Netherlands Journal of Agricultural Science 44 (1996) 357-373

Developments in application of light and scanning electron microscopy techniques for cell wall degradation studies

F.M. ENGELS

Department of Agronomy, Wageningen Agricultural University (WAU), Haarweg 333, NL-6709 RZ Wageningen, The Netherlands
(fax: +31-317-484575; e-mail: ferdinand.engels@users.agro.wau.nl)

Received 14 June 1996; accepted 13 October 1996

Abstract

The objective of this paper is to review mainly the results of recent technological developments in light and scanning electron microscopy closely used for research on forage cell wall degradation in ruminants.

The indigestibility of forages by rumen microorganisms used to be ascribed mainly to an overall presence of lignin in the plant material. However, early light microscopic observations without application of histochemical staining already revealed that some leaf and stem tissues were degraded completely. The early use of lignin detecting dyes, such as acid phloroglucinol or safranin, in light microscopy made it possible to discriminate between lignified undegradable and unlignified degradable plant tissues.

The introduction of the scanning electron microscope enabled a further discrimination between degradable and undegradable cell wall and cell wall layers in plant tissues. As a result of continuous improvement of the techniques used in microscopy, e.g. section to slide, mirror sectioning, microspectrophotometry and cryo-ultramilling, forage indigestibility can now be attributed to the specific deposition and location of cutin/suberin or lignin layers inside the plant cell wall. These structural layers form barriers hindering access of rumen microorganisms to degradable parts of the cell wall.

Keywords: forage, histochemistry, lignification, microscopy, plant tissues, rumen.

Introduction

Scanning Electron Microscopy (SEM) and Light Microscopy (LM)

The chemical composition of forages for animal feed has been investigated for decades. Common agreement indicated that nutritive quality of feed was affected by growth, maturity, harvest and conservation of plant material, but correlations between degradability and different stages of development of the crop were obscure. The early experiments to analyse and determine forage quality were exclusively chemically oriented e.g., Goering & Van Soest (1970) and procedures for analyses

followed chemical extractions and characterization of specific chemically dissolved plant cell wall complexes, mainly polysaccharides (cellulose and hemicellulose) and polyphenolics (lignin). Phenolics clearly increase in relative quantity during growth and maturation of crops and feed quality decreases consequently. Different types of forages had different relative amounts of polysaccharides and phenolics, but a causal relationship between forage compounds, plant structure, development and degradability was difficult to establish.

Early use of microscopes

Before 1970 only a few papers reported on anatomical / morphological structures of plant tissues that were of interest in relation to degradation by rumen microorganisms. Regal (1960) who investigated rumen excrement, found that vascular bundles, epidermis and sclerenchyma were not degraded in the rumen digestive tract. Sakurai (1963) reported differences in the rate of degradation between different plant tissues. Monson & Burton (1972) found an increase in dry matter disappearance when the cuticle of leaf blades was damaged before degradation. Monson *et al.*, (1972) further showed that the cuticle of the leaf formed an effective barrier to degradation by rumen microorganisms. These early reports had great significance because they established the first link between unlignified degradable and lignified undegradable plant tissues by light microscopy, which marked the beginning of the use of microscopic techniques to study forage quality. These first reports also showed that anatomically different plant structures did have their own degradation characteristics and that a more detailed analysis of these structures in relation to their degradation properties should be made.

During the early application of light microscopy (LM) to investigate ruminally degraded material, conventional techniques consisting of fixation, embedding, sectioning and staining for lignin were used. LM, using 10–100 μm sections, provides anatomical and histochemical data on plant tissue organization and composition, respectively. Transmission electron microscopes (TEM) were used with glutaraldehyde and osmium tetroxide as fixatives and resins as embedding media. In TEM studies of ultra-thin sections (40–70 nm), an ultrastructural analysis of tissues and molecular composition could be obtained at a high level of resolution, but the depth of the field is very limited. The LM and TEM techniques provided information from inside the degraded material as a consequence of sectioning of embedded plant material. Later scanning electron microscopy (SEM) was introduced in degradation studies. SEM investigations enables three-dimensional observations of surfaces with a large depth of field. The thickness of sections is not important since SEM provided analysis on the plant surface. In nearly all investigations on rumen degradation of plant material the TEM and SEM were applied after pre-investigations with LM.

Developments during 1970–1980

New information on the types and behaviour of rumen microorganisms and the process of tissue degradation of different types of cells and cell walls in forages were

obtained when LM, TEM and SEM could be applied to the same plant material. Preparative methods were changed and improved for the fragile organisms and the breakable degraded plant material in rumen degradation studies.

Hungate (1966) and Imai & Tsunoda (1972) used the SEM to investigate ciliated rumen protozoa. Rittenberg *et al.* (1977) prepared ciliated rumen protozoa using a 8μm membrane filter method. Many species of ciliated protozoa could be investigated well preserved and cleaned from adhering small plant tissue leavings.

Hanna *et al.* (1973, 1976) studied *in vitro* degradation of 2 cm long leaf sections of the first fully expanded leaf of different grasses. The leaf sections were embedded in paraffin after degradation and a series of transverse 10 μm thick sections were observed by LM. In detailed anatomical investigations of numerous C_3 and C_4 grasses they found that the cuticle, trichomes, xylem, fibers, and bundle sheaths were not degraded. The mesophyll in C_3 grasses was degraded more rapidly than in C_4 pearl millet and bermudagrass. This difference in degradation was attributed to a less compact organization and larger intercellular spaces in the mesophyll of C_3 species (Hanna *et al.*, 1973, 1976). The sequence in degradation of similar tissues in successive growing and maturing leaves was not affected by the age of the leaves. The rate of degradation diminished and degradability of the forage decreased as a result of the development of a band of thick walled cells in stems. Hanna *et al.* (1973, 1976) recorded the different types of leaf and stem tissues but also the cross sectional areas were measured. Data on cell wall degradation could be visualized in the LM histochemically by staining with safranin which stained the partially degraded tissue areas red. Another observation from this anatomical investigation was that the F1 hybrid of pearl millet had a mesophyll arrangement similar to one of the parents. This observation suggested that the anatomical arrangement is genetically controlled and could be subjected to selection.

The LM provides information at the tissue level (e.g., number of cells, lignification pattern, types and amounts of degradable and undegradable tissues). The SEM improved this resolution. Akin & Burdick (1975) used the LM and the SEM and morphometric measurements (cross sectional area) to investigate the amount of degradable and undegradable tissues in C_3 and C_4 grasses and to study tissue degradation at higher magnification. Differences in amounts of lignified tissues between the C_3 and C_4 grass leaves occurred, and results indicated that the microanatomy of the leaf blade and the specific properties of the cell walls of tissues influence the degree of degradation by rumen microorganisms.

