EFFECT OF ENSILAGE AND DIGESTION ON THE COMPONENTS OF THE CELL WALL OF PERENNIAL RYEGRASS, WHITE CLOVER AND LUCERNE

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy in the Faculty of Science

by

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DECLARATION

I hereby declare that this thesis, entitled 'Effect of ensilage and digestion on the components of the cell wall of perennial ryegrass, white clover and lucerne' is entirely my own work and has not been accepted or submitted for any degree.

Signed _

Jacqueline M. Barwick.

SUMMARY

The structure of the components of plant cell walls have been reviewed with respect to their utilisation as energy sources for ruminants. Various *in vitro* methods for assessing forage utilisation were compared, but emphasis was placed on the artificial rumen system Rusitec. The principles of silage production were reviewed and the known effects of ensilage on the plant cell wall components discussed.

The first series of experiments investigated the detailed composition of the cell wall of a variety of perennial ryegrass harvested at four stages of growth. Each stage of growth was ensiled in nylon bags in a farm scale silo and changes in the composition of the cell walls were determined.

The results showed an overall loss of total dry matter from all four stages of growth during ensilage. There were indications that the maturity of the grass could affect the losses of cellulose during fermentation, with greater loss being seen from the more mature cuts of grass. The only loss apparent from all the cuts of grass was of glucose derived from the hemicellulose. Losses of arabinose from the hemicellulose were seen in the third and fourth cuts of grass during ensilage and xylose was lost from the fourth cut during ensilage.

Apparent loss of lignin on ensilage was seen in the third and fourth cut of ryegrass. As it was in these cuts that loss of cellulose and hemicellulose was seen,

it seems likely that there was solubilization of a lignin-carbohydrate complex, which would be lost in the silage effluent.

Since one aim of the programme was to compare members of the Gramineae family with members of the Leguminosae family, a second series of experiments was carried out with white clover. The clover experiments were carried out using both laboratory scale and farm scale silos. Laboratory scale silos using perennial ryegrass had been investigated prior to the start of this programme.

Using laboratory scale silos it was found that additive-treated silages behaved differently to a water-treated (control) silage. Both a formic-acid additive and a formaldehyde additive seemed to increase the loss of cell wall carbohydrates during ensilage. Losses from the side chains of the hemicellulose were seen but no losses of lignin were found.

The clover harvested for the farm scale silos was cut at three stages of growth, and it was possible to investigate changes in cell wall composition as the clover matured as well as when it was ensiled. A steady increase in the concentration of cellulose in the dry matter of the clover as it matured was seen, with a loss of cellulose during ensilage being seen from the second and third cuts of clover. Hemicellulose levels in the dry matter varied as the plant matured, probably due to changes in the leaf:stem ratio in the maturing plant. A

loss of hemicellulose was seen from all cuts on ensilage, with greater loss being seen from the more mature cuts of clover. No loss of lignin was seen from any cut of clover on ensilage.

A final ensilage experiment was carried out on lucerne. This experiment comprised of only one sample and the ensiled material was taken directly from a farm scale silo. During ensilage losses of cellulose and lignin were seen from the lucerne, but there was no loss of hemicellulose.

Although Tilley and Terry type *in vitro* digestions were used, most emphasis was placed on the Artificial Rumen System (Rusitec) to investigate the rate of utilisation and digestion of ensiled forages. These experiments examined the total loss of dry matter as well as the loss of individual components from the ensiled cell wall. The pattern of fermentation of the ensiled forages was determined from the volatile fatty acid profiles and from the gas production, particularly methane.

The water solubility of the silages showed that the clover silage had the highest ratio of cell contents to cell wall, and it was thought that this contributed to the difficulty of maintaining a stable Rusitec system when using clover silage. The grass silage cell walls were more digestible than those of the lucerne silage and clover silage. This was reflected in the higher digestibility of the cellulose and hemicellulose from the

grass silage.

Loss of lignin was seen from all three silages after seventy two hours digestion in the Rusitec system. It was suggested that this was not true digestion, that is no breakdown products would be available to the rumen micro-organisms for metabolism, but represented the formation of a lignin-carbohydrate complex. This would be associated with the loss of hemicellulose from silages. The lignin-carbohydrate complex was thought to move into the liquid phase of the Rusitec system.

The physical properties of forages appear to be changed on ensilage and some preliminary investigations were carried out on the cation exchange capacity of both the ryegrass and legume forages and their ensiled products. Furthermore the effect of digestion on these properties was investigated.

The method used to determine water holding capacity was not sensitive enough to estimate the role of the cell wall in influencing effluent loss from the forage during ensilage. It was found that grass and grass silages had low cation exchange capacity. The legumes had a higher cation exchange capacity, with an increase seen on ensilage. It was suggested that this could prevent the rapid lowering of pH necessary for the proper preservation of silage, and the maintenance of a low pH necessary for the formation of a stable silage.

CONTENTS

CHAPTER 1

REVIEW OF THE LITERATURE

1.1 INTRODUCTION

Most of pastoral farming practice is based on feeding forages, such as grass and clover, to ruminants to produce either milk or meat. To allow this system to operate throughout the year in Britain's climate, forage grown in summer must be conserved to be fed in winter. One method of preserving forage is ensilage: storage under anaerobic conditions which allow naturally occuring microbes to ferment plant carbohydrates to organic acids, thus reducing the pH in the silo, inhibiting further fermentation and preserving the crop (Thomas and Morrison, 1982). Evidence has been found to suggest that early Egyptians made silage (Schukking, 1976). However, forage was not extensively conserved as silage until more interest was taken in the technique in the 1800s and throughout the early 1900s. Major advances were made between 1925 and 1939, including the introduction of additives to aid fermentation and rapid lowering of pH. This development and the reduction of practical problems during the 1950s and 1960s led to an increase in the ease with which it was possible to make silage on a farm scale. One advantage of ensilage is that it is now possible to produce a good quality silage consistently, irrespective of climatic conditions, whereas production of good quality hay in the U.K. is much less reliable. Reflecting this there has been a steady increase in the tonnage of silage made in recent years (Thomas and Morrison, 1982).

The changes which take place during the period of ensilage have been studied by various workers (e.g. McDonald, Henderson and MacGregor, 1968), but little attention has been paid to the changes in the cell wall fraction. These changes may affect digestibility of forage in the rumen, thus altering the production value. It is also possible that some of these changes in the cell wall may affect the water binding capacity of the forage, altering the amount of effluent produced during ensilage. The determination of cell wall composition of forage and any changes on ensilage is thus of importance, and may yield useful information for modern farming systems.

1.2 CELL WALL STRUCTURE

1.2.1 Chemical structure of cell wall components

Cell walls can conveniently be considered to be of two types, a thin primary wall and a thicker secondary wall (Albersheim, 1965). The primary cell wall is laid down by young growing cells. This primary wall is extensible, and this property allows cell expansion during growth. It has been shown that primary walls of many monocotyledon species have similar compositions (Burke, Kaufman, McNeil and Albersheim, 1974). After the cell has stopped growing, the need for extensibility no longer exists. The secondary cell wall is then laid down over the primary wall to give a stronger and more rigid structure. Composition and ultrastructure of secondary cell walls varies considerably both between different cell types and different plant species.

Cellulose, hemicellulose, pectic polysaccharides, structural proteins, lignin, phenolic and acetyl groups have been identified as the major components of cell walls, with lignin only occuring in secondary cell walls.

1.2.1.1 Cellulose

Cellulose is present in the cell walls of virtually all land plants. The amount varies between plant species and cell types, and can be influenced by growing conditions. Cotton linters are virtually pure cellulose (Van Soest and McQueen, 1973), whereas cereal endosperm cell walls contain very little cellulose (Selvendran, Stevens, O'Neill and DuPont, 1983). The cellulose molecule is identical in all plant tissue, consisting of a chain of β-1,4-

linked D-glucopyranosyl residues. The average chain length can vary greatly. For example, in the primary cell wall of the cotton plant it is 2,000 D-glucose residues long, yet values of 14,000 residues length have been reported in the secondary cell wall of the same plant. When a single chain of cellulose is examined, evidence suggests that neighbouring D-glucose residues are twisted at 180° to each other along the chain to give the preferred conformation. This gives a conformation where every second residue is similar. The more correct interpretation of cellulose structure may be to consider the disaccharride cellobiose as the primary unit of cellulose rather than D-glucose (Atalla, 1983). This is supported by cellulolysis results where cellobiose is the smallest fragment from the action of true cellulases and only further hydrolysis yields D-glucose.

The β-1,4-linked glucan chains are linear, and in the primary cell wall aggregate to form fibres, often called microfibrils, made up of about 40 chains. The glucan chains are held together by hydrogen bonding between the hydroxyl group of one sugar residue and an oxygen atom on another unit. The individual hydrogen bonds are weak, but there are a great number between individual glucan chains, giving a very strong link overall. The internal arrangement of the microfibrils has not been established with certainty, but there is evidence that in cellulose from plant sources all the glucan chains align in the same direction, with the reducing ends of all the chains at the same end of the microfibril. It has also been suggested that the glucan chains are arranged with the glucose units

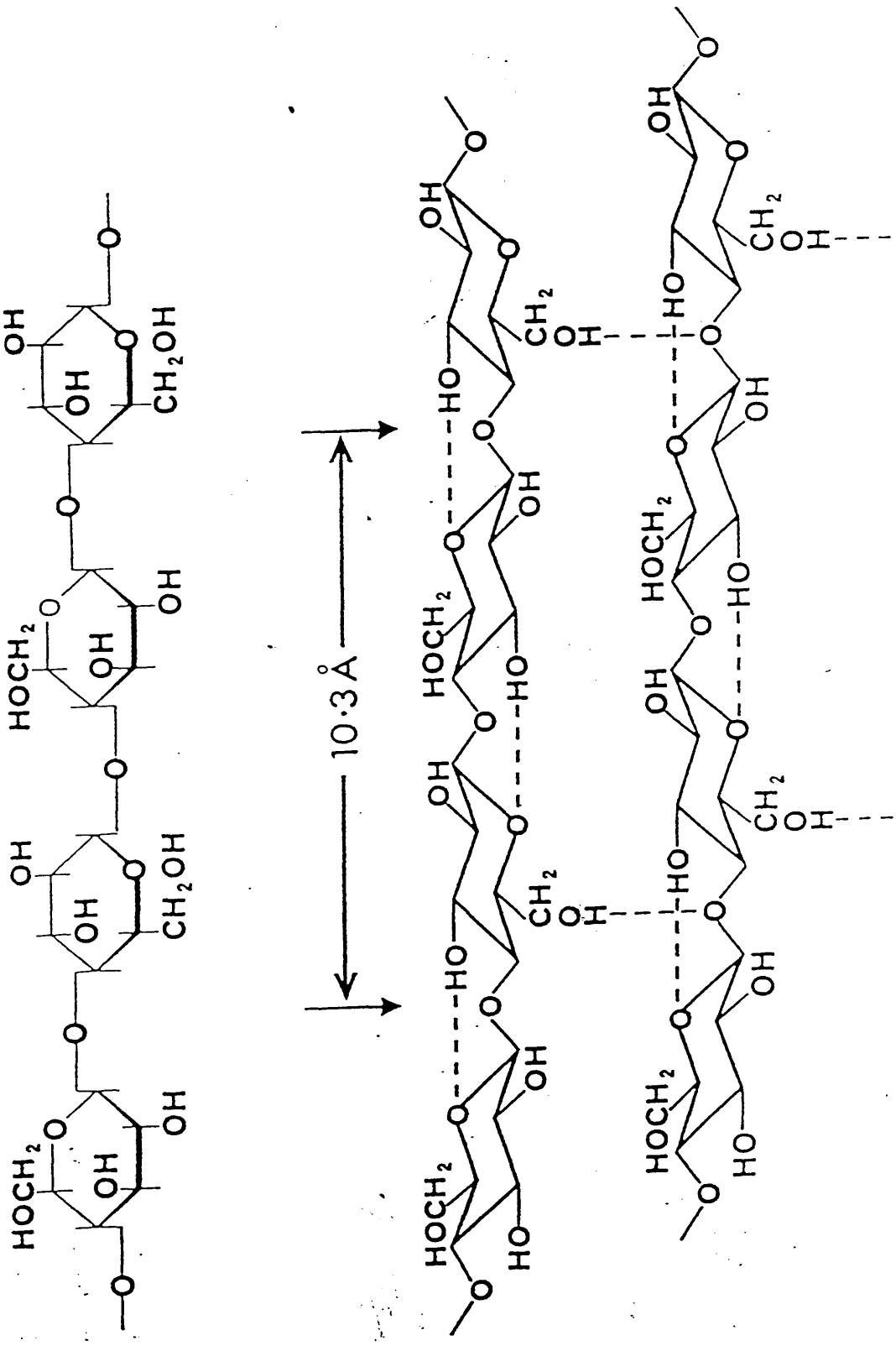


Figure 1.2.1 The structural features of the β-(1-4)-linked D-glucan chains of cellulose (from Selvendran, 1973)

staggered, each being displaced by half a length of a single unit in relation to the adjacent strand (see Selvendran, 1973) (Figure 1.2.1)

The microfibrils have crystalline and amorphous regions. The crystalline regions of the microfibrils may reach lengths of 800-1,200 Å and have a diameter of 50-100 Å; the crystalline core has a diameter of about 40 Å. Individual chain lengths are probably very much longer than this because the degree of polymerisation of cellulose ranges from 8,000 to 12,000 which corresponds to a chain length of 40,000 to 60,000 Å and a molecular weight of more than one million. Therefore, it seems probable that cellulose chains pass successively through highly ordered crystalline regions and amorphous regions of low degree of order (Selvendran, 1983) (Figure 1.2.2).

The ratio of crystalline to amorphous regions can be measured, and the proportion of cellulose in each region is probably of considerable importance in explaining differences in properties between cellulose in primary and secondary cell walls, and also between celluloses isolated from different cells (Selvendran, 1983).

1.2.1.2 Non-cellulosic polysaccharides

Considerable quantities of polysaccharides other than cellulose are found in plant cell walls. These non-cellulosic polysaccharides are traditionally known as the hemicelluloses and the pectic group. Chemical extractions are used to isolate these groups from the cell wall and the fractions can be defined by the extractions used (Blake and

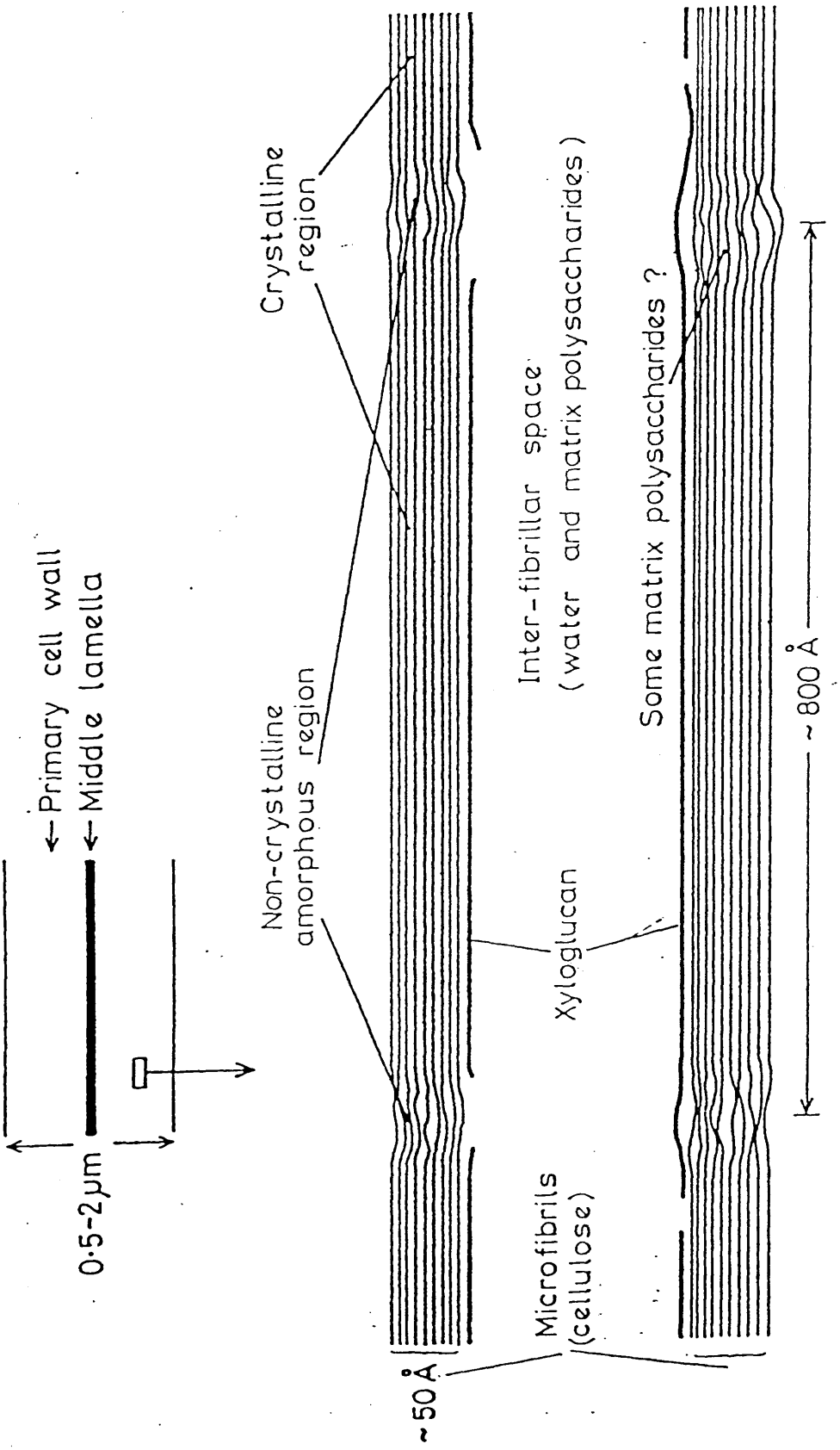


Figure 1.2.2 Diagrammatic representation of the main structural features of a primary cell wall (from Selvendran 1983)

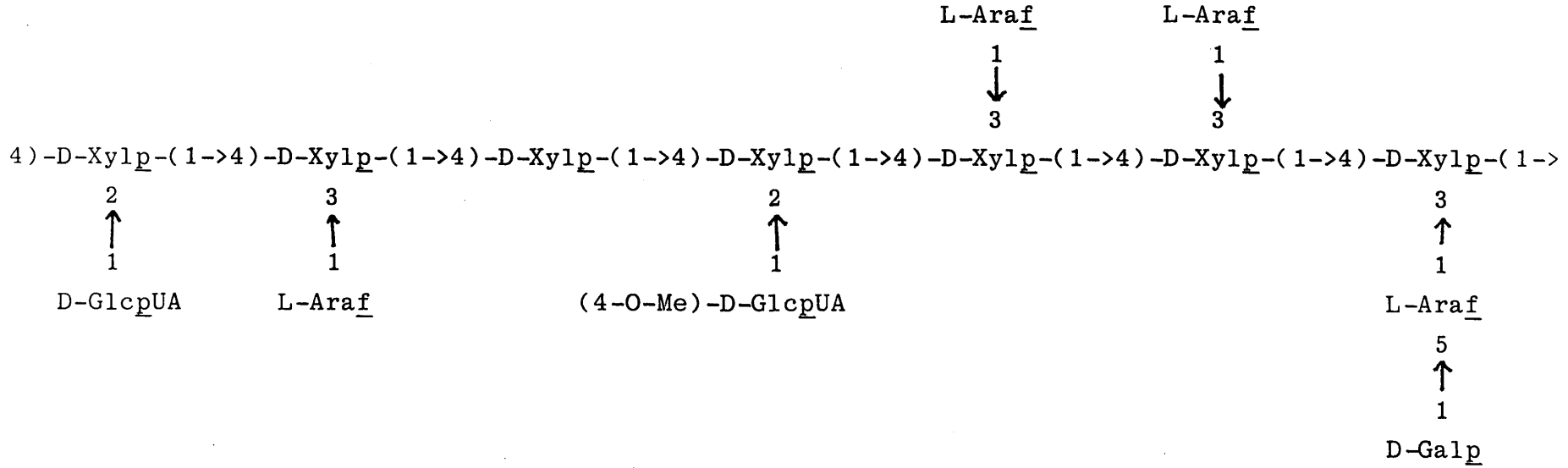


Figure 1.2.3. Structure of a Gramineae hemicellulosic xylan

Gaillard, 1971). Hemicelluloses are usually extracted using alkaline conditions. Variation in hemicelluloses between species is seen, with noticeable differences between monocotyledonous and dicotyledonous plants. In forages, this is seen as differences between the hemicelluloses of grass and of clover and lucerne. Differences are also seen between different grasses, but variation is less than between monocotyledons and dicotyledons.

Unlike cellulose, hemicelluloses are polymers containing more than one type of sugar residue. The xylans are the predominant class of hemicelluloses in grasses, and they exist as linear and branched polymers. In the latter types the branching takes the form of other sugars and uronic acids, such as D-galactose, L-arabinose and D-glucuronic acid (Akin, 1984). The major components of hemicelluloses of the Gramineae are structures based on a linear chain of β-1,4-linked D-xylopyranosyl residues, to which are attached residues of L-arabinofuranose, D-galactopyranose and 4-O-methyl-D-glucopyranosyluronic acid (Wilkie, 1979). Figure 1.2.3 indicates the structure of such a hemicellulose. It has been suggested that most of the arabinose residues appear as terminal units (Anderson and Stone, 1978) but there were indications that more complex hetero- and homo-oligosaccharides were present. Studies of bamboo, a member of the Bambusoidae sub-family of the Gramineae, suggest six possible side chains:

(a) L-arabinofuranose,

(b) O-galactopyranosyl-1,5-L-arabinofuranose,

(c) O-galactopyranosyl-1,4-O-D-xylopyranosyl-1,2-L-arabinofuranose,

(d) O-D-xylopyranosyl-1,2-O-L-arabinofuranosyl-1,2-L-arabinofuranose,

(e) O-(D-glucopyranosyluronic acid)-1,4-O-D-xylopyranosyl-1,4-galactopyranose,

(f) D-glucopyranosyluronic acid and its 4-O-methyl ether.

In addition to a linear xylan, which was found to be the major hemicellulosic polysaccharide, the presence of a non-cellulosic β-glucan and a highly branched galactoarabinoxylan have been reported in ryegrass (Morrison, 1974b). Other workers have isolated acidic arabinoxylans, non-cellulosic β-glucans (Reid and Wilkie, 1969; Buchala and Wilkie, 1973) and both branched and linear xylans (Blake and Richards, 1971) from Graminaceous sources.

The predominant hemicelluloses in dicotyledons are xyloglucans (Selvendran, 1983). These are formed from a β-D-glucan backbone identical in structure to that found in cellulose to which short chains are attached at C-6 of at least 50% of the glucose residues. There appears to be α-D-xylopyranosyl residues directly linked to glucose residues, but these side chains may be extended by the apposition of β-D-galactopyranose, L-arabinofuranose or β-L-fucopyranosyl-1,2-β-D-galactopyranose residues. (Fig. 1.2.4).

The pectic group of structural polysaccharides are the most abundant non-cellulosic components of the primary cell walls of most flowering plants, the exception being the Gramineae, which have cell walls which may contain pectins

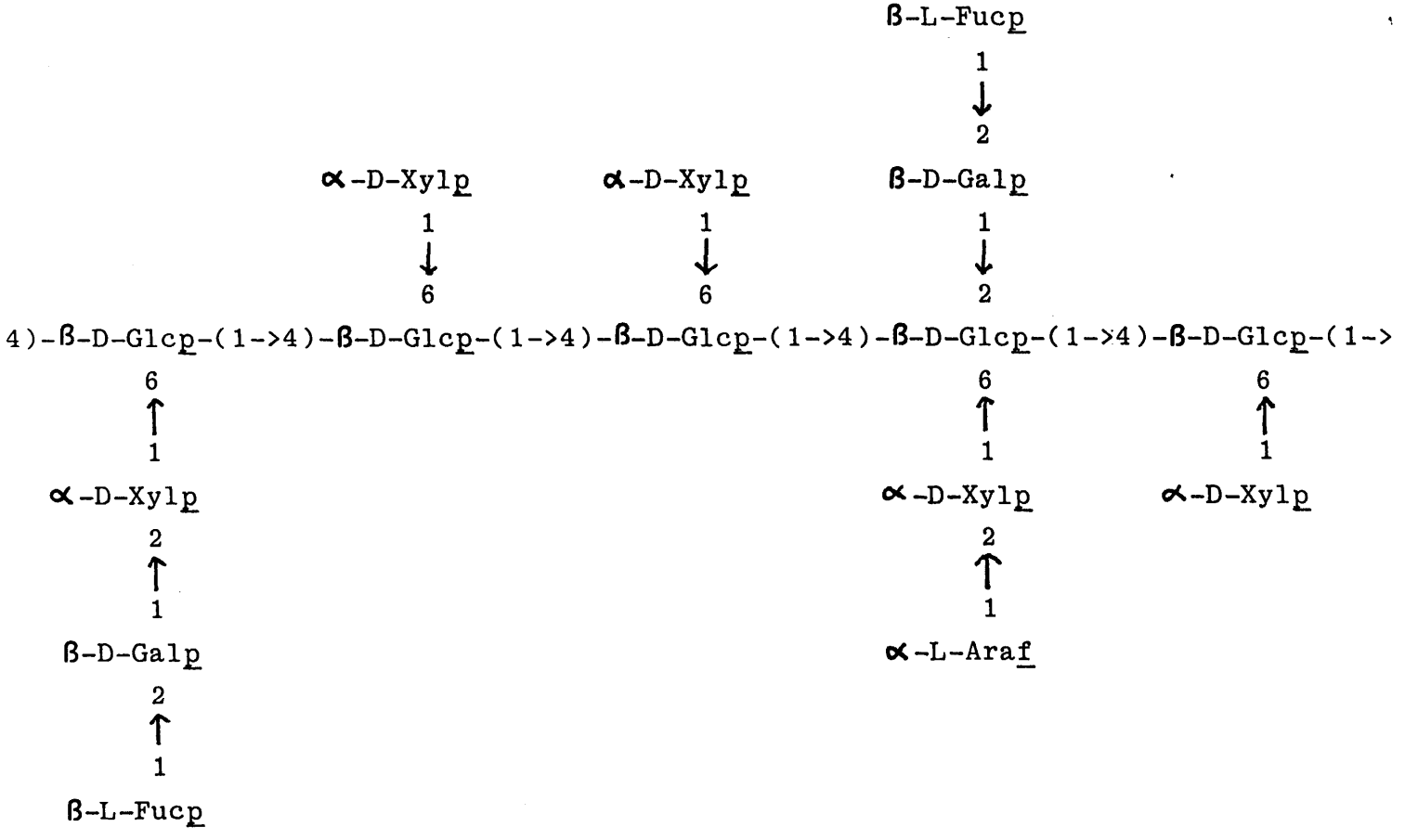


Figure 1.2.4. Structure of a typical hemicellulose from a Dicotyledon

of normal structure but in very small quantities (Jarvis, 1984). In pasture legumes, pectins have been reported to account for between 5% and 12% of the dry weight of the entire plant (Chesson and Munro, 1982). Pectins contain a number of different sugar residues and these are present in rhamnogalacturonan, homogalacturonan, arabinan, galactan and arabinogalactan chains, all of which make up the pectic complex (Hall, 1981). The rhamnogalacturonans are the backbone chains of pectins, and the structural variations superimposed on the dominant α-1,4-linked D-galacturonan chain include interruption of the chain with L-rhamnose residues, attachment of side chains at C-3 of some galacturonic acid residues and at C-4 of some rhamnose residues, varying degrees of esterification of the galacturonic acid residues or occasional acetylation of free hydroxyl groups of galacturonic acid residues (Selvendran, 1983). There are some linkages from the pectic molecules to other cell wall components. Some carry glycosidically linked xyloglucan chains which can bind non-covalently to cellulose (Keegstra, Talmadge, Bauer and Albersheim, 1973). Alkali-labile linkages, either to other pectic molecules, or to hemicellulose or protein are found (Jarvis, 1984) and it has been suggested that some of these linkages are diferulate bridges (Fry, 1983; 1984). Pectins can also form non-covalent gels, which allow them to maintain their position in the cell wall matrix (Jarvis, 1984). Calcium ions are found associated with the pectins, and play a role in the formation of such gels (Selvendran, 1983).

