissues (Akin and Burdick, 1981), changes with maturity in overall and cell wall composition (Journal Paper 1), lignin structure (Buxton and Russell, 1988), and lignin distribution within cell walls (Engels and Schuurmans, 1992).

Implications

Blending cellulose and lignin enhanced cellulose degradation, presumably by changing the polymer conformation (porosity), but this effect was counteracted by the presence of phenolic hydroxyl groups in lignin. Free lignin in the incubation medium inhibited cellulose degradation, most likely by adsorption of microbial enzymes. This effect is enhanced by the availability of phenolic hydroxyl groups in lignin, which allows formation of reactive quinone methide intermediates. The mechanism(s) by which lignin was shown to interfere with cellulose degradation, may not all be equally important with regard to forage cell wall degradation, because the impact of lignin depends on the sequence of events in cell wall deposition and is interdependent with changes in lignin composition and distribution within cell walls, and changes in overall cell wall structure and composition.

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Journal Paper 4. Cell Wall Composition and Digestibility of Corn Stover Fractions in Response to Ammonia Treatment

ABSTRACT: Treatment of corn stover fractions (leaf, upper stem, and lower stem) from drought-stressed corn with 3% aqueous NH_3 and isonitrogenous NH_3 addition was evaluated using a 3 x 3 factorial. For NH_3 treatment, fractions were packed in 4-L cardboard containers double lined with polyethylene and opened after 30 d. Samples taken during the initial preparation of NH_3 treatments were immediately frozen and served as NH_3 additions. Water controls were prepared by addition of H_2O to achieve DM levels similar to NH_3 added. *In vitro* NDF degradation and IVDMD were related to changes in overall and cell wall composition and the linkage pattern of phenolic acids. Ammonia treatment decreased ($P<.05$) NDF and hemicellulose, compared to NH_3 added. Permanganate lignin was higher ($P<.05$) for NH_3 added than NH_3 treatment. Klason lignin was higher ($P<.05$) for NH_3 added than water control and NH_3 treatment. Ammoniation did not affect ($P>.05$) xylan concentration in NDF. Ammonia treatment, compared to NH_3 added, decreased ($P<.001$) arabinan. Glucan in NDF was higher for NH_3 treatment than for NH_3 added. Lignin methoxyl group content was lower ($P<.05$) in leaf than stems, but was not affected ($P>.10$) by ammoniation. Total phenolic acid content was lower

(P<.001) in leaf than stems. Values were lower for ammoniation in upper stem only (treatment x fraction interaction, P<.05). Ammonia treatment increased (P<.05) the ratio of esterified *p*-coumaric acid (*p*-CA) to ferulic acid (FA), and decreased (P<.05) the ratio of etherified *p*-CA to FA. The IVDMD (48 h) and the degradation of NDF beyond 12 h were lower for stems (P<.05) than leaves. The IVDMD and NDF degradation were increased (P<.001) by ammoniation, with higher (P=.03) IVDMD for NH_3 treatment than NH_3 added, especially in leaves (fraction x treatment interaction, P<.001). The variable response to NH_3 treatment among stover fractions of drought stressed corn appears to be related to the high concentration of residual water-soluble carbohydrates in the stalks.

Key Words: Ammonia Treatment, Corn Stover Fractions, Lignin, Phenolic Acids, Digestibility.

Introduction

Crop residues form an abundant but underutilized feed resource for ruminants. They are generally inadequate as sole sources of nutrients (O'Donovan, 1983), due to low CP, low rate and extent of digestion, and imbalanced mineral content (Schiere and Ibrahim, 1989). Botanical fractions differ in composition, leaves generally being of higher quality than stems due to higher protein and lower lignin content (Morrison, 1980). The methoxyl content of lignin affects the formation of intermolecular linkages, e.g., between lignin and hemicellulose (Leary, 1980), and is, therefore, likely to affect digestibility of plant cell walls. Ferulic acid and *p*-CA,

precursors in the biosynthesis of lignin (Sarkanen and Ludwig, 1971) may also interfere with microbial digestion (Jung, 1985; Hartley and Ford, 1989). They are present in plant cell walls in the 'free' (unbound) form, ester-linked to hemicellulose (Smith and Hartley, 1983), or ether-linked to lignin (Scalbert et al., 1985).

Alkali treatment results in saponification of ester linkages between cell wall components (Chesson, 1988), which renders cell wall carbohydrates more accessible to microbial enzymes. Ammonia has the advantage over other alkali of supplying NPN to rumen microorganisms, which is required for the microorganisms to benefit from the increase in potentially available fermentable energy (Orskov and Grubb, 1978), if intake is to be maximized. The added NPN is also thought to be partly responsible for the observed increase in digestibility (Ibrahim, et al., 1989). The effectiveness of NH_3 in hydrolyzing ester linkages has been challenged in relation to the high buffering capacity of many residues (Van Soest et al., 1984).

The objectives of the research were to determine the response of different corn stover fractions (leaf, upper stem, lower stem) to ammoniation; to separate the response to ammoniation into effects of NPN addition and chemical treatment per se; and to relate the response to NH_3 treatment to changes in linkages of phenolic acids with other cell wall components (ester and ether linkages).

Experimental Procedures

Plant Material and Processing. Stover from mildly drought-stressed corn (*Zea mays*) was harvested manually after a dry summer and early fall (average monthly precipitation, June to October: 34 mm) from six replicate plots at approximately 50

% DM on October 22, 1991, from a field near Blacksburg, VA. A 3 x 3 factorial arrangement was used, consisting of three plant fractions (leaf, upper stem, lower stem) and three processing methods (control, NH_3 added, NH_3 treatment). The plants were first separated into leaf blades and stems (including leaf sheaths). Stems were further divided in upper (above ear) and lower (below ear) fractions. Replicate fractions were chopped through a 1-cm screen, and subdivided into two batches each. To one batch, NH_4OH was added to achieve 3% NH_3, DM basis, for all fractions, and H_2O was added to achieve 45% DM for leaf and upper stem. Lower stem was already of a lower DM content, therefore moisture addition to lower stem was minimized by application of undiluted (30%) NH_4OH. After thorough mixing of the NH_4OH with the plant material, initial samples were taken, which served as NH_3 additions, isonitrogenous to the NH_3 treatments. For NH_3 treatment, the ammoniated material was compressed manually into 4-L cardboard containers double-lined with polyethylene bags, which were stored for 30 d at room temperature. Controls were prepared by addition of distilled water to the second batch to achieve DM levels similar to those for NH_3 added and treatment. The water control and NH_3 added samples were frozen immediately at -20 °C after mixing. At sampling of the NH_3 treated fractions, molded material, if present, was removed, and samples were frozen at -20 °C.

Chemical Analyses and In Vitro Degradation. Dry matter, CP (AOAC, 1990), and water soluble carbohydrates (WSC) (Dubois, 1956, modified by Johnson et al., 1966) were determined after thawing. Subsamples were freeze-dried, ground through a 1-mm screen, and analyzed for ash (AOAC, 1990), total silica, fiber components, NDF, ADF, and permanganate lignin (Robertson and Van Soest, 1981). True IVDMD (48 h) and *in vitro* NDF degradation after 12, 24, 48, 72, and 96 h were

determined by incubation in 20% (v/v) rumen fluid in McDougall's buffer (Tilley and Terry, 1963), followed by extraction with neutral detergent (Goering and Van Soest, 1970).

Klason lignin and cell wall carbohydrates were determined by a two-stage acid hydrolysis (TAPPI, 1985, modified by Kaar et al., 1991). as described in Journal Paper 1. Acid soluble lignin (ASL) was quantified spectroscopically by UV absorption at 205 nm (TAPPI, 1989). Lignin methoxyl groups were determined by reaction with 57% hydriodic acid at 145 to 150 °C in a methoxyl apparatus, and titration of the resulting iodine with sodium thiosulphate (TAPPI, 1972).

Free, esterified and etherified p-CA and FA were determined by GLC (modified from Iiyama et al., 1990). For determination of free phenolic acids, 50 to 60 mg NDF were extracted with 6 mL diethyl ether overnight at room temperature and washed twice with 6 mL diethyl ether. Residual ether was evaporated under N_2, the residue stored over desiccant until alkali saponification. The diethyl ether extract was combined with the two washings and evaporated under N_2 to 1 to 2 mL, transferred to a septum vial, further evaporated to dryness, and stored until derivatization.

To release esterified phenolic acids, ether-extracted cell walls were suspended in 4 mL of 1 M NaOH in stoppered tubes of 12 mL capacity. After flushing with N_2 for 1 h, the tubes were kept in a shaking water bath at 20 °C for 24 h. The hydrolyzate was removed with a Pasteur pipette, and the residue was washed twice with 1 mL water. The combined extract and washings were acidified with 6 M HCl to pH 1 and extracted four times with 15 mL diethyl ether. The combined diethyl ether extracts were evaporated under N_2 and derivatized.

To release etherified phenolic acids, saponified cell wall residues (25 mg) were subjected to alkali treatment (1 mL of 4 M NaOH) in a 2-mL stainless steel pressure vessel kept in an oil bath at 168 to 172 °C for 2 h. The contents were collected by rinsing three times with 1 mL 0.2 M NaOH, and were processed as for the room temperature saponified extracts.

Trimethylsilyl-derivatized phenolic acids were separated on a wall-coated open-tubular bonded phase fused silica capillary column (30 m x .53 mm i.d.) in a gas chromatograph (Perkin Elmer) equipped with flame ionization detector. Conditions applied were: injection temperature, 280 °C; oven temperature, 180 °C for 5 min, programmed at 5 °C/min to 230 °C, held for 5 min; linear flow rate of He, 30 mL/min. Standards used for quantification (trans-p-CA, trans-FA) were also subjected to the respective treatments. Derivatization was with 200 μL N,O-bis(trimethylsilyl) acetamide (BSA) at 100 °C for 10 min.

Statistical Analysis. The data were analyzed as a 3 x 3 factorial by two-way ANOVA using the GLM procedures of SAS (1989). Main effects were plant fractions and processing methods. Orthogonal contrasts tested were: leaf vs stems (upper and lower); upper stems vs lower stems. Non-orthogonal (Bonferroni) contrasts (Lowry, 1992) tested were: water control vs NH_3 added; NH_3 treatment vs NH_3 added. Interaction between main effects was included in the model. In case of fraction x ammoniation interaction, the contrasts for ammoniation were tested within fractions by one-way ANOVA.

Fiber digestion kinetics were calculated according to a non-linear model for degradation of NDF (adapted from Mertens and Loften, 1980) using non-linear regression procedures (SAS, 1989). Correlation coefficients and multiple prediction equations were obtained using linear and stepwise regression procedures (SAS, 1989).

Results

Ammonia treatments resulted in products of satisfactory color (medium to dark brown). The treated fractions were almost odorless (leaf) to sweet (stems), and were softer and more pliable than the controls. Minor spoilage due to molding occurred during treatment of leaf.

Composition. Dry matter content of leaf, upper stem, and lower stem at harvest were 81, 59, and 35%, respectively, and 47, 41, and 33% after addition of NH_3. Crude protein increased (P<.01) with ammoniation (Table 12), with a higher increase (P<.001) for NH_3 treatment than NH_3 added. Leaf had a higher (P<.001) NDF content than stem fractions, with lower stem being lower (P<.001) than upper stem (Figure 12). This was associated with accumulation of WSC in lower stem and, to a lesser extent, in upper stem in contrast (P<.001) to leaf. Neutral detergent fiber was reduced (P=.01) by NH_3 treatment, compared with NH_3 added, but was not affected (P=.5) by NH_3 addition, compared to water control (Table 12). The reduction in NDF by NH_3 treatment was by approximately six percentage units (P<.001) in leaf, but was absent in stems (ammoniation x fraction interaction, P<.001). Ammonia treatment tended to increase (P=.06) ADF, compared with NH_3 added. Hemicellulose (NDF - ADF) decreased (P<.001) with NH_3 treatment, but not NH_3 added (data not shown). The reduction in hemicellulose was most apparent in leaf (P<.001), minor in upper stem (P<.05), and absent (P>.15) in lower stem (ammoniation x fraction interaction, P<.001). Permanganate lignin was lower (P<.001) in leaf than in stems, and higher (P<.01) in lower than upper stem. Ammonia treatment resulted in lower permanganate lignin (P<.001) compared with NH_3 added.

Table 12. Composition of corn stover fractions as affected by ammoniation

Fraction	Treatment	Component					
		Ash[a]	Silica[a]	CP[bce]	NDF[acde]	ADF[a]	Lignin[abcdf]
		----------------------- %, DM basis -----------------					
Leaf	Water control	6.0	1.5	6.6	74.8	40.0	4.1
	NH_3 added	6.0	1.5	10.7	73.6	40.5	5.5
	NH_3 treated	6.2	1.5	12.0	67.7	40.2	3.7
Upper stem	Water control	3.6	.2	4.2	70.3	38.2	4.5
	NH_3 added	3.7	.8	11.3	70.6	37.9	5.4
	NH_3 treated	3.8	1.0	13.7	69.7	39.9	4.2
Lower stem	Water control	3.4	.5	4.7	62.0	38.0	5.6
	NH_3 added	3.5	.5	12.1	64.4	38.5	5.7
	NH_3 treated	3.7	.8	13.3	64.8	40.4	4.6
SE		.13	.33	.59	1.00	.76	.23

[a]Leaf differs $(P < .05)$ from stems.
[b]Ammonia added differs $(P < .05)$ from water control.
[c]NH_3 treatment differs $(P < .05)$ from NH_3 added.
[d]Upper stem differs $(P < .05)$ from lower stem.
[e]Fraction x ammoniation interaction $(P < .05)$.
[f]Permanganate lignin.
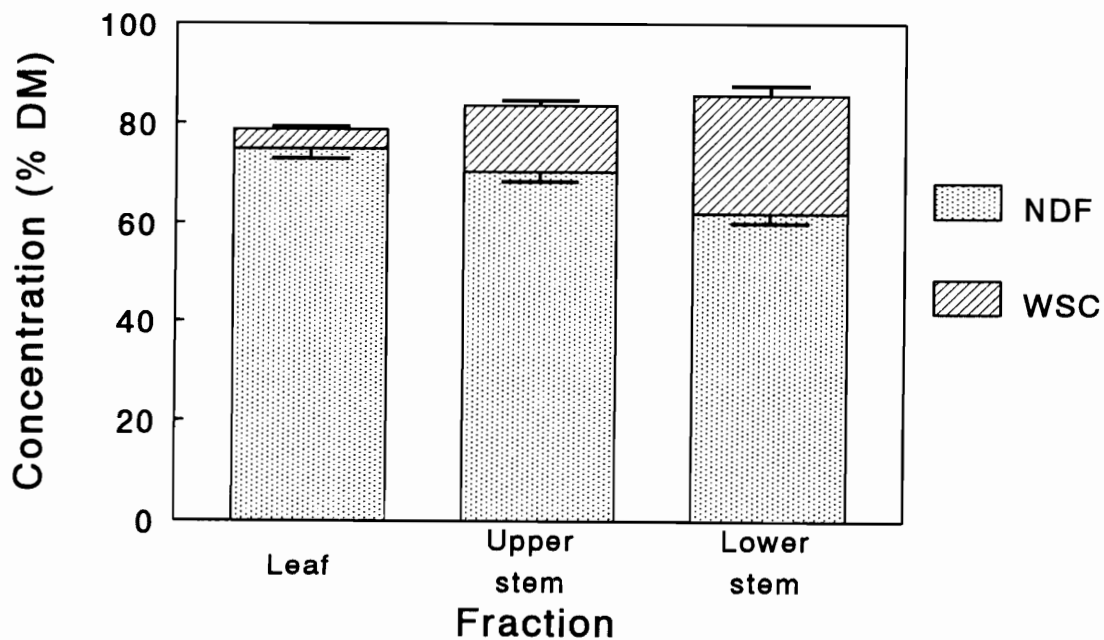
Figure 12. Water soluble carbohydrates (WSC) and NDF in untreated (water control) corn stover fractions. Both components differ (P<.05) between leaf and stems, and between upper and lower stem.

Klason lignin in NDF (Table 13) was lower (P<.001) in leaf than in stems, and higher (P<.01) in lower than upper stem. Klason lignin increased (P<.001) with NH_3 added but not NH_3 treatment. Acid soluble lignin was also different among fractions (P<.001), and decreased (P<.001) with ammoniation.

Glucan in NDF of lower stem was higher (P<.01) than of upper stem. Leaf glucan tended to be lower than stem glucan (P=.08). Hemicellulosic sugars (xylan, arabinan, and galactan) were higher (P<.01) in leaf than in stems, and were lower in lower than upper stem (P=.08, P<.001, and P<.01, respectively). Ammonia treatment increased (P=.016) glucan in NDF, compared with NH_3 added, but did not affect xylan content (P>.15). Arabinan content of NDF was reduced (P<.001) by NH_3 treatment, compared with NH_3 added, and galactan was reduced (P<.001) by ammoniation (both NH_3 added and treatment).

Methoxyl group content, as percentage of Klason lignin, was lower (P<.001) in leaf than in stems (Table 13), but was not affected by ammoniation. The methoxyl concentrations were approximately equivalent to one methoxyl group per lignin monomeric unit for stems, and .75 methoxyl groups per monomeric unit for leaf lignin. As a percentage of NDF, lignin methoxyl content was different among all fractions (P<.05), and tended to increase (P=.11) with NH_3 addition.

Ammoniation did not affect (P>.15) total contents of p-CA and FA (Table 14) and their sum. Phenolic acid contents (FA, p-CA, and sum) were lower (P<.001) in leaf than in stems. Overall, esterified p-CA was not affected (P>.15) by ammoniation. However, within the leaf fraction, ammoniation increased (P<.01) the concentration of saponifiable p-CA. For upper stem, effects of NH_3 addition (vs water control) and NH_3 treatment (vs NH_3 added) were not significant (P>.15), but

Table 13. Concentration of neutral cell wall sugars, lignin, and phenolic acids in NDF of corn stover fractions as affected by ammoniation

Fraction	Treatment	Cell wall component							Methoxyl groups	
		Glucan[ab]	Xylan[c]	Arab-inan[abc]	Galac-tan[bcd]	Klason lignin[bcde]	Acid sol. lignin[abcd]	Phenolic acids[bcef]	% of NDF[bcf]	% of Klason lignin[c]
						% of NDF				
Leaf	Water control	42.8	24.2	3.46	1.49	11.7	2.18	.93	1.36	11.9
	NH₃ added	42.5	23.7	3.38	1.07	13.3	1.94	1.15	1.59	10.4
	NH₃ treated	46.4	23.5	2.94	1.01	13.3	1.85	1.55	1.56	11.1
Upper stem	Water control	43.2	23.1	3.36	1.21	13.4	2.11	3.17	2.02	14.4
	NH₃ added	43.0	22.4	3.50	1.15	12.7	1.94	2.31	2.04	14.5
	NH₃ treated	45.7	22.1	2.58	.81	13.9	1.79	2.00	2.21	14.4
Lower stem	Water control	46.4	21.9	2.48	.99	13.8	1.74	3.43	2.15	14.3
	NH₃ added	46.8	22.2	2.32	.61	16.6	1.7	3.28	2.41	14.3
	NH₃ treated	48.0	20.8	1.59	.63	15.8	1.6	3.13	2.47	14.7
SE		1.28	.63	.20	.12	.31	.061	.20	.115	.34

[a]NH₃ treatment different (P<.05) from NH₃ added.
[b]Upper stem differs (P<.05) from lower stem.
[c]Leaf differs (P<.05) from stems.
[d]Ammonia added different (P<.05) from water control.
[e]Fraction x ammoniation interaction (P<.05).
[f]NH₃ treatment different (P<.05) from water control.