Cross sectional area measurements were then introduced to study differences in amounts of tissues in leaf and stem, and data were correlated with *in vitro* degradation studies. Several papers deal with tissue properties and the mode of action of the rumen microorganisms. Comparisons were made between different plants and plant organs such as the leaf, sheath and stem, and between genotypes within breeding programs. In bermudagrass a negative effect of maturity on degree of degradation (Akin *et al.*, 1977; Hanna *et al.*, 1976) was found. High temperatures during plant growth (Smith, 1977) decreased tissue degradation in tall fescue. The degree of lignification of parenchyma tissue e.g., young and old parenchyma in bermudagrass stems and sheaths (Akin *et al.*, 1977) influenced the extent of degradation of the

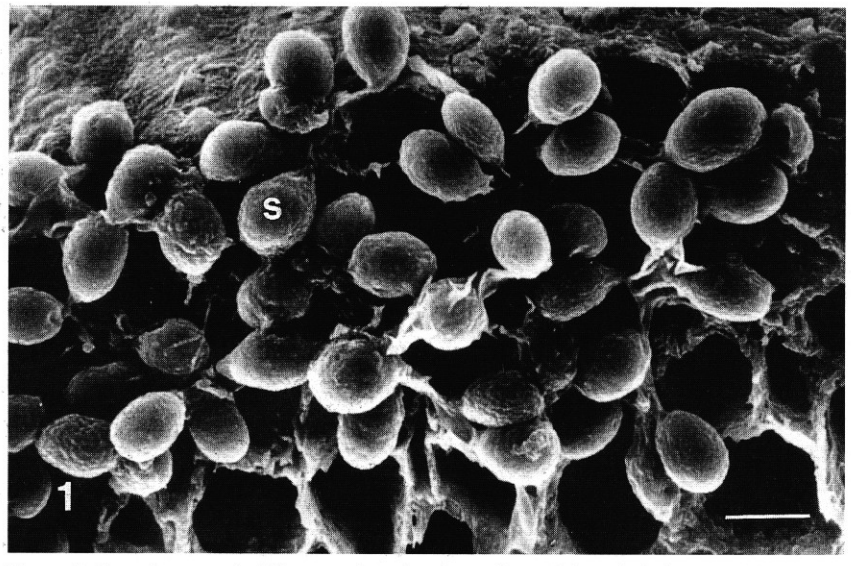


Figure 1. Fungal sporangia (S) are produced at the surface of degraded plant particles. Bar = 10.0 μm.

parenchyma tissue. Brazle & Harbers (1977) showed that the cuticle on stems and leaves of alfalfa formed an effective barrier against degradation. Latham *et al.* (1978) used pure cultures of anaerobic bacteria to investigate typical adhesion of bacteria to the cell walls of different tissues and degradation of these tissues in perennial ryegrass. They observed that rumen bacteria showed some specificity in adhesion to cell wall types of different plant tissues, confirming earlier observations made by Akin (1976a), Akin *et al.* (1974) and Cheng *et al.* (1977). Small gradual differences were found in the rate of degradation between mesophyll, epidermis and phloem compared to sclerenchyma and epidermal tissues. Sclerenchyma had been considered recalcitrant to degradation but Latham *et al.* (1978) demonstrated, using TEM, that sclerenchyma was partly degraded.

By the end of the 1970's numerous LM-SEM-TEM papers appeared on different types of rumen microorganisms and their mode of attachment and attack of plant tissues. The zoospores of the rumen phycomycete *Piromyces sp.* (Orpin, 1977a) and *Neocallimastix sp.* (Orpin, 1977b) showed taxis to specific tissues; fungi principally invaded plant material at damaged sites and through the stomata. After at least 24 hour of degradation fungal sporangia are formed at the outer surface of the degraded plant particle (Figure 1). The rumen protozoa *Isotricha intestinalis* and *I. prostoma* attached to plant tissue with a special organ on the cell surface. These organisms showed chemotaxis to soluble carbohydrates (Orpin & Letcher, 1978). Rumen protozoa ingested partially degraded plant cells (Akin & Amos, 1979). A protozoa is presented (Figure 2) ingesting a small plant cell wall particle heavely occupied with rumen bacteria. Rumen anaerobic fungi were investigated in cattle and sheep (Bauchop, 1979). Akin (1976b, 1980a) found that fibrolytic rumen anaerobic bacteria consisted of two main types: the encapsulated cocci and the irregularly shaped

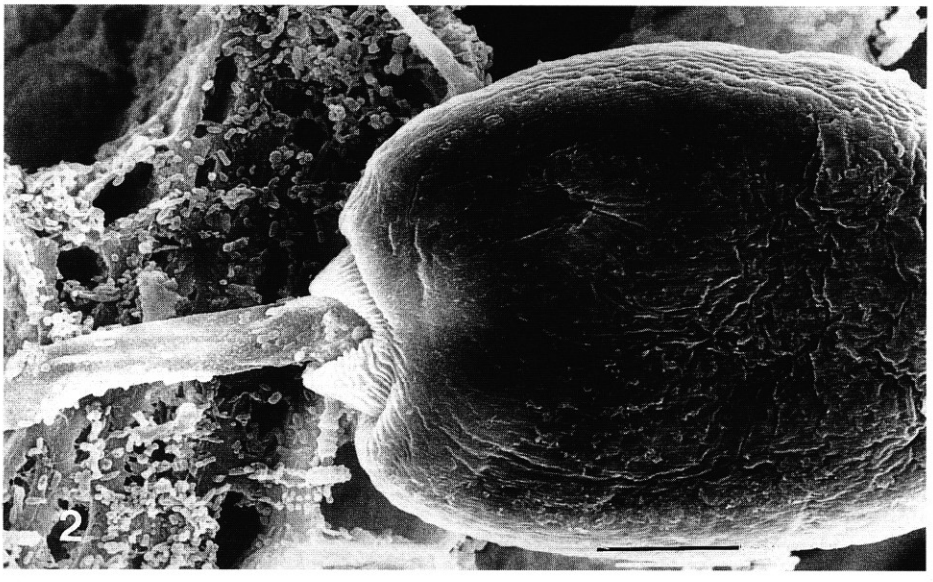


Figure 2. A rumen protozoa swallowing a small plant particle heavely covered with rumen bacteria. Bar = 10.0 μm.

bacteria. He suggested that the availability of cell wall constituents during degradation could have an effect on numbers and types of degrading bacteria on plant tissue. A filamentous facultative anaerobic bacterium was discovered that preferentially attached to lignified tissues (Akin, 1980b). Akin (1980b) showed that rumen microorganisms have preferences to invade different types of plant tissue.

LM and SEM were also used to investigate the effects of pre-treatments of forages and their degradation. Alkali treatment of rice hulls (McManus *et al.*, 1976), rice straw (Kawamura *et al.*, 1973) and bermudagrass leaves (Spencer *et al.*, 1984) resulted in an increase in degradation of the predominantly soft and swollen plant residues. These results could be compared with the results of permanganate treatments of bermudagrass and tall fescue (Barton & Akin, 1977). Although, permanganate delignifies plant tissues, some structures, e.g., vascular bundles, were not degraded. The authors suggested that plant cell wall polysaccharides showed important differences in their rates and extents of degradation. An interaction between cellulolytic bacteria and purified cotton fibers was studied by Berg *et al.* (1972). Lignin free fibers were degraded. The authors concluded that the structure of the cotton fibre was crucial for cellulose decomposition. In addition to conventional SEM investigations energy dispersive X-ray analyzer can be attached to SEMs and elements with an atomic mass larger then 10 can be localized. Investigations of corn and sorghum silage by SEM and SEM X-ray dispersion revealed that less degradable tissues contained remarkable amounts of silica compared to the degraded tissues such as mesophyll and stem parenchyma. Silica was also detected in rice (Kunoh & Akai, 1977) and calcium crystals were found in alfalfa (Ward *et al.*, 1979), indicating that

deposition of relatively large amounts of inorganic crystalline material could negatively affect biodegradation. Modern detectors can detect now elements with atomic mass below 5.