1.2.1.3 Lignin, phenolic monomers and acetyl groups.

Associated with the structural polysaccharides of cell walls are several groups of non-polysaccharide compounds. Of considerable importance in forage crops is the series of phenolic compounds of which lignin is the major component. Lignins are only laid down in secondary cell walls, where they are found in association with structural polysaccharides, especially the hemicelluloses (Morrison, 1973; 1974a,c). The functions of lignin in the cell wall are varied. By decreasing permeation of water across the cell wall into conducting xylem tissues, it plays an important role in the intricate internal transport of water, nutrients and metabolites. Rigidity is imparted to cell walls by lignin, and in woody parts of plants it acts as a binding agent between cells, generating a composite structure resistant towards impact, compression and bending. Lignified tissues effectively resist attack by micro-organisms and can prevent the breakdown of cell walls by fungi and other invasive pests (Sarkanen and Ludwig, 1971).

Its structure is different from other biopolymers as the molecule is globular, with many different types of linkage between the aromatic units, ether bonds being most common (Eggeling, 1983). These bonds are very stable, and resist hydrolytic cleavage (Harkin, 1973). Lignins are polymeric products arising from an enzyme-initiated dehydrogenative polymerisation of three primary precursors: trans-coniferyl, trans-sinapyl and trans-p-coumaryl alcohols.

Lignins are optically active since they contain

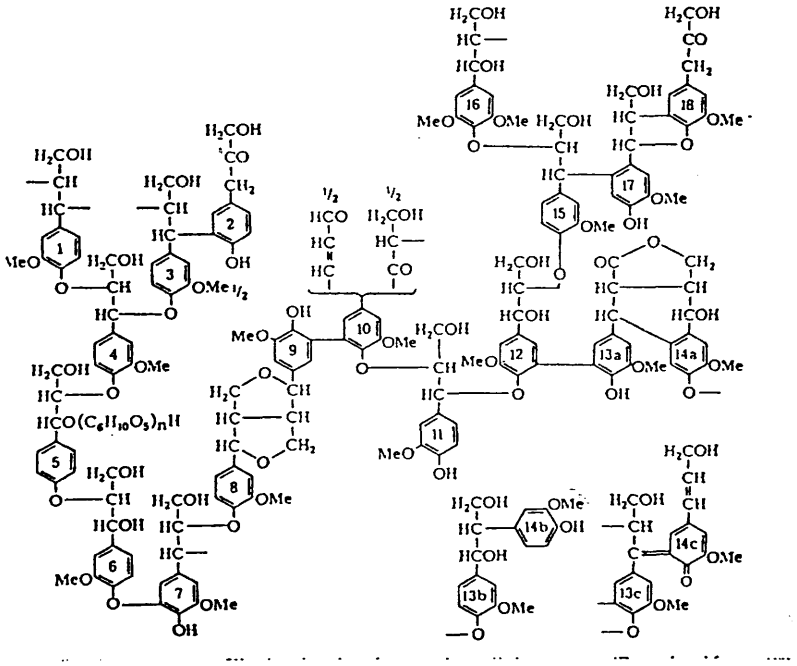


Figure 1.2.5 Typical structure of lignin, showing the prominent linkage types.

asymmetric carbon atoms. This is due to the last non-enzymatic step in lignin synthesis, where radicals of phenylpropane units couple in a random fashion to yield dilignols, trilignols and oligolignols which couple with each other and with the starting monomers. The resulting co-polycondensate is insoluble in water (Eggeling, 1983) (Figure 1.2.5).

The structure shown in Figure 1.2.5 shows lignin isolated from spruce, which is probably representative of gymnosperm wood lignin in general. No formula for a herbage lignin has yet been proposed in such detail. The structure of herbage lignin seems to be more complex than that of softwoods, although it has been suggested that grass lignins may be less polymerised than those in woods (Tanner et al. and Morrison, 1983). Some grass and herbage lignins appear to contain mostly p-coumaryl units, but this may be confused by their high content of esterified cinnamic acids (Harkin, 1973). Differences also occur between grass and lucerne lignins, with lucerne lignins being more highly cross linked than those extracted from grass (Gordon, 1975). In cell walls, lignins are found in close association with the polysaccharides (Neilson and Richards, 1978). When forages are digested, solubilization may occur and a lignin-carbohydrate complex can be recovered from the rumen (Gaillard and Richards, 1975). This work has also led to the idea that lignin measured in cell walls may actually consist of a lignin 'core' and a series of phenolic monomers in association with this core. The major phenolic monomers found are trans-ferulic and trans-p-

coumaric acids, possibly with their cis isomers, along with p-hydroxybenzoic, vanillic and diferulic acids and other unidentified compounds (Hartley and Jones, 1976; Fahey, Al-Haydari, Hinds and Short, 1980). These acids occur in all the plant parts of graminaceous species examined (Hartley, 1981) and are found in lucerne and soybean straw, but in lower concentrations (Jung, Fahey and Garst, 1983). Hartley and Jones (1977) suggested that some, and possibly all, of the ferulic acid is ester-linked to the xylan chain by its carboxyl group. These authors suggest that these phenolic acids are lignin precursors and that they are laid down during the formation of secondary walls. An alternative, or complementary, role suggested for these monomers is that they form a link between lignin and cell wall polysaccharides (Chaves, Moore, Moye and Ocumpaugh, 1982). This hypothesis is supported by observations that only trace amounts of carbohydrate esters of p-coumaric acid have been released from forage cell walls treated with cellulolytic enzymes, and the lignin-carbohydrate complex is resistant to digestion.

Acetyl groups are also found in the cell walls of graminaceous plants. Bacon and co-workers (Morris and Bacon, 1977; Bacon and Gordon, 1980) have shown that acetyl groups account for 1-2% of the cell walls of graminaceous plants. They suggest that acetyl groups may have importance in limiting digestion of the wall polysaccharides.

1.2.1.4 Glycoproteins

Since Albersheim (1965) proposed the existence of extensin in the primary cell wall of sycamore, the presence

of protein in the cell wall has been demonstrated in a wide variety of plant species, although extensin may be confined to dicotyledons. Primary cell walls of dicotyledonous species may contain 5-10% protein, which has a high concentration of L-hydroxyproline (up to 20%), L-alanine, L-serine and L-threonine. Polypeptides released from this protein by hydrolytic methods contain L-arabinosyl and D-galactosyl residues. It has been established that L-hydroxyproline residues are linked to arabino-tetrasaccharides and that D-galactosyl residues are attached to L-serine (Hall, 1981). However, work on the neutral detergent fibre (NDF) residues of mesophyll cells of grasses suggested that very little L-hydroxyproline was present (Gordon, Hay, Dinsdale and Bacon, 1977). Thus, if protein is a structural component of grass cell walls it does not have the same structure as that found in dicotyledonous plants.

In secondary cell walls there is little direct evidence to suggest that protein is a structural component. Protein associated with secondary cell wall isolates may be from enzymes associated with cell wall biosynthesis (Morrison, 1981).

1.2.1.5 Minerals

Several minerals have been found in association with plant cell wall components. Wherever pectin is found in cell wall preparations it is in association with Ca^{2+} ions (Morrison, 1981). Monosilicic acid is taken up by the roots of plants and is transported to the shoots. When water is lost in transpiration, opaline silica, ($SiO_2.nH_2O$)

is deposited in the cell wall, apparently in association with lignin (Jones and Handreck, 1967). The function of other ions in the cell wall is obscure, with no role having been elucidated for some.

1.2.2 Cell wall architecture

1.2.2.1 Primary cell walls

Most of the work on the architecture of primary cell walls has been done using suspension cultured dicotyledon cells. One model for the structure of the primary cell wall was proposed using evidence from sycamore cells (Keegstra, Talmadge, Bauer and Albersheim, 1973). This model suggests that each cellulose fibre is completely coated with a layer of xyloglucan one molecule thick. The polymeric glucan chain of the xyloglucan lies parallel to the axis of the fibre and is hydrogen bonded to the glucan chains in the fibre. At its reducing end each xyloglucan is glycosidically bonded to an arabinogalactan molecule. The D-galactose unit at the reducing end of an arabinogalactan is linked glycosidically to a rhamnogalacturonan chain. Each xyloglucan is bound only to one arabinogalactan and each arabinogalactan terminates at one rhamnogalacturonan chain. These latter chains receive numerous arabinogalactan molecules, including chains radiating from more than one cellulose fibre. Rhamnogalacturonan thus binds the fibre into a more or less rigid matrix (Albersheim, 1973) (Figure 1.2.6).

However, work by other authors on lupin hypocotyl cell walls has suggested flaws in this model (Monro, Penny and

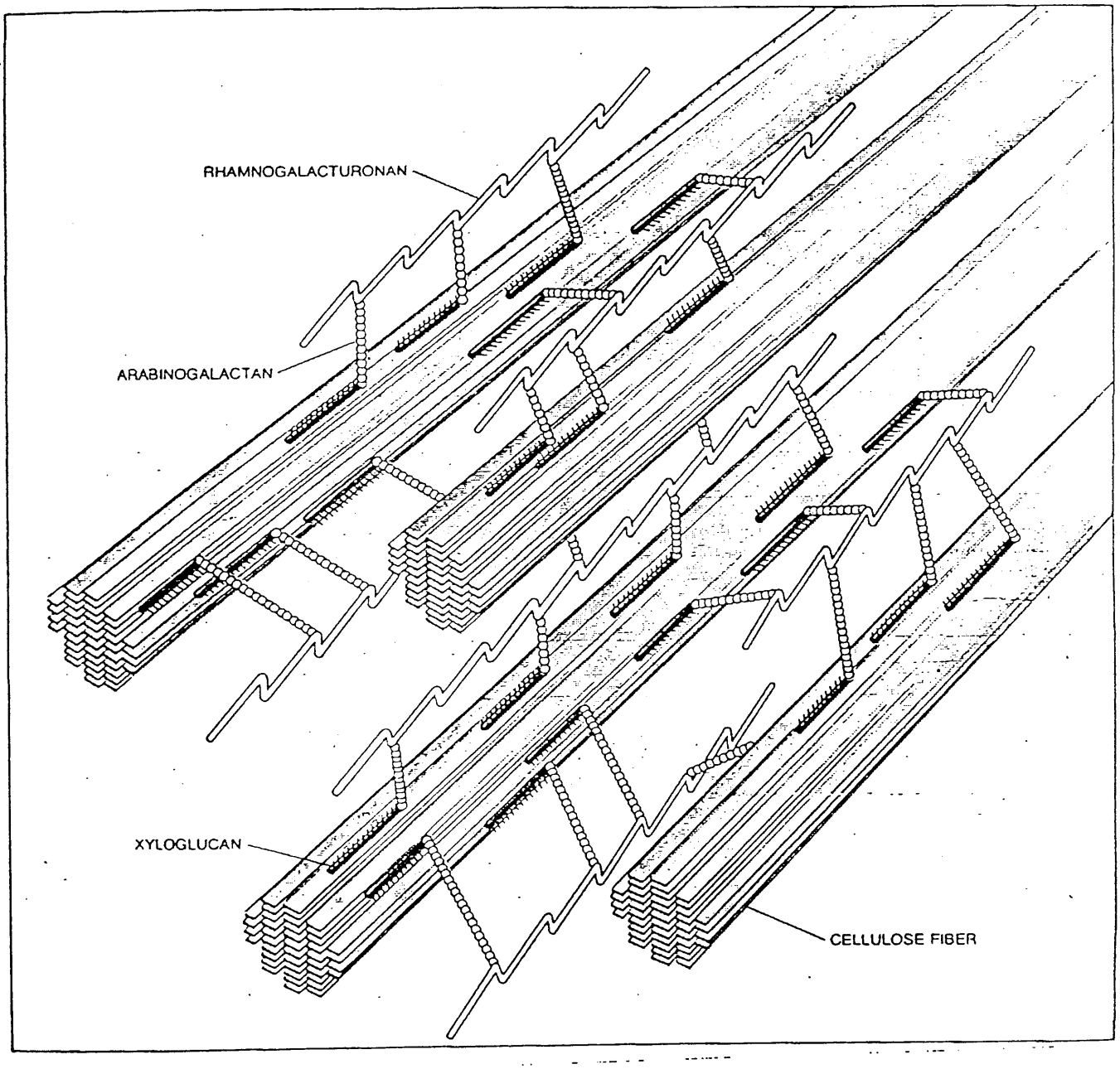


Figure 1.2.6 Model of the cell wall matrix (from Albersheim, 1973)

Bailey, 1976). From this work, a different cell wall model has been proposed. It was suggested that there is a non-covalent interaction of much of the pectin, hemicellulose and glycoprotein, and more direct interaction of the glycoprotein and cellulose microfibrils.

When suspension cultured monocotyledon cells were studied, the primary cell walls of all the species examined were similar, but the structure was quite different from that found in dicotyledons (Burke et al, 1974). Albersheim (1974) suggested that the primary cell wall of monocotyledons may be formed with the same architectural pattern, although the composition of the polysaccharides with functions analogous to those of the xyloglucan, arabinogalactan and rhamnogalacturonan of the dicotyledon model may be different. Although acknowledgement is made of the existence of protein in the cell wall, no role is postulated for this fraction in Albersheim's model.

It is thus apparent that although the composition of the chemical components of the monocotyledon primary cell wall has been studied fairly extensively, very little is known of the arrangement of these components in the wall.

1.2.2.2 Secondary cell wall

During the differentiation of the dicotyledonous secondary cell wall, cellulose, hemicelluloses and lignin are deposited, resulting in considerable thickening of the walls. Lignin is deposited in association with the microfibrillar structure of the wall and forms covalent linkages with the hemicelluloses. The cell wall polymers become a unified rigid matrix and in the process the wall becomes

stratified. From observations in woods, it seems three layers are formed, (i) a thin outer layer (ii) a thick middle layer and (iii) a thin inner layer. In the thin layers the microfibrils are arranged approximately longitudinally with respect to cell length, whereas in the middle layer they are arranged approximately transversely with respect to cell length (Selvendran, 1983).

There has been more study of the secondary cell wall structure of monocotyledons than of the primary cell wall, chiefly due to the interest in the effect of lignification on cell wall digestibility (see 1.4.3.3). The secondary cell wall contains the polysaccharides found in the primary cell wall (cellulose and hemicellulose) with the addition of lignin and other phenolic compounds. It has been established that there are linkages between lignin and cell wall polysaccharides (Morrison, 1974a). A lignin-carbohydrate complex was isolated, with the carbohydrate portion consisting of both cellulose and hemicellulose. It was suggested that phenolic acids may act as cross-linking agents between the components of the complex. Further development of this idea led to the suggestion that there were only two components in the plant cell walls; these being cellulose microfibrils which were embedded in a ligno-hemicellulosic macromolecule to which acetyl and phenolic acid groups are attached (Morrison, 1979b). The polymers of the ligno-hemicellulosic matrix may exist as heteromolecular aggregates in intimate association with each other. The lignin 'core' may form the centre of modules which are interspersed with less dense regions, the whole comprising the

ligno-hemicellulose matrix.

The cellulose microfibrils in the secondary cell walls are laid down in a series of layers. There are several theories regarding the orientation of the microfibrils between the layers. One suggestion is that between successive layers there is a small change in fibre direction. Thus in succeeding layers there is a change in orientation like the steps in a spiral staircase. This is called the helicoidal model (Neville, Gubb and Crawford, 1976). A two layer system for the secondary cell wall has also been suggested from electron microscopy work. A thin electron transparent layer on the inner surface was found below the thicker outer layer. The outer layer was seen to have a fibrillar structure (Gordon, Hay, Dinsdale and Bacon, 1977; Brice and Engels, 1984). There are thus differences in both structure and chemistry of cell walls from monocotyledons and dicotyledons which will probably affect the responses to degradation.

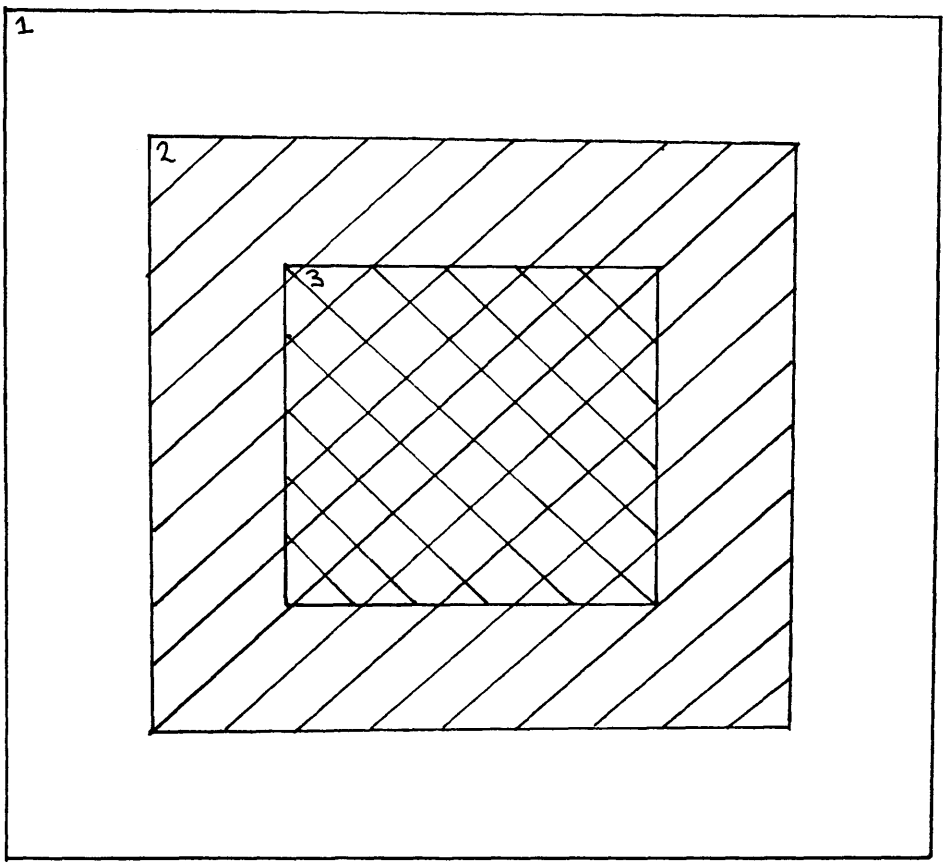
1.2.3 Physical properties of cell wall components

One set of factors often disregarded in the study of cell walls is the physical properties of the walls. These include the water holding capacity and cation exchange capacity. These have been studied in fibre fractions in the human diet (Eastwood, 1983) but may well also have relevance in forage studies, especially with regard to effluent production in the first stages of silage production.

Water has an important role in the cell wall matrix, with polysaccharides holding about 8 to 10% water at normal

humidities as water of hydration. The water content of primary cell walls is generally higher than that of secondary cell walls. The amount of water in the wall matrix is affected by the deposition of matrix polysaccharides which form close intermolecular association, thus excluding water, and deposition of a hydrophobic filler such as lignin. During secondary thickening the space occupied by water in the wall is progressively filled by the lignin-polysaccharide matrix (Selvendran et al, 1983). When a cell is immersed in water, water is distributed throughout the fibre as surface, interstitial and free water (Figure 1.2.7). Swelling of dried cells may occur to a certain extent, with water penetrating the ligno-carbohydrate complex. The water holding capacity is a measurement of the ability of the cell wall to immobilise water in its matrix (Eastwood, 1983).

The cation exchange capacity of a forage wall is an indication of its buffering capacity. This may have significance in the achievement of a low pH in the initial stages of silage fermentation, this being a requisite for satisfactory fermentation. The bonding of Cu^{2+} ions to the cell wall has been used as a measurement of cation exchange capacity (McBurney, Van Soest and Chase, 1983). The constituents of the cell wall polymers contain functional groups such as carboxyl and amino group, the former of which will bind cations. The cation binding capacity can be affected by pH surrounding the cell wall matrix, osmolality, microbial degradation of the wall, and the composition of the fibre (McBurney et al, 1983).



1. Free liquid phase.
2. Liquid associated with solid (surface water).
3. Solid containing interstitial water.

Fig. 1.2.7 Phases of water in association with fibre.

1.2.4 Factors affecting cell wall composition

The composition and structure of the plant cell wall described above gives a generalised model, with information being gained from a number of plant species grown under varying conditions. It has already been suggested that structure may vary between monocotyledons and dicotyledons. There is also evidence to suggest that within these groups differences occur between plant species, and that secondary cell wall composition can be affected by growing conditions and stage of maturity of the plant.

1.2.4.1 Species

Grasses have a higher proportion of hemicellulose and lower lignin to cellulose ratios than broad leaved plants including the legumes. These tend to be highly lignified with a low proportion of hemicellulose.

In a study of five temperate grass species, considerable differences were found in lignin concentration between the species. Lignin and hemicellulose concentrations tended to be higher in cocksfoot and timothy than in perennial ryegrass, and diploid varieties had higher lignin and hemicellulose concentrations than their tetraploid counterparts (Morrison, 1980b). Variation in lignin, cellulose and hemicellulose content of cell walls of eleven temperate grasses, all grown under similarly controlled environmental conditions were seen to vary according to species (Ford, Morrison and Wilson, 1979).

1.2.4.2 Growth conditions

Environmental temperature is dominant over light, fertilization and probably water supply on its effect on

plant composition. Temperature is positively associated with lignification, increased content of cell wall material and cellulose formation (Van Soest, 1973). In a study of the effect of temperature during the growth period on cell wall composition by Ford et al, (1979), it was found that an increase in growth temperature consistently increased the content of lignin, cellulose and hemicellulose in temperate grass species. The ratio of hemicellulose to cellulose also increased with increased growth temperature. It was suggested that cells which have undergone rapid expansion have thinner cell walls than those developed under slower growth conditions which permit more secondary thickening, containing proportionally more cellulose than hemicellulose.

1.2.4.3 Stage of maturity

It has been noted for many years that as forages mature, their digestibility declines.

Some of this change can be attributed to changes in cell wall composition with increasing maturity. Various workers have noted these seasonal trends. There is a general increase in lignin, xylan and cellulose with stage of growth, accompanied by a decrease in the amount of pectic substances (Christian, Jones and Freer, 1970). Of the cell wall carbohydrates it is the hemicellulose fraction whose digestibility declines to the greatest extent with increased maturity. When hemicelluloses were extracted from grasses of differing maturity, it was found that as maturity increased the proportion of xylose residues in the hemicellulose rose, whilst the proportion of arabinose and

galactose residues remained the same (Morrison, 1974b; 1980b). From these observations it has been suggested that the branched xylan component has a similar composition throughout growth. It is considered to be homogeneous and gradually increases in concentration throughout growth. Linear xylan, the major polysaccharide in the hemicellulose of grasses, exhibits considerable polydispersity, in that the D-xylose to L-arabinose ratio increases greatly during growth. However, as a proportion of total hemicellulose, the amount of linear xylan slowly declines during growth (Morrison, 1974b).

The lignin content of the cell walls increases with increasing maturity. This increase does not occur evenly throughout the cell types of the plant or to the same extent in every part of the plant. The stem of grasses generally has a higher lignin content than the leaves, and there is a more marked increase in lignin concentration with increased maturity in stems than in leaves (Wilman and Altimimi, 1982). Increasing lignification occurs at different rates in different plant tissues. Xylem elements and cells surrounding the major vascular bundles were found to be lignified in grasses at all stages of maturity, and lignin giving a positive reaction to phloroglucinol was found at all stages of maturity in the epidermis, sclerenchyma band and vascular bundles. An increase in lignin giving a positive reaction to chlorine-sulphite staining (syringyl-type lignin) was found in mesophyll and parenchyma cells with increased maturity, and this may contribute to the decrease in digestibility seen in more mature

grass samples (Akin, Robinson, Barton and Himmelsbach, 1977). Increased concentration of phenolic and acetyl groups in the cell walls of grasses with increased maturity has also been reported (Theander and Aman, 1981). The situation may differ in other forage plants. In lucerne and probably other legumes, the increase in lignin in the whole plant appears to be due to a drop in the ratio of leaf to stem, associated with a smaller increase in stem lignin than is found in grass (Christian, Jones and Freer, 1970).

1.3 ENSILAGE

1.3.1 Principles of silage production

Silage is the preservation of wet crops under anaerobic conditions by organic acids, principally lactic acid, which are produced by the fermentation of soluble carbohydrates (Crawshaw, 1977). Many exotic materials have been preserved in this way, including sunflowers and fish offal, but the most commonly ensiled forage in Great Britain is grass (Nash, 1978). An increasing interest is being taken in leguminous silages, as legumes have the ability to fix nitrogen, thus reducing the need for artificial fertiliser.