Table 14. Free, esterified, and etherified phenolic in NDF of corn stover fractions as affected by ammoniation

Fraction	Treatment	p-Coumaric acid				Ferulic acid			
		Free[ab]	Ester[cde]	Ether[bcf]	Total[bcd]	Free[af]	Ester[bdg]	Ether[f]	Total[bc]
						NDF			
					mg/g				
Leaf	Water control	2.56	2.62	.83	6.0	.210	1.32	1.76	3.3
	NH_3 added	2.76	4.81	.84	8.4	.025	2.09	1.03	3.2
	NH_3 treatment	4.41	5.32	.66	10.3	.065	2.25	2.93	5.2
Upper stem	Water control	5.56	17.1	1.42	23.8	.213	6.52	1.20	7.9
	NH_3 added	1.60	14.3	1.26	16.2	.008	4.93	2.42	6.9
	NH_3 treatment	1.45	11.7	.82	14.0	.100	2.73	3.55	6.0
Lower stem	Water control	5.82	20.8	1.58	28.2	.208	4.06	1.79	6.1
	NH_3 added	3.50	22.2	1.44	27.2	.083	3.11	2.43	5.6
	NH_3 treatment	1.80	22.5	.59	24.9	.115	3.61	2.63	6.4
SE		.89	1.8	.12	1.7	.025	.49	.53	.57

[a]Ammonia added different (P<.05) from water control.
[b]Fraction x ammoniation interaction (P<.05).
[c]Leaf differs (P<.05) from stems.
[d]Upper stem differs (P<.05) from lower stem.
[e]Water control upper stem differs (P<.05) from NH_3 treatment.
[f]NH_3 treatment different (P<.05) from NH_3 added.
[g]NH_3 treated upper stem differs (P<.05) from NH_3 added.

when compared to water control, NH_3 treatment reduced (P = .045) esterified p-CA. Etherified p-CA was reduced (P < .001) by NH_3 treatment, compared with NH_3 added.

Ammoniation increased (P < .001) the ratio of esterified p-CA to FA (Table 15), but only in stems (ammoniation x fraction interaction, P < .01). Molar ratio of esterified to etherified p-CA increased (P < .001) after NH_3 treatment. The ratio of esterified to etherified FA tended to decrease (P = .054) after NH_3 treatment, due to an apparent increase (P = .023) in etherified FA after NH_3 treatment, compared with NH_3 added.

Digestibility and Fiber Digestion Kinetics. The IVDMD (Figure 13) of leaf was higher (P < .001) than of stems. Values were increased by NH_3 addition (P < .01), with an additional response to NH_3 treatment over NH_3 added (P = .031). The additional response to NH_3 treatment was more significant (P < .01) within the leaf fraction, and absent (P > .15) in stems (ammoniation x fraction interaction, P < .001). Extent of NDF degradation was higher (P < .001) for leaf than for stems (Figure 14) after 24, 48, and 72 h. Degradation of NDF determined at these times was enhanced (P < .001) by ammoniation. Within fractions, NDF degradation was enhanced (P < .001) by ammoniation after 24 and 48 h only for leaf (ammoniation x fraction interaction, P < .001). Ammoniation was effective in increasing NDF degradation of upper (P = .028) and lower (P = .045) stem only after 72 h, with no difference (P > .15) between NH_3 added and NH_3 treatment. Leaf NDF degradation after 48 and 72 h tended to be higher (P = .097) for NH_3 treatment, compared to NH_3 added. Disappearance of original cell wall was, however, increased (P < .05) after 24 and 48 h. These trends are reflected in a higher potential cell wall degradability due to ammoniation for all fractions, and higher rate constants for the leaf fraction only (Table 16).

Table 15. Molar ratios of free, esterified, and etherified *p*-coumaric acid (*p*-CA) and ferulic acid (FA) in NDF of corn stover fractions as affected by ammoniation

Fraction	Treatment	*p*-CA/FA		Ester/ether		Free/bound	
		Ester[abcd]	Ether[ac]	*p*-CA[abcde]	FA[abd]	*p*-CA[b]	FA[ad]
Leaf	Water control	2.41	.73	3.2	.89	.705	.080
	NH₃ added	2.76	1.17	6.0	2.29	.515	.008
	NH₃ treatment	3.17	.25	8.1	.96	.758	.013
Upper stem	Water control	3.09	1.15	12.8	5.94	.323	.025
	NH₃ added	3.44	.99	10.5	2.91	.140	.000
	NH₃ treatment	5.20	.23	17.3	.89	.143	.017
Lower stem	Water control	5.99	1.18	13.3	2.65	.315	.038
	NH₃ added	8.45	1.04	16.6	1.90	.153	.018
	NH₃ treatment	7.57	.30	39.6	1.48	.078	.020
SE		.35	.24	2.8	.74	.129	.011

[a]Ammonia added different (P < .05) from water control.
[b]Leaf differs (P < .05) from stems.
[c]Upper stem differs (P < .05) from lower stem.
[d]Fraction x ammoniation interaction (P < .05).
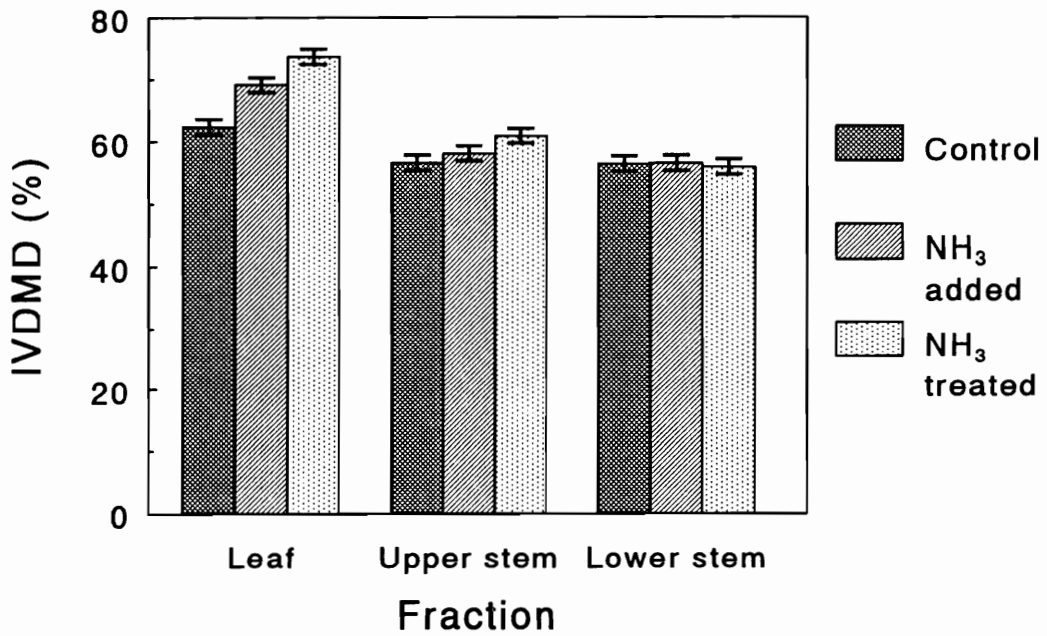[e]NH₃ treatment different (P < .05) from NH₃ added.

Figure 13. *In vitro* dry matter disappearance of corn stover fractions as affected by ammoniation. All contrasts described in the Experimental Procedures and interaction between main effects were significant (P < .05).
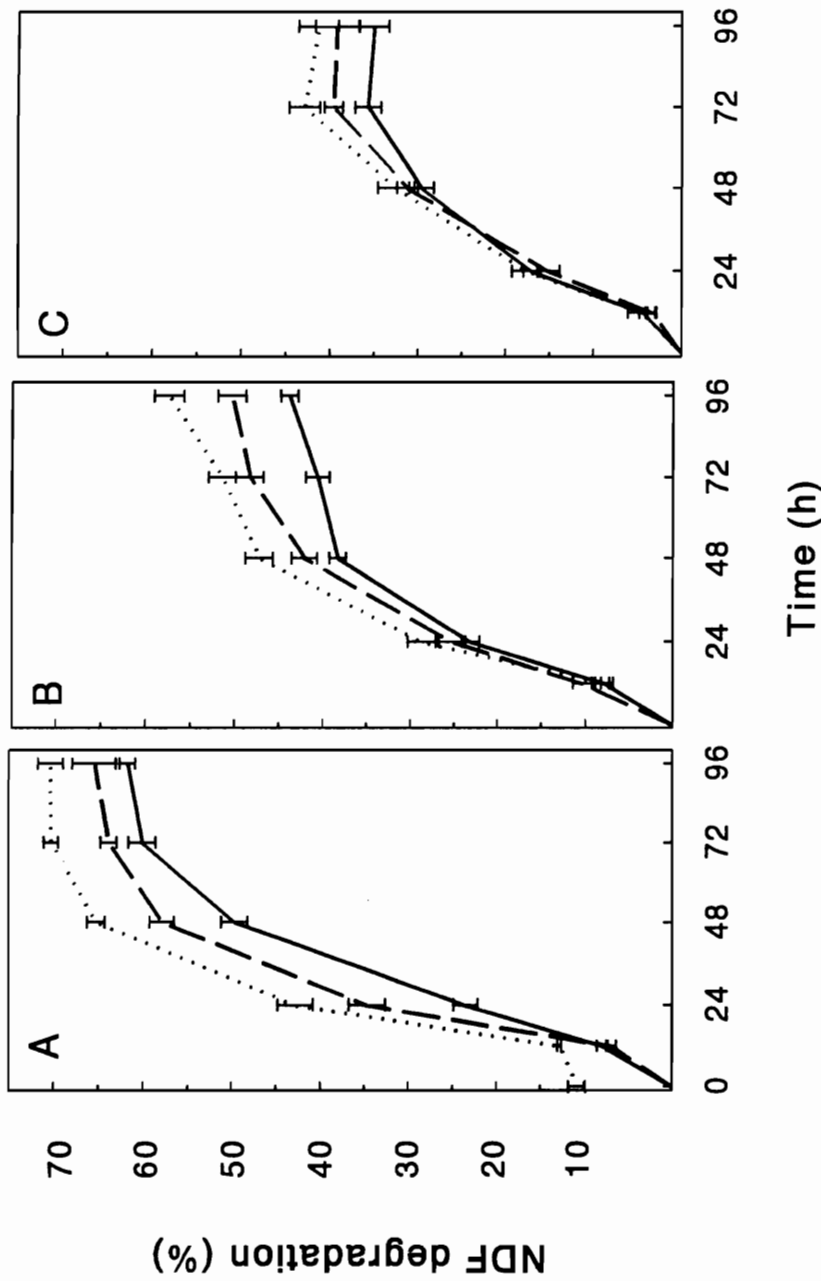
Figure 14. *In vitro* NDF degradation of (A) corn stover leaf, (B) upper stem, and (C) lower stem for control (——), NH_3 addition (———), and NH_3 treatment (·······). Contrasts described in the Experimental Procedures were tested for NDF degradation at 24, 48, and 72 h. Leaf differs ($P<.001$) from stems, and upper stem differs ($P<.001$) from lower stem at all times. Ammonia added differs ($P<.001$) from water control at all times. Trend for difference ($P=.087$) between NH_3 added and NH_3 treatment at 48h. Fraction x ammoniation interaction at 24 h ($P<.001$) and 48 h ($P<.05$). Disappearance of original cell wall differs ($P<.05$) between NH_3 treatment and NH_3 added for leaf at 24 and 48 h.

Table 16. Fiber degradation kinetic parameters as affected by ammoniation

Fraction	Treatment	Digestion kinetics[a]			
		Rapidly digested fraction	Potential digestible fraction	Rate constant	Lag time
		%	%	/h	h
Leaf	Water control	0	67.7	.032	8.7
	NH_3 added	0	66.3	.053	10.0
	NH_3 treatment	0	68.4	.054	10.6
	NH_3 treatment[b]	9.6	70.5	.068	12.8
Upper stem	Water control	0	43.8	.048	8.1
	NH_3 added	0	50.8	.040	7.9
	NH_3 treatment	0	54.3	.044	11.1
Lower stem	Water control	0	35.8	.044	9.1
	NH_3 added	0	42.8	.034	8.7
	NH_3 treatment	0	43.02	.035	9.6

[a] Model (after Mertens and Loften, 1980): $Y = A + B \times (1-e^{-c(t-L)})$, in which

Y = NDF degradation (%),
A = rapidly digested fraction,
B = potentially digestible fraction,
c = first-order digestion rate constant,
L = lag time.

[b] Degradation as percentage of original 'cell wall' (control NDF).

Disappearance of all neutral cell wall sugars and Klason lignin (Table 17) was higher ($P < .01$) for leaf than for stems, with higher values for upper than lower stem for xylan ($P < .01$) and arabinan ($P < .001$). Glucan degradation was not different ($P > .15$) between water control and NH_3 added, or between NH_3 added and NH_3 treatment ($P > .15$). Degradation of xylan in NDF tended to be enhanced ($P = .074$), by NH_3 addition and NH_3 treatment, compared to water control. Lignin disappearance was enhanced ($P < .001$) by NH_3 addition and NH_3 treatment, with no difference between the latter. *In vitro* digestion during 72 h did not alter ($P > .15$) methoxyl content of Klason lignin, for any of the corn stover fractions, irrespective of ammoniation.

Extent of NDF degradation (72 h) was well correlated with lignin methoxyl content ($r = -.87$), esterified p-CA ($r = -.93$), and total phenolic acids ($r = -.93$). Overall, NDF content and IVDMD were not correlated, but when contrasting the processing methods within fractions, NDF and IVDMD appeared well-correlated ($r = -.81$, $-.65$, and $-.71$, within leaf, upper stem, and lower stem, respectively). Contrasting control fractions resulted in high correlation coefficients with NDF degradation for methoxyl content ($r = .-.94$), esterified ($r = -.88$) and total p-CA ($r = -.92$). Apparently, most of the components were highly co-correlated, because stepwise multiple linear regression, using $P < .15$ for entry in the model, resulted in regression equations with only limited number of parameters (Table 18).

Table 17. *In vitro* disappearance (72 h) of NDF, lignin and neutral polysaccharides in corn stover fractions as affected by ammoniation

Fraction	Treatment	Disappearance[a]					
		NDF[bcd]	Glucan[c]	Xylan[cd]	Arabinan[cd]	Klason lignin[bc]	OCH$_3$ groups[e]
		----------------------------- % ----------------------------					% of Klason lignin
Leaf	Water cntrl	60.2	64.9	61.0	66.1	35.3	-.26
	NH$_3$ added	63.9	62.7	60.7	67.1	53.1	1.01
	NH$_3$ treated	66.7	64.3	62.8	76.1	55.1	-.17
Upper stem	Water cntrl	40.6	48.6	37.2	55.7	4.2	-.25
	NH$_3$ added	48.8	51.6	44.8	70.2	26.4	-1.01
	NH$_3$ treated	48.2	44.6	40.8	74.0	28.3	-.28
Lower stem	Water cntrl	35.7	52.3	31.4	38.4	-3.8	-.44
	NH$_3$ added	40.6	45.5	31.8	46.9	29.4	-.79
	NH$_3$ treated	42.2	43.9	38.4	46.6	23.4	-.46
SE		1.8	1.9	2.3	5.5	3.8	.65

[a]NDF degradation determined using six replicates (n=6); disappearance of neutral polysaccharides determined in duplicate using pooled samples (n=2).
[b]Ammonia added different (P < .05) from water control.
[c]Leaf differs (P < .05) from stems.
[d]Upper stem differs (P < .05) from lower stem.
[e]Not different from zero (P > .15) by paired t-test comparison for any treatment.

Table 18. Stepwise regressions predicting IVDMD and rate and extent of NDF degradation of corn stover fractions as affected by ammoniation, using using proximate and cell wall components as independent variables[a][b]

Model	Intercept	Slope		Significance[c]	r^2
		Variable	Coefficient		
IVDMD, %	122	OCH_3	-3.17	< .01	.92
		Free/bound FA	-132	.014	
		$KMnO_4$ lignin	-2.81	.054	
NDF degr., 72 h, %	34	Ester-p-CA	-1.76	< .001	.93
		Klason lignin	2.87	.066	
Degradation rate[d] /h	-.0316	Free/bound p-CA	.068	< .01	.88
		Free/bound FA	-.42	.011	
		OCH_3	.0044	.12	

[a] Independent variables: CP, NDF, ADF, $KMnO_4$ lignin (all in % of DM), Klason lignin, acid soluble lignin, and neutral polysacharides (all in % of NDF), and phenolic acids (in mg/g NDF) and methoxyl (OCH_3) content (in % of Klason lignin).

[b] Entry level: $P < .15$.

[c] Significance of variable in final model.

[d] Rate constant in model by Mertens and Loften (1980).

Discussion

Some of the major changes in composition with NH_3 treatment appeared to be due to chemical treatment per se, others were due in part to the addition of NPN as NH_3. The slight difference in CP between NH_3 added and NH_3 treatment may have been caused by CP concentration due to fermentative losses during NH_3 treatment. However, it is more likely due to volatilization of NH_3 from NH_3 added samples immediately before and during the Kjehldahl procedure.

The partial solubilization of hemicellulose by NH_3 treatment has been observed by others (Kiangi et al., 1981; Givens et al., 1988; Mason et al., 1988), and is caused in part by alkali-induced "peeling" reactions, in which degradation of sugar moieties occurs at the reducing end of hemicellulose chains (Wilkie, 1979). This phenomenon also resulted in the reduced xylan/glucan ratio. Apparently, the pentose sugars are rendered soluble in neutral detergent solution by NH_3 treatment to a larger extent than NDF and xylose, whereby their contents in NDF and their ratio's to xylan are reduced. The reduction in substitution of the xylan backbone of hemicellulose with arabinose side chains is believed to facilitate degradation of hemicellulose by microbial enzymes (Brice and Morrison, 1982).

Differences in disappearance of cell wall carbohydrates and lignin in leaf vs stems followed patterns similar to NDF degradation. Xylan degradation in stems was markedly lower than degradation of glucan and arabinan, in agreement with findings for Caucasian bluestem *(Bothriochloa caucasia)* (Piwonka et al., 1991) and alfalfa *(Medicago sativa)* (Albrecht et al., 1987; Titgemeyer et al., 1992). In leaf, however, the neutral polysaccharides were all degraded to a similar extent in accordance with findings of Amjed et al. (1992) for sugarcane bagasse and wheat straw. The

differential degradation of neutral polysaccharrides in the more recalcitrant corn stover stems does not agree with the suggestion by Chesson (1983) that cell wall components in poorly digestible forages are degraded to a similar extent, which was the basis for postulating that the degradation of all cell wall components was equally affected by lignin, due to accumulation of undigestible lignin at the cell surface. In the present research, however, not all carbohydrates are equally affected by lignin, presumably by the more direct association of hemicellulose with lignin.