By the end of the 1970's the LM and SEM had provided fundamental knowledge on the relationships between types of rumen degrading microorganisms, available plant cell structures and plant cell wall degradation, type and degree of lignification, chemical composition of types of cell walls and the effects of chemical or physical pre-treatment of forage samples. Akin (1979) suggested that anatomical investigations, using LM and SEM, must be incorporated in studies of degradation of plant material.

New developments in LM and SEM techniques during the 1980's

Anatomical data are essential to understand differences in forage quality which is normally estimated by chemical methods that do not discriminate between degradable and non-degradable types of plant tissues. Chemical procedures extract carbohydrate complexes also from inside every cell wall of a plant particle, whereas in rumen degradation enzymatic hydrolyses only takes place on the surface of the plant tissue particles.

The use of the LM and SEM to visualize cell wall degradation became more successful by the development of techniques which minimized artefacts and optimized plant structure images even after heavy degradation. The LM and SEM preparation methods for plant material before or after rumen degradation were based on the use of 15 μm thick sections which were observed in LM, and on 2–3 mm thick sections which were embedded and re-sectioned with loss of the original cut-end. In SEM observations, sections were directly affixed onto stubs with low resistance contact cement, with colloidal silver glue (Harbers & Thouvenelle, 1980) or with a conductive paint (Barton & Akin, 1977).

However, disorder and some loss of tissues from sections could easily take place, especially when degraded sections were handled or when chemical extraction was applied prior to degradation (Barton *et al.*, 1981, Harbers *et al.*, 1982). Barton *et al.* (1981) reported that also critical point drying of SEM material resulted in some damage to the mesophyll tissues. McManus *et al.* (1976) used double-coated adhesive tape to fix representative samples of feed and faeces on SEM stubs.

Akin (1982) published the LM section-to-slide technique which was based on placing of 16 μm thick frozen cut sections on double adhesive tape that was already fixed on top of a specimen slide. The frozen sections were then air dried and thereby fixed on the tape. The technique enabled rapid and easy comparison and investigation of large numbers of sections before and after rumen degradation. The advantage of this method was that once the plant tissues were fixed to the adhesive tape, they could be easily handled. Moreover, the undegradable tissues in a section were held in their anatomical arrangement. The results of LM investigations of 16 μm thick sections made from bermudagrass and orchardgrass (Akin, 1982) showed that the new technique gave degradation of tissues similar to that found earlier (Akin & Burdick, 1975, Hanna *et al.*, 1973). In addition to differences in degradability between cell

wall types more detailed information was obtained on cell wall substructures that were degradation barriers for rumen microorganisms.

It had already been noted that chemically resistant suberized lamellae occur in the bundle sheath of wheat, oat and maize leaves (O'Brien & Carr, 1970). In a research on suberized lamellae that occur in bundle sheath of C_4 grasses, Wilson & Hattersley (1983) suggested that this chemically inert layer could also be undegradable by rumen microorganisms. Because of its cutin-suberin-like properties this layer could be compared to the undegradable cuticle of epidermal cells. In a series of experiments on the ammoniation of cereal straws (Theander *et al.*, 1984) barley straw was investigated using LM, SEM and TEM and the section to slide technique. To investigate bacterial and cell wall surface substructures, Engels & Brice (1985) used the replica technique from TEM studies. This technique can be used to investigate cell wall substructures, e.g. cellulose microfibrils (Engels, 1974). A platinum-carbon coating was evaporated onto the surface of plant material and this layer was cleaned from adhering plant material by strong chemical agents. The replica was observed in a TEM where cell wall degradation of plant tissues and typical bacterial structures could be observed (Figure 3). Modern SEMs allow direct observation of the platinum carbon coated surface. With the new SEMs it is not necessary to practice the very complicated procedure of plant tissue disintegration with strong chemical agents to clean the platinum-carbon layer which often destroyed a great part of the breakable replica's.

The section-to-slide technique had only been used in LM sofar. Engels & Brice (1985) modified this technique for SEM applications. (Figure 4 and 5). The sample (tape and plant tissue) was critical point dried and gold coated. Small parts of the tape with tissue sample were removed and mounted on SEM stubs. A small drop of conducting paint which connected the gold layer directly to the stub surface was then

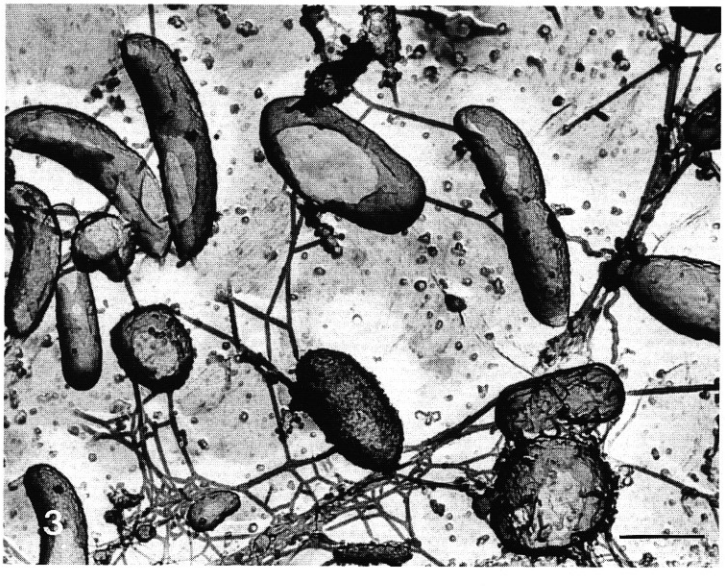


Figure 3. Replica micrograph made from a degraded barley straw section. The clean gold-carbon coat is observed in a transmission electron microscope. Different bacterial types are found and can be studied in great detail. Bar = 0.50 μm.

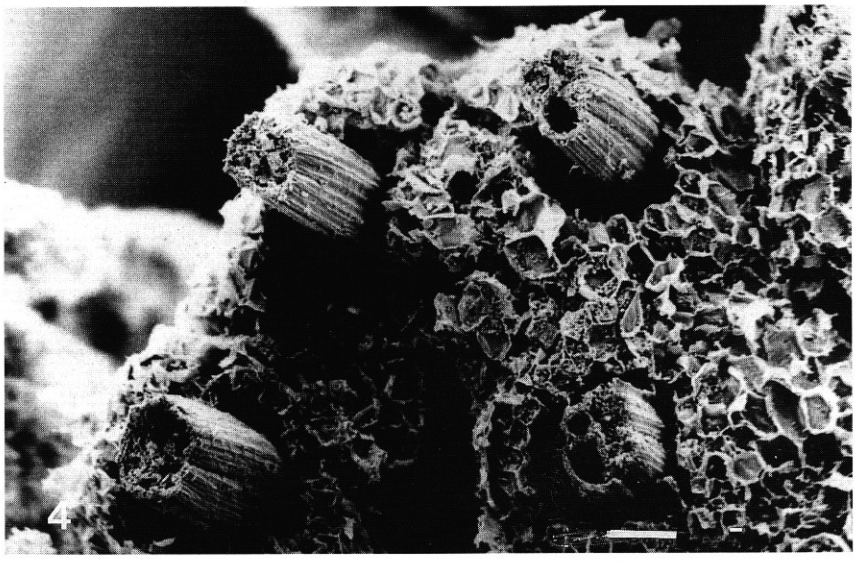


Figure 4. SEM picture of a thick section of a maize stem degraded for 48 hours. The stem parenchyma has been degraded partially and undegradable vascular bundles are left. Bar = 100.0 μm.