To produce a good silage, with a minimum loss of nutrients or depression of potential dry matter intake, anaerobic conditions must be rapidly achieved in the silo and maintained throughout fermentation. These anaerobic conditions are created in the sealed silo as respiration by the plant cells and any aerobic micro-organisms present

uses available oxygen. Anaerobic bacteria begin to multiply when oxygen levels drop and in a successful fermentation the lactate producing bacteria (both hetero- and homofermentative) become the dominant population. The end products of fermentation of the soluble sugars by these organisms, principally lactic acid, reduce pH in the silo to around 4. Fermentation then ceases, giving a stable preserved silage (Thomas and Morrison, 1982).

If these conditions do not occur, other microorganisms may become dominant. In particular, a clostridial fermentation can occur. These organisms break down soluble sugars to butyric acid, and degradation of amino acids to ammonia may also take place. This produces a highly unpalatable silage with lowered feeding value.

1.3.2 Methods of silage production

There are a number of different systems used to produce silage. The most basic method is to pack forage into the silo, seal it and allow fermentation to occur naturally. This method relies on lactic acid bacteria establishing themselves as the dominant population. This may not be the case if the pH does not drop rapidly, or if it does not fall low enough to inhibit clostridial activity. Clostridial activity is inhibited by both high hydrogen ion content and the toxic nature of the ions produced. The amount of acid necessary to inhibit clostridial action depends on the dry matter of the grass. Clostridia require high water availability and have a reduced tolerance of organic acids and hydrogen ions at high osmotic pressures (Whittenbury, McDonald and Bryan-

Jones, 1967). Leguminous plants have high buffering capacity and this increases the amount of acid necessary to lower the pH to a level at which clostridial fermentation is inhibited.

To reduce water availability in the forage, it can be wilted in the field before ensilage. Production of consistently high quality silages has been achieved when grass was ensiled at around 250g.kg^{-1} DM (Castle, 1982).

An alternative method of inhibiting undesirable fermentation is to apply an additive to the grass at ensilage. These can be applied to wilted or unwilted silages. Additives can be grouped according to their mode of action:

(i) Stimulant type additive - increases the rate of formation of a stable silage.

(ii) Acid additives - rapidly lower the pH to inhibit undesirable fermentation.

(iii) Preservative type additives - prevent fermentation and act as a preserving agent.

The stimulant type additive most widely used in the U.K. in recent years is molasses. Addition of molasses will increase the amount of water soluble carbohydrate available to the bacteria, allowing rapid establishment of a lactic type fermentation (Anderson and Jackson, 1970b). Inocula of lactic acid producing bacteria have been tried as a stimulant to fermentation. It was thought that an initial increase in the population of these bacteria would give a more rapid establishment of fermentation and lowering of pH. In practice, it seems a build up of lactic acid

producing bacteria occurs on the machinery used to harvest the crop, and this provides a sufficient inoculum (McDonald and Whittenbury, 1973).

The action of acids added to ensiled grass depends on both the nature of the grass and the type of acid used. Hydrochloric, sulphuric, orthophosphoric and glycollic acids appear to have no specific antimicrobial action, but act by reducing pH of the crop to a level at which clostridial organisms cannot grow (Woolford, 1978). Other acids have an antimicrobial action and will either partially or totally inhibit fermentation. Among these, hexanoic, acrylic and sulphonic acids all appear to have antimicrobial action (Woolford, 1978 ; McDonald and Whittenbury, 1974). The action of formic acid on silage is dependant on both the concentration of acid and the dry matter of the grass (Henderson and McDonald, 1976). On unwilted grass, when applied at the commercially recommended level, it does not appear to totally inhibit fermentation (Barry, 1976), and Wilson and Wilkins (1973) found that stable silages made using formic acid as an additive contained lactic acid, although at lower levels than in silages made without an additive. Addition of formic acid will lower the initial pH, and may preserve WSC during the period between harvest and ensilage (Henderson and McDonald, 1971). At high dry matter levels and high application rates, formic acid will give a preserved type silage, with high residual WSC levels (Carpintero, Henderson and McDonald, 1979).

The use of formaldehyde to produce a preserved type

silage has also been investigated (Barry and Fennessy, 1972). Formaldehyde appears to have an initial bacteriocidal effect, which decreases with time as the formaldehyde breaks down (Petersen, Olsen and Gutterman, 1973). This can lead to an unstable silage, with a high pH and later fermentation by clostridial bacteria. When formaldehyde was applied with formic acid, fermentation was more inhibited than when formaldehyde was applied alone, producing a preserved silage (Valentine and Brown, 1973).

1.3.3 Changes in forage composition during ensilage

During the process of ensilage, changes in the composition of the forage occur due to the action of lactic acid producing micro-organisms. The water soluble carbohydrates present are fermented by the homofermentative bacteria to produce lactic acid and by heterofermentative bacteria to produce lactic acid, ethanol, acetic acid, mannitol and carbon dioxide (McDonald, Greenhalgh and Edwards, 1973). Also present in grasses are the organic acids citrate and malate. These acids form an important part of the buffering system in plants, but are broken down by both homo- and hetero-fermentative lactic acid type bacteria to give various products, including acetic acid, acetoin, lactic acid and formic acid (Whittenbury *et al*, 1967). The overall effect of these reactions and the production of lactate from water soluble carbohydrates is to give an apparent increase in buffering capacity during ensilage.

Very little work has been done on the fate of cell wall components on ensilage. Plant hemicellulases act on hemicellulose to give D-xylose, L-arabinose and D-galactose

(Dewar, McDonald and Whittenbury, 1973). These sugars can be detected in small amounts in silages (Henderson and McDonald, 1971; Anderson and Jackson 1970b). In work with ryegrass ensiled in laboratory scale silos, Morrison (1979a) found that the amount of core lignin remained unchanged while cellulose content only decreased by up to 5%. Large losses of acetyl residues and alkali-labile phenolic acids were observed in all the silages and losses of 10-20% of hemicelluloses were found. The losses of hemicellulose were not uniform, with the L-arabinose side chains being preferentially cleaved relative to the D-xylose main chain. He suggested that part of the loss of carbohydrate could have been caused by the acidic conditions in the silage, as well as by microbial activity.

1.4 FORAGE UTILISATION

1.4.1 Ruminant digestion: rumen function

The ruminants are a group of large ungulates, which have evolved a complex stomach system to allow them to utilise the low quality forages which form a large part of their diet. The fore stomach is greatly enlarged, to give three compartments, the rumen, the reticulum and the omasum. The fourth compartment, the abomasum, corresponds to the simple stomach of non-ruminants.

The rumen and reticulum are often considered together as a single unit called the reticulo-rumen. This has a large volume (about 100 litres in mature cows) and contains a complex population of bacteria, protozoa and fungi. Bacteria number about 10^9 - 10^{10} ml^{-1} of rumen contents and protozoa about 10^6 ml^{-1} of rumen contents (McDonald *et*

al, 1973). The breakdown of food in the rumen is by both chemical and physical means. Food is diluted with very large amounts of saliva during mastication and passes into the rumen, where it can be considered as three phases:
(i) lower liquid layer in which the finer food particles are suspended, containing a large proportion of the microbial population,
(ii) an intermediate fibrous layer, floating on (i), containing large food masses and the microbial fraction closely associated with them,
(iii) a gas phase above (i) and (ii), containing mainly carbon dioxide and methane, produced by microbial fermentation of the feed.

Excess gas is removed by eructation two or three times per five minutes (Demeyer, 1981). The contents of the rumen are continually mixed by contractions of the rumen wall. During rumination, material at the anterior end of the rumen is drawn into the oesophagus and returned to the mouth. The coarse material is thoroughly chewed before being swallowed again. Chemical breakdown of food in the reticulo-rumen is by enzymes produced by the microbial population. These enzymes hydrolyse and breakdown many constituents of the food, including the cell wall polysaccharides. The main products of this fermentation are volatile fatty acids, methane, carbon dioxide and an increased microbial biomass. Many soluble products of fermentation are absorbed into the bloodstream through the rumen wall.

The rumen contents, including the undigested portion

of the forages and microbial matter, pass through the omasum, where the water content is reduced, and enter the abomasum. Here further breakdown of the digesta occurs by enzymes secreted by the abomasum wall (McDonald et al, 1973).

1.4.2 Methods of studying rumen digestion

1.4.2.1 In vivo

The complexity of the microbial ecology of the rumen and the variety of end products makes study of digestion in the rumen difficult. Basic data on overall digestiblity of a single forage can be obtained by feeding trials with accurate measurement of intake and faecal output. This method has many limitations. The number of animals required to give statistically significant results is large, trials take a great deal of time and the apparatus needed to restrain the animals is cumbersome. Digestibility of grazed forages cannot be determined since intake cannot be measured accurately (Prins, Cline-Theil and Van'T Klooster, 1981). To alleviate the difficulty in quantitative collection of the faeces, marker or indicator substances can be included in the diet. For a substance to be suitable for use as a marker in nutritional studies, it should be inert and not influence digestion or the normal microflora of the gut, and must not be either absorbed or metabolised in the alimentary tract. It should mix easily with the usual food without adding excessive bulk and remain uniformly distributed in the digesta. Finally, it should be easy to measure quantitatively throughout the digestive process

(Kotb and Luckey, 1972). These criteria make it very difficult to select a suitable marker for digestibility studies. An internal marker, such as a plant constituent would be most convenient. Lignin is often regarded as being indigestible and thus would seem to be a good marker, but there is now evidence that lignin may be made soluble as a lignin-carbohydrate complex in the rumen (Gaillard and Richards, 1975). Assay of lignin may also present problems. Fahey and Jung (1983) suggested that lignin should only be used as a marker where it can be shown that faecal recovery is high.

Surgical techniques can give access to the rumen or abomasum through fistulation or cannulation. Suspension of feedstuffs in Dacron bags in the rumen through fistula can give information on the degradation of these feedstuffs. This method was first described by Quin, Van der Wath and Myburg (1938), but requires the maintenance of fistulated animals, and a specialized unit for this purpose. There are a number of factors inherent in this technique which may affect the results, and its indiscriminate use could lead to serious errors in the interpretation of data (Lindberg and Varvikko, 1982). Thus the study of digestion *in vivo* is often a difficult procedure, and it is for this reason that *in vitro* techniques have been developed.

1.4.2.2 *In Vitro*

Many methods have been proposed for the study of digestion *in vitro*. The system adopted will be determined by the type of work to be performed and the facilities available. Each of the main areas of work will require

different systems. Routine analysis needs a simple method where numerous incubations can be performed. Speculative work (e.g. investigation of a particular biochemical reaction and the effect of changing certain parameters) will require more complicated apparatus. Simulation of the rumen will demand an artificial rumen system, which can maintain a state similar to the rumen for a certain length of time.

To determine digestibility on a routine basis the system developed by Tilley and Terry (1963), with or without modifications, is most often used. In this system, dried forage is incubated anaerobically with rumen liquor in a glass tube for forty eight hours, to simulate rumen digestion, followed by digestion with acid pepsin, simulating digestion in the abomasum and small intestine. This method gives good estimation of digestibility, especially if results are related to _in vivo_ digestibility results. A further refinement replaces incubation with rumen fluid with an incubation using a fungal cellulase (Jones and Hayward, 1975). This gives a rapid, consistent and precise method of predicting digestibility, and removes the need for donor animals to provide rumen fluid. A system of this type obviously has a place to play in routine digestibility determination.

The need for a suitable long term artificial rumen system was identified following a series of experiments investigating rumen metabolism (Czerkawski, Christie, Breckenridge and Hunter, 1975). A system was developed where reaction vessels contained both a solid phase and a

liquid phase in physical contact with each other, but not homogeneous. The system can be agitated slowly and the reaction vessels are gas tight, allowing any gas produced to be collected quantitatively. In addition, the apparatus is robust and reliable, allowing fermentation for long periods with the minimum of maintenance to the hardware, and is relatively simple to construct. The RUmen SImulation TEChnique (RUSITEC) was developed by Czerkawski and Breckenridge (1977). As food is added once each day, diurnal variation of concentration of metabolites is found. However, if a steady state is defined as when daily output of products of fermentation does not change significantly between days, this can be obtained within four to six days from the beginning of an experiment. The effect of changing certain parameters or adding supplements to the system can then be studied with a degree of control that is not possible *in vivo*.

1.4.3 *Ruminal degradation of cell wall components*

1.4.3.1 *Rumen micro-organisms*

From work done using both *in vivo* and *in vitro* observations of the breakdown of cell wall components, the microbial and biochemical processes involved in this degradation can be suggested. Of the major types of micro-organisms present in the rumen (bacteria and protozoa), the bacteria have been found to be the major source of enzymes for the degradation of cell walls. The major cellulolytic species in the rumen are *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Bacter oides succinogenes* and possibly

Butyrivibrio fibrisolvens. Many cellulolytic species also produce enzymes active against other carbohydrates in plant fibre, and all but B.succinogenes are capable of utilising hemicellulose components of forages. Many non-cellulolytic rumen bacteria degrade xylans and pectins. Synergistic interactions between cellulolytic and non-cellulolytic species have been shown to enhance degradation (Akin and Barton, 1983).

Protozoa also have a role in cell wall degradation. The number of protozoa in the rumen is lower than the number of bacteria, but their greater size means they may form 50% of the microbial mass (Baldwin and Allison, 1983). Both large and smaller entodiniomorph protozoa have been shown to be cellulolytic (Demeyer, 1981) and Epidinium have been seen to degrade mesophyll and portions of the parenchyma bundle sheath (Amos and Akin, 1978). Protozoa can only maintain themselves in the rumen when a matrix for sequestration is present, with attachment to plant particles providing such a matrix (Demeyer, 1981). When ruminants are defaunated overall fibre digestibility is decreased. This may be due to both the direct breakdown of fibre by protozoa and their effect on bacterial population as it has been suggested that there may be an increase in the number of cellulolytic bacteria in the rumen when protozoa are present.

Synergism may occur between rumen bacteria and protozoa. It has been suggested that ciliate protozoa participate directly in cellulose degradation when the diet has a low cellulose content (15% of DM), and when the diet

has a higher cellulose content (30% of DM) improvement of digestion following an inoculation of ciliates into the rumen is probably due to a protozoal and bacterial synergistic effect (Jouany and Senaud, 1979).

Recent research has shown that fungi exist in the rumen population (Orpin, 1977) and that they can colonize forage fragments in the rumen (Bauchop, 1981). Plant material entering the rumen is rapidly colonized by flagellated zoospores which attach and produce hyphae which then grow and penetrate deeply into the plant tissues. The major route of fungal invasion is via areas of damaged epidermis. The extent of colonization and growth by fungi on fibrous plant fragments indicates that they may play a significant part in cellulose digestion in the rumen. The ability of fungi to penetrate deeply into tissues not normally accessible to bacteria may suggest that anaerobic fungi have a role in increasing the digestibility of these tissues.

1.4.3.2 Mechanisms of cell wall degradation.

Electron microscopy of plant material has shown that a significant number of bacteria adhere to the cell walls during the degradation process (Akin and Barton, 1983), although rumen bacteria appeared to degrade the mesophyll, and in some cases phloem, of fescue without prior attachment to the walls. The physical association between plant material and rumen bacterial cells during degradation apparently varies with tissue types. Bacterial attachment seems to be required prior to degradation of more resistant

tissue types (Akin and Amos, 1975). Ciliate protozoa have been observed to ingest particles of tissue from lucerne stem, indicating that microbial breakdown may occur at larger scale than molecular breakdown (Bauchop, 1979).

The bacteria in the rumen degrade plant cell walls by producing extracellular carbohydrases. These can either be released from the bacteria attached to the cell walls and act in the immediate area surrounding the bacteria, or can be released into the phase surrounding the bacteria and act on structural carbohydrates some way from the bacteria. Cellulose hydrolysis by B.succinogenes was studied by Groleau and Forsberg (1981), who identified it as an endo-β-1,4-D-glucanase and a β-1,4-D-glucosidase (cellobiase). It was found that more than 80% of the endoglucanase was released into the culture fluid from B.succinogenes cells, even though the cells appeared to be intact. In the gram positive rumen bacterium Clostridium thermocellum 95% of the cellulase is secreted into the medium, while Cytophaga possess only cytoplasmic, periplasmic or membrane bound endoglucanases. Between these extremes there are gram negative bacteria (including R.flavefaciens) in which the proportion of bound glucanase depends on the organism and the state of growth (Forsberg, Beveridge and Hellstrom, 1981). The celluloytic enzymes have different actions. Endo-β-1,4-D-glucanase acts by random cleavage anywhere along the straight β-1,4-D-glucopyranoside chain. Non-glycosidic cross links in cellulose derivatives will inhibit its digestive action (Harkin, 1973). Exo-β-1,4-D-glucanases digest the cellulose by removing cellobiose

units from the non-reducing end of the polymer, and these units are split to two glucose molecules by a β-1,4-D-glucosidase (cellobiase) (Harkin, 1973).

Other polysaccharides behave in the same way as cellulase. Morrison (1975c) isolated an enzymatic complex which was capable of randomly hydrolysing xylan chains at the bond between 2 D-xylose residues providing these residues were not substituted by L-arabinose residues. Exo-xylanases, which cleave the main xylan chain in sequence have been identified (Harkin, 1973). Isolation of bacteria with hemicellulase activity indicated that the organisms had the facility to utilise fully the polysaccharide components of the plant cell wall. Strains of bacteria with α-L-arabinofuranosidase activity also had activity against β-D-galactopyranoside, β-D-fucopyranoside and β-D-xylopyranoside. This activity may reflect non-specific activity of an enzyme with a wide substrate specificity (Williams and Withers, 1981).

It has been suggested that cellulolytic bacteria produce a constitutive extra-cellular enzyme that can degrade or solubilize isolated hemicellulose. This enzyme shows a similarity to cellulase enzymes and degradation may be due to a non specific cleavage of the β-1,4-D-xylosidic linkage of the hemicellulose (Dehority, 1973).

Pectinolytic activity is found in many species of rumen bacteria and protozoa, including B.succinogenes, Bacteroides ruminocola, B.fibrisolvens, Streptococcus bovis, Lachnospira multiparus, ruminal spirochetes and several genera of protozoa (Baldwin and Allison, 1983).

Certain strains of bacteria were also found with the ability to degrade, but not to utilise, pectins (Gradel and Dehority, 1972). It has been shown that pectic substances from red clover and lucerne cell walls were degraded by rumen micro-organisms at rates in excess of those shown for other structural polysaccharides (Chesson and Munro, 1983).

There are many reports that lignin is indigestible in the rumen (e.g. Crampton and Maynard, 1938), yet more recent work has suggested that positive digestibility values may be obtained for lignins (Fahey, McLaren and Williams, 1979). Porter and Singleton (1971) suggested that up to 10% of lignin may disappear from the alimentary tract of sheep. Chemical analysis indicated that demethoxylation of lignin took place. It was concluded that the site of this action was the stomach, as the chemical composition and quantity of lignin passing through the duodenum closely matched that of the faecal lignin. From this and other studies it seems that the main site of lignin digestibility is the rumen. Gaillard and Richards (1975) isolated a soluble lignin carbohydrate complex from the rumen, and suggested that the formation of these complexes by the action of rumen micro-organisms may account for the dissolution of about half the total lignin intake. The complex was not significantly affected by further digestion in the rumen after passing into solution, was found to precipitate at the low pH of the abomasum and was present in the solid fraction of the faeces (Neilson and Richards, 1978). Allinson and Osbourne (1970) noted that changes in the lignin fraction of sainfoin, as well as lucerne, occurred

in the rumen. It appeared that simple non-conjugated phenolic units in the lignin fraction were digested or modified in some way in the rumen, although conjugated phenolics did not appear to be affected. Rumen digestion of lignin appears to be partially a result of microbial activity, as Akin (1980) has reported the isolation from the rumen of a bacterium which degraded lignified vascular tissue.

There is an apparent digestion of phenolic monomers from plant cell walls in the rumen. Rumen micro-organisms reduced the ferulic acid content of timothy grass cell walls to a greater extent than the p-coumaric acid content (Theander, Uden and Aman, 1981), and similar results were found for lucerne and smooth bromegrass hay (Jung, Fahey and Garst, 1983). There are several possible explanations for the apparent digestibility of phenolic monomers and lignin. In vitro work has shown that phenolic monomers can be fermented as a source of energy by rumen microbes, although the growth rate was very slow (Akin, 1980). Production of soluble glycosides of ferulic acid after cellulase treatment of grass cell walls occurs (Hartley, Jones and Wood, 1976). Negative digestion coefficients are more difficult to explain, but may represent increased extractability of phenolic monomers from plant material due to physical or chemical modifications during the digestive process, or a precipitation of soluble phenolics during passage through the gut (Jung, Fahey and Merchen, 1983). Free phenolic monomers may also bind to low molecular weight materials such as metabolic products of carbohydrate

and protein digestion, the complexes assaying gravimetrically as lignin in faecal residues, giving negative digestion coefficients (Fahey and Jung, 1983).

1.4.3.3 Factors affecting cell wall degradation in the rumen

From the complex nature of the processes involved in the breakdown of cell walls in the rumen, it can be seen that many factors may influence this breakdown. Cell wall structure and composition, plant species and the type of plant tissue are all inter-related factors affecting breakdown.

In grasses the digestibility of cell wall carbohydrates declines with increasing maturity, characterized by an increase in the lignin content of the wall. In lucerne, lower digestibilities were associated with higher stem contents and higher proportions of lignified xylem cells in the stems (Akin and Barton, 1983).

The rate of digestion of cellulose seems to be related to the amount of lignin in the wall, with cellulose digestion decreasing as the ratio of lignin to cellulose increases (Van Soest, 1973). The presence of lignin may prevent the entry of water and swelling of the plant fibre to a condition suitable for penetration by the microbial polysaccharidases. Covalent links between hemicellulose and lignin may explain why hemicellulose digestion falls at a greater rate than cellulose digestibility (Demeyer, 1981). In a study of pangola grass (Digitaria decumbens), Ford (1978) found the apparent digestibility of the individual monosaccharides in grass varied considerably. In

the hemicellulose of untreated plant samples, the L-arabinose residues were digested to a greater extent than the D-xylose residues. However, after delignification, D-xylose residues showed a greater relative increase in digestibility, suggesting the lignin had a greater protective effect on the D-xylose units than on the L-arabinose units. The removal of buffer soluble phenolic material appeared to increase the in vitro digestibility of lucerne (Jung and Fahey, 1981). The effect of lignin on xylanases has been studied using wood as a model, where the presence of a cross link from xylan through a glucuronic acid derivative to lignin has been shown to inhibit the enzyme's ability to break down the xylan. This 'obstructive' effect will leave the area of xylan close to the linkage undigested as shown in Figure 1.4.1 (Harkin, 1973).

The binding of phenolic compounds other than lignin (e.g. p-coumaric, ferulic and diferulic acids) to the cell wall carbohydrates is also thought to limit microbial degradation. When added to in vitro systems, p-coumaric acid will inhibit the degradation of cellulose and intact cell walls by micro-organisms and interferes with the growth of protozoa and bacteria (Akin and Barton, 1983). Similar results have been found with pure cultures of rumen bacteria and phenolic compounds, but microbial transformation of these potential toxins occurs (Chesson, Stewart and Wallace, 1982).

The cell wall structure depends on the species of forage and the lignin content influences digestibility, with more heavily lignified varieties of grass generally

Figure 1.4.1 Obstructive effect of lignin on xylanase breakdown of xylan.

having a lower digestibility. However, at equal concentrations of lignin, legumes have higher digestibilities than grasses. Legume lignins thus appear to have a smaller effect on the digestion of hemicellulose and cellulose than do grass lignins (Gordon, 1975). A more rapid disappearance of cell wall constituents and cellulose from lucerne than from grasses during the early stages of fermentation occurs, but fermentation of the grasses continues over a longer period (Van Soest, 1973). The initial composition of cell walls of rye, lucerne, orchardgrass and timothy varied widely, but after 72 hours of digestion the composition of the indigestible residues was more similar (Smith, Goering, Waldo and Gordon, 1971). These authors suggested that the degree of crystallinity of the structural polysaccharides of legumes was different to that of grasses.

Different micro-organisms tend to adhere to different cell types within the plant. In a comparison of B.succinogenes and R.flavefaciens, both were found to have a preference for cut or damaged plant cell walls, but distinct differences were found between the two bacterial types in affinity to plant cell wall types. A predominance of B.succinogenes was found on mesophyll walls, with R.flavefaciens preferring cut edges of epidermal cell walls. Both species colonized lignified sclerenchyma cell wall and partially digested it (Latham, Brooker and Pettifer, 1978). A general pattern for ease and extent of tissue degradation by rumen micro-organisms has been suggested to be:

mesophyll, phloem epidermis, parenchyma bundle sheath sclerenchyma lignified wood tissues (non degradable) (Akin, 1979).

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS AND SOLVENTS

Chemicals and solvents were purchased from BDH Chemicals Ltd., Poole, England with the exception of those listed below.

Diethyl ether, methanol, acetone, chloroform, hydrochloric acid (37% w/v) and sulphuric acid (98% w/v): May and Baker, Dagenham, England.

Ammonium sulphate, sodium chloride, cupric nitrate, potassium dihydrogen orthophosphate, sodium sulphate, hydroxyammonium chloride, D-glucose, D-galactose, barium carbonate, sodium hydroxide, propan-2-ol, acetic acid: Fisons, Loughborough, England.

Casein, cuprizon, L-arabinose, D-xylose: Sigma Chemicals, Poole, England.

Acetic anhydride: Aldrich Chemicals, Gillingham, England.

2.2 FORAGE ANALYSIS

2.2.1 Preparation and ensilage of forage samples

2.2.1.1 Grass samples

Experiment 1

Samples of perennial ryegrass (*Lolium perenne* cv S23) were harvested using hand shears, from a field reserved for silage making, at four stages of primary growth during the early summer of 1976. The dates of harvest were 25th May, 10th June, 1st July and 1st August. Each sample (approx. 5 kg) was divided into five equal subfractions. One fraction

was weighed, deep-frozen, lyophilized, reweighed and milled to pass a 1.0 mm screen. The other four fractions were weighed into nylon bags of pore size ca. 0.5 mm x 1.0 mm. The bags were stored at -20°C. The field was cut for silage using a drum mower and after wilting for 24 hours picked up using a precision chop forage harvester and formic acid (ADD-F) added at the rate of 2.25 $l T^{-1}$. The bagged samples of grass were tied together in groups of four, with one bag from each stage of growth in each group, and the four groups placed in the silo as it was being filled and consolidated. The groups of bags were arranged as shown in Figure 2.2.1. When the bags were recovered, as the silage was being used for feeding trials, they were weighed, deep frozen and lyophilized and reweighed. After reweighing the samples were milled to pass through a 1.0 mm screen and stored at -20°C.