The increase in concentration of Klason lignin in NDF with ammoniation could be explained by the observed decrease in NDF content after NH_3 treatment for the leaf fraction, although a similar increase in Klason lignin also occurred with NH_3 addition. Part of the discrepancy may be due to condensation reactions occurring between NH_3-N and lignin, resulting in Maillard products (Van Soest and Mason, 1991). These condensation products may be formed during NH_3 treatment, or, alternatively, during the preparatory steps prior to lignin determination. If the increase in Klason lignin is due to the formation of Maillard products, these products are apparently included in the Klason lignin but are unlikely to be fully oxidized by permanganate, which excludes them from permanganate lignin. A possible cause for the general discrepancy between permanganate and Klason lignin may be the solubilization of lignin-carbohydrate complexes in acid detergent solution preceding the permanganate oxidation, and loss of these complexes during filtration using coarse porosity crucibles. The two-step acid hydrolysis involved in the Klason lignin procedure may not solubilize the complexes to the same extent as acid detergent, and overall lignin recovery is likely to be improved by the use of fine porosity crucibles.

Ammoniation had no direct effect on methoxyl groups in Klason lignin, the observed increase in methoxyl content as percentage of NDF being due to

concentration of Klason lignin in NDF. However, the difference between leaf and stems in methoxyl content is of potential interest in relation to ruminal fiber degradation, as discussed in Journal Papers 1 and 2. Lignin of a high methoxyl content is particularly prone to formation of quinone methide intermediates during lignin formation and aging (Glasser and Kelley, 1987). These intermediates will readily react with cell wall components bearing carboxyl or hydroxyl groups, thereby establishing ester- or ether linkages with these components (Leary, 1980).

With respect to the amount of esterified phenolic acids released by room temperature alkali saponification of corn stover cell walls, the results of this experiment differ from previous research (Hartley and Jones, 1978), presumably due to the pre-extraction of 'unbound' phenolic acids. A considerable portion of the p-CA appeared not to be covalently linked to any other cell wall components as reflected in the relatively high content of ether-extractable p-CA (up to 43% of total p-CA in leaf). Free phenolic acids in lower concentrations have been reported earlier in wheat and rice plants (Lam et al., 1990), but these were obtained by extraction with water instead of ether. Another unexpected finding was the partial release of etherified p-CA by NH_3 treatment in all fractions, whereby the resulting ratio of etherified p-CA to FA decreased. This was not expected since the premise for NH_3 treatment is saponification of ester-linkages exclusively (Chesson, 1981). Finally, the increasing ratio of esterified p-CA to FA after NH_3 treatment of stems implies that esterified FA is more easily saponified by NH_3 than esterified p-CA. This may be related to the fact that p-CA is predominantly ester-linked to lignin, whereas FA is mainly esterified to arabinose residues in hemicellulose (Atsushi et al., 1984). The difference in alkali-stability between the esters linking FA and p-CA has been demonstrated by Hartley and Morrison (1991), who were able to release most of the

FA present in tall fescue and bermudagrass cell walls with 0.1 M NaOH, whereas 1-2 M NaOH was required to release p-CA.

Contrary to general findings with regard to the inverse relationship between initial quality and treatment response (Kiangi et al., 1981; Givens et al., 1988; Ibrahim et al., 1989; Goto et al., 1991), the differential response to NH_3 treatment among corn stover fractions was not inversely related to initial quality. However, it could be related, in part, to initial differences in composition. The contribution of NPN addition to the response to NH_3 treatment was higher for stems than for leaves, which may be related to the lower CP content of control stems than of leaves. Another major difference in initial composition was that of the WSC concentration between leaf, upper stem, and lower stem. Maturity of corn stover is normally associated with an increase in NDF concentration due to the loss of cell solubles (Weaver et al., 1978; Berger et al., 1979; Russell, 1986) after deposition of sugars as starch in the grain (Phipps and Weller, 1979). The stover used in this research, however, was from mildly drought-stressed corn, in which concentration of soluble carbohydrates in the stalks remained relatively high. A similar observation was made by Russell (1986) in a season of inadequate precipitation. He also observed an inverse relationship between NDF and total non-structural carbohydrates. In the present research, the NDF in stems remained below that of leaves, which caused a poor correlation of NDF with IVDMD in the overall data set, whereas within fractions, NDF and IVDMD were reasonably well correlated. The high concentration of WSC in the stems, especially the lower portion, is also a likely reason why chemical treatment showed no (lower stem) or marginal (upper stem) improvement in digestibility beyond that for NPN addition.

No appreciable saponification of phenolic acids by NH_3 occurred in the lower stem (in contrast to the upper stem), which was presumably due to neutralization of alkali by the excess of sugars in the lower stem fractions. The effectiveness of NH_3 in hydrolyzing ester linkages has been challenged before, in relation to the high buffering capacity of plant fibers (Van Soest et al., 1984), but in our research the high level of water soluble sugars seems reason enough. Fiber degradation and IVDMD of leaf was enhanced by NH_3 treatment beyond the effect of NPN addition, which seemed attributable to chemical treatment per se. However, NH_3 treatment of leaves did not lead to appreciable saponification of phenolic acids, of which the initial content was much lower than in the stem fractions. It seems that esterification of phenolic acids (and their involvement as bridging units between lignin and hemicellulose) is not a major limitation to degradation of leaf cell walls.

Xylan degradation from NDF was enhanced by ammoniation, indicating a response not only in initial xylan solubilization from NDF, but also in microbial degradation of residual xylan in NDF. Whether the release of esterified phenolic acids is a good model for the effect of alkali on direct ester linkages between lignin and arabinoxylan, is still subject to speculation (Chesson, 1988). If so, it seems that the improvement in digestibility of the leaf fraction by NH_3 treatment is rather due to a better distribution or absorption of NPN in the fiber matrix, where cellulolytic microorganisms can utilize it. Contrary to the leaf fraction, extent of cell wall degradation in stems was associated with saponification of ester linkages, as exemplified by the enhanced degradation of fiber in upper stem. Distribution or absorption of NPN in the fiber matrix is not a likely factor of importance to the degradation of stem fiber, since NH_3 treatment of lower stem, in the absence of saponification of ester linkages, did not enhance fiber degradation compared with

NH$_3$ addition. Differences between plant fractions in the total content of phenolic acids and the ratio's of free-to-bound phenolic acids seem to be of importance in limiting fiber degradation. Indirect evidence related to the extent of interlinkage of lignin with hemicellulose and/or phenolic acids also points to the importance of the methoxyl content of lignin as a factor interfering with cell wall degradation.

Parameters of fiber digestion kinetics show that for the stem fractions, both NH$_3$ treatment and NH$_3$ addition result in higher potential fiber degradation but not higher rate of degradation. Since rate of degradation was not increased by NH$_3$ addition or -treatment, the practical importance of increased potential degradation is minimal. Ammoniation of leaf, however, improved both rate and potential extent of degradation, with partial response to NH$_3$ addition compared with NH$_3$ treatment. The increase in rate of degradation was probably due to the initial solubilization of NDF by NH$_3$ treatment. Improvement in leaf IVDMD at 48 h due to NH$_3$ addition amounted to 74% of the improvement due to NH$_3$ treatment, whereas 57% of the improvement in leaf NDF degradation (72 h) by NH$_3$ treatment was due to isonitrogenous supplementation provided by the NH$_3$ addition. These trends compare well with those for *in vivo* digestibility of urea-ammonia treated versus urea-supplemented straw (Djajanegara and Doyle, 1989).

Implications

The response to NH$_3$ treatment of corn stover can vary considerably among plant fractions. Differences in protein content, as well as composition of cell wall carbohydrates and lignin, and the linkage pattern of phenolic acids in the cell wall

are likely to affect the response to NH_3 treatment. Most importantly, the lack of additional response to NH_3 treatment of stems beyond NH_3 addition was associated with a high concentration of WSC, presumably responsible for neutralizing the applied alkali. The response to NH_3 treatment could not be conclusively linked to saponification of ester linkages. Further research into the effect of drought stress (especially WSC concentration in stalks) on the response to NH_3 treatment of corn stover is warranted. Possibilities to benefit from differences among stover fractions include fractionated harvesting, e.g. by varying cutting height, and(or) NH_3 treatment of selected fractions.

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Journal Paper 5. Response to Ammoniation in Cell Wall Degradation of Corn Stover Fractions Harvested in Different Years

ABSTRACT: The response to treatment with 3% aqueous NH_3 on *in vitro* cell wall degradation of corn stover fractions harvested in 1991 and 1992 was compared to untreated controls and NH_3 added controls in 2 x 3 factorials, separately for leaf, upper stem, and lower stem fractions. Ammonia added controls consisted of initial samples taken after NH_3 application. The 1991 corn crop was mildly drought stressed, but not the 1992 crop. The response to ammoniation was evaluated using both N-sufficient and N-limiting *in vitro* buffer systems. *In vitro* NDF degradation after 24 h and 72 h was related to changes in CP, detergent fiber components, and the solubility of phenolics as measured by UV absorbance at 280 nm. Overall, cell wall disappearance from 1992 samples was lower (P<.05) than from 1991 samples. Ammoniation (both NH_3 treated and NH_3 added) increased (P<.05) IVDMD and rate and extent of cell wall degradation. For the leaf fraction, the overall increase in degradation was accompanied by higher values for NH_3 treatment than for NH_3 added. Ammonia treatment resulted in partial solubilization of hemicellulose, resulting in lower (P<.001) NDF than NH_3 added. However, degradation of residual

NDF was however not (P>.05) increased by NH_3 treatment. The response to ammoniation was higher (P<.001) when measured in N-limiting medium, compared with N-sufficient (50 mg N/100 mL) medium. However, even in the N-sufficient medium, the response to ammoniation (both NH_3 added and NH_3 treatment) was substantial. Rate of cell wall degradation (24 h incubation) was enhanced by 3 to 11 percentage points by ammoniation in 1991 and by 4 to 14 percentage points in 1992. Extent of cell wall degradation (72 h) was increased by 2 to 8 percentage points in 1991 and by 5 to 10 percentage points in 1992. Ammonia treatment of 1992 stover resulted in a larger response above NH_3 added for leaf (both rate and extent), and enhanced rate of cell wall degradation of stems, compared to the 1991 stover, especially in the N-limiting medium.

Key Words: Ammonia Treatment, Corn Stover Fractions, Soluble Phenolics, Cell Wall Degradation, Buffer Systems

Introduction

Crop residues such as straws and stovers can be regarded as overmature forages in which the cell soluble material has been translocated into the harvested product or deposited in the cell wall. Utilization of corn crop residues by livestock is limited by the low concentration of available nutrients (O'Donovan, 1983; Givens, 1987). The low rate and extent of cell wall degradation in the rumen can be attributed to suboptimal protein, and to interactions between cell wall carbohydrates and phenolic compounds (Jung, 1989, 1990).

The nutritive quality of corn stover can be improved by alkali treatment (Berger et al., 1979), which saponifies the alkali-labile ester linkages (Chesson, 1988). Ammonia is a weak alkali, but is capable of releasing ester-linked acetyl groups (Morrison and Brice, 1984), phenolic acids (Mason et al., 1988) and of partially solubilizing hemicellulose (Kiangi et al., 1981; Givens et al., 1988). Ammonia treatment of corn stover results in increased intake and digestibility (Morris and Mowat, 1980). The high buffering capacity of many crop residues, may, however, interfere with effective NH_3 treatment (Van Soest et al., 1984; Dias-da-Silva and Guedes, 1990). The enhancement in ruminal fiber degradation has been attributed, at least in part, to the additional NPN provided with NH_3 treatment to the rumen microorganisms (Djajanegara et al., 1989a,b).

In many NH_3-treatment experiments, an inverse relationship has been observed between initial quality and the response to NH_3 treatment (Kiangi et al., 1981; Givens et al., 1988). Interactive effects between maturity and the response to alkali treatment have been demonstrated (Berger et al., 1979). Differences among straw species and varieties in DM distribution among botanical fractions contribute to the difference in initial quality (Doyle and Champongsang, 1990; Wales et al., 1990) and may also affect the response to NH_3 treatment (Goto et al., 1991).

In a previous experiment (Journal Paper 4), fractions of drought-stressed corn stover were ammoniated, resulting in a variable response. As the response was highest for the leaf fraction, the variation in response could not be related to initial quality. Instead, substantial consumption of NH_3 may have been due to neutralization, especially in the lower stem portion, probably by the large amount of water soluble carbohydrates contained in the lower stems after the drought stress. A second harvest of non-drought-stressed corn stover was ammoniated the following

year. The objective of this experiment was to compare the *in vitro* response in cell wall degradation to NH_3 treatment of different corn stover fractions harvested in different years. Secondary objectives were to evaluate the relation between treatment response and initial quality, and to separate the overall response to NH_3 treatment into NPN supplementation and chemical treatment effects, interrelated with rumen environment, as simulated by the use of different buffer systems.

Experimental Procedures

Plant Material and Processing. Corn (*Zea mays*) stover was harvested manually at full maturity (grain harvest) on 18 October, 1991 and 30 October, 1992. Varieties used in yr 1 and 2 were, SS 728 and SS 844 (Southern States Cooperative, Richmond, Virginia), respectively. The corn in yr 1 was mildly drought-stressed, after dry summer and early fall, whereas precipitation in yr 2 was adequate (average monthly precipitation, June to October, 34 mm and 123 mm in yr 1 and 2, respectively). A 2 x 3 x 3 factorial arrangement was used, consisting of two yr, three plant fractions (leaf, upper stem, lower stem) and three processing methods (water control, NH_3 added, NH_3 treatment). Fraction separation and detailed processing methods were as described in Journal Paper 4. Treatment conditions were: 30 g NH_3/kg stover DM, applied as NH_4OH solutions of different strengths to balance for moisture; 45% DM in the product, except for lower stem in 1991 (30 % moisture), because it was already of a lower than desired initial DM content. Treatment duration was 30 d, at room temperature (approximately 23 °C).

To analyze samples of both years simultaneously, new subsamples were taken from the frozen batch samples of 1991 and freeze-dried simultaneously with samples of yr 2. Subsamples of approximately 20 g DM were freeze-dried under vacuum for 48 h.

Chemical Analyses. Dry matter was determined by oven-drying at 100 °C for 24 h. Freeze-dried samples were ground to pass a 1-mm sieve. Crude protein was determined by the Kjehldahl procedure (AOAC, 1990). Detergent fiber components were determined according to sequential procedures of Robertson and Van Soest (1981). Lignin was determined in ADF as $KMnO_4$ lignin and ADL.

In Vitro Experiment. Ruminal fluid was taken from a steer equipped with rumen fistula after an overnight fasting. The steer had been fed a diet of 50% corn stover and 50% corn silage (wt/wt, DM basis), supplemented with a concentrate/mineral mixture (.9 kg/d), consisting of 90% soybean meal, 6% common salt (NaCl) and 4% $Ca_3(PO_4)_2$. The ruminal fluid was strained through four layers of cheesecloth, and mixed with reduced buffer of pH 6.8 (20% ruminal fluid, vol/vol).

Two different buffers were used, adapted from the mixed phosphate/bicarbonate (NaH_2PO_4/$NaHCO_3$/NH_4HCO_3) buffer described by Goering and Van Soest (1970). In the first buffer, all NH_4HCO_3 was replaced with $NaHCO_3$, to create a N-limiting medium. In the second buffer, the NH_4HCO_3 contribution to the bicarbonate portion of the buffer was adjusted to provide 50 mg N/100 mL medium. Tryptone was not added as protein source, but micromineral additives and reducing agents as described by Goering and Van Soest (1970) were added to both buffers.

At inoculation, 35 mL of buffered ruminal fluid were added to 50 mL-tubes containing approximately .35 g of sample. The tubes were purged with CO_2 and

closed immediately with rubber stoppers equipped with Bunsen valves. All incubations were replicated six times. After 24 or 72 h, the tube contents were extracted with neutral detergent solution as described in Journal Paper 2. Cell wall disappearance (CWD) of NH_3 added and NH_3 treated fractions was calculated by substituting NDF content of the water controls for NDF content of the ammoniated fractions in the following formula:

$$\text{NDF disappearance} = 100 \times \frac{(\text{sample wt x DM x NDF}) - \text{residual wt (NDF)}}{\text{sample wt x DM x NDF}}$$

in which DM and NDF are expressed as fractions.

Statistical Analysis. Analyses of variance of the data were performed using the General Linear Models procedures of SAS (1989). Concentrations of CP, NDF, ADF, hemicellulose, cellulose, $KMnO_4$ lignin and ADL were analyzed as 2 x 3 x 3 factorials, with factors year, ammoniation, and plant fraction. Significant differences were determined using the orthogonal contrasts: leaf vs stems (upper and lower); and upper vs lower stems. Separation of NH_3 effects was accomplished using the non-orthogonal (Bonferroni) contrasts (Lowry, 1992): NH_3 treatment vs NH_3 added; and NH_3 added vs water control. Due to consistent three- and two-way interactions among main effects, effects of ammoniation and year were further analyzed by two-way ANOVA within each fraction, using the above non-orthogonal contrasts.

In vitro data were initially analyzed as a 2 x 3 x 3 x 2 factorial; factors were *in vitro* medium, fraction, ammoniation, and year. The full model was as follows:

$A_{ijklm} = \mu$ + medium$_i$ + fraction$_j$ + ammoniation$_k$ + year$_l$ + (medium x fraction)$_{ij}$ + (medium x ammoniation)$_{ik}$ + (medium x year)$_{il}$ + (fraction x ammoniation)$_{jk}$ + (fraction x year)$_{jl}$ + (ammoniation x year)$_{kl}$ + (fraction x ammoniation x year)$_{jkl}$ + (medium x fraction x ammoniation x year)$_{ijkl}$ + error$_{ijklm}$.

in which

A^{ijklm} = the measured extent of cell wall degradation;

μ = the overall mean;

medium$_i$ = the effect of the ith medium (i=1,2);

fraction$_j$ = the effect of the jth fraction (j=1,2,3);

ammoniation$_k$ = the effect of the kth processing (NH$_3$) method (k=1,2,3);

year$_l$ = the effect of the lth year (l=1,2).

Contrasts tested were as above. Because of consistent four- and three-way interactions, data were further analyzed by two-way ANOVA as 2 x 3 factorials within each medium x fraction combination. The reduced model used was:

$A_{ijklm} = \mu_{ij}$ + ammoniation$_{ijk}$ + year $_{ijl}$ + (ammoniation x year)$_{ijkl}$ + error$_{ijklm}$,

in which

A_{ijklm} = the measured extent of cell wall degradation for the ij[th] medium x fraction subclass;

μ_{ij} = the overall mean for the ij[th] medium x fraction subclass;

ammoniation$_{ijk}$ = the effect of the kth processing (NH$_3$) method within the ij[th] medium x fraction subclass;

year$_{ijl}$ = the effect of the lth year within the ij[th] medium x fraction subclass.

Results

Ammonia treatments resulted in products of satisfactory color (medium to dark brown). The fractions treated in 1991 were almost odorless (leaf) to sweet (stems), whereas all treated fractions in 1992 had a slight NH_3 smell. The treated fractions were softer and more pliable than the controls. Minor spoilage due to molding occurred during treatment of leaf in 1991, but no mold was visible in any of the ammoniated batches in 1992.