applied. This method combined with low and high magnifications of the SEM enabled investigations on plant tissue degradation (Figure 4 and 5) and on the level of bacterial cell wall degradation (Figure 6). Using this technique an undegradable layer was found covering the secondary cell walls in fully differentiated sclerenchyma

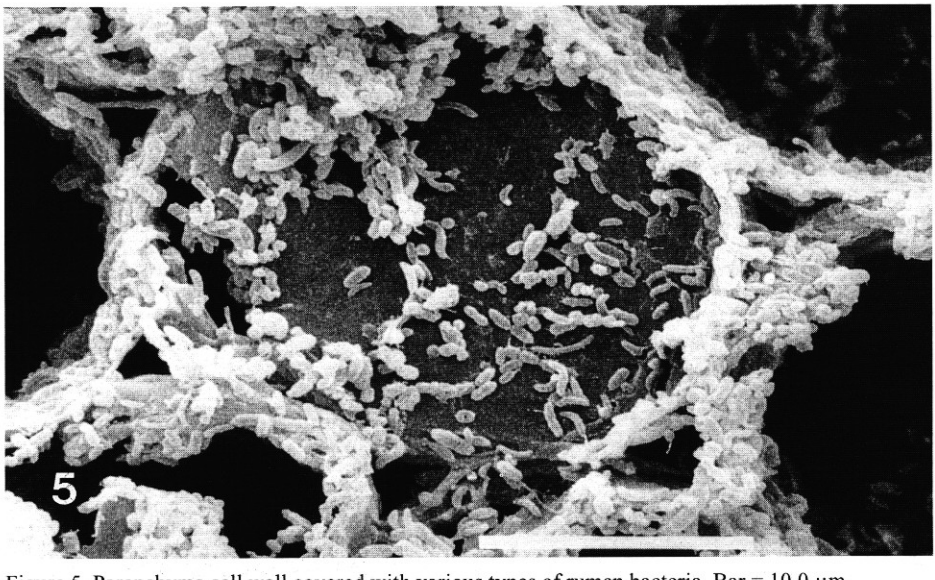


Figure 5. Parenchyma cell wall covered with various types of rumen bacteria. Bar = 10.0 μm.

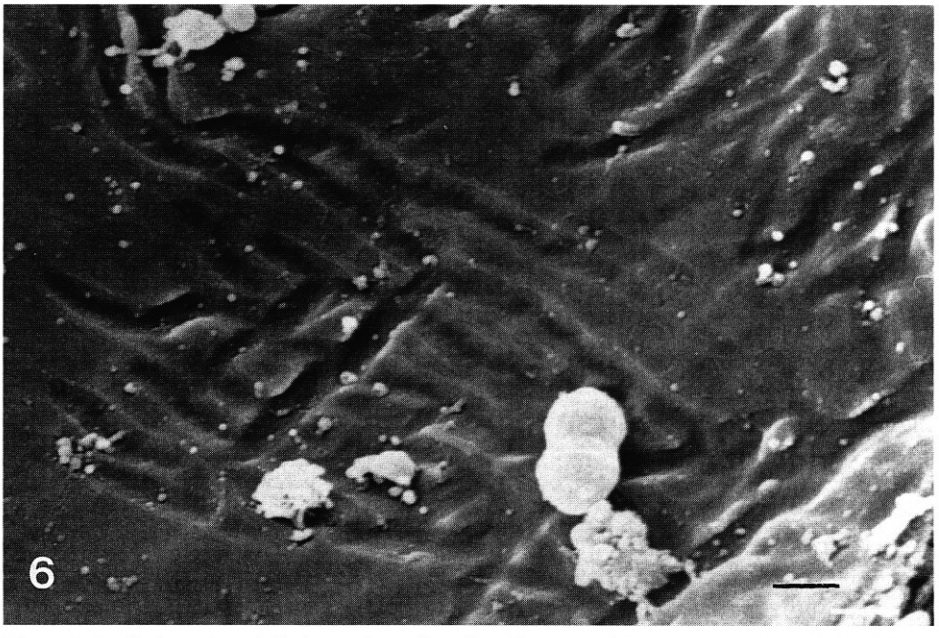


Figure 6. Typical pattern of ditches at the cell wall surface caused by enzymatic degradation of rumen bacteria. Bar = 1.0 μm.

cells (Engels & Brice, 1985). This layer, which restricted bacterial access to the secondary cell wall, was called the warty layer because of its structural appearance and its resistance to degradation by bacteria and fungi (Liese, 1963).

In a parallel series of experiments, LM and SEM were used to investigate the effect of alkali extraction on cell wall degradation and ultraviolet fluorescence in bermudagrass. Harris & Hartley (1976) had previously found UV fluorescence and the presence of ferulic acid in highly degradable grass cell walls. In bermudagrass stems stainability of the cell walls with phloroglucinol and their fluorescence at 510 nm decreased considerably after permanganate and alkali extraction and degradation of partly delignified tissues increased (Akin *et al.*, 1985). These experiments were performed with excitation at 320 nm and measurement of fluorescence at 510 nm. In maize chemical treatments of cell walls of various tissues effected different changes in the intensity of autofluorescence in the tissues (Willemse & Emons, 1991). A much better method which combined the UV-absorption microscopy with type of cell wall phenolics was developed by Hartley *et al.* (1990), Akin & Hartley (1992), and Goto *et al.* (1992). Feruloyl and *p*-coumaryl arabino-xylan isolated from bermudagrass cell walls were used to produce UV control absorption spectra. A small aperture, 1.56 μm in diameter (Akin & Hartley, 1992; Akin & Rigsby, 1992), was placed over thick (up to 3.0 μm) cell walls providing different absorption levels across the cell wall. The highest absorption was found in the middle lamella which proved to be indigestible depending on the stage of maturity of the tissue. In another

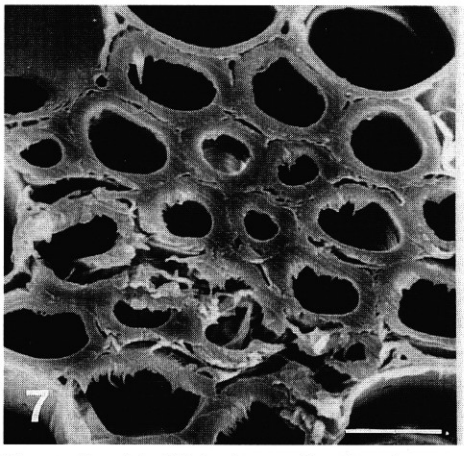
investigation Akin & Rigsby (1992) used microspectrophotometry and scanning electron microscopy during degradation of some warm and cool season grasses. They found high levels of UV absorption in sclerenchyma middle lamella-primary wall complex which proved to be undegradable even after 7 days of rumen fluid incubation. Microspectrophotometry (Akin & Hartley, 1992) allows one to correlate the amounts of coumaric and ferulic acid present in parenchyma and sclerenchyma cell walls with an estimation of the rate of their respective degradabilities. This method looks promising for investigations of aromatics, tissue maturity and digestibility. With histochemical staining reactions of cell walls known phenolic acids, esters and aldehydes and lignin extracts showed clear colour reactions with numerous lignin staining agents (e.g., Akin *et al.*, 1990). Although these reactions are specific for the separate compounds, their presence in the cell wall is still questionable because interactions with other stainable compounds cannot be excluded. Comparisons between degradation and histochemical staining suggest that histochemical staining only detects the presence of phenolic compounds in cell walls (Cone & Engels, 1990, 1993; Wilson *et al.*, 1991) and cannot be correlated with degree of degradation.