2.2.1.2 Clover samples and silages

Experiment 2

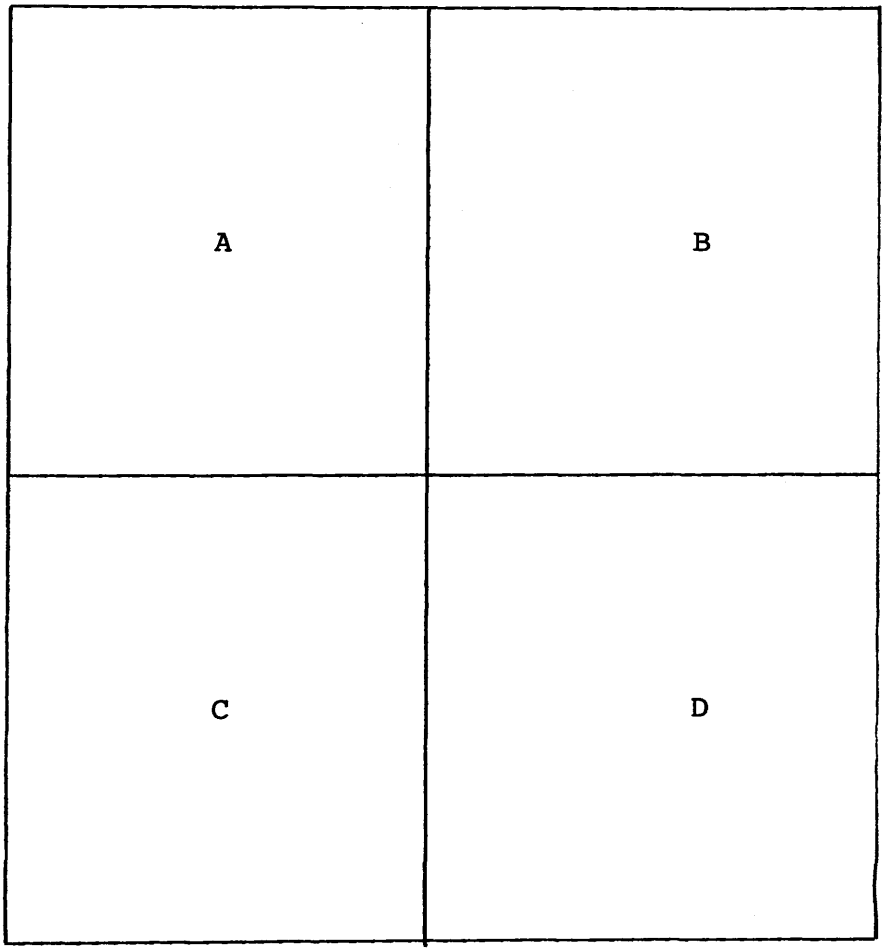
A pure sward of white clover (Trifolium repens var Blanca) was cut with a drum mower and wilted for 48 hours in sunny conditions. Approximately 5 kg of this material was collected and divided into four subfractions. One fraction was immediately deep frozen, and subsequently lyophilized and milled to pass through a 1.0 mm screen. Three treatments were imposed on the remaining fractions:

(i) 1M formic acid at 50 ml kg^{-1}

(ii) 1M formaldehyde at 50 ml kg^{-1}

(iii) Distilled water at 50 ml kg^{-1}

REAR OF SILO



FRONT OF SILO

Figure 2.2.1 Position of bags in ryegrass silage silo.

The treatments were applied using a Shandon Laboratory Spray Gun (Shandon Southern Products Ltd, Cheshire, England). For each of the three treatments eight pre-weighed plastic bottles (nominal volume 250 ml) were packed with material to give airtight conditions. The bottles were reweighed and stored at 25°C. Two bottles from each treatment were removed after two, twenty, sixty and one hundred and fifty days. These were weighed, frozen, lyophilized and milled to pass through a 1.0 mm screen before storage at -20°C.

Experiment 3

White clover (Trifolium repens var Blanca) was harvested at three stages of primary growth. Hand shears were used to collect approximately 4 kg at each harvest. The dates of harvest were 7th May, 19th May and 1st June 1984. Each harvest was divided into four subfractions. One subfraction was immediately deep frozen and subsequently lyophilized and milled to pass through a 1.0 mm screen. The other fractions were weighed into nylon bags as in Experiment 1, and deep frozen until filling of a farm-scale silo with white clover began. The bags were then tied into three groups each containing one bag from each harvest and placed in the silo as it was being filled and consolidated. The bulk clover being used to fill the silo had been cut, wilted for 48 hours and lifted using a precision chop forage harvester and treated with formic acid (ADD-F) at 2.25 $l T^{-1}$.

2.2.1.3 Lucerne and lucerne silage

Experiment 4

Lucerne (Medicago sativa var Europe) was cut using a drum mower, wilted for 24 hours, and picked up using a precision chop forage harvester and treated with formic acid (ADD-F) at 2.25 lT^{-1}. Samples were taken from each forage wagon as it arrived at the silage pit, bulked, oven dried at 100°C and milled to pass through a 1 mm screen. A subsample of this material was used for analyses. Lucerne silage was taken directly from the silo, samples being collected at random points along a fresh silage face. This material was immediately deep frozen and subsequently lyophilized and milled to pass through a 2 mm screen.

2.2.1.4 Rusitec Samples

Silages were taken from random points of a fresh face of farm scale silos. Three types of silage were collected; ryegrass, white clover and lucerne silage. The ryegrass used was made from perennial ryegrass (cv S23), wilted for 48 hours and treated with formic acid (ADD-F) at 2.25 lT^{-1}. The white clover silage was from the silo into which the bags in Experiment 3 were placed. The lucerne silage was taken from the silo sampled in Experiment 4. The samples were deep frozen immediately after collection and subsequently lyophilized. The grass silage was milled to pass through a 1 mm screen, the white clover silage and the lucerne silage were milled to pass through a 2 mm screen.

2.2.2 Preparation and Hydrolysis of Cell Wall Fractions

2.2.2.1 Solvent Extraction

Preweighed, milled forage samples were extracted continuously with diethyl ether in a soxhlet extractor until no colour was seen in the ether surrounding the thimble. The ether extract was discarded and the residue air-dried overnight in a fume cupboard. The residues were then re-extracted in the soxhlet extractor with 95% aqueous ethanol until the ethanol around the thimble was no longer coloured. The ethanol extract was retained for estimation of water soluble carbohydrate composition (2.4.2.1). The forage residue was air-dried in a fume cupboard until no detectable ethanol remained and then oven-dried overnight at 105°C and weighed.

2.2.2.2 Preparation of cellulose fraction

The method used was adapted from Crampton and Maynard (1938). A sample of forage (1 g) was weighed into a pre-weighed glass centrifuge tube and 80% acetic acid (15 ml) and concentrated nitric acid (1.5 ml) added. The tube was heated in a boiling water bath for twenty minutes, removed, and 20 ml of 95% aqueous ethanol added. After centrifugation at 6,000 rpm for ten minutes, the supernatant was discarded and the residue washed repeatedly with ethanol and diethyl ether before oven drying at 105°C. The residue left in the tube was weighed and taken as corresponding to the cellulose fraction of the cell wall. This fraction was not ashed as it was retained for further analysis.

2.2.2.3 Hydrolysis of Polysaccharides

For analysis of the cell wall polysaccharides of the grass and grass silages of Experiment 1 and of the residues from digestion in Rusitec unextracted milled samples were used. For Experiments 2 and 3, and analysis of forage samples before digestion in Rusitec, residues after solvent extraction were used. Total cell wall polysaccharides were hydrolysed by the two stage technique using 72% and 1 M sulphuric acid originally described by Saeman, Moore, Mitchell and Millet (1954) and adapted by Sloneker (1971). About 30 mg of sample was dispersed in 1.0 ml of 72% sulphuric acid at room temperature for one hour with the aid of a glass rod in a wide necked McCartney bottle. 11.5 ml of distilled water and 1 ml of internal standard (myo-inositol; 2 mg ml^{-1}) was added to the sample and the mixture sealed and heated at 105°C overnight, or at 121°C for one hour. The hydrolysed sample was neutralized with barium carbonate, filtered through glass fibre filter paper (Whatman GF/A) and traces of Ba^{2+} ions removed by the addition of a small amount of cation exchange resin (Dowex 50W X8 H^{+}). The solution was removed to a Quickfit B24 test tube and evaporated to dryness using a rotary evaporator with the water bath maintained at 40°C.

Hemicellulosic polysaccharides were hydrolysed with 2.0 M trifluoroacetic acid (TFA) as described by Albersheim, Nevins, English and Karr (1967). The sample (10 - 20 mg) was weighed into a Teflon capped glass vial and 4 M TFA (1 ml) and myoinositol standard (1 ml; 2 mg ml^{-1}) were added. The vial and contents were heated overnight at

105°C or at 121°C for one hour. The vials were centrifuged in a bench centrifuge for five minutes at 2,000 rpm, and the supernatant removed to a Quickfit B14 test tube, and evaporated to dryness using a rotary evaporator with the water bath maintained at 40°C.

2.2.3 Determination of non-carbohydrate cell wall components

2.2.3.1 Lignin analysis

Lignin was determined by a modification of the method described by Morrison (1972a;b). In Experiment 1, milled samples were washed prior to analysis to remove cell contents. 20 - 30 mg of sample was weighed into a Quickfit B14 test tube and 15 ml of water added. The test tube was then heated at 70°C for thirty minutes. The contents of the tube were filtered through glass fibre filter paper (Whatman GF/A;2.5 cm) and washed consecutively with water, ethanol, acetone and diethyl ether until no colour was seen in the filtrate in each case. The filter paper and washed sample were transferred to a screw top vial left unsealed and heated gently in a heating block in a fume cupboard to remove any remaining diethyl ether.

In subsequent experiments either residues after extraction or residues from Rusitec digestion were used, and these did not require pretreatment to remove cell contents. In this case 20-30 mg of sample was weighed directly into the screw capped vial.

To determine lignin, 1 ml of 25% acetyl bromide in acetic acid was added to the vial and it was sealed with a

cap containing a Teflon lined septum. The vial was placed in a heating block at 70°C for 30 minutes and shaken at 5 minute intervals. A reagent blank was run at the same time. A 25 ml volumetric flask containing 0.45 ml of 2M sodium hydroxide and 5 ml acetic acid was prepared and after cooling the contents of the vial were transferred quantitatively to this flask using acetic acid. The volume was then made up to about 20 ml using acetic acid and 0.8 ml of 0.5M aqueous hydroxylammonium chloride was added before making up to the mark with acetic acid.

Optical density was read at 280 nm using 10 mm silica cells against a water blank. Absorbance was determined from the equation:

$$A = \frac{\text{O.D. sample - O.D. blank}}{\text{concentration D.M. (g } l^{-1})}$$

For grasses and grass silages lignin (%DM) was calculated as:

Lignin (%DM) = 3.36A - 1.11

For legumes and legume silages, lignin was calculated as:

Lignin (%DM) = 5.12A - 0.72

2.2.3.2 Esterified Phenolic acid determination

Residues from solvent extraction (100 mg) were extracted by stirring overnight with 0.5M sodium hydroxide (5 ml). The mixture was centrifuged in a bench centrifuge at 2,000 rpm for 20 minutes and a 2.0 ml aliquot removed to a stoppered test tube. This was neutralised by addition of

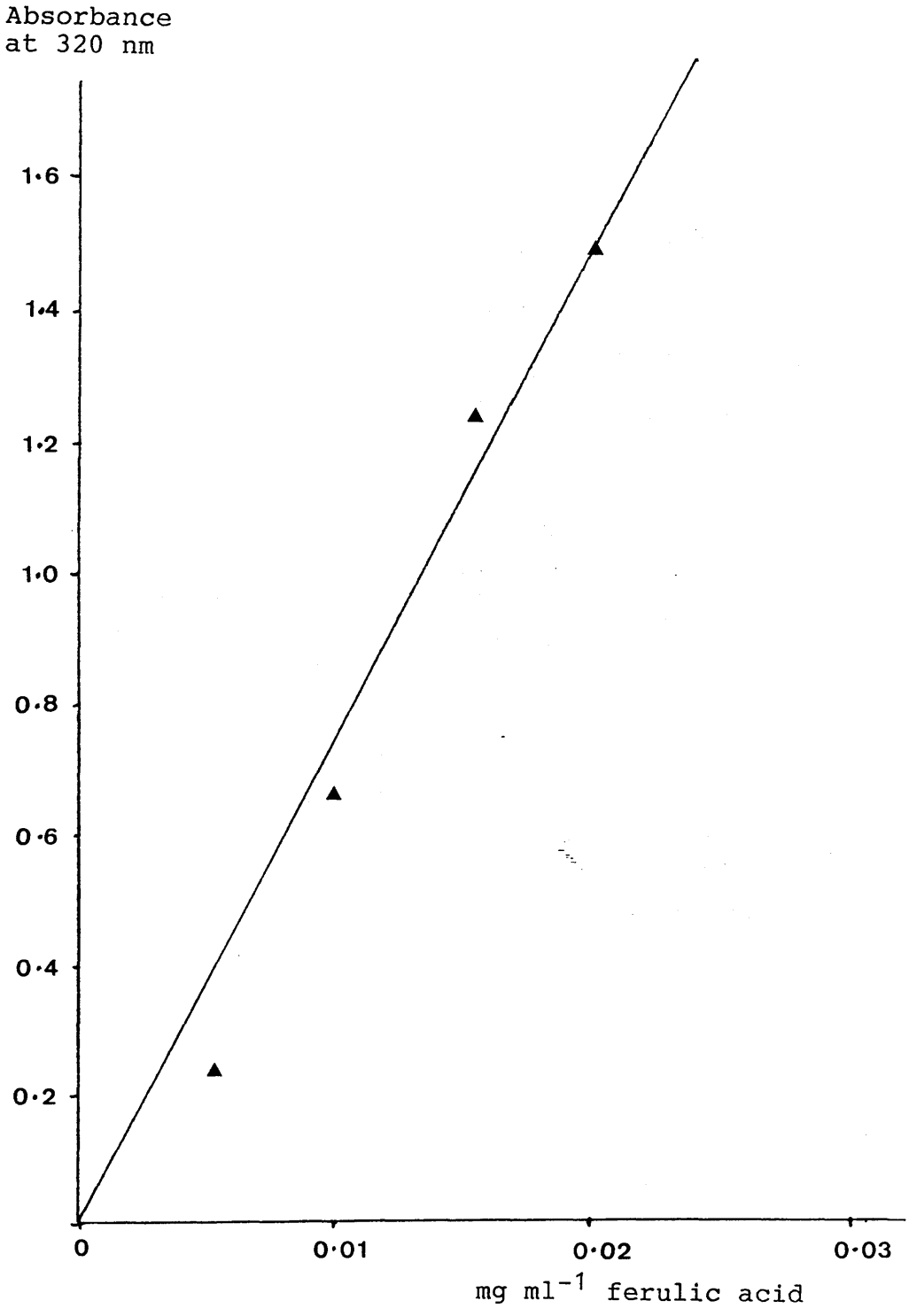


Figure 2.2.2 Graph used to calculate ferulic acid concentration from absorbance.

1.1 ml of 1M hydrochloric acid and extracted with 5x5 ml diethyl ether, the washings being transferred directly to a volumetric flask. After removal of the diethyl ether by evaporation, the phenolic acids extracted were taken up in methanol. A series of ferulic acid standards were made, and the absorbance of these at 320 nm measured (Figure 2.2.2). The phenolic acid content of the cell walls was estimated by determining absorbance at 320 nm, using a methanol blank, and relating this to the absorbance of the ferulic acid standards.

2.2.4 Determination of non cell wall components

2.2.4.1 Starch determination

Starch was determined using an adaptation of the method of Salomonsson, Theander and Aman (1980). Duplicates of unextracted sample (100 mg) were suspended in 25 ml 0.1M sodium acetate-acetic acid buffer (pH 5.0). The starch was gelatinised on a boiling water bath for one hour. This also brought into solution any water soluble carbohydrate present in the sample. The starch in one of each of the duplicates was hydrolysed to glucose with 100 ul amyloglucosidase (from Aspergillus niger E.C.3.2.1.3, suspended in 3.2M ammonium sulphate (Boehringer Mannheim)) for 16 hours at 60°C. All the samples were centrifuged and the glucose contents of 0.5 ml aliquots of the supernatant determined by the glucose oxidase method, using Sigma test kit 510 (Sigma Chemicals Ltd., Poole, England). This utilises the reactions:

(i) glucose + $2H_2O$ + O_2 $\xrightarrow{\text{glucose oxidase}}$ gluconic acid + $2H_2O_2$

(ii) H_2O_2 + O-dianisidine $\xrightarrow{\text{peroxidase}}$ oxidised O-dianisidine (brown)

The intensity of the brown colour was measured at 450 nm, and is proportional to the original glucose concentration. A glucose standard was included in each set of determinations and from this the concentration of glucose in the samples was determined. The tubes to which amyloglucosidase was not added gave the amount of water soluble glucose; starch glucose was determined in the tubes to which the enzyme was added.

2.2.4.2 Nitrogen determination

Nitrogen was determined by the Kjeldahl method, using the semi-automated Kjeltec system. 100 mg of sample was placed onto a one inch square of Whatman No 1 filter paper, and placed in a digestion tube. On each day analysis was performed two blank tubes were run containing only filter paper. To each tube was added a catalyst tablet (K_2SO_4 3.5g; $CuSO_4.5H_2O$ 0.4g), three glass beads and 3 ml of 95% sulphuric acid, and the tubes were heated at 420°C in a thermostatically controlled block for twenty minutes. Exhaust caps were fitted to the tubes to remove the fumes. After removal from the heating blocks, 75 ml of distilled water was added to each tube immediately fuming stopped.

The tubes were then transferred to a steam distillation unit, alkali added and steam passed through until 200 ml of distillate had been collected. 25 ml of mixed methyl

red and methylene blue indicator was added to the distillate and the mixture titrated to the endpoint using 0.01M hydrochloric acid.

% N was calculated as:

$$\frac{a-b \times N \times 14.008}{\text{wt. of sample (g)}}$$

where a is titre value of sample

b is titre value of blank

N is normality of acid.

2.2.4.3 Dry matter determination

To determine dry matter, metal dishes with lids were dried in an oven at 100°C for three hours, placed in a desiccator and allowed to cool. The metal dish and lid were weighed and the material weighed directly into them. The sample was heated at 100°C overnight and then removed from the oven, the lid placed on the dish, which was then transferred to the desiccator to cool. The loss of weight from the sample represents the water in the sample.

$$\% \text{ dry matter} = \frac{\text{sample weight after drying}}{\text{sample weight before drying}} \times 100$$

2.2.4.4 Determination of ash

At least 1 g of sample was weighed into a silica crucible and the weight recorded. The sample was heated in a muffle furnace at 650°C overnight and the weight of the residue recorded.

$$\% \text{ ash} = \frac{\text{residue weight after heating}}{\text{sample weight before heating}} \times 100$$

2.2.5 Physical properties of cell walls

2.2.5.1 Water holding capacity

Water holding capacity (WHC) was estimated using a modification of the centrifugation method described by Robertson, Eastwood and Yeoman (1980). Milled samples (0.5 g; (a) in calculation) were weighed into 50 ml centrifuge tubes. Distilled water (ca. 40 ml) was added and the contents of the tubes mixed by a magnetic stirrer overnight. The stirrer bar was removed and the tubes centrifuged on a bench centrifuge at 1,500 rpm for fifteen minutes. Excess water was decanted off and the tube was left to drain for one hour. WHC was estimated by weighing the tube containing the saturated material (weight b) and reweighing after drying at 105°C overnight (weight c). From the three weights the amount of water held by the original dry matter could be calculated:

$$\text{WHC} = \frac{\text{wt. of saturated material(b)} - \text{wt. of oven dried material(c)}}{\text{dry weight of sample (a)}}$$

2.2.5.2 Copper Exchange Capacity

Measurement of copper exchange capacity (CEC) was performed as described by McBurney, Van Soest and Chase (1983). CEC was measured on whole samples rather than NDF samples as described by the original authors, as it was felt that the pectic fraction of the white clover and the lucerne should remain intact for this measurement. 1.0 g of sample was weighed into a 25 ml (40 um pore size) Gooch crucible. The crucible and sample were placed inside a 100 ml beaker and 1.0M copper (II) sulphate (40 ml) was added. The sample was stirred with a glass rod to break up aggre-

gates, ensuring complete sample saturation. The glass rod and the sides of the crucible were then rinsed clean with 1.0M copper (II) sulphate (10 ml). After a sixty minute incubation the crucible was removed from the beaker to a vacuum filtration rack, where it was washed with distilled deionised water (5x40 ml) to remove unbound Cu^{2+} ions. The base of the crucible was wiped dry and placed on a filtration rack over a 100 ml beaker. The physically bonded Cu^{2+} ions were then washed off the sample by gravity filtration with three separate rinses (25 ml/wash) of 0.6M hydrochloric acid in 70% (v/v) propan-2-ol. The combined washings were brought to a constant volume of 100 ml using distilled deionised water and subsampled.

This solution (5 ml) was added to a 16 cm x 12 mm test tube and brought to pH 8-9 with 2.0M ammonium hydroxide. A 0.5% cuprizon solution was made by heating 2.5 g cuprizon (oxalic acid bis(cyclohexylidene hydroxide)) in 250 ml of ethanol and then making this to 500 ml with distilled deionized water. The cuprizon solution (2.5 ml) was added to a 25 ml volumetric flask and the test tube contents were washed into the volumetric flask with distilled, deionized water bringing the volume to 25 ml. To allow for colour development, absorbance was measured after 30 mins at 590 nm. Prepared copper nitrate standards ranging from 0-125 mg l^{-1} were similarly brought to pH 8-9 with 2.0M ammonium hydroxide and treated with 2.5 ml of 0.5% cuprizon as above.

2.3 DIGESTIBILITY STUDIES

2.3.1 Water solubility

Unextracted milled samples (1.0 g) were weighed into 25 ml conical flasks, five flasks being weighed for each sample. Distilled water (20 ml) was added to the flasks and the flasks shaken at 37°C. One flask from each series was removed after 3, 6, 24, 48 and 72 hours. The contents of each flask were transferred into a preweighed centrifuge tube and centrifuged at 2,000 rpm in a bench centrifuge. The supernatant was decanted. The residue was centrifuged twice with water at 37°C then dried overnight at 105°C and the dry weight of this residue recorded.

2.3.2 In-vitro digestion

In-vitro digestion in Experiment 1 was performed using a modification of the technique of Tilley and Terry (1963), with the omission of the second (pepsin) digestion. 0.5 g of sample was weighed into 100 ml centrifuge tubes. Rumen fluid was collected from a donor animal fed on a grass diet and filtered through two layers of muslin. 10 ml of strained rumen fluid were added to each tube and the tubes sealed using a one-way valve constructed from rubber tubing and glass rod (Figure 2.3.1)

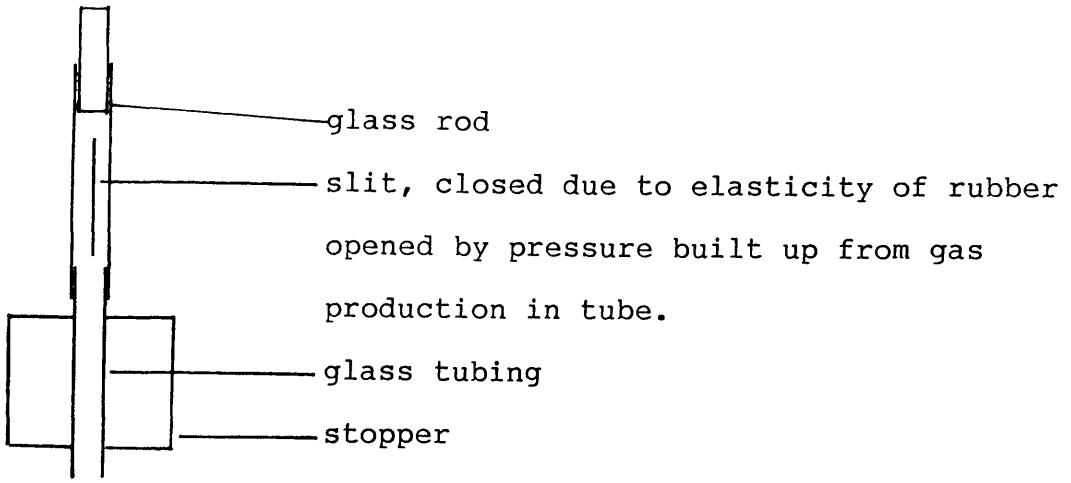


Figure 2.3.1 One-way valve used for in-vitro digestibility studies

The tubes were incubated at 37°C for forty eight hours, with shaking twice daily. The estimations were performed in three batches. One sample (1st cut grass) was included in all three batches, and the digestibilities of all samples corrected to give a constant digestibility for this sample. This eliminates any variation between batches due to variation in the rumen liquor. Fermentation was stopped by the addition of 1 ml saturated mercuric chloride. 2 ml of 0.5M sodium carbonate was also added to aid sedimentation during centrifugation. The tubes were centrifuged at 14,000 rpm for fifteen minutes in a MSE high speed centrifuge and the supernatant discarded. The residue was resuspended in distilled water, centrifuged under the same conditions, the supernatant again being discarded. The residue was transferred to a preweighed metal drying dish, and dried overnight at 105°C. The dry weight of the residue was recorded.

In vitro digestibility =

$$\frac{\text{weight of original DM - weight of residue DM}}{\text{Weight of original DM}}$$

2.3.3 Rusitec digestion

2.2.2.1 Equipment

The rumen simulation technique (Rusitec) used for these experiments was based on apparatus described by Czerkawski and Breckenridge (1977) adapted to provide for the differing aims of this work. The apparatus used in these experiments can be seen in Figure 2.3.2 and numbers in the text refer to this figure.