Composition. Effect of ammoniation on cell wall sugar monomer composition, ester- and ether-linked phenolic acids, and digestion kinetics of the fractions collected in 1991 has been reported as part of the initial study (Journal Paper 4). Differences in initial composition between years have been discussed in Journal Paper 2. Irrespective of ammoniation, leaves were higher ($P < .001$) in CP and lower ($P < .05$) in NDF in yr 2 than in yr 1 (Table 19). Stems were higher ($P < .001$) in NDF and ADF in yr 2. Ammoniation increased ($P < .05$) CP in all fractions, but CP of NH_3 treated fractions was higher ($P < .05$) than of NH_3 added as observed earlier (Journal Paper 4). Ammonia treatment, compared to NH_3 added, decreased ($P < .05$) NDF content of leaf in both years, but of stems only in yr 2 (yr x ammoniation interaction, $P < .05$). Overall, ADF content increased ($P < .05$) with ammoniation, but the response differed ($P < .05$) between NH_3 treatment and NH_3 added. The response also differed among fractions (processing x fraction interaction, $P < .05$) and between years (ammoniation x yr interaction, $P < .05$).

Initial cell wall composition (Table 20) was similar between years. In both years, leaf cell walls contained more ($P < .05$) hemicellulose than stems, and upper stem more ($P < .05$) than lower stem. In all fractions, NH_3 treatment decreased

Table 19. Crude protein, NDF, and ADF content of corn fractions harvested in different years as affected by ammoniation

Year and treatment	CP[abcdefg]			NDF[abcdefhi]			ADF[abcdeghi]		
	Leaf[bcdi]	Upper[bdi] stem	Lower[bcd] stem	Leaf[bd]	Upper[bdi] stem	Lower[bdi] stem	Leaf[bci]	Upper[bdi] stem	Lower[bi] stem
				%, DM basis					
1991									
Water control	6.59	4.16	4.74	72.2	70.6	63.1	33.7	36.8	39.8
NH₃ added	9.18	9.94	9.34	73.1	69.3	62.8	38.7	36.2	37.9
NH₃ treated	10.81	13.91	13.40	66.4	69.4	63.6	40.2	40.9	41.3
1992									
Water control	12.50	4.74	3.95	69.5	76.3	79.1	33.3	40.6	47.2
NH₃ control	17.34	8.31	8.19	69.5	79.0	80.6	33.5	43.2	50.6
NH₃ treated	21.34	11.12	12.56	61.2	73.0	74.2	33.5	42.1	49.3
SE	.355	.347	.234	.52	.97	.95	1.02	.76	.90
		.318			.84			.90	

[a]Leaf different from stems (P<.05).
[b]Year effect (P<.05).
[c]NH₃ control different from water control (P<.05).
[d]NH₃ treatment different from NH₃ control (P<.05).
[e]Year x fraction interaction (P<.05).
[f]fraction x ammoniation interaction (P<.05).
[g]Year x fraction x ammoniation interaction (P<.05).
[h]Upper stem different from lower stem (P<.05).
[i]Year x ammoniation interaction (P<.05).

Table 20. Cell wall composition of corn stover fractions harvested in different years as affected by ammoniation

Year and treatment	Hemicellulose[abcdefg]			Cellulose[abcdefghi]			KMnO$_4$ lignin[abcdfghi]			ADL[abeghi]		
	Leaf[cdhi]	Upper stem[di]	Lower stem[di]	Leaf[cdhi]	Upper stem[cdhi]	Lower stem[d]	Leaf[cdhi]	Upper stem[cdi]	Lower stem[cdhi]	Leaf[h]	Upper stem[j]	Lower stem[j]
								— % of NDF —				
1991												
Water control	53.4	48.0	36.9	42.2	44.3	52.7	6.03	6.43	9.02	4.35	7.73	10.35
NH$_3$ added	47.1	48.8	39.6	48.5	47.5	53.8	3.94	4.11	6.22	4.86	5.26	7.74
NH$_3$ treated	39.4	41.1	35.1	54.4	51.6	56.3	6.33	6.86	8.25	6.10	7.59	9.02
1992												
Water control	52.2	46.7	40.3	41.0	47.7	52.5	6.11	6.03	7.76	7.67	5.07	6.59
NH$_3$ added	51.8	45.3	37.3	41.2	48.4	53.1	5.64	5.64	9.04	8.94	6.83	10.23
NH$_3$ treated	45.3	42.3	33.6	47.0	51.4	57.9	6.97	6.31	8.57	8.52	6.17	8.52
SE	1.24	.66	.67	1.14	.57	.50	.215	.263	.276	.396	.558	.723
		.98			.89			.253			.573	

[a] Leaf different from stems (P<.05).
[b] Upper stem different from lower stem (P<.05).
[c] NH$_3$ control different from water control (P<.05).
[d] NH$_3$ treatment different from NH$_3$ control (P<.05).
[e] Year x fraction interaction (P<.05).
[f] fraction x ammoniation interaction (P<.05).
[g] Year x fraction x ammoniation interaction (P<.05).
[h] Year effect (P<.05).
[i] Year x ammoniation interaction (P<.05).

(P<.05) hemicellulose, compared with NH_3 added, irrespective of year. However, for lower stem in yr 1, the difference between NH_3 treatment and NH_3 added seemed due mainly to a slightly higher hemicellulose for NH_3 added. Cellulose content of cell walls of all fractions increased (P<.05) with NH_3 treatment, compared with both water control and NH_3 added. Lignin, as measured by $KMnO_4$ oxidation, was similar between years. Permanganate lignin was higher (P<.05) in lower stem than in upper stem. The values were lower (P<.05) for NH_3 added versus NH_3 treated and water control, which may have resulted from interference of unreacted NH_3 with $KMnO_4$ oxidation. Stems were higher in ADL than leaf in yr 1, but not in yr 2 (year x fraction interaction, P<.05). Acid detergent lignin of leaf was higher (P<.05) in yr 2 than yr 1. Although initial ADL of stems appeared lower in yr 2, overall stem ADL was not different (P>.05) between years (year x ammoniation interaction, P<.05).

In Vitro Experiment. Cell wall disappearance after 24 h was about 50 % lower (P<.001) when samples were incubated in the N-limiting medium, compared to the N-sufficient medium (Table 21). The difference was largely compensated for after 72 h incubation, although after 72 h disappearance of NDF, cell wall (Table 22) and DM (Table 23) was still higher (P<.001) for the N-sufficient medium. At both incubation times, effects of ammoniation on cell wall degradation were smaller for the N-sufficient medium than for the N-limiting medium (medium x ammoniation interaction, P<.001). Within each medium, however, the effects of ammoniation followed similar trends. Interactions between type of medium and fraction, and between type of medium and year were stronger after 24 h incubation (both P<.001) than after 72 h incubation (P=.14, and P<.01, respectively), and were mainly related to the different CP contents of leaf in yr 1 and 2.

Table 21. Cell wall and NDF disappearance (%) after 24 h *in vitro* incubation of corn stover fractions harvested in different years as affected by ammonia treatment and incubation medium[a,b,c,d,e,f,g,h]

Component by medium and fraction

Year and treatment	N-sufficient medium Leaf CW[efgh]	NDF[efg]	Upper stem CW[ef]	NDF[efgh]	Lower stem CW[efh]	NDF[efg]	N-limiting medium Leaf CW[efgh]	NDF[efh]	Upper stem CW[efh]	NDF[efh]	Lower stem CW[efh]	NDF[efh]
	%											
1991												
Water control	48.2		38.4		30.6		21.9		14.8		13.5	
NH$_3$ added	52.8	53.3	48.9	48.1	40.4	40.1	37.2	38.0	45.7	44.7	35.8	35.6
NH$_3$ treated	54.3	50.3	48.9	48.0	33.6	34.2	40.6	35.3	45.0	44.1	34.2	34.8
1992												
Water control	32.8		34.8		25.0		28.7		19.7		15.1	
NH$_3$ added	38.8	38.7	40.7	42.7	28.6	30.1	34.7	34.6	31.8	34.2	23.7	25.3
NH$_3$ treated	46.6	39.4	41.7	39.2	31.6	27.3	46.3	39.0	35.0	32.1	30.3	25.9
SE	.99	1.04	1.18	.85	1.38	1.02	1.24	1.23	1.54	1.30	1.34	.93

[a] N-sufficient medium different from N-limiting (P<.001).
[b] Medium x ammoniation (P<.001), medium x fraction (P<.001), medium x year (P<.001), fraction x ammoniation (P<.001), fraction x ammoniation x year (P<.001), and four-way (P<.001) interactions.
[c] Leaf different from stems (P<.01).
[d] Upper stem different from lower stem (P<.01).
[e] Year effect (P<.05).
[f] NH$_3$ control different from water control (P<.01).
[g] NH$_3$ treatment different from NH$_3$ control (P<.01).
[h] Year x ammoniation interaction (P<.01).

Table 22. Cell wall (CW) and NDF disappearance after 72 h *in vitro* incubation of corn stover fractions harvested in different years as affected by ammonia treatment and medium type

Component by medium and fraction[abcdefg]

%

Year and Ammoniation	N-sufficient						N-limiting					
	Leaf		Upper stem		Lower stem		Leaf		Upper stem		Lower stem	
	CW[efgh]	NDF[efg]	CW[ef]	NDF[efg]	CW[efg]	NDF[efg]	CW[efgh]	NDF[efg]	CW[efg]	NDF[efg]	CW[ef]	NDF[efg]
1991												
Water control	75.0		65.3		59.8		70.2		56.6		52.9	
NH$_3$ added	77.2	77.4	72.1	71.6	61.6	61.4	74.6	74.9	70.9	70.4	58.7	58.5
NH$_3$ treated	79.0	77.2	73.4	73.0	62.5	62.8	77.4	75.4	72.3	71.9	60.9	61.2
1992												
Water control	56.4		60.8		51.0		53.9		56.8		47.6	
NH$_3$ added	61.8	61.8	69.0	70.1	58.3	59.1	61.8	61.8	66.7	67.9	57.5	58.4
NH$_3$ treated	66.0	61.4	67.7	66.3	60.3	57.9	65.2	60.6	65.0	63.5	58.6	56.0
SE	.45	.45	.68	.58	.98	.75	.62	.60	.93	.80	.98	.78

[a]N-sufficient medium different from N-limiting (P<.001).
[b]Medium x ammoniation (P<.001), medium x year (P<.01), year x fraction (P<.001), fraction x ammoniation (P<.001), and year x fraction x ammoniation (P<.001) interactions.
[c]Leaf different from stems (P<.001).
[d]Upper stem different from lower stem (P<.001).
[e]Year effect (P<.01).
[f]NH$_3$ control different from water control (P<.01).
[g]Year x ammoniation interaction (P<.05).
[h]NH$_3$ treatment different from NH$_3$ control (P<.01).

Table 23. Dry matter disappearance after 72 h *in vitro* incubation of corn stover fractions harvested in different years as affected by ammonia treatment and type of medium

Year and treatment	Fraction by medium[a,b,c,d,e,f,g,h]					
	N-sufficient			N-limiting		
	Leaf[e,f,g,h]	Upper[e,f] stem	Lower[e,f,h] stem	Leaf[e,f,g,h]	Upper[e,f,h] stem	Lower[e,f] stem
	--------------------------------- % ---------------------------------					
1991						
Water control	82.0	75.3	74.6	78.5	69.3	70.3
NH$_3$ added	83.5	80.3	75.8	81.6	79.5	73.9
NH$_3$ treated	84.8	81.2	76.3	83.7	80.5	75.3
1992						
Water control	69.7	70.1	61.2	67.9	67.1	58.6
NH$_3$ added	73.5	76.4	67.1	73.4	74.6	66.5
NH$_3$ treated	76.4	75.4	68.7	75.8	73.3	67.4
SE	.32	.51	.81	.46	.77	.84

[a]Medium effect $(P < .001)$.
[b]Medium x ammoniation, year x fraction, fraction x ammoniation, and year x fraction x ammoniation interactions $(P < .001)$.
[c]Leaf different from stems $(P < .001)$.
[d]Upper stem different from lower stem $(P < .001)$.
[e]Year effect $(P < .001)$.
[f]NH$_3$ control different from water control $(P < .01)$.
[g]NH$_3$ treatment different from NH$_3$ control $(P < .01)$.
[h]Year x ammoniation interaction $(P < .05)$.

After 24 h, disappearance of NDF and original cell wall was increased (P<.01) by ammoniation in all fractions, for both years, irrespective of N-sufficiency in the incubation medium. In leaf, disappearance of original cell wall but not residual NDF after ammoniation was further increased (P<.01) by NH_3 treatment above NH_3 added in both years and both media. For stems, disappearance of original cell wall but not NDF was increased (P<.05) by NH_3 treatment versus NH_3 added, only for 1992 samples (year x ammoniation interaction, P<.01), and the effect was more pronounced in the N-limiting medium. Overall, CWD from 1992 samples was lower (P<.05) than from 1991 samples. The effect was more pronounced when incubated in N-sufficient medium (medium x yr interaction, P<.001).

Results after 72 h incubation were similar to 24 h incubation (Tables 22 and 23). After 72 h incubation, the N-deficiency imposed on the microorganisms by incubation in N-limiting medium was largely overcome by ammoniation, as evidenced by the relatively small differences between media in cell wall degradation of ammoniated versus control fractions (medium x ammoniation interaction, P<.001). The overall advantage of ammoniation over water control was slightly smaller after 72 h, compared to 24 h. Ammonia treatment, compared with NH_3 added, consistently increased CWD of leaf but not stems (fraction x ammoniation interaction, P<.001). The same trends were observed for DM disappearance after 72 h, but the differences were even smaller than for cell wall and NDF disappearance.

Within water control and NH_3-treated samples, solubilization of phenolics as measured by the optical density at 280 nm, was moderately correlated with cell wall degradation after 24 and 72 h (Figure 15). However, the response to NH_3 treatment in solubilization of phenolics was poorly correlated with the corresponding response
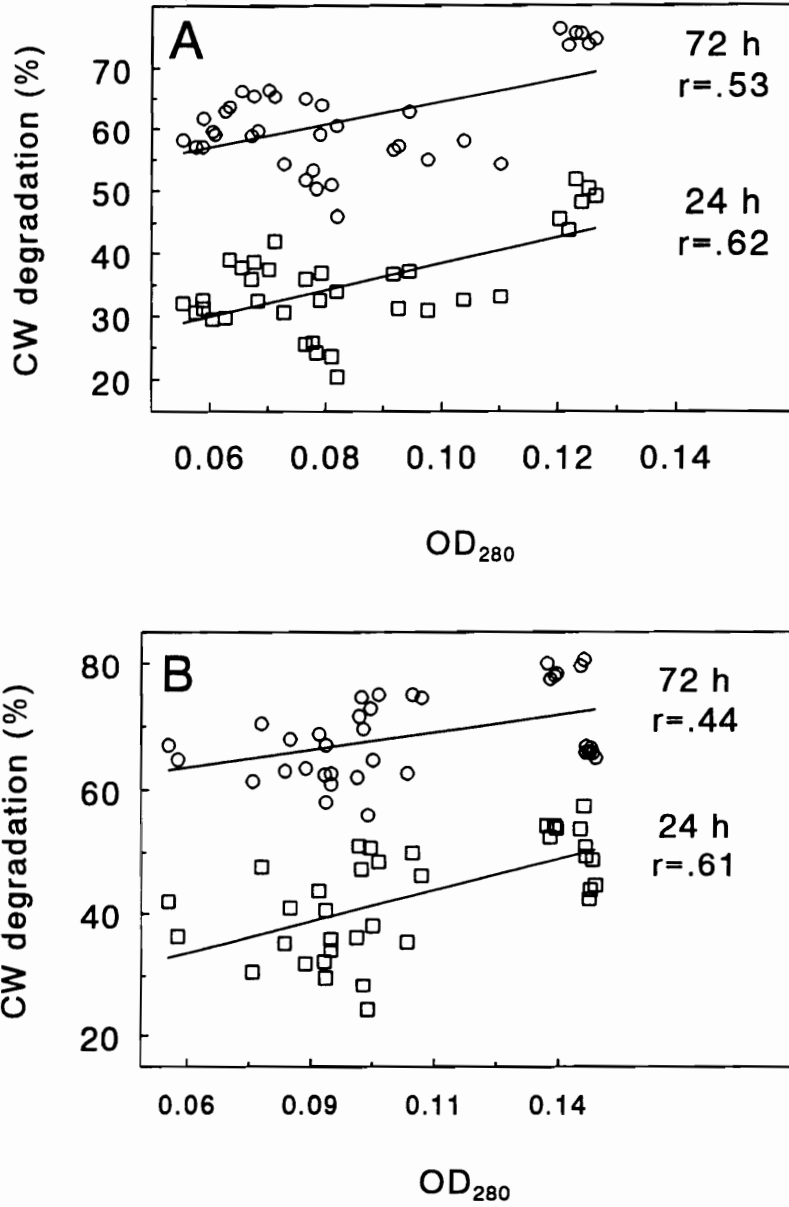
Figure 15. Relation of soluble phenolics (OD_{280}) with cell wall degradation in N-sufficient medium of (A) water control and (B) NH_3 treated corn stover fractions.

in cell wall degradation (Figure 16). Overall, cell wall degradation at 24 h was negatively correlated with concentrations of $KMnO_4$ lignin, ADL, and ADF (Table 24), and positively correlated with CP and the optical density at 280 nm (OD_{280}). The correlations with lignin and OD_{280} were higher for the 1991 samples than for the 1992 samples, perhaps due to more uniformity in initial composition and in response to ammoniation among fractions in 1992. However, NDF and ADF were less correlated with cell wall degradation for the 1991 samples, due to drought stress effects. In general, correlations were slightly lower for cell wall degradation after 72 h (data not shown).

Within the leaf fraction, ADL was negatively correlated with rate (-.56) and extent ($r = -.69$) of cell wall degradation, and the ratio of cellulose to hemicellulose ($r = .61$ for both) and OD_{280} ($r = .63$, and $r = .52$, respectively) were positively correlated (Table 24). In upper and lower stem, NDF content was the component most negatively correlated with rate of cell wall degradation ($r = -.54$, and $r = -.65$, respectively). Crude protein in upper stem ($r = .72$) and lower stem ($r = .45$) and OD_{280} of upper stem ($r = .60$) were positively correlated with rate of cell wall degradation.