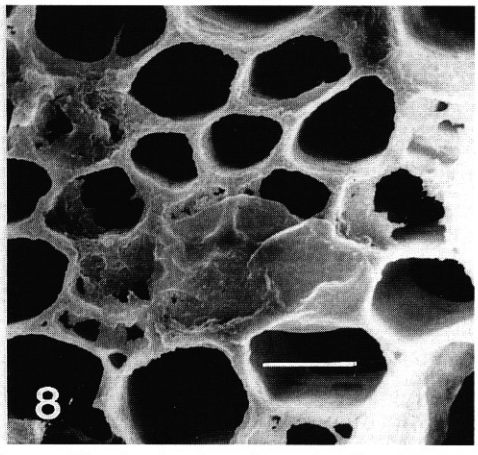
Cross sectional area measurements of tissues were used over a long period (e.g., Hanna *et al.*, 1976, Ehlke & Casler, 1985) and data have been correlated with differences in plant type and tissue degradation. However, primary and secondary cell walls were found to be degradable to different levels depending on grass species, tissue and cell wall type and stage of plant development. In sorghum the percentage of tissue types in the brown midrib mutant was similar to the normal genotype (Akin *et al.*, 1986a). However, in the brown midrib type microbial degradation was improved in some tissues (parenchyma and sclerenchyma) compared to the normal genotype. In a subsequent paper (Akin *et al.*, 1986b) permanganate delignification was followed by LM observation and phenolic compound composition was analyzed using nuclear magnetic resonance. These results indicated that brown midrib sorghum contained less phenolic acids and reduced levels of syringyl groups in the lignin. Engels & Schuurmans (1992) isolated tissues from maize stem. The dry weight was estimated and cross sectional area measurements were made. Stem parenchyma and sclerenchyma formed about 7% and 80% of the dry-weight but about 80% and 20% of the cross area, respectively. However, in cross sectional area measurements the amount of cell wall per unit area is very low in thin walled (0.2 μm) parenchyma compared to the thick walled (2–3 μm) sclerenchyma. Additionally, both tissues showed positive phloroglucinol staining and were considerably degraded by rumen microorganisms. Cross sectional area measurements of tissues are not synonymous with mass of cell walls of the tissues. A new approach could be developed if the dry weight of tissues could be related with the cross cell wall area. This needs a careful quantitative isolation and dry weight measurement of each tissue. Parenchyma and sclerenchyma tissue have been successfully isolated by Gordon *et al.* (1985) and Grabber & Jung (1991). Wilson (1993), who reviewed organization of forage plant tissues, warned that tissue proportions do not take into account intercellular spaces and the thickness and degree of lignification of cell walls. Cross sectional area measurements of tissues cannot explain small differences in digestibility observed be-

tween species and cultivars. Measuring cell wall thickness in sections with an eyepiece micrometer in several plant tissues is very time consuming and the sites of measurement are subjectively taken as representative for the cells in tissues. Travis *et al.* (1993) developed an image analysis system that automatically measured mean cell wall thickness and cell diameters in plant tissues. The results of application of this method to a series of sections made from the bottom to the top of one internode of maize showed the steady increase in cell wall thickness of epidermal, sclerenchymal and vascular cell wall types. Earlier observations (Engels, 1989) showed an increase in phloroglucinol staining from the bottom to the top of the maize internode. However, staining intensity is determined by cell wall thickness and section thickness as well. Image analysis on cell wall level provided more accurate information on the cell wall amounts of tissue before and after degradation than the cross sectional area method because cell lumina are excluded. Cell wall amounts of tissues can be compared during plant development and changes in degradation of tissues can be estimated. However, as stated by Travis *et al.* (1993) due to shrinking by chemical procedures during fixation for microscopy cell wall thickness of parenchyma is overestimated when values are compared to electron microscopic observations, but the values are in accordance with standard measurements when obtained by the light microscopic micrometer line method.

Although image analysis is a quantitative method which allows a rapid mean thickness value measurement for cell walls, it cannot be used when a cell wall (double) is formed from two cells of different tissues located next to one another, a situation which frequently occurs in stem and leaf. In stems of maize where vascular bundles just underneath the epidermis are separated by sclerified parenchyma or in the stem centre where they are separated by thin walled parenchyma, mean cell wall thickness can not be used to show clear differences in cell wall thickness and degradability. Furthermore, Cone & Engels (1990) found thin walled stem parenchyma that varied in cell wall degradation. In case of changes in cell wall degradability during cell wall development, Engels & Schuurmans (1992) stated that it is very essential to obtain data on single cell walls. For this purpose the section-to- slide technique from Akin (1982) was modified into the mirror sectioning technique (Engels, 1989). In principle two serial sections (A and B) from 100 μm are cut. The A section is turned over 180^0 before it is mounted on the double adhesive tape. The B section is fixed on the tape in the same position as it was on the cutting knife. With this technique the same cross sectioned cell wall surface can be investigated with different types of pre-treatments.

The mirror sections when treated as described by Engels & Brice (1985) can be investigated by SEM. When degradation incubation time of one section is prolonged to at least 48 hours then the degradable part of the cell wall has been removed by the rumen bacteria and bacteria leave behind a clean undegradable cell wall residue (Figures 7 and 8). Measurements of the cell wall thickness in both sections gives valuable data on degradable and undegradable part of one cell wall. Using the mirror sections and light and scanning microscopy Engels & Schuurmans (1992) found that the middle lamella was lignified first, before secondary cell wall synthesis started and that this thin specifically placed layer of lignin was responsible for the decrease





Figures 7 and 8. SEM micrographs of a mirror section set of maize. A sclerenchyma bundle with thick secondary walls (Figure 7) showed reduced cell wall thickness after rumen degradation (Figure 8). Bar = 15.0 μm.

in degradation of tissues during plant development. It was suggested that the degradation of lignified cell walls of sclerenchyma tissue could be of interest for plant breeders.