The complete unit consisted of four identical vessels. Each vessel (1) had a capacity of 1 litre (up to the overflow) and was immersed in a Perspex water bath (2), maintained at 39°C. The vessels were secured in the water bath by a recess in the tank floor and a metal clamp (3) which fitted across the top of each vessel from lips on either side of the water bath. Each reaction vessel consists of a perspex cylinder (270 x 88 mm) on a square perspex base (105 x 105 mm) with an inlet port near the base of the cylinder (4). The vessel is sealed with a flat perspex cover (5) fitted with a screw flange and a rubber seal. The cover has two ports, one for a sampling tube fitted with a three way plastic tap (6) and the other for the overflow tube (7). The overflow tube passes through a rubber stopper in a 1 litre conical flask stored on a refrigerated tray. A 5 litre gas bag (8) is connected to an outlet tube from the rubber stopper, with a glass wool moisture trap in line to prevent water vapour entering the

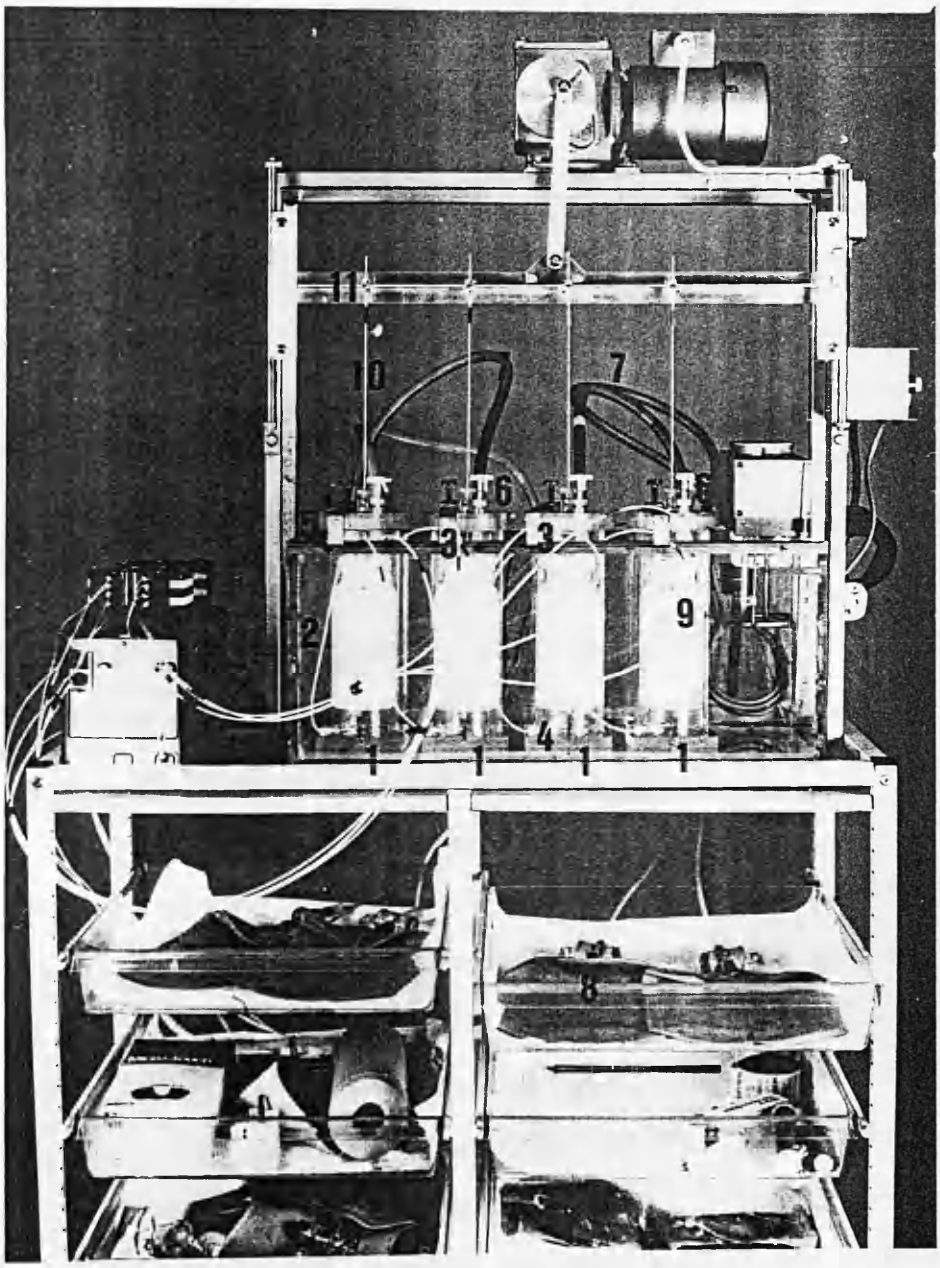


Figure 2.3.2 Apparatus used in Rusitec experiments.

gas bag.

The inner container for the feed bags was a 500 ml polypropylene reagent bottle with a screw cap of the same material (9). This bottle, which fits closely into the outer container has 8 12 mm holes drilled in the base, shoulder and cap to allow the liquid phase to pass through the bottle. A stainless steel rod (10) is connected to the centre of the cap. This passes through the Perspex cover of the vessel via a sealed gland packed with petroleum jelly.

The plastic container is moved up and down in the vessel by means of the stainless steel rod. This is held in place in a slot in a horizontal bar 300 mm above the vessel by two nuts (11). The bar is moved vertically by a split phase Normand motor. The motor speed is 9 rev/min. which produces a vertical stroke of 50 mm at 9 cycles/min.

The food is weighed into preweighed Dacron bags (Henry Simon Ltd., Stockport, 170 x 80 mm), the pore size used being appropriate to the form of the food. A stiffener is placed in the bags to maintain the shape of the bag when the container was being agitated. These were either sections of plastic centrifuge tubes with the base removed, to give pieces 15 mm x 90 mm, or in later experiments 6 mm diameter Perspex rod cut to 80 mm lengths. The bags are closed by means of a plastic tie (RS Components Ltd., USA) attached to which was a 15 mm circular coloured plastic disc, numbered to allow identification of individual bags.

2.3.3.2 General incubation procedure

The day before a Rusitec run was due to start, the water bath was filled with clean water and the heater unit switched on to allow time for the water temperature to stabilise. The refrigerated tray was also switched on.

The incubation was started using rumen fluid from a donor animal maintained on a roughage only diet. The rumen fluid was strained through a double layer of muslin, and 300 ml of strained rumen fluid was placed into each vessel. A further 300 ml of strained rumen fluid was diluted to 600 ml with artificial saliva (Table 2.3.1) and added to the vessel. Solid rumen contents (80 g) were weighed into a 1 mm mesh nylon bag made from mesh bags used for wine making (The Boots Company plc, Nottingham), and one of these was placed in the inner container of each vessel together with two bags of forage or silage. The cap was screwed onto the food container and the assembly was lowered into the vessel and the volume made up to approximately 1 litre using artificial saliva. Petroleum jelly was applied to the rim of the vessel and the sealing ring, and the vessel was closed. All this was done outside the water bath to avoid contamination of the bath. The vessel was then wiped dry to remove any spillage, and lowered into the water bath, with the base fitting into the recess in the bottom of the tank. The clamp was secured across the top of the vessel, and the effluent collection flask connected to the overflow tube. To ensure anaerobic conditions the vessel was flushed via the three way tap with a CO_2:N_2 (5:95, v/v) mixture for one minute, displacing any air trapped in the

Table 2.3.1

Composition of artificial saliva (McDougal's Buffer)

gl^{-1}

$NaHCO_3$	9.8
Na_2HPO_4	3.68
NaCl	0.47
KCl	0.57
$CaCl_2.2H_2O$	0.052
$MgCl_2.6H_2O$	0.13
Trace Elements	
$FePO_4$	0.007
$MnSO_4.H_2O$	0.012
$CuSO_4.5H_2O$	0.0004
$ZnSO_4.7H_2O$	0.0044
H_3BO_3	0.0004
$CoCl_2$	0.0004

vessel. The three way tap was then closed and an empty gas bag connected to the gas outlet tube. The stainless steel rod was connected to the horizontal bar and the motor turned on. A liquid infusion (Table 2.3.2) was pumped through the inlet port at the base of the vessel via a peristaltic pump (Gilson miniplus 2) set to deliver 0.75 litres per day. The pH of the liquid infusion was adjusted to give a constant vessel pH of 6.95.

2.3.3.3 Daily operation

There are a number of operations that must be performed daily during a Rusitec run. The food bags have to be changed, the gas bags disconnected and gas volume and composition measured and effluent volume measured.

To allow these changes to be effected, the infusion pump and the motor were both switched off. For each vessel the gas in the system was displaced by slowly injecting 1 litre of a CO_2:N_2 (5:95, v/v) mixture through the three way tap. The gas bag tap was closed and the gas bag removed and retained for subsequent analysis. The effluent flask was removed, effluent volume measured and a clean flask reconnected to the overflow tube. The vessel was removed from the water bath and opened. Samples of the liquid phase were removed for VFA analysis (1 ml) and pH (10 ml). The food container was raised to the top of the vessel. On day 1, the bag containing rumen solids was removed and replaced with a bag of the forage under investigation. On subsequent days bags were changed according to a defined rotation to give incubation times of 24, 48 and 72 hours (see individual experiment details). The food bags removed

Table 2.3.2

Composition of Nufood

gl^{-1}

Sodium caseinate	1.0
Casein hydrolysate	1.0
$(NH_4)_2SO_4$	0.05
KH_2PO_4	0.24
NaCl	0.06
$MgSO_4.7H_2O$	0.33

Dissolved in McDougal's buffer.

were placed in a plastic bag for washing and the food container resealed.

Each bag was washed twice with 40 ml of artificial saliva. The artificial saliva was added to the plastic bag, the contents were gently manipulated and excess artificial saliva was removed by squeezing the food bag. The washings were poured into the vessel and the washing repeated.

Any spilt fluid was removed from the top of the vessel and the sealing ring, and petroleum jelly was reapplied to both these parts. The vessel was closed, wiped to remove spillage, replaced in the water bath and secured by the clamp. The vessel was flushed out with the CO_2:N_2 mixture through the three way tap for thirty seconds, the tap closed and an empty gas bag connected to the gas outlet tube.

This procedure was repeated for each vessel. When complete the motor and the peristaltic pump were turned on and infusion recommenced. The liquid infusion was changed each day, the pH being adjusted according to the pH of the sample taken from the vessel.

2.3.3.4 Forage residue

On day 1 of the run the bag containing rumen solids was discarded after washing. On subsequent days, wet weight of the bags was recorded and the bags then oven dried at 105°C for twenty four hours and oven dry weight recorded. The forage residue was retained and pooled samples used for further analysis.

2.3.4.5 Individual experiment details

Experiment R1. Grass Silage

3 g of dried milled silage was weighed into each of 32 Dacron bags (pore size 56 μm). Two Rusitec vessels were set up as described and the food bags changed according to Table 2.3.3

Table 2.3.3

GRASS SILAGE RUSITEC RUN

Pattern of changing of forage bags

Day	Vessel 1			Vessel 2		
0	Charge	O W1	O W2	Charge	O W3	O W4
1	O Y22	1	1	O Y23	1	1
2	1	2 N13	2	1	2 N14	2
3	2 W5	1	3 W6	2 W7	1	3 W8
4	1	2	1 Y24	1	2	1 Y25
5	2	3 N15	1	2	3 N16	1
6	3 W9	1	2 W10	3 W11	1	2 W12
7	1 Y26	2	1 Y27	1 Y28	2	1 Y29
8	1	3 N17	1	1	3 N18	1
9	2	1 W1	2 W2	2	1 W3	2 W4
10	3 Y30	1	1	3 YB	1	1
11	1	2	2	1	2	2

Key: on day 5, vessel 1 bag N13 was removed and replaced with N15

Table 2.3.4

Pattern for changing forage bags in clover silage RUSITEC runs

Day	Vessel 1			Vessel 2		
0	C	O R1	O R2	C	O R3	O R4
1	O B15	1	1	O B16	1	1
2	1	2 Y26	2	1	2 Y27	2
3	2 R5	1	3 R6	2 R7	1	3 R8
4	1	2	1 B17	1	2	1 B18
5	2	3 Y28	1	2	3 Y29	1
6	3 R9	1	2 R10	3 R11	1	2 R12
7	1 B19	2	1 B20	1 B21	2	1 B22
8	1	3 Y30	1	1	3 Y	1
9	2	1 R13	2 R14	2	1 R1	2 R2
10	3 B24	1	1	3 B25	1	1
11	1	2 GY22	2 GY23	1	2 GY24	2 GY25
12	2 W1	1 W2	1	2 W3	1 W4	1
13	1	1	2 GN13	1	1	2 GN14
14	2	2 GY26	1	2	1 GY27	1
15	3 W5	1 W6	2	3 W7	1 W8	2
16	1 GN15	1	3 GN16	1 GN17	1	3 GN18
17	1	2 GY28	1	1	2 GY29	1
18	2	3 W9	2	2	3 W10	2
19	3 B19	1	3 B20	3 B21	1	3 B22
20	1	2 Y27	1 Y28	1	2 Y29	1 Y30
21	2 R1	1	1	2 R2	1	1
22	1	2	2	1	2	2

Table 2.3.5

Pattern for changing forage bage in lucerne silage

RUSITEC runs

Day	Vessel 1			Vessel 2		
0	C	0	0	C	0	0
		1	2		3	4
1	0	1	1	0	1	1
	5			6		
2	1	2	2	1	2	2
	7	8		9	10	
3	1	1	3	1	1	3
			11			12
4	2	2	1	2	2	1
		13	14		15	16
5	3	1	1	3	1	1
	17			18		
6	1	2	2	1	2	2
	19	20		21	22	
7	1	1	3	1	1	3
			23			24
8	2	2	1	2	2	1
		25	26		27	28
9	3	1	1	3	1	1
	29			30		
10	1	2	2	1	2	2
	31	32		33	34	
11	1	1	3	1	1	3
			35			36
12	2	2	1	2	2	1
		37	38		39	40
13	3	1	1	3	1	1
	41			42		
14	1	2	2	1	2	2
	43	44		45	46	
15	1	1	3	1	1	3
			47			48
16	2	2	1	2	2	1
		49	50		51	52
17	3	1	1	3	1	1
	53			54		
18	1	2	2	1	2	2
	55	56		57	58	
19	1	1	3	1	3	3
			59			60
20	2	2	1	2	2	1
		61			62	
21	3	1	2	3	1	2

Experiment R2. Clover silage

6g of dried, milled silage was weighed into 56 μm Dacron bags. Two Rusitec vessels were set up as described and food bags changed as in Table 2.3.4. The infusion in this experiment had reduced protein levels; 0.5 gl^{-1} sodium caseinate replaced 1 gl^{-1} sodium caseinate and 0.5 gl^{-1} casein hydrolysate replaced 1 gl^{-1} casein hydrolysate.

Experiment R3. Lucerne silage

6 g of dried, milled silage was weighed into 56 μm Dacron bags, two Rusitec vessels set up and the bags changed according to Table 2.3.5. Reduced protein levels were used in the liquid infusion in this experiment, the levels used being the same as in Experiment R2.

2.4 GAS-LIQUID CHROMATOGRAPHY (G.L.C) METHODS

2.4.1 Determination of neutral sugars by g.l.c.

2.4.1.1 Gas-liquid chromatography of aldononitrile acetate derivatives of neutral sugars

Two sets of gas liquid chromatographic (g.l.c.) apparatus were used in the experiments. The first system used consisted of a Pye model 104 gas chromatograph fitted with dual flame ionisation detectors, linked to a Hewlett Packard model 3373B integrator and a Kipp and Zonen recorder. Glass columns (4 mm internal diameter, length 2 m) were used packed with 5% OV225 on Chromosorb W.AW.DMCS 100/120 mesh. The g.l.c. was operated at an oven temperature of 210°C, detector temperature of 250°C and an injection temperature of 250°C. The carrier gas was nitrogen, with a flow rate of 50 ml/minute. Hydrogen and 21% oxygen

in nitrogen were used for the flames, with flow rates of 50 ml/minute and 500 ml/minute respectively.

The second set of g.l.c. apparatus was based on a Packard model 427 gas chromatograph, fitted with dual flame ionisation detectors linked to a LDC model 308/9 integrator and a Kipp and Zonen chart recorder. Glass columns of length 2 m, i.d. 2 mm were packed with 5% OV225 on Chromosorb W.AW.DMCS 100/120 mesh. The g.l.c. was operated at an oven temperature of 210°C, detector temperature of 250°C and injection temperature of 250°C. The carrier gas, nitrogen, was maintained at a flow rate of 40 ml/minute. Hydrogen and 21% oxygen in nitrogen were used for the flames, with flow rates of 40 ml/minute and 400 ml/minute respectively.

2.4.1.2 Analysis of standard sugar mixtures

The aldononitrile acetate derivatives were prepared of both separate solutions and of mixtures of the following sugars: D-xylose, L-arabinose, L-rhamnose, D-mannose, D-glucose and D-galactose. Myo-inositol was included with the mixtures as an internal standard. The peaks were identified using derivatives of single sugars. Retention times (R_T) relative to myo-inositol and the molar response factor (R_F) for the sugars was calculated using the derivatives of the mixtures of sugars.

2.4.1.3 Derivatisation of monosaccharides from cell wall hydrolysis.

The method used for the derivatisation of neutral sugars for injection into the g.l.c. have been described by Morrison (1975). The aldononitrile acetates were prepared

according to the modification, by Varma, Varma and Wardi (1973), of a method described by Eastwood and Huff (1971). 0.5 ml of 5% hydroxylammonium chloride in dry pryidine was added to the dried hydrolysate (see section 2.2.2.3) and the tube was heated at 100°C for fifteen minutes. The mixture was cooled slightly and 0.5 ml of acetic anhydride added. The test tube was heated at 100°C for one hour. The tubes were removed from the heat and allowed to cool.

The acetylated aldononitriles were purified from the reaction mixtures by addition of chloroform (2 ml) to dilute the mixture and 2M NaOH solution (5 ml), which hydrolysed and neutralised the excess acetic anhydride. Each tube was vigorously shaken and the aqueous layer removed and discarded. The chloroform layer was washed once with 1.0M HCl (5 ml), to remove pyridine as its hydrochloride, then twice with water and finally dried with anhydrous Na_2SO_4. The chloroform solution was pipetted into a clean tube, evaporated to dryness and diluted to a known volume (200 ul) with chloroform. Small aliquots (3-5 ul) of this solution were then injected onto the g.l.c. Typical peaks produced by each set of apparatus are shown in Figures 2.4.1 - 2.4.2 and R_T and R_F values shown in Tables 2.4.1 - 2.4.2

2.4.2 Determination of Water Soluble Carbohydrate content using G.L.C

2.4.2.1 Derivatisation and analysis of ethanol extracts

The ethanol extract from solvent extraction (section 2.2.2.1) was used for water soluble carbohydrate determination. Aliquots (1 ml in duplicate) were placed in Teflon

Table 2.4.1

Molar response factor (R_F) and retention time (R_T) for aldononitrile acetate derivatives of sugar mixtures using Pye model 104 gas chromatograph.

Sugar	R_F	R_T (seconds)
L-rhamnose	1.35	637
L-arabinose	1.39	933
L-xylose	1.38	1126
D-mannose	1.30	2144
D-glucose	1.45	2643
D-galactose	1.48	2879
Myo-innositol	1.00	3571

Table 2.4.2

Molar response factor (R_F) and retention time (R_T) for aldononitrile acetate derivatives of sugar mixtures using a Packard Model 427 gas chromatograph.

Sugar	R_F	R_T (seconds)
L-rhamnose	1.44	283
L-arabinose	1.42	398
L-xylose	1.39	470
D-mannose	1.37	860
D-glucose	1.49	1062
D-galactose	1.47	1135
Myo-inositol	1.00	1516

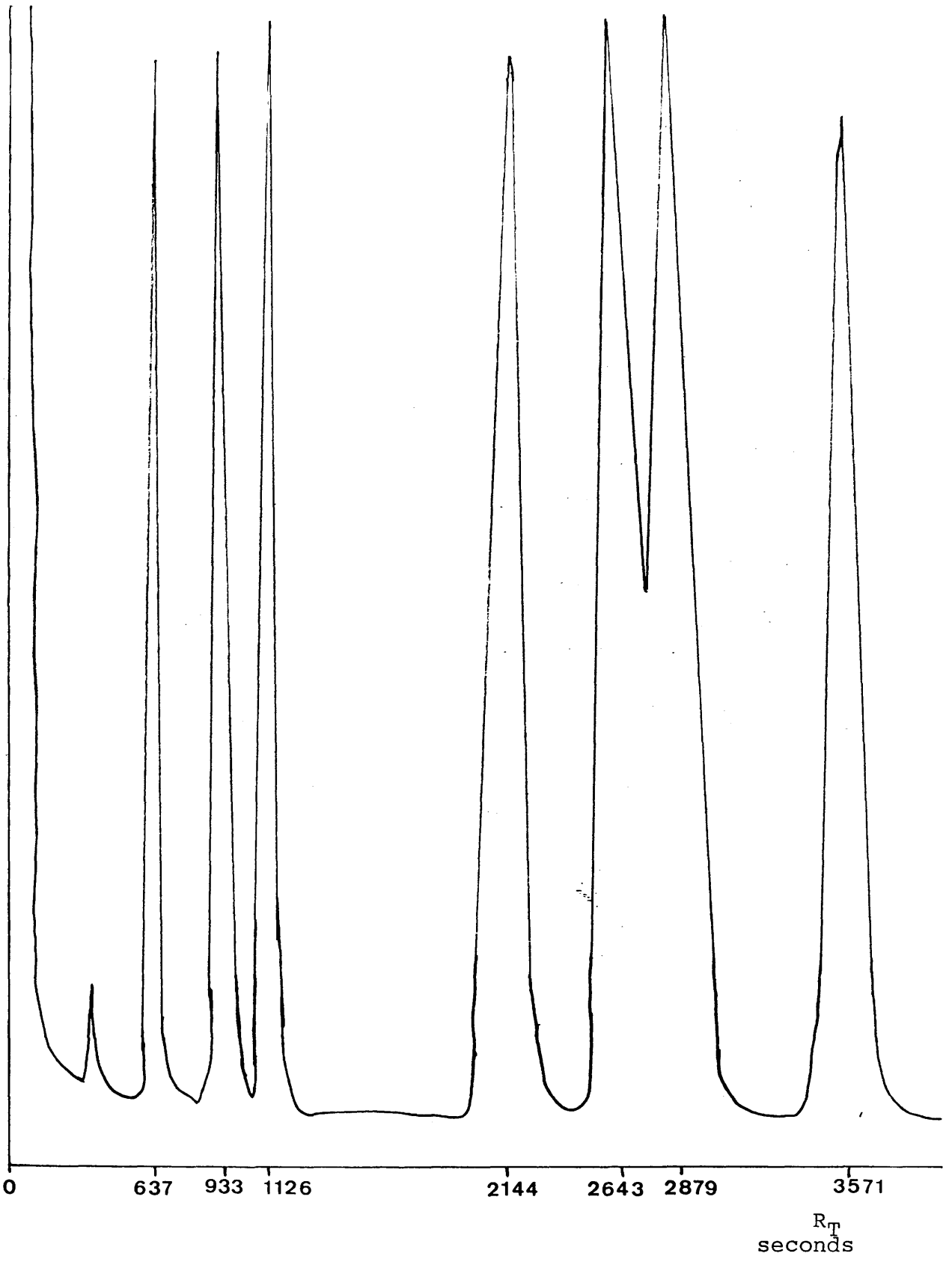
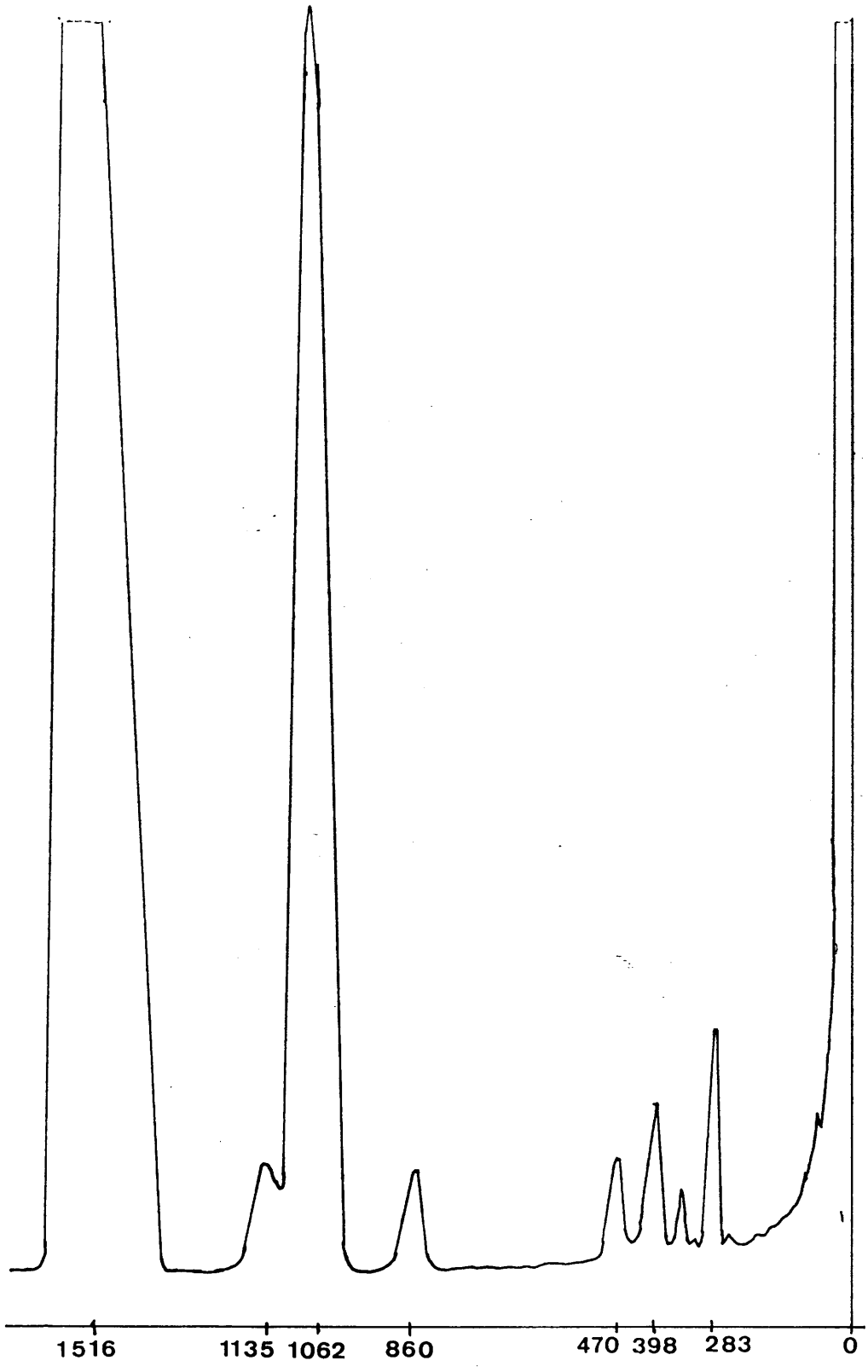


Figure 2.4.1 Example of peaks produced after injection of aldonitrile acetate derivatives into a Pye model 104 gas chromatograph.