Overall, the correlation of treatment response in extent of cell wall degradation and initial cell wall degradation (water control) was -.54 (Table 25). The inverse relation of treatment response (solubilization of phenolics and NDF, and cell wall degradation) with initial quality (initial rate and extent of degradation) was most obvious within the leaf fraction.
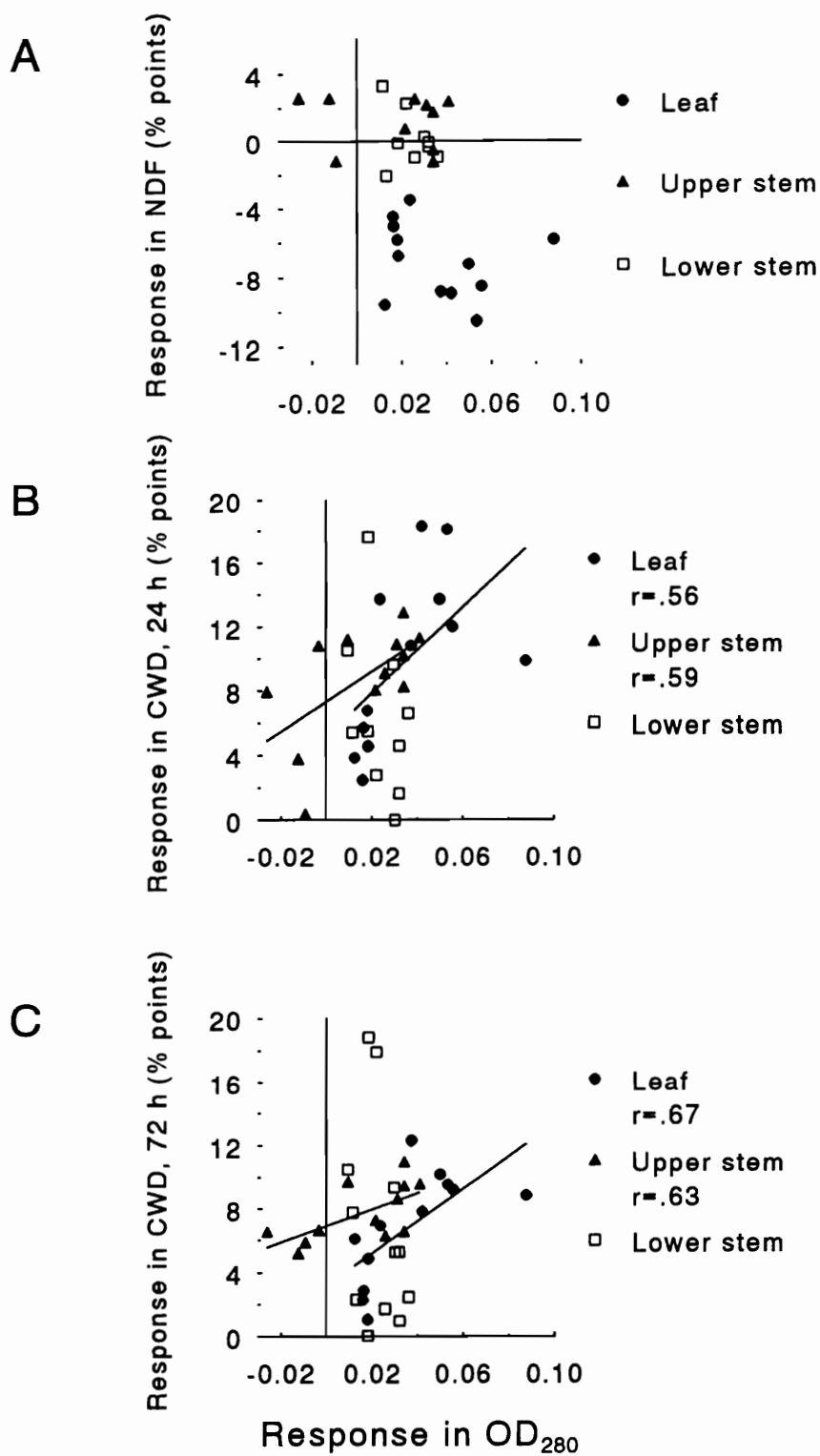
Figure 16. Correlations between response to NH_3 treatment in solubilization of phenolics and (A) NDF solubilization, (B) cell wall degradation after 24 h, and (C) cell wall degradation after 72 h in N-sufficient medium.

Table 24. Correlation coefficients of composition and optical density at 280 nm with rate (24 h) and extent (72 h) of cell wall degradation using N-sufficient medium

	Correlation coefficients					
		By year		By fraction		
Item	Overall	1991	1992	Leaf	Upper stem	Lower stem
24 h degradation						
CP[a]	.28	NS[b]	.61	NS	.72	.45
NDF[a]	-.32	.51	-.62	NS	-.54	-.65
ADF[a]	-.46	NS	-.58	.53	NS	-.57
Hemicellulose[c]	.32	.40	.37	-.53	NS	NS
Cellulose[c]	NS	NS	-.34	.66	NS	NS
Cellulose/hemicell. ratio	-.30	-.38	-.40	.61	NS	NS
KMnO$_4$ lignin[c]	-.62	-.70	-.51	NS	-.35	-.48
ADL[c]	-.42	-.61	NS	-.56	NS	NS
Soluble phenolics (OD_{280})[d]	.63	.87	.48	.63	.60	NS
72 h degradation						
CP[a]	NS	.29	.39	-.55	.79	.65
NDF[a]	NS	.59	-.28	NS	-.40	-.62
ADF[a]	-.30	NS	NS	.61	NS	-.42
Hemicellulose[c]	.22	.43	NS	-.48	NS	-.35
Cellulose[c]	NS	NS	NS	.66	.35	NS
Cellulose/hemicell. ratio	-.19	-.40	NS	.61	.37	.36
KMnO$_4$ lignin[c]	-.54	-.61	-.41	NS	-.35	-.48
ADL[c]	-.43	-.62	NS	-.69	NS	NS
Soluble phenolics (OD_{280})	.55	.89	.31	.52	.70	NS

[a] %, DM basis
[b] $P > .05$
[c] % of NDF
[d] Optical density at 280 nm.

Table 25. Correlation Coefficients of initial quality (*in vitro* cell wall degradation after 24 or 72 h) with the response to NH_3 treatment in *in vitro* cell wall degradation after 24 and 72 h, NDF solubilization, and water soluble phenolics

NH₃ treatment response	Correlation coefficients					
	Overall	By year		By fraction		
		1991	1992	leaf	upper	lower
Initial CW degr., 24 h						
Cell wall degradation, 24 h	NS[a]	NS	NS	-.84	NS	-.77
Cell wall degradation, 72 h	-.45	NS	-.60	-.83	NS	-.82
Soluble phenolics $(OD_{280})^b$	NS	-.69	NS	-.83	NS	.68
NDF solubilization	NS	-.87	NS	NS	NS	.91
Initial CW degr., 72 h						
Cell wall degradation, 24 h	NS	NS	NS	-.72	.57	NS
Cell wall degradation, 72 h	-.54	NS	-.63	-.90	NS	-.85
Soluble phenolics (OD_{280})	NS	-.55	NS	-.82	.64	NS
NDF solubilization	NS	-.86	NS	.58	NS	.82

[a] $P > .05$
[b] Optical density at 280 nm

Discussion

Due to the large number of samples resulting from the initial 2 x 3 x 3 x 2 factorial design of the *in vitro* experiment, a limited number of meaningful incubation times were selected. An incubation period of 48 h, which is the standard incubation time in the Tilley and Terry (1963) IVDMD procedure, is too long to estimate differences in rate of degradation, and may not always be suitable to detect differences in potential degradability (Grant and Mertens, 1992). In a previous experiment (Journal Paper 4), in which NDF degradation was determined after 12, 24, 48, 72, 96 h, differences in cell wall degradation after 24 h incubation of corn stover fractions were representative of differences in rate of cell wall degradation calculated according to a first order model (Mertens and Loften, 1980). Although the potential degradability in that study was not reached until 96 h, the differences in CWD at 72 h were representative of differences in potential degradability. An incubation period of 72 h also reflects more closely the retention time of stover fractions in the rumen. Therefore, 24 and 72 h were chosen as incubation times in this experiment, and the differences observed at these times are discussed as differences in rate and extent of degradation.

Rate and extent of cell wall degradation were enhanced by ammoniation, both by NH_3 addition and by NH_3 treatment, in agreement with generally observed *in vivo* responses to NH_3 treatment and NPN supplementation in intake and digestibility (Djajanegara and Doyle, 1989a,b). The small improvement in cell wall degradation of leaf after 24 h by NH_3 treatment over NH_3 control in yr 1 was maintained until 72 h incubation. The larger additional improvement in yr 2 in initial degradation rate resulted in only minor improvement of extent of degradation. The enhancement in

CWD of leaf by NH_3 treatment versus NH_3 added appears due mainly to partial hemicellulose solubilization prior to incubation, rather than to a change in degradability of the residual NDF. A slightly enhanced rate but not extent of cell wall degradation of 1992 stems could also be attributed to a chemical treatment effect, notably the solubilization of NDF. Degradation of residual NDF after NH_3 treatment of stems was actually slightly lower than after NH_3 addition, associated with the solubilization of more easily degradable hemicellulose. The lack of enhancement of NDF degradation by NH_3 treatment over NH_3 added for all fractions in both years implies that the chemical treatment effect is limited to an initial solubilization of NDF, rather than an improvement in degradability of the residual fiber, in agreement with findings of Graham and Aman (1984). These authors reported an increase in barley straw digestibility of 23 percentage points, mainly due to solubilization of cell wall constituents rather than microbial degradation of residual fiber.

The increase in rate and extent of NDF degradation by both NH_3 addition and NH_3 treatment can be interpreted in different ways. Initially, it appears that a major contribution of NH_3 treatment to the enhancement of cell wall degradation is the supply of NPN to the rumen microorganisms, as suggested by Djajanegara and Doyle (1989a,b). This is in accordance with the lower response to ammoniation in the N-sufficient incubation medium. However, the fact that there is an apparent 'NPN-effect' even in N-sufficient incubation medium, implies there is at least one additional factor, involving either a nutritional or purely chemical mechanism. Apparently, a more efficient use is made of the added NH_3 than of NH_3 in solution. A likely reason is absorbance of NH_3 into the fiber matrix and binding to matrix components, resulting in available NPN in the fiber where adhering cellulolytic bacteria can utilize it. An alternative mechanism would be the availability of

unreacted NH_3, especially in NH_3 control samples, for saponification and(or) nucleophilic addition reactions during the *in vitro* incubation, similar to those postulated for sulphur (Journal Paper 2). Evidence for the availability of NH_3 for saponification in NH_3 added samples was provided by the extent of release of soluble phenolics similar to NH_3 treated samples.

Overall, the magnitude of the response to NH_3 treatment was only moderately related to initial quality, as has been observed for cereal straws (Dias-da-Silva and Guedes, 1990). However, within the leaf fraction, in which the chemical treatment response was highest, the overall response seemed inversely related to initial quality. For stems, the lack of inverse relationship between initial quality and response to ammoniation is probably related to interference of WSC with ammoniation in yr 1.

Implications

The findings of this *in vitro* experiment can be extrapolated, with some caution, to the practical feeding situation. The major NH_3 treatment effect, besides adding NPN, was a modest initial fiber solubilization, resulting in a faster rate of degradation of corn stover, which should result in higher *ad libitum* feed intake. However, spraying with NH_3 prior to feeding, or perhaps feeding urea, would be nearly as effective as NH_3 treatment in increasing digestibility of corn stover. Furthermore, the effectiveness of NH_3 treatment, compared to NH_3 spraying depends heavily on the concentration of buffering constituents of cell walls and cell solubles, and in the case of drought-stressed corn the high concentration of WSC in the stems is likely to interfere with effective NH_3 treatment.

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General Discussion

It is well established that nutritive quality of forages decreases with increasing maturity, associated with the transition from the rapidly growing, vegetative stage to stem elongation and the formation of a seed head, resulting in a decrease in leaf to stem ratio, and in changes in composition of the respective fractions. Leaves are of higher quality than stems, due to higher protein, lower cell wall content, and different cell wall structure and composition, associated with the different functions of leaf and stem. This is generally true, with certain exceptions such as rice (*Oryza sativa*) straw, in which a high silica concentration reduces digestibility of the leaf fraction (Doyle et al., 1986), and timothy (*Phleum pratense*), in which the basal stem serves as a storage organ of non-structural carbohydrates. The changes in composition of corn stover fractions with increasing maturity described in the first paper indicate that the quality difference between corn stover leaves and stems decreases with increasing maturity, and more so before than after physiological grain maturity (early dent).

The response to maturity in the 1991 corn crop was affected by drought stress in August and September, resulting in high concentration of WSC in the stalks, especially in the lower stem. The high concentration of WSC resulted in a relatively

high IVDMD, similar to that of leaf, although the cell wall digestibility remained very low, especially that of lower stem. This was confirmed by comparison of the 1991 stover with a crop harvested at similar maturity stages in 1992 (Journal Paper 2). The high concentration of WSC in the 1991 lower stems resulted in suboptimal conditions for NH_3 treatment, as evidenced by the inability to solubilize hemicellulose or phenolic acids from lower stem cell walls, and the lack of difference in cell wall degradation between NH_3 addition and NH_3 treatment when applied to the drought stressed stover. In practical terms, it seems that in case of drought stressed corn stover, pretreatment of the lower stalks should not be attempted, but instead the lower stalks could be ensiled effectively. Drought-stressed corn stover has been successfully ensiled (Irlbeck et al., 1993), although some fermentative loss of WSC occurs.

Apart from the overall difference in quality between leaf and stems, the difference in cell wall digestibility between upper and lower portion of the stem was remarkable, and was mainly associated with differences in cell wall carbohydrate composition (hemicellulose) and lignin composition (methoxyl groups), in which upper stem appeared intermediate to lower stem and leaf. This may be attributed, in part, to the higher contribution of leaf sheath to the total mass of upper stem, even though leaf sheaths are less easily degradable than leaf blades, associated with their function as support tissue. It is likely that the actual variation in composition and degradability within the stem fraction is even larger than measured in this research, since the cell wall composition probably gradually changes from bottom to top, and in the present research the whole stem was separated in only two portions.

One of the components that was consistently negatively correlated with cell wall digestibility, whether compared within botanical fractions, or within maturity

stages, was the lignin methoxyl content. Although the lignin content was not always highly correlated with digestibility, combination of lignin methoxyl content, and Klason lignin in NDF, giving the lignin methoxyl content as percentage of NDF, resulted in a powerful predictor of IVDMD (Journal Paper 1) and cell wall degradability (Journal Paper 2). Research in the 1980's (Reeves, 1985, 1987; Buxton and Russell, 1988; Buxton, 1989) showed a negative correlation between concentration of syringyl lignin (dimethoxylated) in grasses and legumes with digestibility. On the other hand, lignin in recalcitrant residues such as peanut hulls consists mainly of guaiacyl (monomethoxylated) lignin. It can be assumed that both types of lignin may play a role in limiting cell wall degradation by rumen microorganisms.

This assumption is supported by close examination of the results on microbial degradation of mature cell walls obtained by electron microscopy (Spencer and Akin, 1980; Akin, 1989; Engels, 1989; Engels and Schuurmans, 1992; Mulder et al., 1992). The research of Engels and Schuurmans (1992) and Mulder et al. (1992) has shown that the most recalcitrant cell wall layer of intact cells was the middle lamella/primary wall. The outer cell wall layer is known to contain a high concentration of guaiacyl-rich lignin (Terashima et al., 1988; He and Terashima, 1990; Wallace et al., 1991), and is relatively resistant to delignification with $KMnO_4$. Guaiacyl lignin is more condensed, and interacts less with other cell wall components than syringyl lignin (Gordon, 1975). However, the high lignin concentration renders the primary wall into a physical barrier to microbial enzymes, provided the cell wall is not mechanically ruptured (Engels and Schuurmans, 1992).

The research of Akin's group (Spencer and Akin, 1980; Akin, 1989) has shown consistent lower degradability of sclerenchyma and xylem tissues stained by acid

phlorogucinol, indicating guaiacyl lignin, than of parenchyma tissue stained by chlorine sulfite, indicating predominance of syringyl lignin. The predominance of syringyl lignin in the partially degradable parenchyma indicates that syringyl lignin interferes with cell wall degradation by a mechanism that results in slower degradation but not necessarily lower potential degradability. Furthermore, treatment with weak alkali improved degradability of stem parenchyma substantially, contrary to that of the most recalcitrant tissues (Hartley and Morrison, 1991; Akin et al., 1992), indicating that the impact of syringyl lignin is mainly through its interactions with cell wall carbohydrates, rather than due to a high concentration.

The reason syringyl lignin interferes more with carbohydrate degradation through interlinkages than guaiacyl lignin must be sought in the difference in methoxyl group content, as discussed in the review of literature in this volume. In syringyl lignin, fewer sites are available for radical coupling reactions than in guaiacyl lignin, which results in a higher contribution of ß-linkages, and therefore, a higher likelihood formation and(or) regeneration of highly reactive quinone methide (QM) intermediates. It is therefore postulated that the negative association of syringyl lignin with digestibility is related to the formation of quinone methides, both in the aging cell, and possibly in the rumen. In the aging cell wall, the progressive negative impact of syringyl lignin may continue even after lignification is completed. Quicke and Bentley (1959) showed that digestibility of several grasses decreases linearly with advancing maturity, even after lignification slowed down. Therefore, the negative association of lignin content with digestibility was poor. These authors also characterized the lignin by its methoxyl content, but in interpreting the relation of lignin with digestibility focused on lignin content. Reinterpretation of their data shows a narrow association between digestibility and methoxyl content.

Determination of methoxyl content in Klason lignin was the method of choice in this research. Of the methods to determine lignin content ($KMnO_4$ lignin, ADL, Klason lignin, Acetyl bromide lignin), Klason lignin was found to be less subject to non-specific variability, and is a better estimate of both condensed and uncondensed lignin. Determination of methoxyl groups (by iodometry or ^{13}C NMR spectroscopy) is preferable over attempts to determine syringyl/guaiacyl ratio of lignin (e.g. nitrobenzene oxidation and thioacidolysis), because the latter suffer from low yields and side reactions (see Review of Literature) and are not able to characterize both uncondensed and condensed regions of lignin. Nuclear magnetic resonance spectroscopy is more suitable for exact determination of specific bonds, particularly in combination with ^{14}C labeling, rather than for syringyl/guaiacyl ratios.

Although reducing conditions prevail in the rumen, evidence of oxidative degradation exists in the form of stable radicals in lignin-carbohydrate complexes isolated from rumen fluid. Under the mild conditions (pH 6 to 7) in the rumen of animals fed high fiber diets, QM intermediates would be stable enough to allow opportunistic addition of nucleophilic compounds. Ester- and ether-linkages formed by addition of carboxyl groups and hydroxyl groups are reversible under pulping conditions (Glasser, 1981). They may thus not be totally undegradable in the rumen either, but formation of these linkages would at least decrease the apparent rate of cell wall degradation.

The deductions based on the microscopic observations described above and the biochemical processes occurring in lignin biosynthesis and degradation were confirmed in the present research. As stated earlier, lignin methoxyl content was highly negatively correlated with cell wall degradation, and the hypothesis of formation of QM intermediates during rumen fermentation was confirmed by the

observed S incorporation into NDF via nucleophilic addition. The amount of S incorporated was only moderately correlated with digestibility, possibly due to the beneficial effect of S in preventing cross-linkages between QM intermediates and carbohydrates.

The beneficial effect of adding cysteine-HCl and Na$_2$S to *in vitro* incubations has been ascribed to their potency as reducing agents, which would aid in establishing sufficiently reducing conditions for the rumen microorganisms. Their reducing power made them likely candidates to test the QM hypothesis. The addition of sulphide or sulfhydryl groups to QM intermediates is irreversible (Glasser, 1981). Sulphur addition to quinone methides may also provide a partial explanation of beneficial *in vivo* effects of S supplementation on digestibility, especially when provided as cysteine. Based on the S/N ratio in microbial protein, a 15:1 ratio of rumen degradable S to degradable N is recommended by ARC (1980). However, for optimal animal performance, a N:S ratio of 10 is regarded more appropriate (Moir et al., 1968), which may be related to the potential of S as an anti-cross-linking agent. Both S supplementation and fertilization result in improved digestibility of corn silage (Buttrey et al., 1986), with an additional response in N retention for S-fertilized over S-supplemented corn silage. The additional response may be explained by incorporation of S into the corn fiber during lignification, when quinone methide intermediates are formed, which would limit the extent of cross-linkage of lignin with hemicellulose and proteins, thereby limiting the formation of ADIN and enhancing CP availability and N-retention.