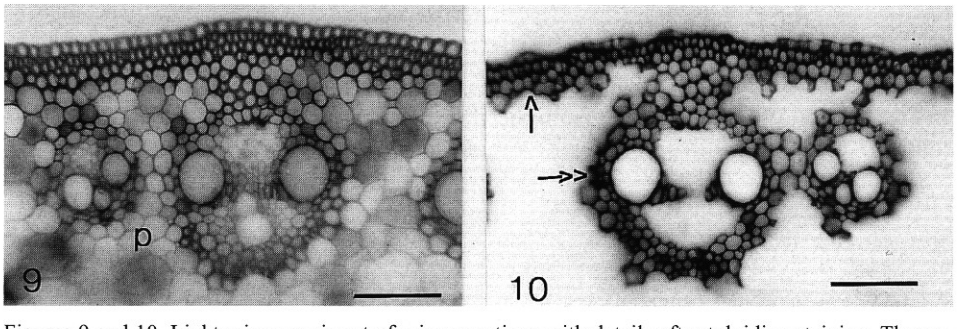
Judkins & Stobart (1988) showed that addition of enzyme preparations to the diet feed intake and time of passage were increased, meat and milk production were raised, but total degradability of the feed was not changed. The mirror sectioning technique has been used in experiments to investigate the mechanism of the increase in rate of degradation when an enzyme cocktail from fungal origin was added *in vivo* or *in vitro* to rumen fluid (F.M. Engels, unpublished). In our experiments we found that both additions advanced degradation of the parenchyma and resulted in more rapid disintegration of the tissue. It was postulated (F.M. Engels, unpublished) that the anatomical distribution of parenchyma in stem and leaf are of crucial importance. Parenchyma and sclerenchyma will be degraded (e.g., Wilson *et al.*, 1991; Engels & Schuurmans, 1992) to the same level assuming that in developing tissues, parenchyma cell walls and sclerenchyma secondary walls are not lignified and equally degradable. At the time that the parenchyma cell walls (thickness approx. 0.5 μm) are degraded completely, the sclerenchyma tissue walls are reduced in thickness with the same 0.5 μm (originally 2 μm thick). Thus sclerenchyma tissues (e.g. vascular and sclerenchyma bundle, Mulder *et al.*,1992) are set free from their surrounding parenchyma (in fact particle size reduction) and will leave the rumen. Mulder *et al.* (1992) found that the carbohydrate composition of partly degraded tissue was different from the control due to the disappearance of primary cell walls (parenchyma). In maturing tissues cell wall degradation slows down or is no longer possible as a result of lignification as could be shown for parenchyma in maize by phloroglucinol-LM and SEM observations (Cone & Engels, 1990) and in other grasses (Wilson *et al.*, 1989). The same process will occur in sclerenchyma after completion of sec-

ondary cell wall synthesis (Engels & Schuurmans, 1992; Wilson *et al.*, 1991; Wilson & Mertens, 1995; Wilson & Kennedy, 1996). This observation implies that degradation slows down, that particle size reduction will slow down and that also intake and passage will slow down consequently. Using the mirror sectioning technique and histochemical staining with toluidine blue, we found perfect mapping between degradable maize stem parenchyma and the presence of red purple stained cell walls (F.M. Engels, unpublished) (Figures 9 and 10).

In some cases when immature, partly degraded materials are used to investigate degradation events in depth, the SEM and LM preparation methods gave unsuitable results. In our case (F.M. Engels and J. Ton, unpublished) plant material (maize) was soft and tissues were separated as a result of cell wall degradation; any movement would disturb the location of the residual material. Van Doorn *et al.*, (1991) published a cryo-ultramilling procedure for SEM. In this technique the material is immediately frozen on top of a small stub. At the surface of the frozen material high speed rotating diamond knives remove a thin layer of material. Under continuous low temperature conditions the flat surface is freeze-etched and covered with a thin gold film and investigated in a SEM. If a further in depth observation is needed, the procedures can be repeated because the sample is kept at low temperature. The gold layer is milled away and a new one is sputtered on the freshly exposed freeze-etched surface located deeper in the tissue. In our experiments (Figure 11) some disadvantages were found. Freezing produced large ice crystals but this can be overcome by incubation with cryoprotectants. Observation in the SEM may result in further sublimation of ice which may deform the palladium gold layer.

Light microscopy and scanning electron microscopy have been employed in a wide range of investigations to study cell wall properties during degradation by the rumen microorganisms. Light and scanning electron microscopy should be used together. What is questionable in the LM can be elucidated by SEM/TEM and vice versa.

Developments of new techniques and methods in types of microscopy have resulted in a better understanding of the properties of plant organs, tissues and cell walls



Figures 9 and 10. Light microscopic set of mirror sections with details after toluidine staining. The undegraded mirror section (Figure 9). The degraded section (Figure 10) showed the diminished wall thickness of sclerenchyma (-->) and vascular tissue (-->>). Parenchyma cells (Figure 9: p) were completely degraded. The degradable parenchyma had a red-purple colour in the control mirror section. Bar = 100.0 μm.

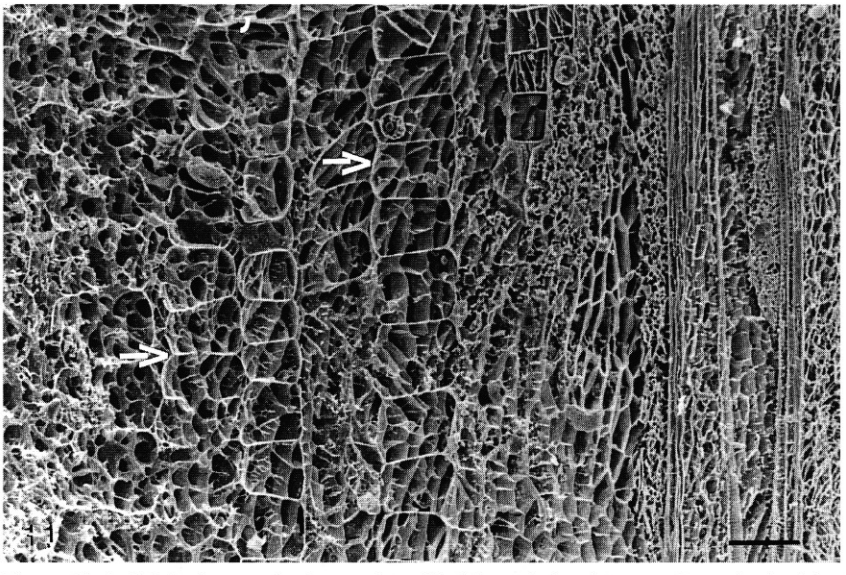


Figure 11. A SEM micrograph of cryo-ultramilled degraded maize stem. Parts (-->) of the parenchyma cell walls proved to be undegradable. Bar = 100.0 μm.

and the correlations with the possibilities of degradation of the various rumen microorganisms.

Fundamental research on biodegradability of forages will require the application of microscopes and further development of techniques to obtain a complete picture of the problems of microbial degradation of forages with the potential to improve feed value for animals.

Acknowledgements

The author gratefully acknowledges Prof. L. 't Mannetje and Prof. P.C. Struik (Department of Agronomy, Wageningen Agricultural University) for reading the manuscript and Mr K. Hulstein from the IPO-DLO, Wageningen, for technical assistance in cryo-ultramilling.

References

Akin, D.E., 1976a. Ultrastructure of rigid and lignified forage tissue degradation by a filamentous rumen microorganism. *Journal of Bacteriology* 125:1156–1162.

Akin, D.E., 1976b. Ultrastructure of rumen bacterial attachment to forage cell walls. *Applied and Environmental Microbiology* 31:562–568.