Figuire 2.4.2 Example of peaks produced after injection of aldonitrile acetate derivatives of monosaccharides from cell wall hydrolysis into a Packard model 427 gas chromatograph.

stoppered vials, myo-inositol standard added (1 ml; 2 mg/ml) and the mixture dried at 60°C in a stream of air.

The trimethylsilyl (TMS) derivatives of the samples were prepared for injection onto the g.l.c. 1 ml of pyridine was added to the vial followed by 0.2 ml of hexamethyldisilazane and 0.1 ml of chlorotrimethylsilane. The vial was tightly stoppered and shaken vigorously on a vortex mixer for thirty seconds. The sample was left to stand at room temperature for one hour before injection into the g.l.c.

The gas chromatograph used was a Packard model 427 fitted with dual flame ionisation detectors linked to a LDC model 308/9 integrator and a Kipp and Zonen chart recorder. Glass columns of length 2 m, i.d. 2 mm were packed with 5% OV225 on Chromosorb W.AW.DMCS 100/120 mesh. A temperature programmed run was required to obtain separation of the derivatised sugar components. The initial phase of 130°C for ten minutes was followed by a temperature increase of 3°C per minute to 210°C, maintained for ten minutes. The carrier gas, nitrogen, was maintained at a flow rate of 50 ml/minute. Hydrogen and 21% oxygen in nitrogen were used for the flames, with flow rates of 50 ml/minute and 500 ml/minute respectively.

2.4.2.2 Analysis of standard sugar mixtures

A mixture of 100 mg D-glucose, 100 mg D-fructose and 500 mg sucrose was made up in 50 ml water. 1, 2, 3 and 4 ml samples were taken into vials and 1 ml of myo-inositol (2 mg ml^{-1}) was added to each. After standing overnight to allow the sugars to equilibrate in solution, the mixtures

were evaporated to dryness under a stream of air at 50°C. When the samples were dried, 1 ml of pyridine was added followed by 0.2 ml hexamethyldisilazane and 0.1 ml chlorotrimethylsilane. The vials were capped using teflon seals and shaken vigorously on a vortex mixer for thirty seconds. The sample was left to stand at room temperature for one hour before injection into the g.l.c. An example of the peaks obtained is shown in Figure 2.4.3.

2.4.3 Volatile fatty acid determination by G.L.C.

The concentration and proportions of the volatile fatty acids in the liquid phase of Rusitec were analysed by a method similar to that of Cottyn and Boucque (1968). An internal standard, t-butylacetic acid (4.234 mMol 100 ml^{-1}) was mixed with the preservative (25% w/v metaphosphoric acid) in the proportion of 4 ml internal standard to 100 ml preservative. 0.35 ml of this mixture was added to 1 ml of sample and centrifuged at 2,000 rpm for thirty minutes in a bench centrifuge. 5 ul of the supernatant was injected into a Pye model 104 gas chromatograph fitted with dual flame ionization detectors, connected to a Hewlett Packard 3373b integrator. The chromatograph was fitted with a glass column (i.d. 4 mm, 2 m long) packed with 5% carbowax 20M/TPA on Chromosorb 80-100 mesh. The oven temperature was set at 135°C, argon carrier gas flow rate was 60 ml/minute and the detector flame was maintained at hydrogen and air pressures of 1.8 kg cm^{-2} with port detector temperatures of 260°C.

2.4.4 Determination of composition of gas mixtures by G.L.C.

An aliquot (20 ml) of gas was removed from the gas collection bag (section 2.3.3.3). This was analysed for carbon dioxide, hydrogen, nitrogen, oxygen and methane using a Pye 104 gas liquid chromatograph fitted with a thermal conductivity detector and a disc integrator, following the method described by Czerkawski and Clapperton (1968).

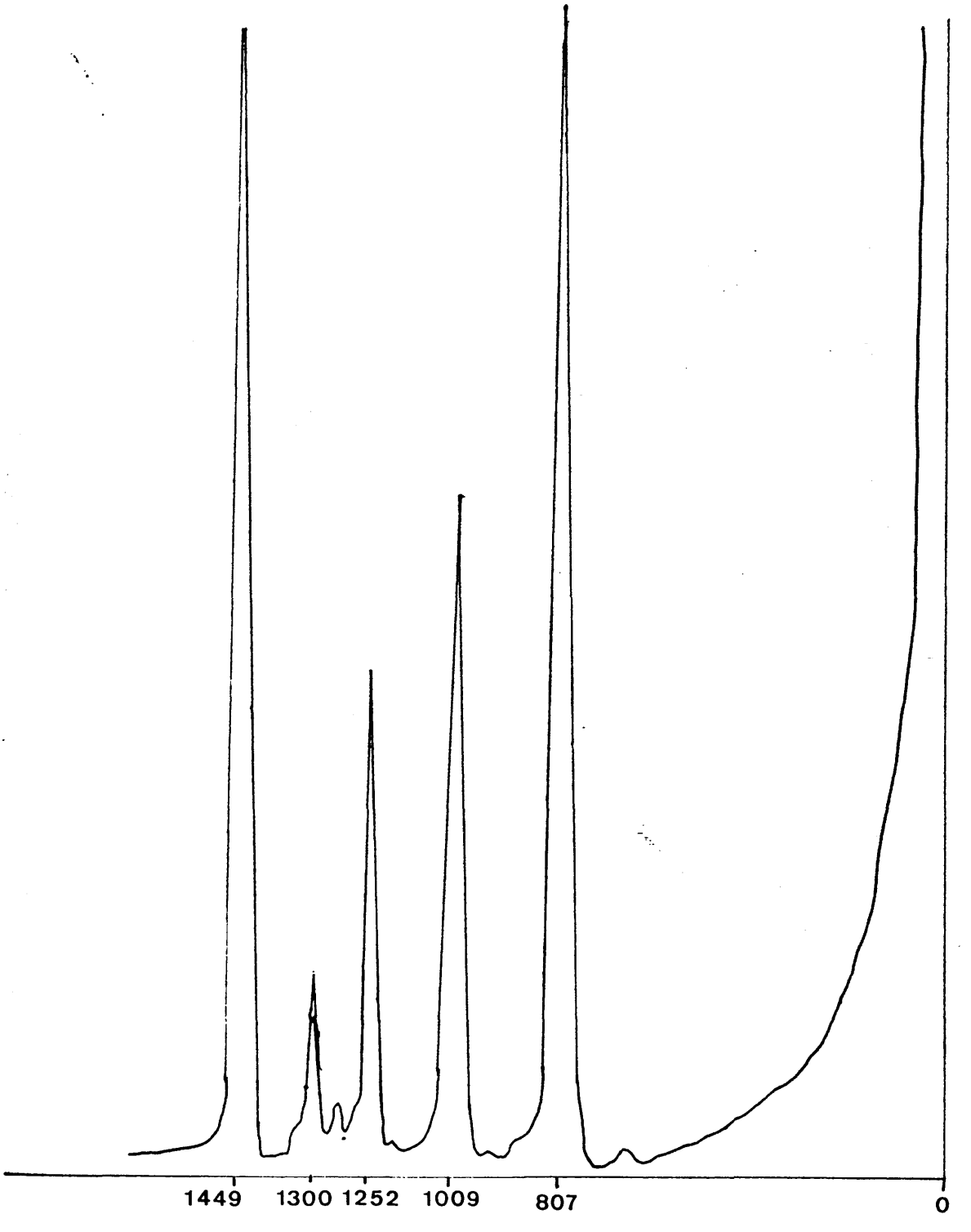


Figure 2.4.3 Example of peaks produced after injection of trimethylsilyl derivatives of water soluble carbohydrates into a Packard model 427 gas chromatograph.

CHAPTER 3

CELL WALL COMPOSITION OF PERENNIAL RYEGRASS AND CHANGES IN COMPOSITION ON ENSILAGE

3.1 INTRODUCTION

It has been well documented that composition of grass cell walls changes with increasing maturity. In the experiment described in this chapter the changes in cell wall composition with increasing maturity were investigated. However, the main aim was to expand on previous work by Morrison (1979) documenting the changes in cell wall composition when perennial ryegrass is ensiled. Morrison (1979) ensiled perennial ryegrass in laboratory scale model silos containing less than 1 kg of material in each silo. In this experiment, grass was weighed into nylon mesh bags which were included in a farm scale silo (see 2.1.1.1 for full details of the method). This grass was thus exposed to the pH changes, to the range of temperatures and to the microbial population found in a farm scale silo.

The bags containing the ensiled ryegrass were removed from the silo by the farm staff as soon as they were uncovered whilst the silo was emptied for feeding trials, eliminating the problem of aerobic fermentation of ensiled material. The yield of ensiled material was recorded and the bags immediately deep frozen. The frozen material was then lyophilized and freeze dried yield recorded before milling. Oven dry matter was determined on the ensiled material, and with the dry matter

of the original grasses this was used to determine the dry matter recovery of the material ensiled. Ash content of the grasses and silages was also determined to estimate contamination by soil during collection from the field.

All results have been corrected for losses during ensilage, and are quoted as g kg^{-1} oven dry matter. When the grass was harvested, samples were taken from a number of sites on the field. These samples were bulked and divided into five equal portions, four of which were ensiled (see 2.2.1.1). The remaining grass was lyophilized, giving one sample representative of that cut which was subsampled for each analysis. All four bags of each cut of grass were ensiled but one bag from the 1st cut was not recovered from the silo. The results from the silages are the means of the four bags (three bags in the case of the 1st cut) and the standard deviation from the mean is quoted.

All the methods used in the analysis of these samples have been described in Chapter 2, with any modifications being noted in the text.

3.2 RESULTS

3.2.1 General silage characteristics

Table 3.2.1 DRY MATTER RECOVERY OF GRASS SILAGES

CUT NO	D.M.	(s.d)
1	0.97	(0.03)
2	0.90	(0.06)
3	0.80	(0.07)
4	0.68	(0.08)

Dry matter recovery steadily decreased as the stage of maturity of the grass used for ensilage increased with dry matter recovery of the fourth cut of grass being as low as 680 g kg^{-1} of dry matter ensiled. These values were used to correct all the values found in subsequent analyses, all figures quoted being stated in g kg^{-1} of the plant dry matter ensiled, to allow the amount of any constituent before and after ensilage to be compared directly.

e.g. Second cut silage

Lignin content = 66.67 g kg^{-1}

D.M. recovery of silage = 900 g kg^{-1}

thus Lignin recovered = 66.67 x 0.9 g kg^{-1}

= 60.0 g kg^{-1} grass D.M.

Results were also corrected for the oven dry matter of the freeze-dried samples.

Ash was determined for each of the samples (Table 3.2.2) to check that soil contamination had not occurred, and the similarity of the results for grass and silages indicates that no contamination was present.

Table 3.2.2 ASH CONTENT g kg^{-1}

CUT NO	GRASS	SILAGE	(s.d.)
1	91.5	80.1	(5.3)
2	63.5	72.8	(8.3)
3	60.8	57.4	(1.5)
4	55.9	53.5	(5.6)

3.2.2 Cell wall components

The samples of milled ryegrasses and ryegrass silages were hydrolysed by the two methods described in Chapter 2 section 2.3.3. The hydrolysis with 72% sulphuric acid released all the neutral sugar residues from both the cellulose and the hemicellulose fractions, whereas the hydrolysis with 2M TFA depolymerised only the hemicelluloses to their constituent monosaccharides. These samples also contained water soluble carbohydrates, as no pre-extraction had been performed. The amount of cellulose was calculated as the difference between the glucose released by hydrolysis with TFA.

This value then has to be corrected since one molecule of water is added for every glycosidic bond hydrolysed. If a polymer contains n sugar residues, then n-1 molecules of water are added when it is fully hydrolysed. When a polymer chain is long, as in cellulose and the hemicelluloses, n-1 can be taken to have the same value as n. Hence every mole of glucose released (180 g) will have arisen from 180-18 g (molecular weight of water) of anhydrous units in the polymer chain. The weight of glucose released on hydrolysis must therefore be reduced by a factor of 162/180, i.e.

$$\text{Cellulose (g kg}^{-1}\text{D.M.)} = [\text{glc(72\% } H_2SO_4\text{)-glc(TFA)}] \times 162/180$$

The amount of hemicellulose in the samples was calculated as the sum of all the neutral sugar residues released by hydrolysis with 2M TFA, also corrected in a

similar manner to above for the difference between the weight of the hydrolysed neutral sugars and the weight of the residue as present in the polysaccharide chain, with consideration for the presence of hexose, pentose and deoxyhexose sugars. The amount of glucose was also corrected for water soluble glucose present. These calculations gave the following equation:

glc (value used) = (glc released after hydrolysis with TFA - glc found in water soluble extract)

Hemicelluloses (g kg^{-1}) = (xyl x 132/150) + (ara x 132/150) + (rha x 146/164) + (glc x 162/180) + (gal x 162/180)

Table 3.2.3

CELLULOSE CONTENT OF GRASS AND GRASS SILAGES g kg^{-1} DM

CUT NO	GRASS	SILAGE	(s.d.)
1	169.2	233.9	(13.2)
2	239.4	262.5	(10.9)
3	233.1	222.1	(21.9)
4	233.3	177.5	(15.7)

The cellulose content of the grass rises between the first two cuts, but then remains constant as the grass matures. The amount of cellulose in the silage follows an unusual pattern. There is an apparent increase in cellulose content between the first cut grass and the silage produced from it. This apparent increase was confirmed using the cellulose extraction method described by Crampton and Maynard (1938).

Table 3.2.4

COMPARISON OF METHODS OF CELLULOSE ANALYSIS

	Cellulose by neutral sugar analysis ($g\ kg^{-1}$ DM)	Cellulose by Crampton and Maynard method ($g\ kg^{-1}$ DM)
1st cut grass	169.2	276.1
1st cut silage	239.4	384.8
(s.d.)	(13.2)	(18.6)

This gave higher figures for the amounts of cellulose present in both the grass and silage but still showed an apparent increase in the cellulose content of the silage relative to grass. The higher values obtained are partly due to contamination of the "cellulose" by neutral sugar residues other than glucose derived from the hemicelluloses. Hydrolysis of the "cellulose" extracted by this method with 72% sulphuric acid showed that appreciable amounts of xylose were present, along with low levels of arabinose, mannose and galactose. Together, these "non-glucose" sugars made up nearly ten percent of the fraction extracted (Table 3.2.5).

Table 3.2.5

NEUTRAL SUGAR COMPOSITION OF THE "CELLULOSE" ISOLATED BY CRAMPTON AND MAYNARD METHOD (mg sugar g^{-1} residue)

	Ara	Xyl	Man	Glc	Gal	Total
1st cut grass	6.64	49.95	6.41	676.65	8.77	748.42
1st cut silage	9.20	51.18	10.02	630.63	8.15	709.19
(s.d.)	(1.85)	(6.11)	(0.89)	(40.26)	(2.96)	(39.81)

The remaining cuts of grass do not present as confusing a picture as the first cut. The second cut of grass also showed a slight increase in the amount of cellulose in the silage relative to the grass, although this was not large and was probably not significant. There is a slight loss of cellulose between the third cut grass and the silage, although this too is probably not significant. In the fourth cut grass, a large loss of cellulose on ensilage is seen. These results produce a trend of increasing amounts of cellulose in the grass with increasing maturity, associated with a corresponding decrease in the amount of cellulose in the silage. This suggests that degradation of cellulose on ensilage does occur in some situations.

The results obtained from the hydrolysis of the samples with 2M TFA are presented for each individual sugar residue (xylose, arabinose, glucose and galactose), and as ratios between xylose and both arabinose and glucose, and as the amount of hemicellulose present calculated from the equation above.

Table 3.2.6

XYLOSE RELEASED BY HYDROLYSIS WITH 2M TFA g kg^{-1} DM

CUT NO	GRASS	SILAGE	(s.d.)
1	101.7	111.4	(13.7)
2	100.3	97.1	(8.8)
3	108.6	110.4	(19.7)
4	156.1	90.2	(20.6)

The xylose content remained constant in the first three cuts of ryegrass, with no change in the level of xylose between the grasses and the silages. However, the fourth cut of grass appeared to have different characteristics. The amount of xylose is higher in the fourth cut grass than in the first three cuts, and there is a loss of xylose on ensilage to give a level close to that in the silages from the first three cuts of grass.

Table 3.2.7

ARABINOSE RELEASED BY HYDROLYSIS WITH 2M TFA g kg^{-1} DM

CUT NO	GRASS	SILAGE	(s.d.)
1	40.3	37.8	(0.8)
2	32.3	27.3	(3.0)
3	33.4	23.8	(3.9)
4	45.6	29.7	(8.8)

Arabinose content of the grass follows a similar pattern, except the first cut of grass seems to have a higher amount of arabinose than the second and third cuts. The fourth cut of grass also has higher amounts of arabinose. The differences between the grasses and the silages also follow a similar pattern to that of xylose, with little change between the first and second cut grasses and the respective silages. However, there is a decrease in the amount of arabinose in both the third and fourth cut silages compared with the respective grasses. This decrease is greatest in the fourth cut.

Table 3.2.8

GLUCOSE RELEASED BY HYDROLYSIS WITH 2M TFA g kg^{-1} DM

CUT NO	GRASS	SILAGE	(s.d.)
1	46.2	20.4	(1.8)
2	71.9	16.9	(1.3)
3	72.7	16.9	(5.8)
4	76.9	15.2	(3.8)

The amount of glucose released by TFA hydrolysis of the grass samples shows a rise as the stage of maturity of the grasses increases. The largest increase is between the first and second cuts, with a slight increase between the third and fourth cuts. The level of glucose in the hydrolysate of the first cut silage is less than 0.25 of that in the grass. As the level in the grass increases with increasing maturity, these different losses bring the amount of glucose in all the silages to about the same amount.

Table 3.2.9

GALACTOSE RELEASED BY HYDROLYSIS WITH 2M TFA g kg^{-1} DM

CUT NO	GRASS	SILAGE	(s.d.)
1	20.2	27.9	(7.4)
2	23.5	20.9	(8.0)
3	23.6	29.2	(11.2)
4	31.1	26.8	(10.8)

The level of galactose in the hydrolysates of all the silages appears to be very similar, but this arises

as there is comparatively little change in the galactose levels of the grasses as maturity increases, and very little change in levels on ensilage.

Table 3.2.10

HEMICELLULOSE CONTENT OF GRASS AND GRASS SILAGE AT FOUR STAGES OF MATURITY g kg^{-1} DM

CUT NO	GRASS	SILAGE	(s.d.)
1	161.1	162.4	(12.9)
2	159.3	151.3	(13.9)
3	176.0	158.6	(18.7)
4	257.1	160.9	(37.5)

When these sugars are considered together as the hemicellulose fraction, a steady increase in the amount of hemicellulose with increasing maturity can be seen. The greatest increase is seen between the third and fourth cut. This follows the trend seen for cellulose and indicates the fourth cut grass was physiologically considerably more mature than the other cuts. Examination of the amount of hemicellulose in the silages shows that similar amounts are found in all four, at about the levels found in the first cut grass silage. There are thus increasing losses of hemicellulose on ensilage with increasing maturity. This may indicate a core of hemicellulose which is not degraded on ensilage, and may be similar to the hemicellulose present in the first cut grass silage. However, this seems unlikely when the xylose:arabinose and xylose:glucose ratios are studied.

Table 3.2.11

XYLOSE:ARABINOSE RATIOS IN HEMICELLULOSE OF GRASS AND GRASS SILAGES

CUT NO	GRASS	SILAGE
1	2.5	2.9
2	3.1	3.5
3	3.3	3.3
4	3.4	3.2

Table 3.2.12

XYLOSE:GLUCOSE RATIOS IN HEMICELLULOSE OF GRASS AND GRASS SILAGES

CUT NO	GRASS	SILAGE
1	2.2	5.5
2	1.4	6.1
3	1.5	6.5
4	2.0	6.3

The xylose:arabinose ratio steadily increases as the grass matures, but the situation in the silages is more complicated. There is no clear pattern discernable in the silage xylose:arabinose ratios, reflecting the variability in the arabinose values of the silages. The xylose:glucose ratios also suggest that a change occurs in the hemicellulose composition during ensilage. The ratios in the grass, although variable, are all below 2.25, whereas the xylose:glucose ratios in the silages are all greater than 5.40. There appears to be an increase in the xylose:glucose ratio in the silages with

increasing maturity of the grass ensiled; this is not consistent as the ratio for the third cut is greater than that for the fourth cut. An overall pattern can be stated for these samples of an increased hemicellulose content of grasses with increased maturity, associated with loss of hemicellulose on ensilage.

Table 3.2.13

RATIO OF GLUCOSE FROM CELLULOSE TO XYLOSE FROM HEMICELLULOSE OF GRASS AND GRASS SILAGES

CUT NO	GRASS	SILAGE
1	1.88	2.68
2	2.79	3.26
3	2.57	3.04
4	1.89	3.61

The ratio of the glucose derived from cellulose to xylose derived from hemicellulose will indicate the relative amounts of cellulose and hemicellulose present in the cell walls. The variability in the ratio reflects the different patterns of cellulose and hemicellulose deposition and losses. In the grasses the ratios are distorted by the low cellulose found in the first cut, but otherwise a steady decrease in the ratio of cellulose to hemicellulose is indicated as the plant matures. The ratios in the silages reflect the greater loss of hemicellulose than cellulose, with greater relative losses of hemicellulose occurring as the grass matures.

Table 3.2.14

LIGNIN CONTENT g kg^{-1} DM

CUT NO	GRASS	SILAGE	(s.d.)
1	53.9	76.3	(13.7)
2	55.3	60.0	(5.4)
3	77.0	62.3	(7.1)
4	89.7	46.0	(5.6)

As would be expected, lignin concentration in the ryegrass samples increased steadily as maturity of the grasses increased. When the silages are studied, the situation appears similar to that seen with cellulose. There is an apparent increase in lignin content on ensilage of the first cut grass, virtually no change in the second cut grass, and a loss of lignin on ensilage in the third and fourth cut grasses, this being greatest in the fourth cut. The apparent increase in the first cut is an anomaly that, like the changes in cellulose content, is difficult to explain. It is unlikely that lignin synthesis has occurred to this extent during ensilage; the most likely explanation being that the analytical method used (Chapter 2, section 2.3.1) is measuring some component released from the grass or produced by micro-organisms on ensilage as lignin. Since there is no great change in the phenolic acid levels between the first cut grass and silage, the former seems unlikely. If a microbial product is being measured as lignin in this cut, it would probably also be present in the other silages, reducing the apparent lignin content

quoted to a true lignin of approximately 15 g kg^{-1} lower; this being the apparent gain of lignin in the first cut silage. This would give a very low lignin value for the fourth cut silage (approximately 31 g kg^{-1}) which seems unlikely.

Excluding this anomaly, there is a pattern of increasing apparent lignin loss on ensilage with increasing maturity of the grass ensiled. No decrease in apparent lignin is seen when second cut grass is ensiled, approximately 20% of apparent lignin is lost when the third cut is ensiled and about 50% of apparent lignin is lost on ensilage of the fourth cut.

Table 3.2.15

ESTERIFIED PHENOLIC ACID CONTENT

g ferulic acid equivalent kg^{-1} DM

CUT NO	GRASS	SILAGE	(s.d.)
1	12.0	16.1	(1.2)
2	12.4	14.3	(1.7)
3	11.9	13.0	(1.6)
4	11.6	8.7	(2.1)

Esterified phenolic acid content of the grasses and silages was determined to find whether this could be related to the changes in lignin content. The phenolic acid content of the grasses remained constant while the phenolic acid content of the silages decreased with increasing maturity of the grasses. There is a slight increase in the phenolic acid content of the first cut

grass on ensilage.

3.2.3 Non cell wall components

To allow for correction for the amount of glucose in the samples to be made when calculating the hemicellulose content, the samples were extracted with ether and 95% ethanol as in Chapter 2, section 2.2.1. The residue left after extraction is shown in Table 3.2.16.

Table 3.2.16

RESIDUE AFTER EXTRACTION WITH ETHER AND ETHANOL AS FRACTION OF WHOLE SAMPLE

CUT NO	GRASS	SILAGE	(s.d.)
1	0.69	0.72	(0.02)
2	0.75	0.71	(0.05)
3	0.78	0.69	(0.02)
4	0.79	0.74	(0.05)

In the grasses there is an increase in the fraction of the sample which is left after extraction reflecting the increase in the amount of cell wall present relative to cell contents as the grass matures. This trend is also seen in the silages.

Table 3.2.17

TOTAL WATER SOLUBLE CARBOHYDRATE (W.S.C)

OF GRASS AND GRASS SILAGES g kg^{-1} DM

CUT NO	GRASS	SILAGE (s.d.)
1	223.8	Not detectable
2	173.0	Not detectable
3	153.9	Not detectable
4	130.7	Not detectable

Table 3.2.18

COMPOSITION OF W.S.C. OF GRASS g kg^{-1} DM

CUT NO	FRUCTOSE	GLUCOSE	SUCROSE
1	71.8	48.6	103.5
2	59.4	64.6	49.0
3	45.0	58.9	32.0
4	39.1	46.2	45.4

Water soluble carbohydrates were present in the ryegrass samples, but none was detectable in the silages. During ensilage, the water soluble carbohydrates are preferentially metabolised by micro-organisms. The amount of water soluble carbohydrate found in the grasses decreased with increasing maturity, and the proportion of sucrose in the total decreased, being lowest in the second and third cuts of grass, accompanied by an increase in the proportion of glucose. Fructose levels remained around the same proportion of the total water soluble sugar content at all stages of maturity.

Nitrogen content of the grasses and silages was measured by the Kjeldahl method and apparent protein

calculated as N x 6.25.