In addition to possible cross-linking by quinone methide intermediates, this research has demonstrated various other potential effects of lignin on microbial degradation of structural carbohydrates. The model study (Journal Paper 3) revealed

that lignin can actually enhance cellulose degradation, when amorphous cellulose and lignin are blended together. The positive influence of lignin, when blended with cellulose, is unlikely to occur in maturing forage cell walls for three reasons. First, lignification starts only after deposition of the carbohydrates (Northcote, 1972). Before the onset of lignification, cellulose has already been organized into microfibrils. Secondly, hemicellulose surrounds the microfibrils, forming the cell wall matrix in which lignin is deposited (Terashima et al., 1988). Therefore, lignin does not interact directly with cellulose in nature, and even if it did, only after establishment of cellulose crystallinity. Thirdly, lignin is not inserted into the cell wall as a pre-existing polymer, but is polymerized within the cell wall (Northcote, 1972). During the peroxidase/H_2O_2-induced, radical-propagated polymerization of lignin, cross-linkages are formed through quinone methide intermediates to a larger extent than when two pre-existing polymers are blended. Contrary to maturing plant cell walls, a direct association of lignin and cellulose does occur during pulping of wood (Glasser and Barnett, 1979).

Available phenolic hydroxyl groups reduced the enhancing effect of lignin blending on cellulose degradation, through the formation of QM intermediates. A similar difference between hydroxypropylated and unmodified lignin was observed when 'free' lignin was added to the incubation medium. Addition of both reduced the degradation of cellulose, but the effect of hydroxypropylated lignin was probably limited to hydrophobic binding of microbial enzymes. The mechanisms by which lignin inhibits cellulose degradation are more likely to occur during ruminal fermentation of forage cell walls. Formation of quinone methide intermediates and establishment of cross-linkages, whether occurring during lignification (Freudenberg, 1965), aging of lignin in maturing cell walls (Leary, 1980) or during ruminal

fermentation (Journal Paper 2), will have a negative impact on forage cell wall degradation. The extent of cross-linking is highest for lignin of high methoxyl content, hence the negative correlation of syringyl content with forage digestibility.

The extent to which each mechanism contributes to the negative impact of lignin is interdependent with structural differences among plant tissues (Akin and Burdick, 1981), and changes with maturity in overall and cell wall composition (Journal Paper 1), lignin structure (Buxton and Russell, 1988), and lignin distribution within cell walls (Engels and Schuurmans, 1992).

Interlinkages in forage cell walls are initially established during lignin polymerization. The extent of cross-linking depends on lignin composition (Gordon, 1975), the rate of lignin deposition and the composition of the polysaccharide matrix gel in which lignin is deposited (Terashima et al., 1988), and is different for primary and secondary walls. In the primary wall/middle lamella, lignin of a low methoxyl content (guaiacyl and *p*-hydroxyphenyl lignin) is deposited at a high rate in a gel consisting mainly of pectin. In the secondary wall, lignin of a higher methoxyl content (mostly syringyl lignin) is deposited at a lower rate in a matrix of arabinoglucuronoxylans and bound phenolic acids. Overall, this results in a high concentration of condensed lignin in the primary wall/middle lamella (Terashima et al., 1988), forming a physical barrier to microbial penetration (Engels and Schuurmans, 1992), and a relatively low concentration of lignin in the secondary wall, which is highly cross-linked with hemicellulose. The differences in lignin concentration and properties of lignin in primary and secondary walls explain why forage digestibility is often more highly correlated with lignin composition than with lignin content (Journal Paper 2).

The negative impact of free lignin by adsorption of enzymes is probably less

prominent during ruminal degradation of forage cell walls. In this study, relatively high concentrations of 'free' lignin were used, and the concentration of unbound lignin is unlikely to reach a level equivalent to the concentration within the forage. However, high local concentrations of lignin may be exposed at the cell wall surface during cell wall degradation (Chesson, 1981), which may immobilize extracellular microbial enzymes.

Alkali lignin preparations have been shown to inhibit the biological activities of animal enzymes and extracellular microbial products such as enzymes, toxins and antibiotics (Farstad and Naess, 1977). The potential ability of hydrophobic binding to microbial enzymes is in agreement with its capacity of hydrophobic binding of large molecules such as cholesterol and taurocholate (Balmer and Zilversmit, 1974). Lignin is known as a strong adsorbent of cholesterol (Story, 1977) and bile acids (Eastwood and Hamilton, 1968). In bile acid adsorption, a hydrophobic bonding mechanism aslo appears to be involved, since it was enhanced by methylation or acidity (Eastwood and Hamilton, 1968), and affinity for bile acids was not related to methoxyl content (Kay et al., 1979). Binding of enzymes would interfere to a certain extent with fiber degradation in the rumen and lower intestinal tract. Lignin-mineral interactions, such as with S, may interfere with general mineral metabolism, whereas binding of bacterial toxins and antibiotics may actually benefit the host animal.

Ammonia treatment was able to partially overcome the nutritional limitations imposed by advanced plant maturity and lignin-carbohydrate interactions. When comparing the different stover fractions, the response to NH_3 treatment was not inversely related to initial quality, especially for the drought-stressed stover, in which the principal effect of NH_3 treatment was the provision of NPN. In addition to providing NPN, NH_3 treatment caused a partial solubilization of hemicellulose from

leaf cell walls in both years, and from the stems of non-drought-stressed stover, associated with enhanced rate of degradation. When comparing the treatment response between years, the inverse relation between initial quality and response to ammoniation in terms of cell wall degradation was more apparent, especially for leaf and lower stem.

The small treatment response in the drought-stressed stover could not be positively attributed to saponification of phenolic acids, due, in part, to the inefficient treatment (stems), and to the low concentration of phenolic acids in the leaf. In the comparison of NH_3 treatment between years, the UV absorbance at 280 nm after solubilization of phenolics by extraction in water was measured as a rapid indicator of treatment effectiveness (Lau and Van Soest, 1981). Ammonia treatment released phenolics from all fractions in both years, but to a larger extent from leaf than stems, and more from the non-stressed than from the drought-stressed stover, which agreed well with the observed responses in digestibility. However, similar amounts of soluble phenolics were released from the NH_3 controls, indicating saponification activity by unreacted NH_3 present in the fiber during the overnight extraction. It is not unlikely that this may also have occurred during the *in vitro* incubations, which would explain the absence of difference in NDF degradation between NH_3 treatment and NH_3 control. In addition, the added NH_3 may be absorbed by the fiber matrix, both in NH_3 treated stover and in NH_3 controls, where it can be more efficiently utilized by attached cellulolytic bacteria. This effect was demonstrated in the *in vitro* comparison of buffer systems, in which the response to ammoniation, although larger in the N-limiting medium, was still significant in the N-sufficient medium. An alternative mode of action of NH_3 bound to the fiber would be its participation in nucleophilic addition reactions to QM intermediates. Although not a very strong nucleophile, NH_3

is abundant in treated corn stover, and its involvement in nucleophilic addition reactions would explain the small difference in NDF degradation between NH_3 treatment and NH_3 addition for the drought-stressed stover fractions for which the response could not be related to initial solubilization of hemicellulose or saponification of esterified phenolic acids.