Akin, D.E., 1979. Microscopic evaluation of forage digestion by rumen microorganisms. A review. *Journal of Animal Science* 48:701–709.

Akin, D.E., 1980a. Evaluation by electron microscopy and anaerobic culture of types of rumen bacteria associated with digestion of forage cell walls. *Applied and Environmental Microbiology* 39:242–252.

Akin, D.E., 1980b. Attack on lignified grass cell walls by a facultatively anaerobic bacterium. *Applied and Environmental Microbiology* 40:809–820.

Akin, D.E., 1982. Section to slide technique for study of forage anatomy and digestion. *Crop Science* 22:444–446.

Akin, D.E. & H.E. Amos, 1979. Mode of attack on orchardgrass leaf blades by rumen protozoa. *Applied and Environmental Microbiology* 37: 332–338.

Akin, D.E. & D. Burdick, 1975. Percentage of tissue types in tropical and temperate grass leaf blades and degradation of tissues by rumen microorganisms. *Crop Science* 15:661–668.

Akin, D.E., D. Burdick & G.E. Michaels 1974. Rumen bacterial interrelationships with plant tissue during degradation revealed by transmission electron microscopy. *Applied and Environmental Microbiology* 27:1149–1156.

Akin, D.E., W.W. Hanna & L.L. Rigsby, 1986a. Normal-12 and brown midrib-12 sorghum. I. Variations in tissue digestibility. *Agronomy Journal* 78:827–832.

Akin, D.E., W.W. Hanna, M.E. Snook, D.S. Himmelsbach, F.E. Barton II & W.R. Windham, 1986b. Normal-12 and brown midrib-12 sorghum. II. Chemical variations and digestibility. *Agronomy Journal* 78:832–837.

Akin, D.E. & R.D. Hartley, 1992. UV absorption microspectrophotometry and digestibility of cell types of bermudagrass internodes at different stages of maturity. *Journal of the Science of Food and Agriculture* 59:437–447.

Akin, D.E., R.D. Hartley, W.H. Morrison III & D.S. Himmelsbach, 1990. Diazonium compounds localize grass cell wall phenolics: Relation to wall digestibility. *Crop Science* 30:985–989.

Akin, D.E. & L.L. Rigsby, 1992. Scanning electron microscopy and ultraviolet absorption microspectrophotometry of plant cell types of different biodegradabilities. *Food Structure* 11:259–271.

Akin, D.E., E.L. Robinson, F.E. Barton II & D.S. Himmelsbach, 1977. Changes with maturity in anatomy, histochemistry, chemistry, and tissue digestibility of bermudagrass plant parts. *Journal of Agricultural Food Chemistry* 25:179–186.

Akin, D.E., M.T.M. Willemse & F.E. Barton II, 1985. Histochemical reactions, autofluorescence, and rumen microbial degradation of tissues in untreated and delignified bermudagrass stems. *Crop Science* 25:901–905.

Barton, F.E. II. & D.E. Akin, 1977. Digestibility of delignified forage cell walls. *Journal of Agricultural Food Chemistry* 25:1299–1303.

Barton, F.E. II., D.E. Akin & W.R. Windham, 1981. Scanning electron microscopy of acid detergent fiber digestion by rumen microorganisms. *Journal of Agricultural Food Chemistry* 29:899–903.

Bauchop, T., 1979. Rumen anaerobic fungi of cattle and sheep. *Applied and Environmental Microbiology* 38:148–158.

Berg, B., B. Van Hofsten & G. Pettersson, 1972. Electronmicroscopic observations on the degradation of cellulose fibers by *Cellvibrio fulvus* and *Sporocytophaga myxococcoides*. *Journal of Applied Bacteriology* 35:215–219.

Brazle, F.K. & L.H. Harbers, 1977. Digestion of alfalfa hay observed by scanning electron microscopy. *Journal of Animal Science* 46:506–512.

Cheng, K.J., D.E. Akin & J.W. Costerton, 1977. Rumen bacteria: interaction with particulate dietary components and response to dietary variation. *Feed Proceedings* 36:193–197.

Cone, J.W. & F.M. Engels, 1990.The influence of ageing on cell wall composition and degradability of three maize genotypes. *Animal Feed Science and Technology 40:331–342.*

Cone, J.W. & F.M. Engels, 1993. Influence of growth temperature on anatomy and *in vitro* digestibility of maize tissues. *Journal of Agricultural Science,* Cambridge, 114:207–212.

Ehlke, N.J. & M.D. Casler, 1985. Anatomical characteristics of smooth bromegrass clones selected for *in vitro* dry matter digestibility. *Crop Science* 25:513–517.

Engels, F.M., 1974. Function of Golgi vesicles in relation to cell wall synthesis in germinating Petunia pollen: III. The ultrastructure of the tube wall. *Acta Botanica Neerlandica* 23:201–207.

Engels, F.M., 1989. Some properties of cell wall layers determining ruminant digestion. In: A. Chesson & E.R. Örskov (Eds.), Physico-chemical characterization of plant residues for industrial and feed use. Elseviers Applied Science, London, New York, pp. 80–87.

Engels, F.M. & R.E. Brice, 1985. A barrier covering lignified cell walls of barley straw that restricts access by rumen microorganisms. *Current Microbiolology* 12:217–224.

Engels, F.M. & J.L.L. Schuurmans, 1992. Relationship between structural development of cell walls and degradation of tissues in maize stems. *Journal of the Science of Food and Agriculture* 59:45–51.

Goering, H.K. & P.J. Van Soest, 1970. Forage Fibre Analysis.(Apparatus, reagents, procedures and some applications). Agricultural Handbook No. 379. United States Department of Agriculture, Washington DC, pp. 1–20.

Gordon, A.H., J.A. Lomax, K. Dalgarno & A. Chesson, 1985. Preparation and composition of mesophyll, epidermis and fibre cell walls from leaves of perenial ryegrass (*Lolium perenne*) and Italian ryegrass (*Lolium multiflorum*). *Journal of the Science of Food and Agriculture* 36:509–519.

Goto, M., K. Takabe, O. Morita & I. Abe, 1992. Ultraviolet microscopy of lignins in specific cell walls of barley straw fractions with different rumen degradability. *Animal Feed Science and Technology* 36:229–237.

Grabber, J.H. & G.A. Jung, 1991. Isolation of parenchyma and sclerenchyma cell types from the plant parts of grasses. *Crop Science* 31:838–842.

Hanna, W.W., W.H. Mons & T.P. Gaines, 1973. Histological examination of fresh forage leaves after *in vitro* digestion. *Crop Science* 13:98–102.

Hanna, W.W., W.G. Monson & G.W. Burton, 1976. Histological and *in vitro* digestion study of 1- and 4-week stem and leaves from high and low quality bermudagrass genotypes. *Agronomy Journal* 68:219–222.

Harbers, L.H., G.L. Kreitner, G.V. Davis Jr, M.A. Rasmussen & L.R. Corah, 1982. Ruminal digestion of ammonia hydroxide-treated wheat straw observed by scanning electron microscopy. *Journal of Animal Science*, 54:1309–1319.

Harbers, L.H. & M.L. Thouvenelle, 1980. Digestion of corn and sorghum silage observed by scanning electron microscopy. *Journal of Animal Science*, 50:514–526.