Table 3.2.19

NITROGEN CONTENT g kg^{-1} DM

CUT NO	GRASS	SILAGE	(s.d.)
1	31.3	21.9	(2.5)
2	20.0	22.1	(2.5)
3	15.4	18.2	(1.7)
4	13.4	21.3	(2.9)

Table 3.2.20

APPARENT PROTEIN CONTENT g kg^{-1} DM

CUT NO	GRASS	SILAGE	(s.d.)
1	195.6	136.9	(13.1)
2	125.0	138.1	(17.0)
3	96.3	113.8	(10.0)
4	83.8	133.1	(19.1)

A steady decline in the nitrogen content was seen as the grass became more mature. The fourth cut grass has a low nitrogen content. The level of nitrogen in all the silages was very similar, with apparent loss of nitrogen in the first cut grass on ensilage, and apparent gain of nitrogen in the third and fourth cut grasses on ensilage. This latter result is contrary to those found in other constituents of the ryegrasses, where losses were generally greater from the third and fourth cuts than from the first two cuts. One possible explanation for this may be the microbial contribution to the nitrogen measured in

the silages made from the first two cuts, distorting the values obtained.

3.3 DISCUSSION

The changes in the pattern of cell wall components of grass on ensilage will be discussed here, and at a later stage these will be compared with those obtained from similar experiments with clover and lucerne.

The results from this experiment present an overall picture of loss of material from grass during fermentation in the silo. Cellulose content of the grass and grass silage presents a complicated pattern, with apparent increase in the cellulose content of the silage made from first cut grass relative to that grass. This apparent gain of cellulose was investigated using a different method of cellulose determination, but the same trends were seen using the second method. The difference in actual cellulose levels found between the two methods illustrates the difficulty in measuring cellulose accurately when using extraction methods designed to remove all constituents except the cellulose. Further analysis of the residues after extraction by the method of Crampton and Maynard (1938) showed significant amounts of non-glucose monosaccharides. Obviously, this type of contamination must be considered when comparing levels of cellulose in cell walls determined by different methods. However a definite loss of cellulose from the fourth cut grass is seen on ensilage. This suggests that maturity can affect the losses of cellulose during fermentation in

the silo.

The only loss apparent in all the cuts of grass is of glucose derived from the hemicellulose. The major hemicellulosic polysaccharides in ryegrass are the xylans, with branched and linear polymers found. However, Morrison (1974b) has reported the presence of a non-cellulosic β-glucan in ryegrass, and it is possible that this is undergoing degradation in the conditions found in the silage pit.

There is no loss of galactose residues from the xylan in any of the cuts of grass on ensilage. Losses of arabinose from the hemicellulose are also seen in the third and fourth cut on ensilage and xylose is lost from the fourth cut on ensilage.

Apparent loss of lignin on ensilage is seen in the third and fourth cut grasses. It is in these cuts that a loss of hemicellulose and cellulose is seen, so it seems likely that this effect is due to the solubilization of a lignin-carbohydrate complex. Morrison (1973) isolated a lignin-carbohydrate complex from perennial ryegrass with a monosaccharide composition of 50% glucose, 30% xylose, 12% arabinose and 5% galactose, which is similar to the composition of the cell wall. Extraction of this complex was with dimethyl sulphoxide which will break the hydrogen bonds between polysaccharide chains. It seems unlikely that the conditions found in the silo (pH 4-5 and temperatures of about 30°C in the early stages of fermentation) would break the bonds, releasing the lignin-carbohydrate complex.

The loss of lignin as a complex with the carbohydrate seems the most likely explanation of the fate of the lignin as no increase in the phenolic acid content was seen on ensilage. If breakdown of lignin was occurring independantly of the carbohydrate, an increase in phenolic acid levels would have been expected.

Study of the cellular components of the grasses and silages highlights some of the trends seen in the cell wall components. As would be expected, the water soluble carbohydrate content of the silages was negligible. Within the grasses, there were some noteworthy trends in water soluble carbohydrate content, with the third and fourth cut grasses having considerably lower levels than the first two cuts. When nitrogen level of the samples were examined there was an apparent increase in nitrogen content on ensilage in the third and fourth cut grasses.

When all the results are studied, the fourth cut grass appears to behave differently to the other three cuts of grass. From consideration of all the constituents measured it would appear to be a very different material to the first three cuts, probably being considerably more mature and having characteristics more closely resembling straw than a typical ryegrass sample. This disturbs the patterns of loss from the grasses on ensilages and will be highlighted when these results are compared at a later stage with those from clover and lucerne.

CHAPTER 4

CELL WALL COMPOSITION OF WHITE CLOVER AND CHANGES IN COMPOSITION ON ENSILAGE

4.1 LABORATORY SCALE ENSILAGE OF CLOVER

4.1.1 INTRODUCTION

Considerable interest has arisen recently in the possibility of an increase in the amount of clover in mixed clover/grass swards, and in the possibility of pure clover swards, as a means of fixing greater amounts of nitrogen and thus reducing fertilizer application. Traditionally, legumes have been hard to conserve as winter forage, with leaf shatter being the main problem associated with haymaking, and buffering capacity and difficulty in achieving a low pH being problems when ensilage is attempted. There are also several agronomy related problems associated with growing pure clover swards.

In the light of the renewed interest in the crop, it was decided to run a programme of research at the Hannah based on a pure white clover sward to be harvested and ensiled as winter forage for the dairy herd. This provided the opportunity to investigate more closely the changes that occurred during ensilage of clover, and the possibility of comparing the results with those found in the grass silage experiment (Chapter 3). The experiment was therefore split into two parts, the first being based on the experiments of Morrison (1979), and the second based on ensilage of bagged forage samples in a farm scale silo as in Chapter 3.

The laboratory silos were used to follow the changes

in the forage over the time usually taken for ensilage to occur. Silos were opened after 2, 20, 60 and 150 days and the partially or completely ensiled clover removed. Three treatments were applied to the clover, to highlight any differences during ensilage due to silage additive and that the effectiveness of additives at different stages of ensilage could be seen.

To give results which could be compared with those obtained from the experimental ensilage of ryegrass samples, the analytical methods used were the same as those used for the grass and grass silages. The only exception was that during the analyses, hydrolysis with 2M TFA and 72% H_2SO_4 was performed on the residue after extraction of water soluble carbohydrates, thus no correction had to be made when calculating cellulose values.

4.1.2.1 RESULTS

4.1.2.1 General silage characteristics

Table 4.1.1

DRY MATTER RECOVERY FROM BOTTLES

DAYS ENSILED	TREATMENT					
	WATER	(s.d.)	FORMALDEHYDE	(s.d.)	FORMIC ACID	(s.d.)
0	1.00		1.00		1.00	
2	0.93	(0.02)	0.97	(0.03)	0.99	(0.06)
20	1.02	(0.18)	0.97	(0.01)	0.94	(0.03)
60	0.87	(0.03)	0.97	(0.01)	0.87	(0.05)
150	0.84	(0.13)	0.62	(0.19)	0.68	(0.08)

These results can be used to measure the effectiveness of the laboratory silos as a model for the large farm scale silo. It can be seen that dry matter recovery is very high for all treatments up to day 20, and for the formaldehyde treated samples up to day 60. There is a lower dry matter recovery with the other treatments at day 60, and for all the treatments at day 150. This is indicative of some aerobic deterioration of the silages as aerobic or clostridial fermentation in the bottles will generally lead to a greater loss of dry matter than a lactic acid fermentation. Some mould growth was seen in these bottles and to reduce the influence this had on the analyses the material affected by mould growth was not included in the samples bulked for analysis.

4.1.2.2 Cell wall components

Table 4.1.2

CELLULOSE CONTENT OF SILAGES

DAYS ENSILED	TREATMENT					
	WATER		FORMALDEHYDE		FORMIC ACID	
	g kg^{-1} DM forage ensiled (s.d.)					
0	116.64		116.64		116.64	
2	87.39	(9.67)	92.37	(0.62)	92.27	(1.32)
20	102.25	(1.75)	99.87	(10.5)	91.00	(1.85)
60	98.67	(3.32)	70.18	(0.52)	86.05	(9.13)
150	115.36	(0.86)	54.04	(1.85)	53.97	(18.1)

The pattern of cellulose content of the silages

shows a variation between the water-treated and the other two silages. A slight loss of cellulose is seen in the water-treated silages over 150 days, but this does not seem to be consistent, with the level in the 150 day silage being the same as in the unfermented clover. In the preservative treated clover samples, loss of cellulose during the fermentation period was seen, with the 150 day silages containing about half the cellulose present in the original clover. This loss occurred mainly after day twenty, with about the same loss between days 20 and 60 as days 60 and 150. The rate of loss of cellulose was the same in both treatments.

Table 4.1.3

HEMICELLULOSE CONTENT OF SILAGES

DAYS ENSILED	TREATMENT		
	WATER	FORMALDEHYDE	FORMIC ACID
	g kg^{-1} DM forage ensiled		
0	54.47	54.47	54.47
2	44.39	40.51	34.25
20	41.51	36.68	49.70
60	37.17	39.09	40.68
150	42.62	29.70	29.76

Table 4.1.4

RHAMNOSE CONTENT OF HEMICELLULOSES

DAYS ENSILED	TREATMENT					
	WATER		FORMALDEHYDE		FORMIC ACID	
	g kg^{-1} DM forage ensiled (s.d.)					
0	9.60		9.60		9.60	
2	8.54	(0.47)	8.32	(0.31)	6.97	(0.15)
20	8.73	(1.84)	7.19	(0.66)	8.57	(1.31)
60	7.74	(6.59)	8.61	(0.72)	6.59	(2.19)
150	7.30	(1.21)	5.89	(1.41)	5.41	(0.67)

Table 4.1.5

ARABINOSE CONTENT OF HEMICELLULOSES

DAYS ENSILED	TREATMENT					
	WATER		FORMALDEHYDE		FORMIC ACID	
	g kg^{-1} DM forage ensiled (s.d.)					
0	14.60		14.60		14.60	
2	12.05	(1.35)	10.38	(3.36)	9.62	(1.08)
20	10.51	(0.32)	9.31	(1.21)	11.95	(1.91)
60	8.45	(3.98)	9.58	(2.13)	8.12	(1.76)
150	11.40	(4.60)	5.42	(1.13)	6.48	(0.83)

Table 4.1.6

XYLOSE CONTENT OF HEMICELLULOSES

DAYS ENSILED	TREATMENT					
	WATER		FORMALDEHYDE		FORMIC ACID	
	g kg^{-1} DM forage ensiled (s.d.)					
0	11.10		11.10		11.10	
2	8.87	(1.80)	9.02	(0.96)	6.32	(0.44)
20	9.53	(2.58)	7.63	(0.32)	11.30	(3.36)
60	10.16	(1.63)	7.95	(0.65)	11.28	(2.01)
150	12.00	(1.21)	10.30	(0.69)	9.62	(1.49)

Table 4.1.7

GLUCOSE CONTENT OF HEMICELLULOSES

DAYS ENSILED	TREATMENT					
	WATER		FORMALDEHYDE		FORMIC ACID	
	g kg^{-1} DM forage ensiled (s.d.)					
0	12.90		12.90		12.90	
2	10.16	(0.60)	8.50	(2.67)	7.88	(1.83)
20	8.90	(0.93)	8.93	(0.76)	13.46	(2.37)
60	8.59	(1.01)	9.94	(2.13)	9.31	(1.60)
150	9.49	(1.19)	5.48	(0.56)	6.65	(0.07)

Table 4.1.8

GALACTOSE CONTENT OF HEMICELLULOSES

DAYS ENSILED	TREATMENT					
	WATER		FORMALDEHYDE		FORMIC ACID	
	g kg^{-1} DM forage ensiled (s.d.)					
0	13.00		13.00		13.00	
2	10.16	(0.60)	9.31	(3.57)	7.70	(0.81)
20	8.99	(1.39)	7.82	(0.38)	10.55	(1.63)
60	6.86	(1.98)	7.84	(0.56)	10.40	(1.91)
150	7.77	(1.13)	4.10	(0.21)	5.32	(1.08)

A similar situation was seen in the hemicellulose as in the cellulose. The water-treated sample lost less hemicellulose than the two preservative treated samples. The hemicellulose levels in the preservative treated samples are about half those found in the original forage.

The losses of the constituent sugars of the hemicellulose can be seen in Tables 4.1.4 to 4.1.8. There is a loss of rhamnose from the preservative treated clover and there appears to be a small loss from the water treated clover. This pattern is repeated in the losses of arabinose, with even greater losses from the preservative treated silages than seen for rhamnose. The losses of arabinose from the water treated clover was small. There is very little apparent loss of xylose from any of the treated clovers on ensilage. The pattern for glucose and galactose follows that for rhamnose and arabinose

with losses from all three treatments on ensilage and greater losses from the preservative treated silages than the water treated silage. The greatest loss was of galactose from the formaldehyde treated clover.

Table 4.1.9

XYLOSE TO GLUCOSE RATIO OF CLOVER AND CLOVER SILAGES

DAYS ENSILED	TREATMENT		
	WATER	FORMALDEHYDE	FORMIC ACID
0	0.86	0.86	0.86
2	0.87	1.06	0.80
20	1.07	0.85	0.84
60	1.18	0.80	1.21
150	1.26	1.88	1.45

Table 4.1.10

XYLOSE TO ARABINOSE RATIO OF CLOVER AND CLOVER SILAGES

DAYS ENSILED	TREATMENT		
	WATER	FORMALDEHYDE	FORMIC ACID
0	0.76	0.76	0.76
2	0.74	0.66	1.18
20	0.91	0.94	0.82
60	1.20	1.39	0.83
150	1.05	1.48	1.90

Table 4.1.11

XYLOSE FROM HEMICELLULOSE TO GLUCOSE FROM CELLULOSE RATIO OF CLOVER AND CLOVER SILAGES

DAYS ENSILED	TREATMENT		
	WATER	FORMALDEHYDE	FORMIC ACID
0	0.095	0.095	0.095
2	0.101	0.098	0.065
20	0.090	0.076	0.124
60	0.013	0.113	0.131
150	0.104	0.191	0.178

The losses of hemicellulose constituent sugars gave rise to changes in the xylose and glucose ratios of the hemicellulose. In water-treated clover, the xylose:glucose ratio rises steadily with increasing time ensiled. The ratio in the formaldehyde-treated clover remains about the same up to 60 days ensiled, then rises sharply between 60 and 150 days ensiled. When the clover is treated with formic acid, the ratio remains the same up to day 20, then rises steadily after this. The xylose:arabinose ratio in the hemicellulose shows more variation than the xylose:glucose ratio, and it is difficult to see a clear pattern in the water-treated and formic acid-treated clovers. There appears to be an increase in the ratio as ensilage progresses, but this is not clear. In the formaldehyde treated clover a clear increase in the ratio over the ensilage period is seen.

The xylose from hemicellulose to glucose from cellu-

lose ratio is calculated as an indication of the relative amounts of cellulose and hemicellulose lost during ensilage. It appears that the ratio does not change for the water-treated clover during ensilage, but a steady increase in the ratio is seen in the preservative-treated silages. This indicates a greater relative loss of cellulose than hemicellulose from these clovers during the ensilage period.

Table 4.1.12

LIGNIN CONTENT OF SILAGES

DAYS ENSILED	TREATMENT					
	WATER		FORMALDEHYDE		FORMIC ACID	
	g kg^{-1} DM forage ensiled		(s.d.)			
0	48.9		48.9		48.9	
2	46.6	(4.00)	42.6	(2.56)	44.6	(7.11)
20	64.0	(10.7)	52.8	(5.43)	52.9	(2.29)
60	42.9	(7.24)	43.5	(3.49)	47.8	(5.91)
150	59.1	(4.37)	45.2	(7.01)	53.6	(2.88)

There appears to be no significant loss of lignin in any of the clover samples on ensilage, with a slight increase in lignin being seen in some treatments. These apparent increases are probably due to the measurement of some microbial product as lignin. There is no drainage from the laboratory silos, thus any products of digestion, with the exception of gaseous products, are retained in the container. It is possible that during the

microbial fermentation the lignin-carbohydrate complex is solubilized and this allows the reaction with acetyl bromide to proceed more easily, giving higher apparent lignin values.

4.1.2.3 Non cell wall components

Table 4.1.13

RESIDUE AFTER EXTRACTION WITH ETHER AND ETHANOL

DAYS ENSILED	TREATMENT					
	WATER		FORMALDEHYDE		FORMIC ACID	
	(s.d.)		(s.d.)		(s.d.)	
0	0.80		0.80		0.80	
2	0.72	(0.02)	0.70	(0.02)	0.78	(0.06)
20	0.72	(0.01)	0.68	(0.01)	0.69	(0.03)
60	0.71	(0.02)	0.68	(0.06)	0.71	(0.01)
150	0.70	(0.03)	0.71	(0.03)	0.71	(0.02)

The day 0 fraction appears to have a high residue content. This was wilted clover and it is possible that the plant had metabolized some of the water soluble carbohydrate fraction during the wilting period. There is a lower level of dry matter from the residue in the silages, which would appear to be anomalous as it would be expected that during ensilage the W.S.C. fraction would be metabolized, leaving a higher proportion of residue in the sample. However, it is possible that this is a reflection of the labora-

tory silo system. The fractions solubilised during ensilage is retained in solution in the container and when the silage is lyophilized this is dried onto the residue fraction. When the sample is extracted with the solvents the soluble fraction will be removed, giving an apparently low level of residue fraction in the silages.

Table 4.1.14

NITROGEN CONTENT OF CLOVER AND CLOVER SILAGE

DAYS ENSILED	TREATMENT		
	WATER	FORMALDEHYDE	FORMIC ACID
	g kg^{-1} DM forage ensiled (s.d.)		
0	37.8	37.8	37.8
2	39.5 (0.5)	42.0 (2.0)	39.0 (2.3)
20	40.9 (1.1)	41.3 (4.3)	38.2 (2.7)
60	39.3 (3.3)	41.4 (1.1)	46.1 (4.7)
150	37.5 (4.4)	29.7 (1.4)	41.6 (1.4)

Table 4.1.15

APPARENT PROTEIN CONTENT OF CLOVER AND CLOVER SILAGE

DAYS ENSILED	TREATMENT		
	WATER	FORMALDEHYDE	FORMIC ACID
	g kg^{-1} DM forage ensiled (s.d.)		
0	236.3	236.3	236.3
2	246.9 (3.1)	262.5 (1 2.5)	243.8 (1 4.4)
20	255.6 (6.9)	258.1 (2 6.9)	238.8 (1 6.9)
60	245.6 (2 0.6)	258.8 (6.9)	288.1 (2.9.4)
150	234.4 (2 7.5)	185.6 (8.8)	260.0 (8.8)

There is no degradation of the nitrogen between the wilted clover and the silage, with the exception of the formaldehyde-treated clover after day 60 of ensilage. This could be due to the penetration of fungal hyphae into the container. Any apparent mould growth was removed before lyophilization, but the hyphae may have developed deeper in the silage, although no growth was visible to the naked eye.

4.1.3 DISCUSSION

The results of this experiment show the pattern of losses during ensilage of clover using three different treatments. The additive-treated silages behaved differently to the water treated silage, indicating the effect that these additives can have on ensilage beyond the level of simply lowering the pH or acting as a sterilant. Both additives seem to increase the loss of cell wall carbohydrates from the clover during ensilage. This was reflected in a greater loss of dry matter from the additive treated clovers during ensilage.

The losses from the hemicellulose during ensilage were from the sugars forming the side chains of the hemicellulose. There did not seem to be loss of xylose from the β-D-xylan backbone of the hemicellulose. There was also no loss of lignin during ensilage. Any disappearance of lignin would be thought to be associated with the solubilization of the lignin-carbohydrate complex as described by Gaillard and Richards (1975). As this is not seen, it suggests the lignin is either linked

to a part of the cell wall carbohydrate that is not lost during ensilage, or that no complex is formed in clover. However, there is little detailed knowledge about the chemistry of lignins from clover, or legumes in general. Some compositional differences to grass may be responsible for the differences in behaviour.

4.2 ENSILAGE OF WHITE CLOVER IN A FARM SCALE SILO

4.2.1 INTRODUCTION

From the results of the experiments in Chapter 3 and 4.1, combined with those from the preliminary laboratory silo experiment with ryegrass, it was decided to ensile clover of different maturities held in nylon bags in a farm scale silo using a similar method to that employed in the experiment described in Chapter 3. It was hoped that the experiment would add further results to those found from the laboratory scale silos, and comparison between the grass and clover silages would explain some of the many differences found between the two forages in both ensilage characteristics and animal performance obtained from the silage. As this was a relatively new crop for the Hannah Research Institute, its growth pattern was not fully known, so it was not possi ble to time the harvests to produce cuts of four different maturities. Three cuts were taken, evenly spaced throughout the growing season, the last being taken at the same time as the crop was cut for farm scale ensilage. Two further cuts were made during the season, one immediately before the first regrowth was harvested

for ensilage and one immediately before the second regrowth was harvested for ensilage, and results for these two cuts are presented separately. Bagged samples of these cuts were ensiled in the same manner as for the others, but unfortunately only the silage made from the first regrowth was recovered.

During the analyses of the clover and clover silage, hydrolysis with 2M TFA and 72% H_2SO_4 was performed on samples from which water soluble carbohydrate had been extracted by the method described (Chapter 2 section 2.1.1), thus no correction for water soluble carbohydrate had to be made when calculating the hemicellulose figures. Apart from this, all analytical methods and calculations were identical to those previously described.

4.2.2 RESULTS

4.2.2.1 General silage characteristics

Table 4.2.1

MEAN DRY MATTER RECOVERY FROM THE SILO

CUT	MEAN	(s.d.)
1	0.82	(0.03)
2	0.80	(0.02)
3	0.76	(0.02)

Table 4.2.2

ASH CONTENT OF CLOVER AND SILAGES

g kg^{-1} DM

CUT	CLOVER	(s.d.)	SILAGE	(s.d.)
1	110.91	(7.81)	98.14	(3.00)
2	-		96.00	(2.97)
3	98.17	(2.91)	88.70	(11.13)

The dry matter recovery from the silo decreased as the maturity of the clover samples increased, although this decrease was not large. Ash values for all these samples except the second cut clover were determined. It seems probable that this result would be similar to that found for the other two cuts, as these samples were not harvested. It can be seen from Table 4.2.2 that these indicate that no soil contamination was present.

4.2.2.2 Cell wall components

Table 4.2.3

CELLULOSE CONTENT OF CLOVER AND CLOVER SILAGES

g kg^{-1} DM

CUT	CLOVER	SILAGE	(s.d.)
1	61.4	69.4	(5.4)
2	82.1	70.5	(3.8)
3	97.8	73.3	(3.5)

The cellulose content of the clover samples increased steadily with increasing maturity, with the third cut containing 1.5 times as much cellulose as the first

cut. All three silages contained about the same amount of cellulose, this being similar to that found in the first cut of clover. There is a 15% loss of cellulose from the second cut clover on ensilage and a 25% loss of cellulose on ensilage in the third cut of clover.

The cellulose and hemicellulose contents of the clover and clover silages were calculated using the same method as described previously. The hemicellulose was taken as the sum of all the neutral sugar residues released by hydrolysis with 2M TFA, and this includes monosaccharides derived from pectic material as well as monosaccharides derived from hemicellulosic material. It is likely that some of the galactose and arabinose attributed to the hemicellulose is actually derived from pectin.

Table 4.2.4

XYLOSE RELEASED BY HYDROLYSIS WITH 2M TFA

g kg^{-1} DM

CUT	CLOVER	SILAGE	(s.d.)
1	8.4	7.7	(4.9)
2	9.6	6.3	(2.3)
3	15.7	6.0	(0.8)

Table 4.2.5

ARABINOSE RELEASED BY HYDROLYSIS WITH 2M TFA

g kg^{-1} DM

CUT	CLOVER	SILAGE	(s.d.)
1	20.5	12.4	(3.8)
2	16.9	8.1	(0.5)
3	21.4	6.0	(0.8)

Table 4.2.6

GLUCOSE RELEASED BY HYDROLYSIS WITH 2M TFA

g kg^{-1} DM

CUT	CLOVER	SILAGE	(s.d.)
1	21.3	13.6	(2.0)
2	16.0	11.6	(0.5)
3	24.7	10.2	(1.3)

Table 4.2.7

GALACTOSE RELEASED BY HYDROLYSIS WITH 2M TFA

g kg^{-1} DM

CUT	CLOVER	SILAGE	(s.d.)
1	12.8	6.4	(0.8)
2	11.0	5.3	(1.2)
3	13.7	3.9	(0.6)

The neutral sugar residues released by 2M TFA hydrolysis are shown in Tables 4.2.4 to 4.2.7. Xylose content of the clovers increases with increasing maturity, with the third cut of clover having almost twice as much as

the first cut. There is a loss of xylose on ensilage in all the cuts of clover, with very little difference in the amount of xylose in all the silages, the level in all being slightly lower than the amount found in first cut clover. Arabinose content of all the clovers is similar, with the second cut containing slightly less than the other two. In the silages, however, the arabinose content decreases with increasing maturity of the clover ensiled, giving a pattern of increasing losses on ensilage with increasing maturity of the clover. The first cut silage has an arabinose content of 0.6 that of the clover, whereas the third cut silage has an arabinose content of 0.3 that of the clover. Glucose released by 2M TFA hydrolysis follows a similar pattern to that shown by arabinose. The clover cuts all contain similar amounts of glucose derived from hemicellulose, with the second cut having lower levels than the other two cuts. A loss of glucose derived from hemicellulose on ensilage is seen, this loss increasing with increasing maturity of the cut of clover ensiled. These losses are not as great as are seen for arabinose. The first cut silage has 0.64 as much glucose derived from hemicellulose as is found in the clover. This pattern is also seen in the galactose, with steady galactose levels in all three clover cuts, the levels being slightly lower in the second cut. Greater loss of galactose than glucose is seen on ensilage, the fraction of galactose found in the silage relative to the grass being 0.5, 0.48 and 0.28 for the 1st, 2nd and 3rd cuts respectively. These losses are

larger than those seen for any of the other neutral sugar residues from hemicellulose.

The difference in the pattern of xylose change in the clovers and on ensilage compared with arabinose and glucose is reflected in the xylose:arabinose and xylose:glucose ratios.