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Appendix
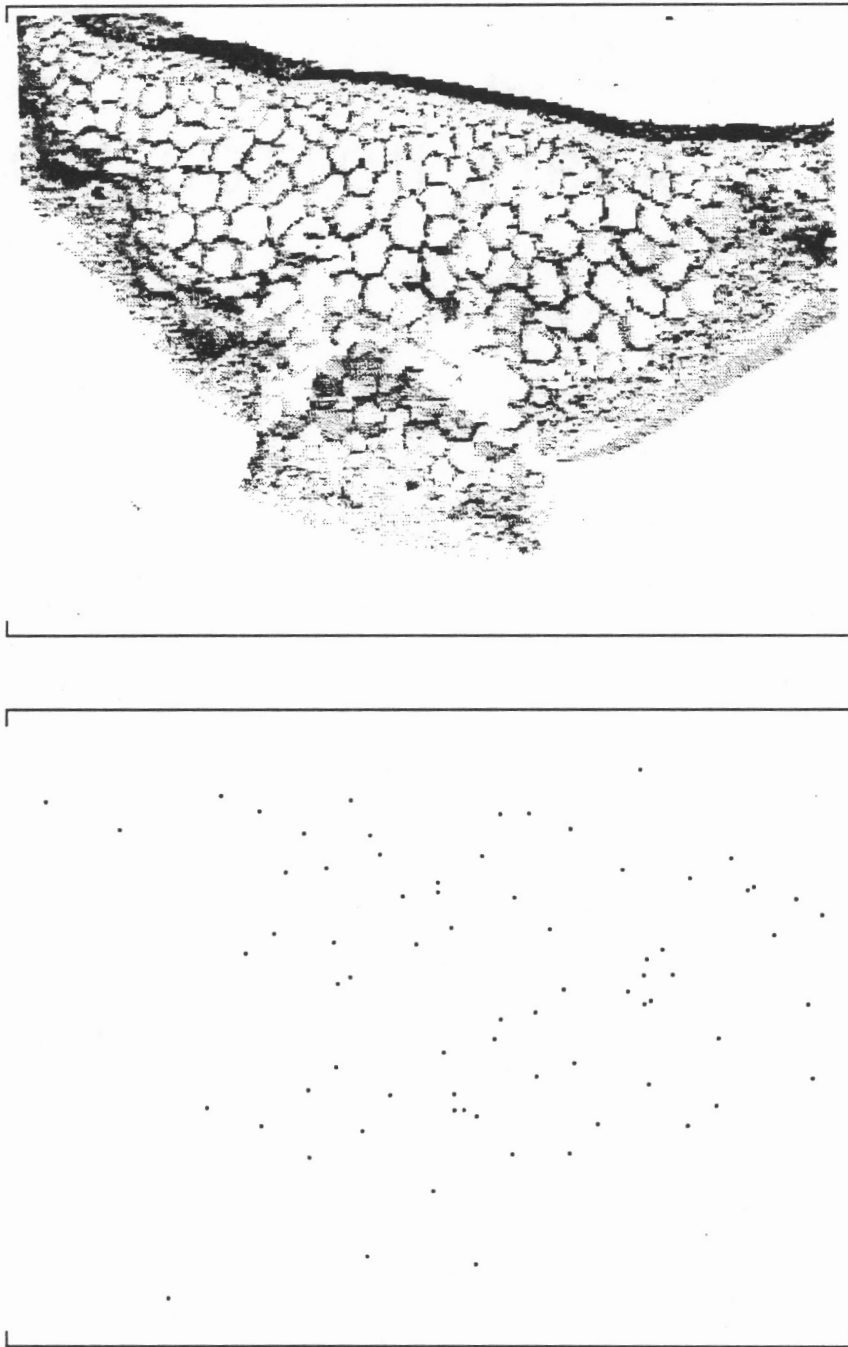
Appendix Figure 1. Image scan (top) and bromine map (bottom) of leaf midrib, early dent (x 37, dwell time 1 msec).
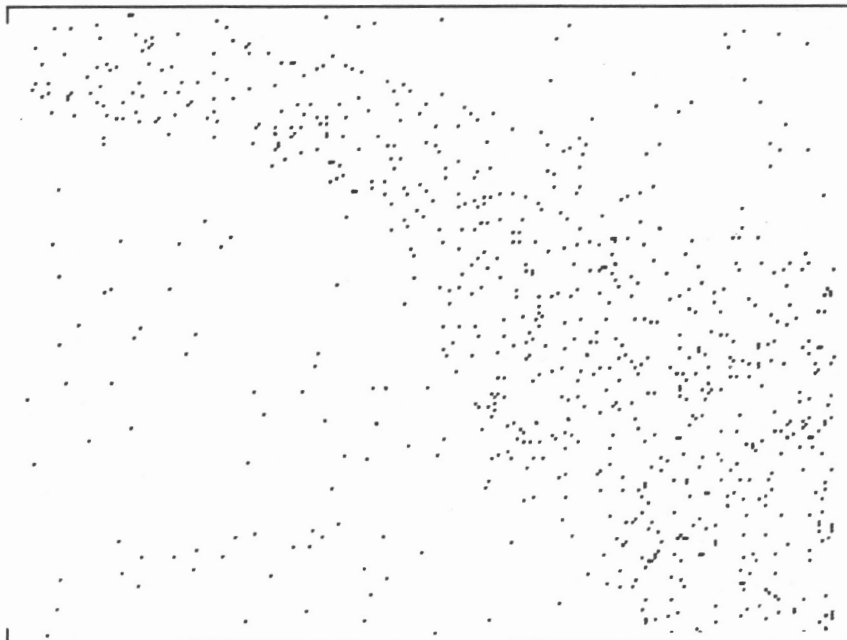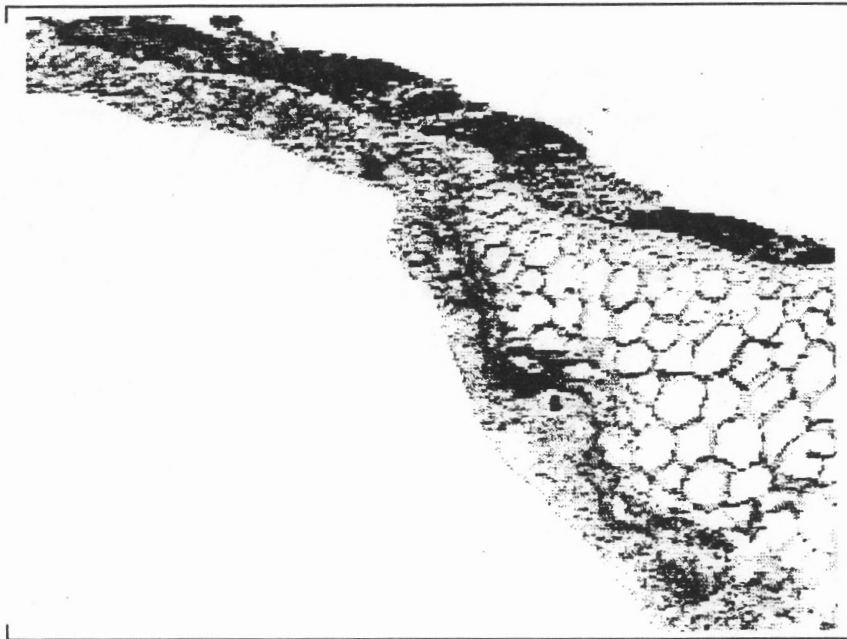
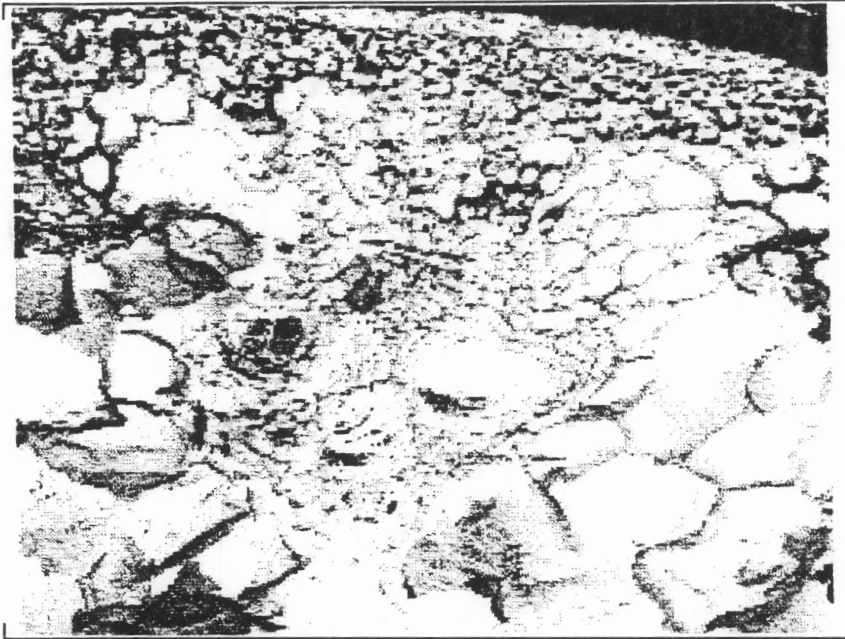Appendix Figure 2. Image scan (top) and bromine map (bottom) of leaf, early dent (x 44, dwell time 10 msec).
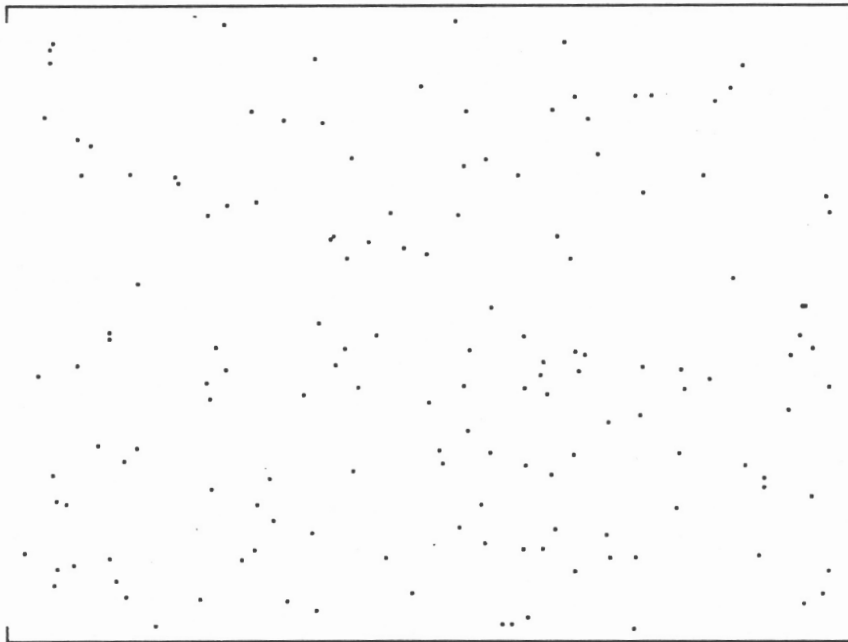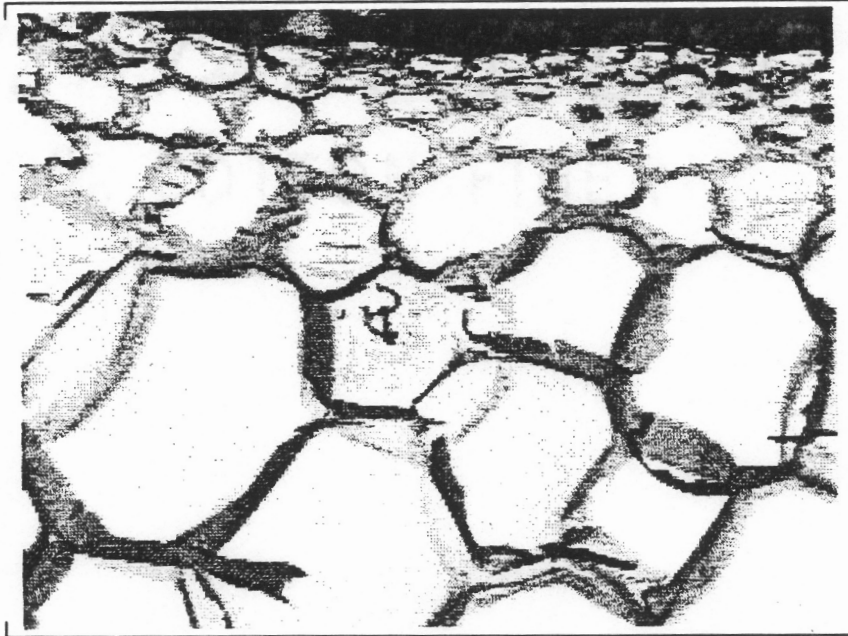
Appendix Figure 3. Image scan (top) and bromine map (bottom) of leaf midrib, early dent: epidermis and parenchyma, (x 294, dwell time 10 msec).
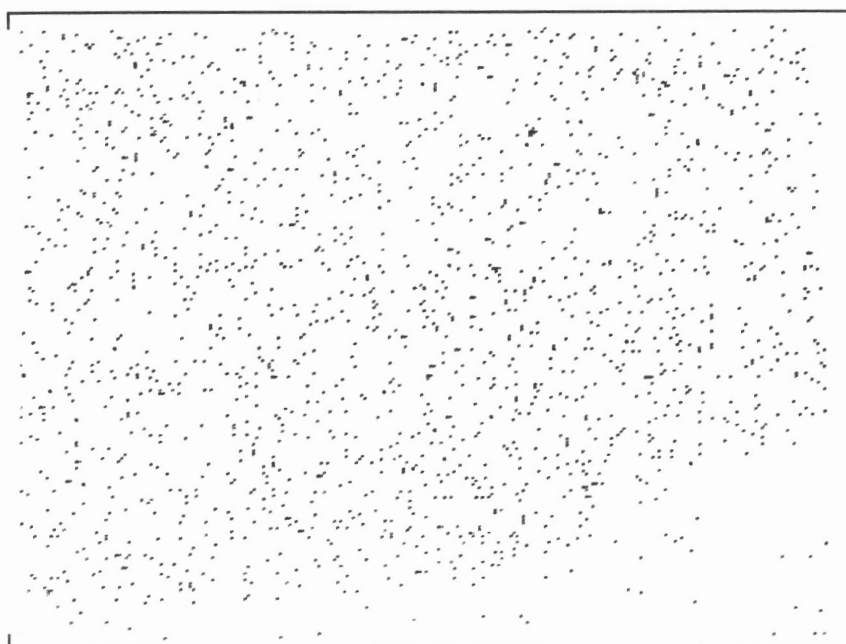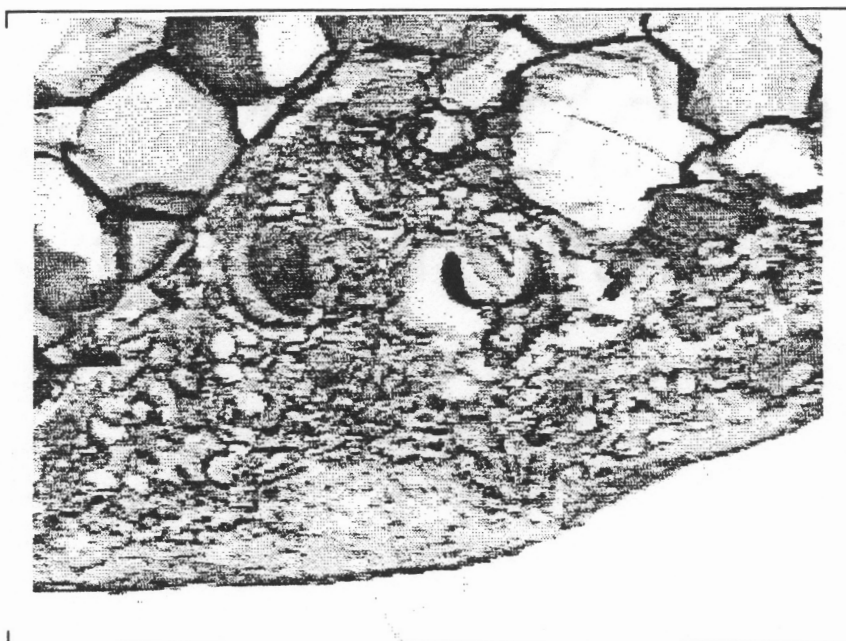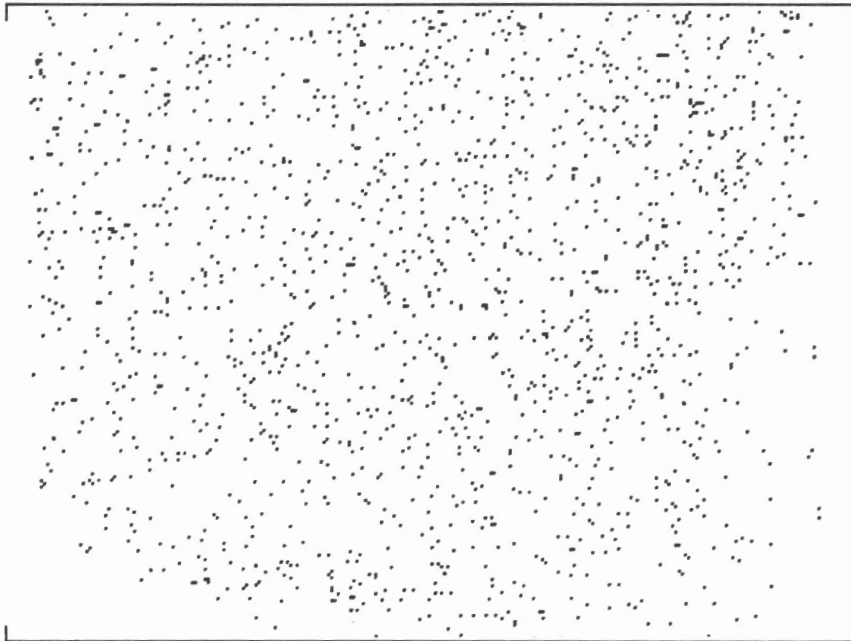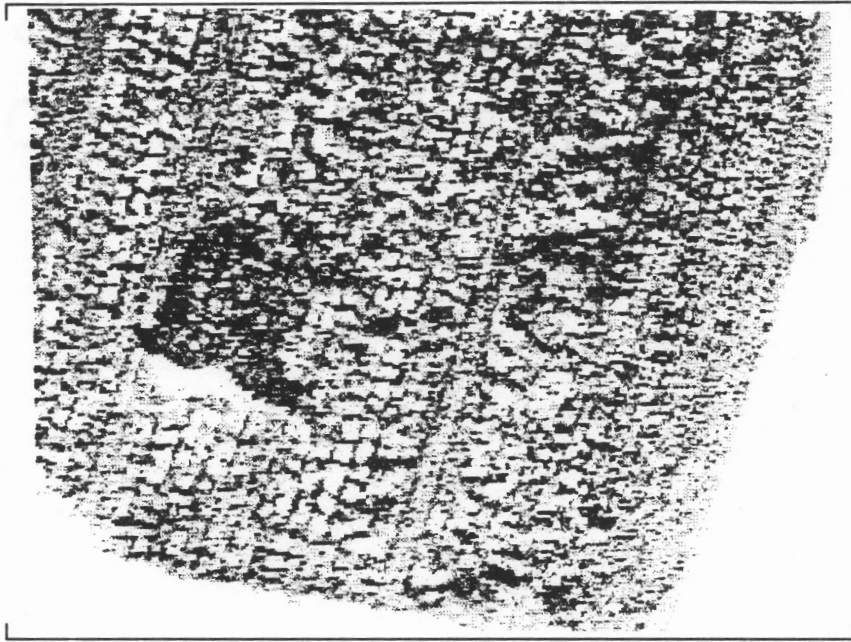
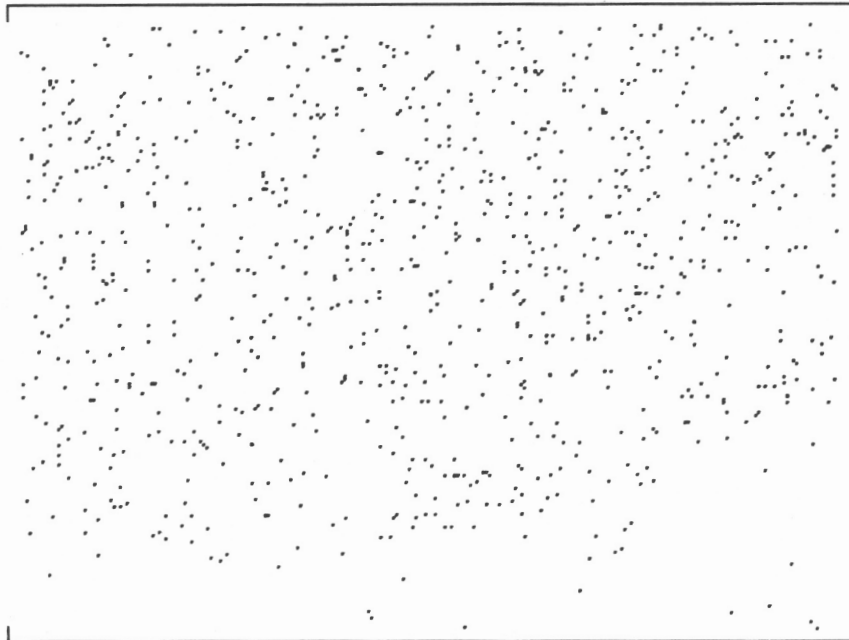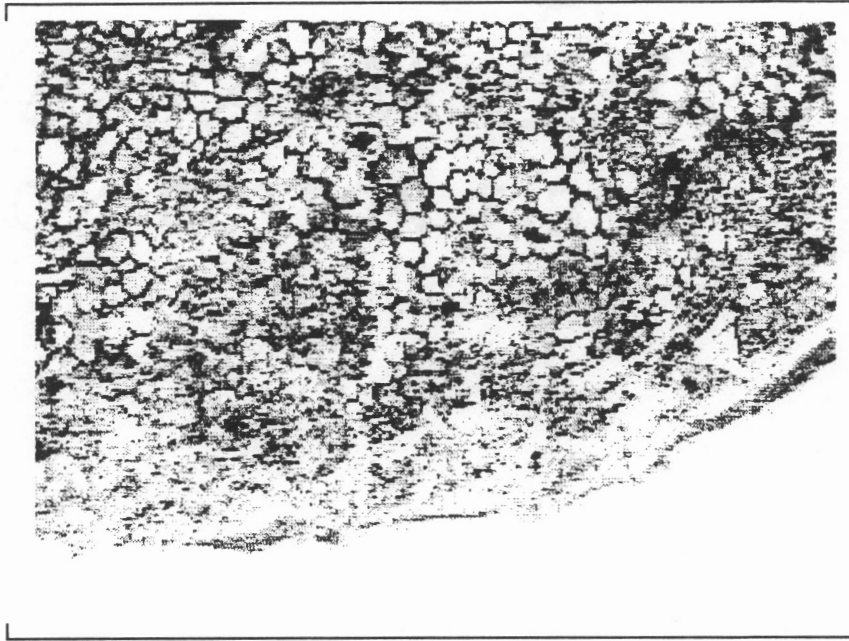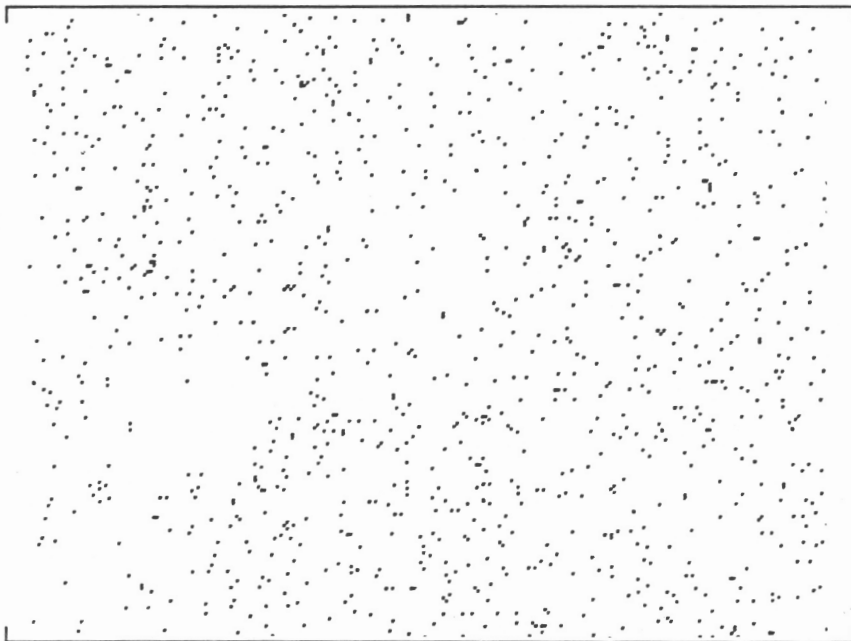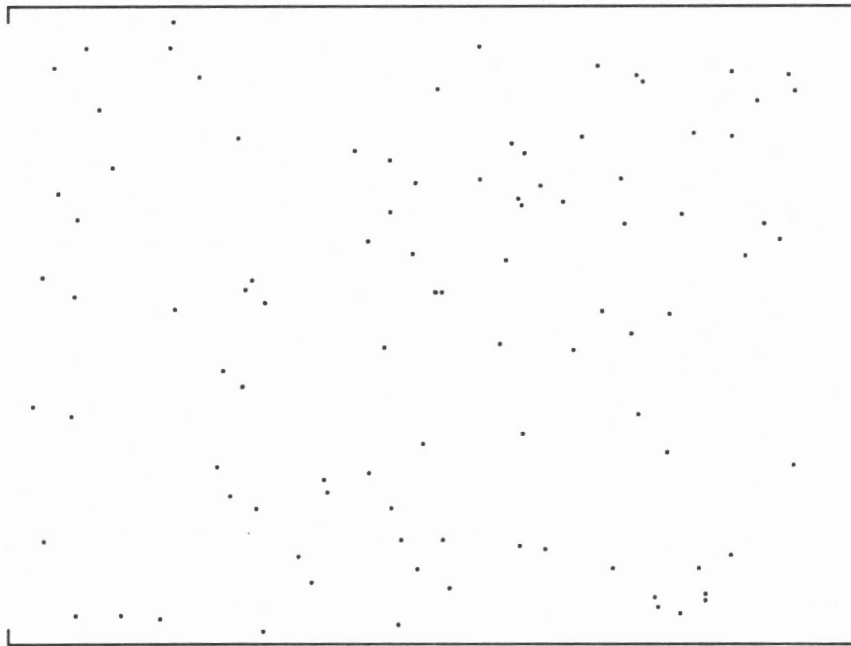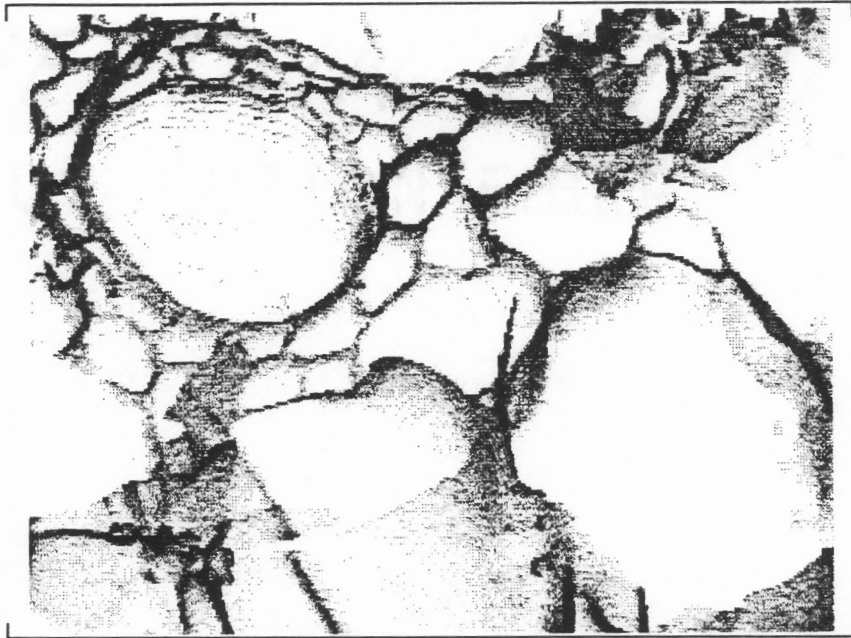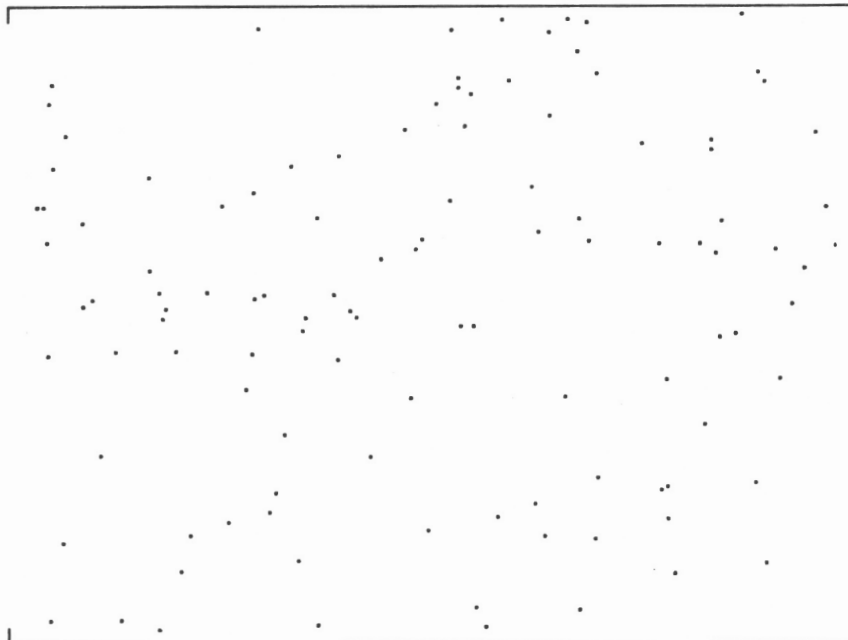Appendix Figure 4. Image scan (top) and bromine map (bottom) of leaf midrib, early dent: epidermis, vascular tissue and parenchyma (x 200, dwell time 10 msec).
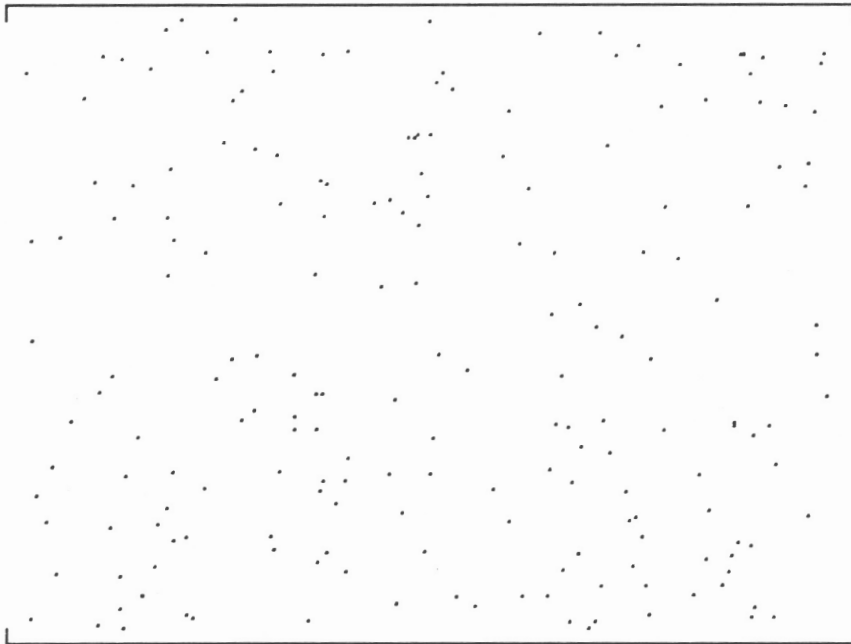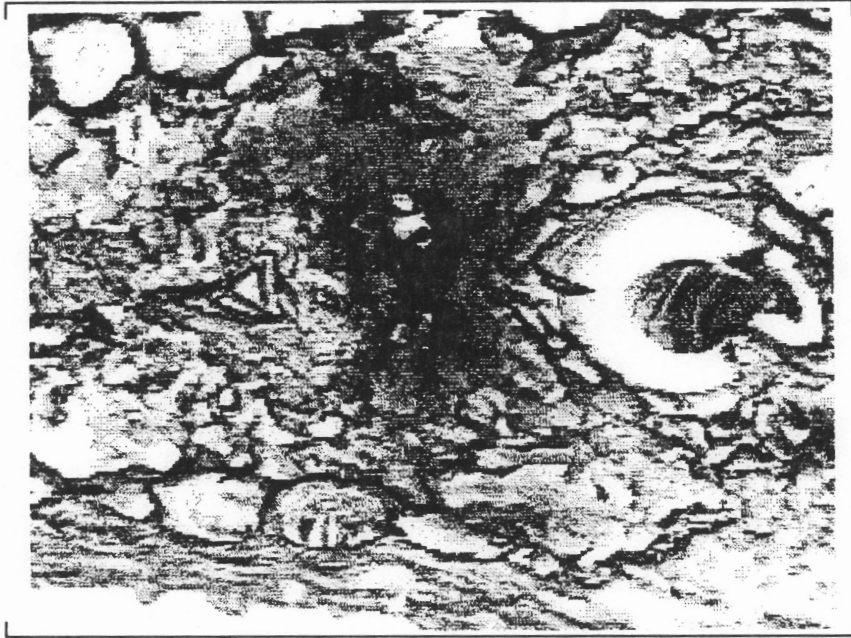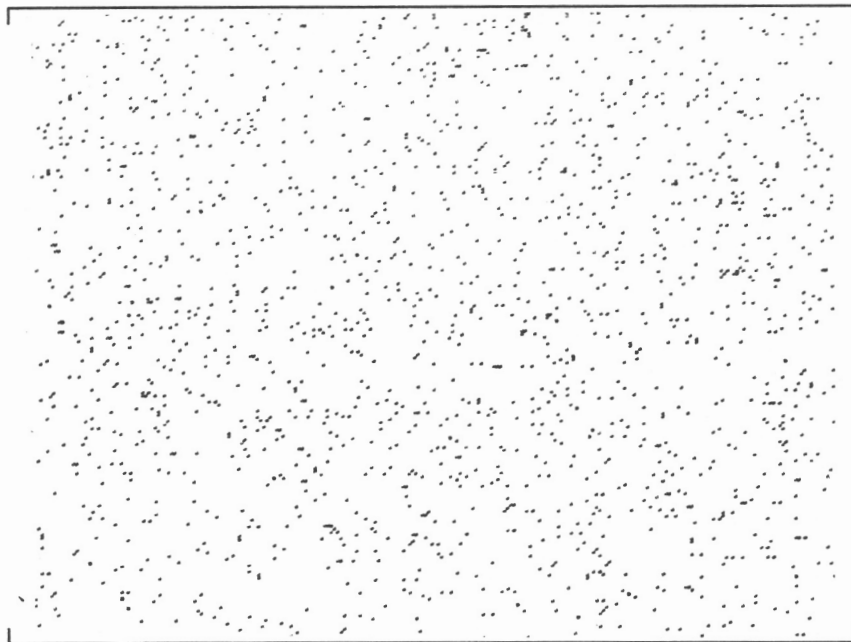
Appendix Figure 5. Image scan (top) and bromine map (bottom) of leaf, early dent: epidermis, vascular bundle, and phloem (x 400, dwell time 1 msec).
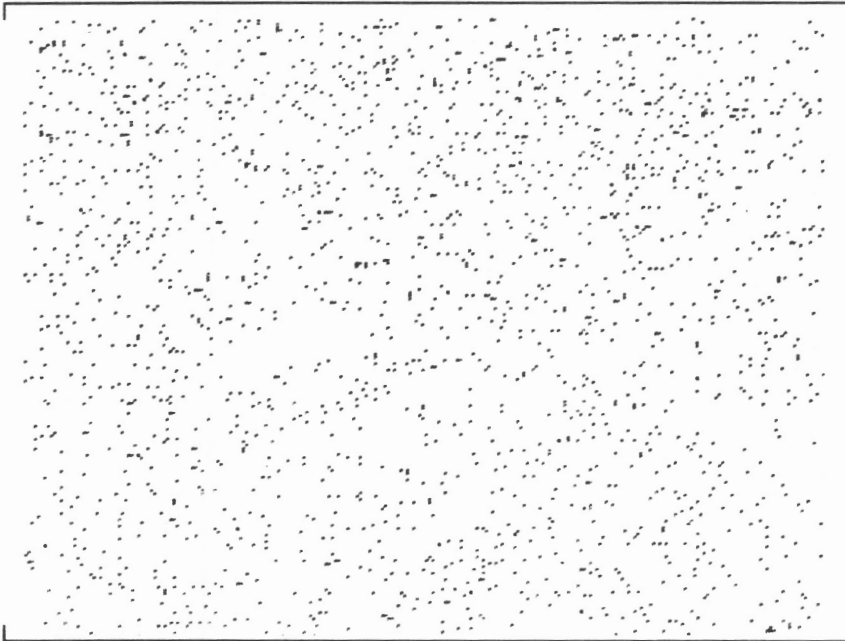
Appendix Figure 6. Image scan (top) and bromine map (bottom) of leaf midrib, full maturity; epidermis, vascular tissue, and parenchyma (x 200, dwell time 10 msec).