Harris, P.J. & R.D. Hartley, 1976. Detection of bound ferulic acid in cell walls of Gramineae by ultraviolet fluorescence microscopy. *Nature*, 259: 508–510.

Hartley, R.D., D.E. Akin, D.S. Himmelsbach & D.C. Beach, 1990. Microspectrophotometry of bermudagrass (*Cynodon dactylon*) cell walls in relation to lignification and wall biodegradability. *Journal of the Science of Food and Agriculture*, 50:179–189.

Hungate, R.E., 1966. The rumen protozoa. In: R.E. Hungate, (ed.), The rumen and its microbes. Academic Press Inc, New York, pp.91–147.

Imai, S. & K. Tsunoda, 1972. Scanning electron microscopic observations on the surface structures of ciliated protozoa in sheep rumen. *National Institute of Animal Health*, 12:74.

Judkins, M.B. & R.H. Stobart, 1988. Influence of two levels of enzyme preparation on ruminal fermentation, particulate and fluid passage and cell wall digestion in wether lambs consuming either a 10% or 25% grain diet. *Journal of Animal Science*, 66:1010–1015.

Kawamura, O., T. Senshu, M. Horiguchi & T. Matsumoto, 1973. Histochemical studies on the rumen digestion of rice straw cell wall and on the chemical determination of its non nutritive residue. *Tokohu Journal of Agricultural Research*, 24:183–191.

Kunoh, H. & S. Akai, 1977. Scanning electron microscopy and x-ray microanalysis of dumbbell-shaped bodies in rice lamina epidermis (*Oryza sativa*). *Bulletin Torrey Botanical Club* 104:309–313.

Latham, M.J., B.E. Brooker, G.L. Pettipher & P.J. Harris, 1978. *Ruminococcus flavefaciens* cell coat and adhesion to cotton cellulose and to cell walls in leaves of perennial ryegrass (*Lolium perenne*). *Applied Environmental Microbiology* 35:156–165.

Liese, W., 1963. Tertiary wall and warty layer in wood cells. *Journal of Polymer Science*, C, Polymer symposium no. 2, pp. 213–219.

McManus, W.R., C.C. Choung & V.N.E. Robinson, 1976. Studies on forage cell walls. 4. Flow and degradation of alkali-treated rice hull digesta in the ruminant digestive tract. *Journal of Agricultural Science*, Cambridge, 87:471–483.

Monson, W.G. & G.W. Burton, 1972. Effect of length of cut and leaf surface treatment on digestibility of fresh forage. *Agronomy Journal* 64:405–406

Monson, W.G., J.B. Powell & G.W. Burton, 1972. Digestion of fresh forage in rumen fluid. *Agronomy Journal* 64:231–233.

Mulder, M.M., F.M. Engels, J.L.L. Schuurmans & J.J. Boon, 1992. *In vitro* digested and potassium per-

manganate delignified maize internode sections studied by histochemistry and analytical pyrolysis mass spectrometry. *Animal Feed Science and Technology* 39:335–346.

O'Brien, T.P, & D.J. Carr, 1970. A suberized layer in the cell walls of the bundle sheath of grasses. *Australian Journal of Biological Science* 23:275–287.

Orpin, C.G., 1977a. The rumen flagellate *Piromonas communis*: Its life-history and invasion of plant material in the rumen. *Journal of General Microbiology* 99:107–117.

Orpin, C.G., 1977b. Invasion of plant tissue in the rumen by the flagellate *Neocallimastix frontalis*. *Journal of General Microbiology* 98:423–430.

Orpin, C.G. & A.J. Letcher, 1978. Some factors controlling the attachment of the rumen holotrich protozoa *Isotricha intestinalis* and *I. prostoma* to plant particles *in vitro*. *Journal of General Microbiology* 106:33–40.

Regal, V., 1960. The evaluation of the quality of pasture grasses by the microscopic method. Proceedings VIIIth International Grassland Congress, Reading U.K., pp. 522–524.

Rittenberg, J.H., R.C. Bayer & M.D. Stern, 1977. A technique for preparing ciliated rumen protozoa for scanning electron microscopy. *Journal of Animal Science* 44:710–712.

Sakurai, M., 1963. Histological studies on the decomposition of pasture grasses by livestock digestion. Grassland Division Kaoto-Tosan, Agricultural Experimental Station, Nisinasuno, Japan, Research Report No 15.

Smith, A.E., 1977. Influence of temperature on tall fescue forage quality and culm base carbohydrates. *Agronomy Journal* 69:745–747.

Spencer, R.R., D.E. Akin & L.L. Rigsby, 1984. Degradation of potassium hydroxyde-treated Coastal bermuda grass stems at two stages of maturity. *Agronomy Journal* 76:819–824.

Theander, O., R.D. Hartley & C.S. Stewart, 1984. A note on the OECD collaborative experiment on ammonia-treated straw. *Animal Feed Science and Technology* 10:89–91.

Travis, A.J., S.D. Murison SD & A. Chesson, 1993. Estimation of plant cell wall thickness and cell size by image skeletonisation. *Journal of Agricultural Science*, Cambridge, 120:279–287.

Van Doorn, W.G., F. Thiel & A. Boekestein, 1991. Examination of occlusions in xylem vessels of cut rose flowers, using cryoscanning electron microscopy and cryo-ultramilling cross sectioning. *Scanning* 13:37–40.

Ward, G.M., L.H. Harbers & J.J. Blaha, 1979. Calcium-containing crystals in alfalfa: Their fate in cattle. *Journal of Dairy Science* 62:715–722.

Willemse, M.T.M. & A.M.C. Emons, 1991. Autofluorescence and HPLC analyses of phenolics in *Zea mays* L. stem cell walls. *Acta Botanica Neerlandica* 40:115–124.

Wilson, J.R., 1993. Organization of forage plant tissues. In: H.G. Jung, D.R. Buxton, R.D. Hatfield & J. Ralph (Eds.), Forage cell wall structure and digestibility. ASA-CSSA-SSSA, 677 S. Segoe Rd., Madison. pp.1–32.

Wilson, J.R., K.L. Anderson & J.B. Hacker, 1989. Dry matter digestibility *in vitro* of leaf and stem of buffel grass *(Cenchrus ciliaris)* and related species and its relation to plant morphology and anatomy. *Australian Journal of Agricultural Research* 40:281–291.

Wilson, J.R., B. Deinum & F.M. Engels, 1991. Temperature effects on anatomy and digestibility of leaf and stem of tropical and temperate forage species. *Netherlands Journal of Agricultural Science* 39:31–48.

Wilson J.R. & P.W. Hattersley, 1983. *In vitro* digestion of bundle sheath cells in rumen fluid and its relation to the suberized lamella and C4 photosynthetic type in Panicum species. *Grass Forage Science* 38:219–223.

Wilson, J.R. & P.M. Kennedy, 1996. Plant and animal constraints to voluntary feed intake associated with fibre characteristics and particle breakdown and passage in ruminants. *Australian Journal of Agricultural Research* 47:199–225.

Wilson, J.R. & D.R. Mertens, 1995. Crop quality & Utilization: Cell wall accessibility and cell structure limitations to microbial digestion of forage. *Crop Science* 35:251–259