Appendix Figure 7. Image scan (top) and bromine map (bottom) of upper stem internode, early dent, longitudinal (x 19, dwell time 10 msec).

Appendix Figure 8. Image scan (top) and bromine map (bottom) of upper stem, early dent: rind and pith (x 35, dwell time 10 msec).
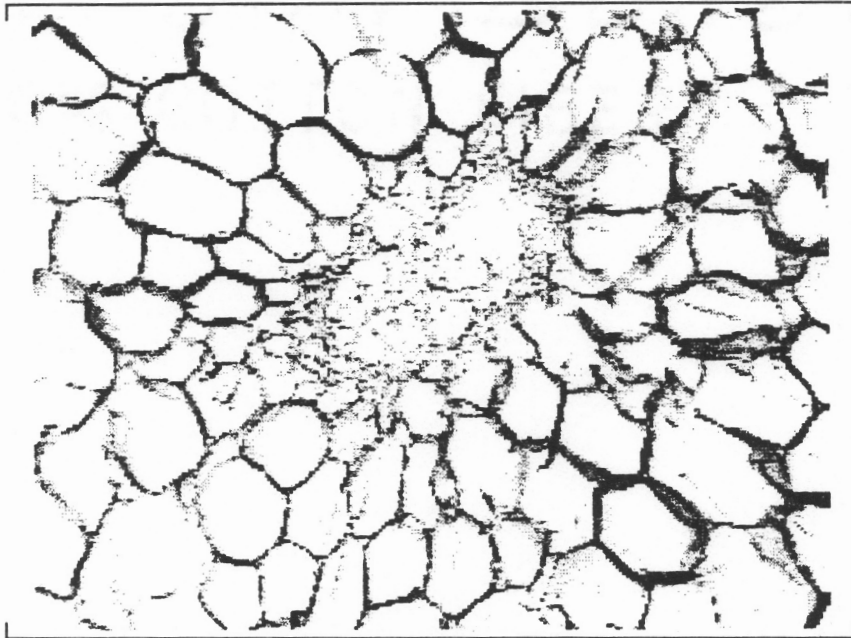
Appendix Figure 9. Image scan (top) and bromine map (bottom) of upper stem pith, early dent; parenchyma and vascular bundle (x 134, dwell time 10 msec).
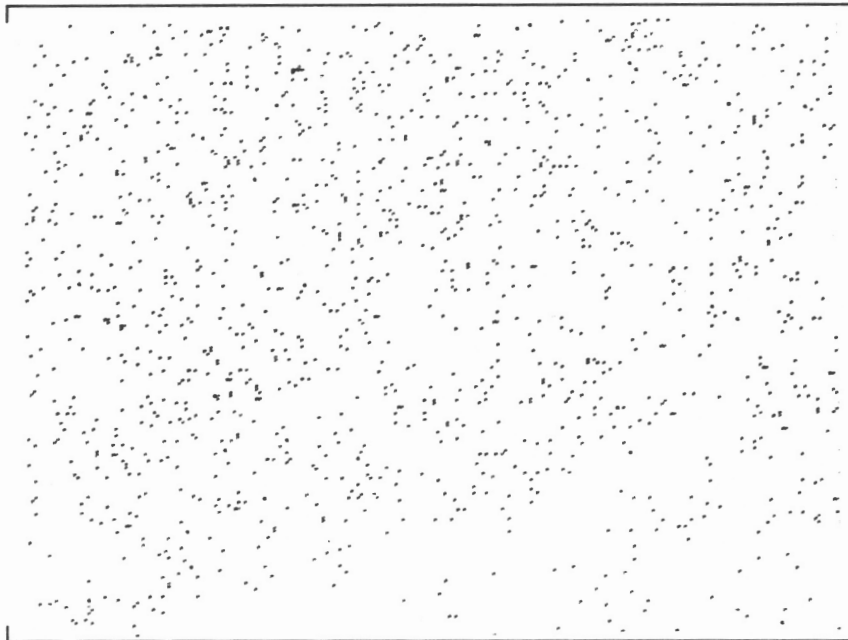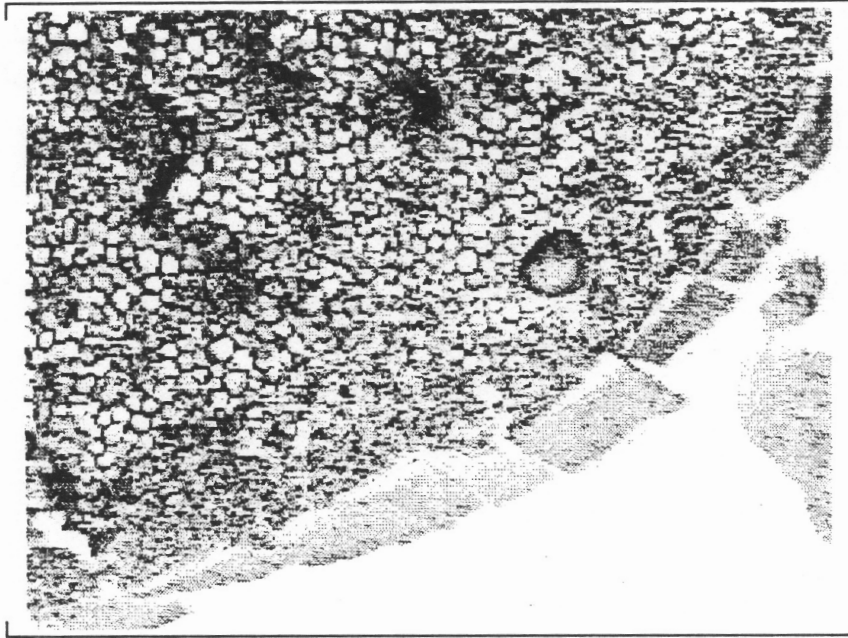
Appendix Figure 10. Image scan (top) and bromine map (bottom) of upper stem, early dent; tracheid and parenchyma, magnified from Appendix Figure 9 (x 641, dwell time 1 msec).
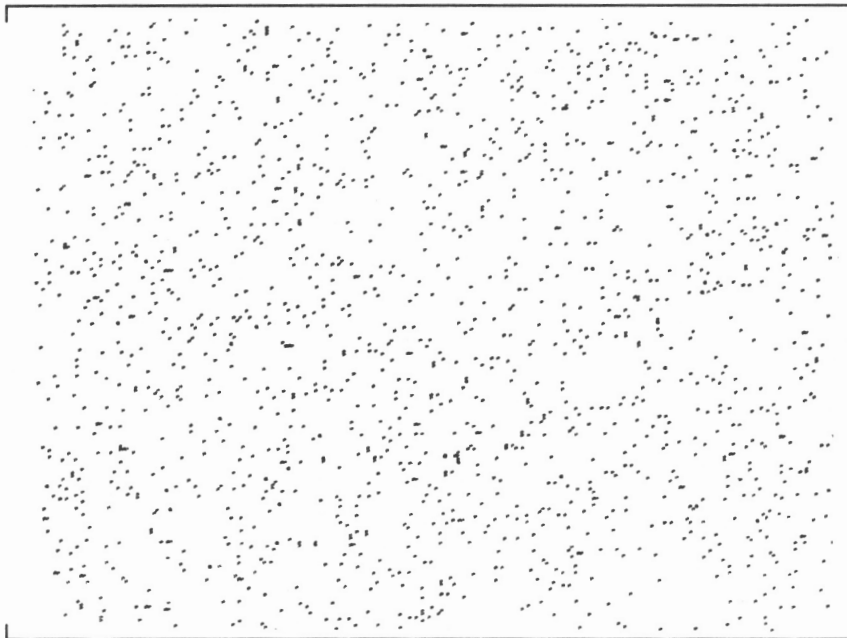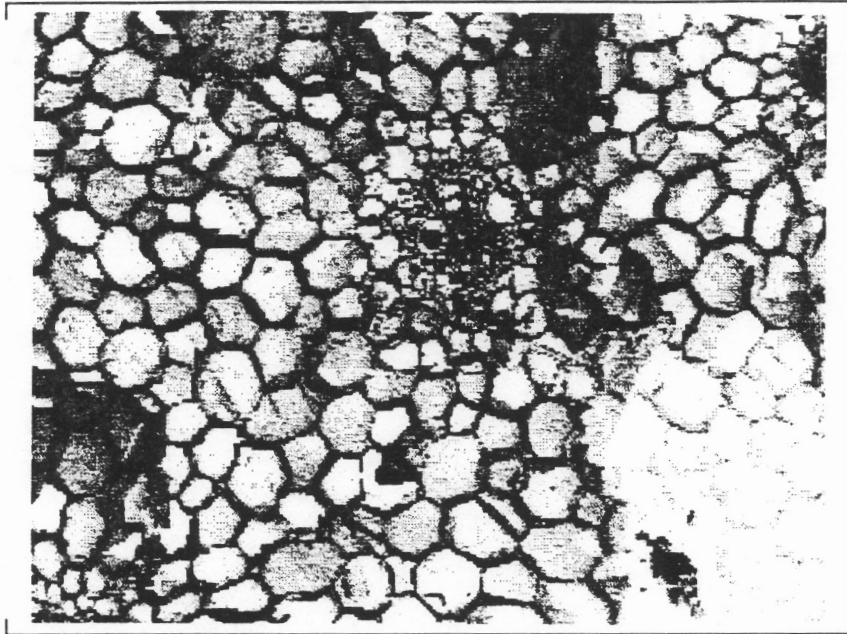
Appendix Figure 11. Image scan (top) and bromine map (bottom) of upper stem, early dent: xylem and sclerenchyma, magnified from Appendix Figure 10 (x6650, dwell time 1 msec).
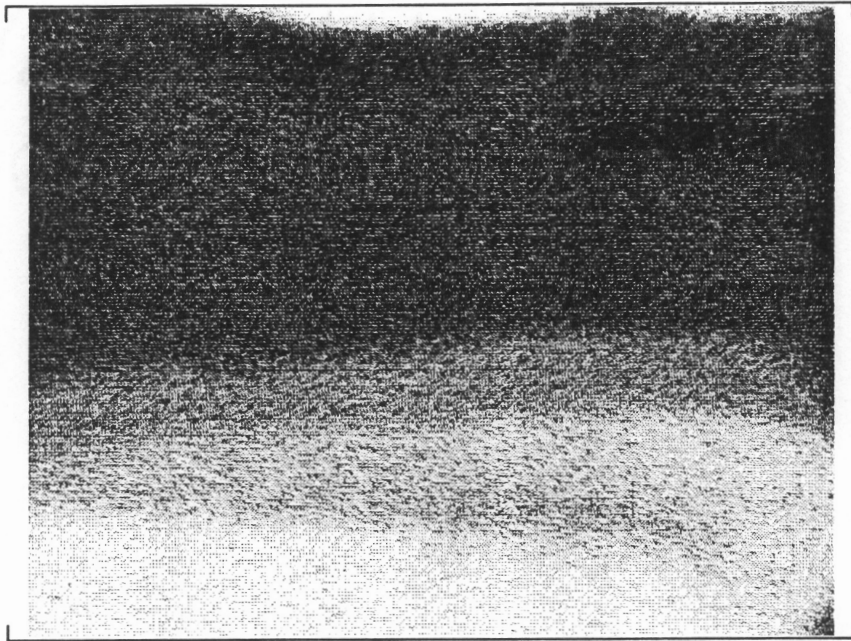
Appendix Figure 12. Image scan (top) and bromine map (bottom) of upper stem, early dent; sclerenchyma wall and cell lumen, magnified from Appendix Figure 11 (x 28300, dwell time 10 msec).

Appendix Figure 13. Image scan (top) and bromine map (bottom) of upper stem, full maturity: rind and pith (x 21, dwell time 1 msec).

Appendix Figure 14. Image scan (top) and bromine map (bottom) of lower stem, early dent; parenchyma and vascular tissue (x 62, dwell time 10 msec).

Vita

Vincent Johannes Hendrikus Sewalt was born from Henk and Antoinette Sewalt on May 13, 1964 in Nijmegen, the Netherlands. He attended the Revius Lyceum in Doorn, before entering Wageningen Agricultural University in 1982. Vincent majored in Animal Husbandry, with specializations in Tropical Animal Production and Grassland Science. In 1985, he received practical training and conducted his major thesis research in Sri Lanka with the Sri Lanka-Netherlands Dairy Development Program. In 1986/87, he conducted grassland research at the University of Santiago (UCCM) in the Dominican Republic, where he also met his wife, Janet Estrella. He obtained his agricultural engineer (Ir.) degree from Wageningen Agricultural University in 1987. From February 1988 to March 1990, Ir. Sewalt worked in several short-term assignments at the Department of Tropical Animal Production, Wageningen Agricultural University.

In May 1990, he commenced his PhD program at Virgina Polytechnic Institute and State University under the direction of Dr. J.P. Fontenot, on factors affecting microbial fermentation of corn stover, with emphasis on the impact of lignification. He is member of the American Society of Animal Science and the American Forage and Grassland Council.

Vincent and Janet have two sons, Jeremy, born February 20, 1989, and Gregory, born May 28, 1990.