Table 4.2.8

XYLOSE:ARABINOSE RATIOS IN HEMICELLULOSE OF CLOVER AND CLOVER SILAGE

CUT	CLOVER	SILAGE
1	0.41	0.63
2	0.57	0.78
3	0.73	1.00

Table 4.2.9

XYLOSE:GLUCOSE RATIOS IN HEMICELLULOSE OF CLOVER AND CLOVER SILAGE

CUT	CLOVER	SILAGE
1	0.39	0.57
2	0.60	0.54
3	0.64	0.59

Increasing xylose levels with increasing maturity compared with steady levels of both arabinose and glucose give an increase in both xylose:arabinose and xylose:glucose ratios with increasing maturity of the

clover. The xylose:arabinose ratio of the silages also shows a increase with increasing maturity of the clover ensiled, as the decrease in arabinose levels with increasing maturity is greater than the slight decrease seen in xylose levels, giving comparatively larger amounts of xylose than arabinose remaining in the silages in the later cuts. The situation is different when the xylose:glucose ratios are studied. Here, the decrease in glucose levels with increased maturity of the silage is not as great, thus there is not as large an increase in the overall xylose:glucose ratio, and a slight drop is seen in the ratio between the first and second cut.

The composite of these neutral sugar figures gives the amount of hemicelluloses in the clovers and silages.

Table 4.2.10

HEMICELLULOSE CONTENT OF CLOVER AND CLOVER SILAGES

CUT	CLOVER	SILAGE	(s.d.)
1	65.7	36.5	(3.1)
2	57.1	34.6	(4.0)
3	86.9	27.3	(2.5)

An overall increase is seen in the level of hemicelluloses as the clover matures. The concentration of hemicelluloses in the silage is considerably lower than in the clover, and decreases with increasing maturity of the clover ensiled.

Table 4.2.11

XYLOSE:GLUCOSE RATIOS IN HEMICELLULOSES AND CELLULOSE OF CLOVER AND CLOVER SILAGE

CUT	CLOVER	SILAGE
1	10.65	14.28
2	11.17	16.08
3	12.02	14.62

There is an increase in the ratio of glucose from the cellulose to xylose from the hemicellulose as the clover matures. This reflects the greater increase of cellulose relative to hemicellulose as the clover matures. There is also an increase in the ratio when the clover is ensiled, indicating that there is a greater loss of hemicellulose than cellulose when the clover is ensiled.

Lignin levels in these samples were measured by the acetyl bromide method as described in 2.2.3.1.

Table 4.2.12

LIGNIN CONTENT OF CLOVER AND CLOVER SILAGES

g kg^{-1} DM

CUT	CLOVER	SILAGE	(s.d.)
1	35.1	37.1	(6.6)
2	37.4	34.7	(3.0)
3	30.1	34.3	(3.0)

The lignin levels in the clover do not increase consistently with increased maturity, with the level being similar in the first two cuts, and slightly lower in the third cut. These results can best be explained by the morphology of the clover plant and the leaf:stem ratio at different ages. The first two cuts would have a lower leaf:stem ratio than the third cut, as leaf expansion occurred between the second and third cuts. Since more of the lignin occurs in the stem of dicotyledonous plants than in the leaves, this change of leaf:stem ratio would explain the slight lowering of lignin levels in the third cut. There is no apparent loss of lignin on ensilage in any of the cuts of clover, all lignin levels remaining virtually unchanged. Very low levels of free phenolic acids are found in the clover, and these are reduced by ensilage to virtually trace levels.

Table 4.2.13

ESTERIFIED PHENOLIC ACIDS IN CLOVER AND CLOVER SILAGE

g kg^{-1} ferulic acid equivalent DM

CUT	CLOVER	SILAGE	(s.d.)
1	3.05	0.73	(0.06)
2	3.37	0.59	(0.06)
3	3.14	0.44	(0.05)

It is probable that phenolics are solubilized on ensilage, leach away and are lost in the silage effluent.

4.2.2.3 Non cell wall components

The samples were extracted with ether and 95% ethanol as described in 2.2.2.1, and the amount of residue left after this extraction is shown in Table 4.2.14.

Table 4.2.14

RESIDUE AFTER EXTRACTION WITH ETHER AND ETHANOL AS FRACTION OF WHOLE SAMPLE

CUT	CLOVER	SILAGE	(s.d.)
1	0.79	0.64	(0.02)
2	0.82	0.64	(0.01)
3	0.76	0.63	(0.02)

The greater amount of residue in the second cut clover supports the idea that this was a relatively stemmy material, as there is a greater ratio of cell walls:cell contents in stem material compared with leaf material. The lower amount of residue in the third cut clover is due to the relatively large amount of leaf material found in this cut. Residues of the silage made from all three cuts after extraction were all about the same fraction of the original material.

Unfortunately, when the extracts were silylated as described in 2.4.2.1, there was a number of unidentifiable peaks seen on the g.l.c. trace, directly interfering with the glucose and sucrose peaks, making measurement of these peaks impossible.

However, the method used for starch analysis allowed

an estimation of the water soluble glucose in the samples.

Table 4.2.15

WATER SOLUBLE GLUCOSE IN CLOVER AND CLOVER SILAGES

g kg^{-1} DM

CUT	CLOVER	SILAGE	(s.d.)
1	11.93	4.18	(0.38)
2	12.95	3.73	(0.40)
3	14.43	4.65	(0.12)

There was a slight increase in the glucose levels in the clover as it matures. Most of the water soluble glucose is metabolised on ensilage, the levels found in the silage being very low, and similar in all three stages.

Table 4.2.16

GLUCOSE DERIVED FROM STARCH IN CLOVER AND CLOVER SILAGES

g kg^{-1} DM

CUT	CLOVER	SILAGE	(s.d.)
1	14.20	8.57	(1.35)
2	10.30	6.13	(2.97)
3	24.63	2.38	(0.40)

The pattern seen in the levels of starch in the clover follows that seen in other constituents of the clover. There is a decrease in starch levels between the

first and second cuts, and a rise between the second and third cuts. Starch is lost on ensilage, with greater losses from the third cut than from the first cut.

Table 4.2.17

NITROGEN CONTENT OF CLOVER AND SILAGES AS MEASURED BY THE KJELDAHL METHOD

g kg^{-1} DM

CUT	CLOVER	SILAGE	(s.d.)
1	41.7	33.3	(0.85)
2	41.9	32.1	(1.16)
3	40.8	28.8	(1.73)

Table 4.2.18

APPARENT PROTEIN CONTENT OF CLOVER AND SILAGES

g kg^{-1} DM

CUT	CLOVER	SILAGE	(s.d.)
1	260.6	207.9	(5.50)
2	261.9	200.8	(7.70)
3	255.0	180.1	(10.98)

The levels of nitrogen found in the clover and clover silages were high. There was no change in nitrogen levels as the clover matures, and there is a consistent loss of nitrogen on ensilage to give the same level of nitrogen in all the silages.

4.2.3 DISCUSSION

From these results several trends can be seen. The changes in the level of cellulose in the different clover samples shows a steady increase in the amount of cellulose as the clover matures, and a loss of cellulose on ensilage from the second and third cuts of clover. The hemicellulose levels present a more complicated picture, with a lower concentration of hemicellulose in the second cut than in the first cut clover. This is possibly due to a decrease in the leaf:stem ratio as the plant matures. The proportion of cellulose to hemicellulose in the stem is greater than in the leaves, which gives an apparent reduction in the concentration of the hemicellulose when the whole plant is considered. As the plant reaches maturity, the concentration of hemicellulose in the total plant material increases. From the results of the individual sugar levels it would seem that this is due mainly to an increase in the xylans, with very little change being seen in the amounts of other neutral sugar residues. There is a loss of hemicellulose from all cuts of clover on ensilage, with most of the xylans which appear with increasing maturity of the clover being lost when the clover is ensiled, and increasing amounts of other hemicelluloses being lost with increasing maturity of the clover being ensiled. It seems unusual that this should occur, but one possible mechanism for this could be disruption of the "polysaccharide matrix" by the loss of the more easily degraded xylans, thus allowing the enzymes attacking the other polysaccharides access to the

sites of degradation. As relatively more xylans are present in the more mature clovers, greater disruption would be caused and the loss of more non-xylan hemicellulose would occur.

The lignin content of the clover increased between the first and second cut, but dropped between the second and third cut. This was also probably due to the changes in the leaf:stem ratio, an increase in the leaf to stem ratio in the third cut producing the drop in lignin concentration. There is no significant loss of lignin from the clover on ensilage. The low levels of esterified phenolic acid are typical of those generally found in clover (Hartley and Jones, 1977) and practically no phenolic acid was found in the silages.

When the non cell wall components are studied, an almost steady level of glucose is seen in the clover as it matures. As would be predicted, a loss of glucose on ensilage was seen. An increase in starch level is seen between the second and third cuts of clover, indicating that the crop was becoming physiologically mature. Most of this starch was lost on ensilage, with the greatest amount being lost from the third cut of clover on ensilage.

The clover and clover silages both had a high nitrogen content, with little nitrogen being lost on ensilage, indicating that these were well preserved silages.

Overall a pattern is seen of losses on ensilage, these being greatest from the third cut clover, although losses are seen from the other two cuts.

CHAPTER 5

CELL WALL COMPOSITION OF LUCERNE AND LUCERNE SILAGE

5.1 INTRODUCTION

The study of white clover at HRI led to the development of the idea of growing lucerne as a legume crop for ensilage. This crop has certain advantages over clover, these being mainly associated with its more upright growth habit. This makes it easier to cut the crop for silage and the more robust stem makes it less susceptible to leaf and stem shatter when it is being mechanically handled. It can also be easily overwintered in the west of Scotland. The best way of doing this is to graze the regrowth with sheep in the autumn.

Unfortunately, it was not possible to run a full scale experiment along the lines of the clover experiments. However, samples of the lucerne and lucerne silage were collected (Chapter 2 section 2.1.3) and these were analysed to give an indication of the overall changes during the ensilage of this crop.

5.2 RESULTS

As the lucerne silage was collected from the silage pit, it was not possible to measure the losses on ensilage, thus the figures quoted are not corrected for loss of dry matter as were the figures for grass and clover silage on ensilage.

Table 5.2.1

CELLULOSE AND HEMICELLULOSE CONTENT OF LUCERNE AND LUCERNE SILAGE

($g\ kg^{-1}$ DM)

	CELLULOSE	HEMICELLULOSE
Lucerne	140.78	67.25
Lucerne Silage	120.81	80.99

The mean dry matter recovery in the clover experiments described in Chapter 4 was 0.80. To allow some estimation of the change in the amount of cellulose and hemicellulose in the lucerne during ensilage it was decided to use this figure as an estimate of the possible dry matter recovery during ensilage of lucerne. When the values in Table 5.2.1 are adjusted using this figure, the following values are obtained:

Silage cellulose 96.65 $g\ kg^{-1}$ DM

Silage hemicellulose 64.80 $g\ kg^{-1}$ DM

The values after correction suggest that there is quite considerable loss of cellulose from lucerne during ensilage, with only 0.69 of the cellulose present in the fresh material remaining in the silage. However, the values for hemicellulose suggest that there is little or no loss of this fraction during ensilage.

Table 5.2.2

NEUTRAL SUGAR COMPOSITION OF HEMICELLULOSE

	Lucerne	(s.d.)	Lucerne Silage	(s.d.)	Silage x 0.80	(s.d.)
Rhamnose	7.03	(1.48)	11.32	(1.52)	9.06	(1.22)
Arabinose	12.36	(1.16)	15.62	(1.93)	12.50	(1.54)
Xylose	21.73	(6.15)	27.88	(6.78)	22.30	(5.42)
Mannose	7.03	(0.64)	11.37	(1.86)	9.10	(1.49)
Glucose	24.98	(0.78)	24.51	(1.96)	19.61	(1.57)
Galactose	9.46	(0.75)	11.75	(3.22)	9.40	(2.58)

The neutral sugar composition values support the suggestion that there is very little loss of hemicellulose during ensilage, with the only change being a small reduction in the amount of glucose in the ensilaged lucerne.

Table 5.2.3

XYLOSE:ARABINOSE RATIO AND XYLOSE:GLUCOSE RATIO

	xyl:ara	xyl:glc
Lucerne	1.76	0.87
Lucerne Silage	1.77	1.12

The xylose:arabinose ratio supports the idea that there is no loss of hemicellulose as there is no change in the ratio between the lucerne and the lucerne silage. The increase in the xylose to glucose ratio in the lucerne supports the suggestion of a slight loss of glucose from the hemicellulose during ensilage.

Table 5.2.4

RATIO OF XYLOSE FROM HEMICELLULOSE

FROM GLUCOSE IN CELLULOSE

Lucerne	0.14
Lucerne Silage	0.21

The higher ratio of xylose from hemicellulose to glucose from cellulose confirms the suggestion that there is a much greater loss of cellulose than hemicellulose from the lucerne cell wall during ensilage. The assumed dry matter recovery figure means that an exact value of the loss of cellulose and hemicellulose cannot be obtained, although the general trend can be seen.

Table 5.2.5

LIGNIN AND PHENOLIC ACID LEVEL OF LUCERNE

AND LUCERNE SILAGE

	LIGNIN g kg^{-1} DM	(s.d.)	PHENOLIC ACID g kg^{-1} DM ferulic acid equiv.	(s.d.)
Lucerne	143.0	(19.0)	2.38	(0.45)
Lucerne Silage	154.1	(7.3)	3.00	(0.53)

Using the same assumed correction value for DM loss (0.80) for the lignin values give figures of:

Lignin in lucerne silage 123.28 g kg^{-1} DM (s.d. 5.84)

Phenolic acid in lucerne silage 2.40 g kg^{-1} DM (s.d. 1.27)

This suggests that there is some loss of lignin from the lucerne during ensilage, with about 14% of the lignin being lost. There is no increase in free phenolic acid during ensilage, so it must be assumed that any lignin lost from the forage is lost from the silo in the effluent.

Table 5.2.6

WATER SOLUBLE RESIDUE AFTER EXTRACTION WITH ETHER AND ETHANOL

(Original Material = 1.00)

Lucerne	0.84
Lucerne silage	0.85 (corrected for DM loss 0.68)

After applying the correction figure, there is a decrease in the water soluble residue after extraction with ether and ethanol showing that some non-soluble fractions of the lucerne had been broken down during ensilage. About one-third of this loss could be attributed to the losses of cellulose already seen, the remainder probably being made up of losses of starch and protein.

Table 5.2.7

STARCH IN LUCERNE AND LUCERNE SILAGE

Lucerne	11.34
Lucerne silage	9.94 (corrected for DM loss 7.95)

The level of starch in the lucerne drops during ensilage although there is a significant amount remaining in the silage.

5.3 DISCUSSION

When lucerne was ensiled losses of cellulose, lignin, starch and other non water-soluble components were found, but there was no loss of hemicellulose. This suggests that there was no digestion of the β-D-xylan chains of the hemicellulose, whereas some of the β-1,4-D-glucopyranosyl bonds of the cellulose were broken. It may be that side chains on the hemicellulose prevent the breakdown of the main chain. The loss of lignin without hemicellulose suggests that there may not be such a close association between these two components in the lucerne cell wall as in the grass cell wall. As stated earlier, confirmation of some of these explanations must await more details of the architecture of legume cell walls.

CHAPTER 6

COMPARISON OF CELL WALLS OF PERENNIAL RYEGRASS, WHITE CLOVER AND LUCERNE, AND OF THE CHANGES ON ENSILAGE.

6.1 INTRODUCTION

The results of the experiments investigating the changes in cell wall structure on ensilage have already been discussed individually for each forage. However, one of the aims of the project was to discover any differences between the changes in the different forages during ensilage and implications they may have for silage production. The individual components of the cell wall will be considered, and any differences in composition, in changes due to maturity and in changes during ensilage will be highlighted.

6.2 CELL WALL COMPONENTS

6.2.1 Variation in cellulose content with age

Table 6.2.1

CELLULOSE CONTENT OF FORAGES

g kg^{-1} DM

CUT NO	RYEGRASS	CLOVER	LUCERNE
1	169.2	61.4	
2	239.4	82.1	
3	233.1	97.8	140.8
4	233.3		

It is clear from the figures that the levels of cellulose in the three forages were very different, with clover having the lowest cellulose levels and grass having the highest levels. The levels of cellulose found

in the forages correspond to those found by other authors. Christian et al (1970) found about 14% cellulose in the lucerne leaf, with slightly higher levels in the stem. Sullivan (1966) found higher levels (27.0%) in lucerne, using a modification of the Crampton and Maynard method of cellulose determination, which may have extracted more than cellulose from the forage. Wilkins (1972) found an increase in levels of cellulose in perennial ryegrass with increased age, with 22% cellulose in the leaf and 24% cellulose in the sheath at six leaf stage and corresponding figures of 25% and 30% at anthesis. The results show an increase in the cellulose levels of grass and clover as the maturity of the forage increased. Christian et al (1970) showed an increase in the cellulose levels in the stem of lucerne with an increase age of the forage, suggesting that lucerne would follow the pattern seen in grass and clover.

6.2.2. CHANGES IN CELLULOSE CONTENT ON ENSILAGE

Table 6.2.2

CELLULOSE CONTENT OF FORAGES

g kg^{-1} DM

RYEGRASS

CUT NO	GRASS	SILAGE
1	169.2	233.9
2	239.4	262.5
3	233.1	222.1
4	233.3	177.5

CLOVER

DAYS ENSILED	TREATMENT		
	WATER	FORMIC ACID	FORMALDEHYDE
0	166.6	166.6	166.6
2	87.4	92.4	92.3
20	106.3	99.9	91.0
60	98.7	70.2	86.1
150	115.4	54.1	54.0

CLOVER

CUT NO	CLOVER	SILAGE
1	61.4	69.4
2	82.1	70.5
3	97.8	73.3

LUCERNE

LUCERNE	SILAGE	
140.8	120.8	(96.7 if corrected)

The results for cellulose content of the ryegrass and ryegrass silage show a baffling pattern with an

apparent increase in cellulose on ensilage in the immature cuts of grass. A small apparent increase in cellulose content (21.9% - 24.7%) was seen in one of the experiments reported by McDonald et al (1968), but as this has not been reported by any other authors, no immediate explanation of this effect seems possible. However, the third cut of grass shows results similar to those obtained by Morrison (1979a) with little or no loss of cellulose during ensilage. Loss of cellulose was seen from the most mature cut of grass in the farm scale silos, suggesting that the maturity of the grass affects the loss of cellulose. The difference in loss of cellulose between the early and later cuts was also seen in clover ensiled in farm-scale silos. There was no apparent loss of cellulose from the young clover, but a loss was seen from the more mature cuts. This clover was ensiled in a pit and had been treated with an acid-type preservative. From the results of the laboratory scale ensilage of the clover it seems that this can have a marked effect on cellulose loss. There was no apparent loss of cellulose in water-treated clover, whereas in the two treated silages the cellulose was reduced by half during ensilage. The pattern in lucerne is not as clear as that seen in clover with a loss of cellulose being seen on ensilage in a pit using a formic acid additive. If this figure is corrected using the 0.80 correction figure then a greater loss of cellulose is apparent, suggesting that lucerne cellulose losses on ensilage are similar to those seen in clover.

It seems that there are trends in the loss of cellulose that are apparent in all forages. More cellulose is lost from the older cuts of the forage, and, in situations where a loss of cellulose would be predicted, this loss is increased if a silage preservative is used. This may have practical consequences as cellulose from silage can provide a large part of the energy supply for the animal.

6.2.3 Variation in hemicellulose content with age

Table 6.2.3

HEMICELLULOSE CONTENT OF FORAGES

g kg^{-1} DM

CUT NO	RYEGRASS	CLOVER	LUCERNE
1	161.2	65.7	
2	159.3	57.1	
3	176.0	86.9	67.3
4	257.1		

In agreement with the cellulose values, the levels of hemicellulose in both the grass and the clover increase as the crop becomes more mature. The figures for hemicellulose content in perennial ryegrass correspond to those found by Morrison (1980b). The level of hemicellulose in the clover and lucerne was much lower than that found in the ryegrass. The difference in level of hemicellulose between the forages is reflected in the difference in composition.

Table 6.2.4

CONSTITUENT SUGARS OF HEMICELLULOSES

g kg^{-1} DM

	RYEGRASS CUT 3	CLOVER CUT 3	LUCERNE
Xylose	108.6	15.7	7.0
Arabinose	33.4	21.4	12.4
Glucose	72.7	24.7	25.0
Galactose	23.6	13.7	9.5
Hemicellulose	176.0	86.9	67.3
Xylose: Arabinose	3.3	0.73	0.57
Xylose: Glucose	1.5	0.64	0.28

When figures for cuts of each forage at similar stages of maturity are studied, it can be seen that as well as different amounts of hemicellulose in the different forages, there were also large differences in the composition of the hemicelluloses between forages. The major components of hemicellulose of the Gramineae are structures based on a linear chain of β-1,4-linked D-xylopyranosyl residues to which are attached residues of L-arabinofuranose, D-galactopyranose and 4-O-methyl-D-glucopyranosyluronic acid (Wilkie, 1979) whereas the predominant hemicellulose in dicotyledons is formed from a β-D-glucan backbone to which short side chains containing xylopyranose residues are attached to at least 50% of

the glucose residues.

The figures show the much larger amount of xylose in grass than in the legumes, with xylose being the major sugar in the hemicellulose (about 0.6 of the total hemicellulose) in the grass, and forming a much smaller proportion in the clover (about 0.18 of hemicellulose) and lucerne (about 0.10 of hemicellulose). The major sugar in legume hemicellulose is glucose. The lucerne hemicellulose has a higher proportion of glucose to other sugars than the clover hemicellulose, suggesting that there are fewer side chains in the lucerne than in the clover, or that the side chains are shorter in the lucerne.

6.2.4 Changes in hemicellulose content on ensilage

Table 6.2.5

CELLULOSE CONTENT OF FORAGES

g kg^{-1} DM

RYEGRASS

CUT NO	GRASS	SILAGE
1	161.1	162.4
2	159.3	151.3
3	176.0	158.6
4	257.1	160.9

CLOVER

DAYS ENSILED	TREATMENT		
	WATER	FORMIC ACID	FORMALDEHYDE
0	54.5	54.5	54.5
2	44.4	34.3	40.5
20	41.5	49.7	36.4
60	37.2	40.7	39.1
150	42.6	29.8	27.7

CLOVER

CUT NO	CLOVER	SILAGE
1	65.7	36.5
2	57.1	34.6
3	86.9	27.3

LUCERNE

LUCERNE	SILAGE	
67.3	81.0	(64.8 if corrected)

There are several trends in losses of hemicellulose on ensilage apparent from these experiments and the work

of Morrison (1979a). The use of additives in silage making leads to an increase in loss of hemicellulose in both perennial ryegrass (Morrison, 1979a) and clover. The use of formic acid as an additive increased the loss of hemicellulose from ryegrass silages compared with formaldehyde (Morrison, 1979a), but both of these additives seem to increase the losses seen from the clover after 150 days were greater than those seen from the ryegrass in laboratory silos (Table 6.2.6).

Table 6.2.6

FRACTION OF ORIGINAL HEMICELLULOSE PRESENT AFTER ENSILAGE

	TREATMENT	
	FORMIC ACID	FORMALDEHYDE
CLOVER	0.55	0.50
GRASS	0.93	0.82

Both the ryegrass and the clover were ensiled in a farm-scale silo using formic acid as a preservative, and from the information from the laboratory scale silos, it would be expected that loss of hemicellulose be seen on ensilage. However, the reaction of the forages of different maturities was not the same. In both grass and clover a greater loss of hemicellulose was seen from the more mature samples than from the younger cuts. In the grass there was virtually no loss of hemicellulose from the first two cuts during ensilage, with a slight loss from the third cut and a large loss from the most mature

cut. Losses were seen from all cuts of clover, increasing as the maturity of the clover increased. The lucerne did not appear to lose hemicellulose on ensilage, even though it was ensiled in an acid-treated farm-scale silo. However, the comparison of the structures of the hemicelluloses suggests fewer or shorter side chains are found in lucerne than in the clover. It is possible that it is these side chains which have acid labile bonds, and the lower proportion of side chains in the lucerne gives less loss of hemicellulose on ensilage.

The losses of the sugars from the ryegrass hemicellulose show a loss of mainly glucose from the second and third cuts. This loss was also seen in the fourth cut, but was accompanied by a loss of xylose as large as the loss of arabinose. The clover results show an almost equal loss of arabinose, glucose and galactose from the hemicellulose and a low loss of xylose in the laboratory scale silos. A similar pattern was seen in the farm scale silos, with the exception of the third cut of clover, where an additional loss of xylose was found. In the lucerne, the only loss seen from the hemicellulose was of glucose.

From these results it seems likely that the losses from the hemicellulose of the less mature cuts of the forages occurred mainly from the side chains. The more mature cuts of forages seem to have losses from the main chain of the hemicellulose.

Table 6.2.7

LOSSES OF CONSTITUENT SUGARS OF HEMICELLULOSES OF FORAGES

($g\ kg^{-1}$ DM)

RYEGRASS

CUT NO	XYLOSE	ARABINOSE	GLUCOSE	GALACTOSE
2	3.2	5.0	55.7	2.6
3	-1.8	9.6	55.8	-5.6
4	65.9	16.0	61.7	4.3

CLOVER (loss between 0 and 150 days)

TREATMENT	XYLOSE	ARABINOSE	GLUCOSE	GALACTOSE
Water	-0.9	3.2	3.4	5.2
Formic Acid	-1.5	3.1	6.3	7.7
Formaldehyde	0.8	9.2	7.5	8.9

CLOVER

CUT NO	XYLOSE	ARABINOSE	GLUCOSE	GALACTOSE
1	0.7	8.1	7.7	6.4
2	3.3	8.8	4.4	5.7
3	9.7	15.4	14.5	9.8

LUCERNE (using corrected figures)

XYLOSE	ARABINOSE	GLUCOSE	GALACTOSE
-0.6	-0.1	5.4	0.1

6.2.5 Variation in lignin content with age

Table 6.2.8

LIGNIN CONTENT OF FORAGES

g kg^{-1} DM

CUT NO	RYEGRASS	CLOVER	LUCERNE
1	53.9	35.1	
2	55.3	37.1	
3	77.0	30.1	140.3
4	89.7		

The clover samples have the lowest levels of lignin and the lucerne has the highest levels. The lignin levels found in this experiment are similar to those found by Wilkins (1972) and Morrison (1980b). As the grass matured, the amount of lignin increased, this being found by most other workers in this area (e.g. Lindgren, Theander and Aman, 1980; Wilman and Altimimi, 1982). The increase in lignin levels with increase in maturity was not seen in white clover. Similar results were found for red clover (Trifolium pratense) for corresponding dates of harvest (Aman and Nordkvist, 1983).

The lignin values in the lucerne correspond to the values found by Christian et al (1970) for lucerne. These authors also found an increase in the lignin content of the lucerne with increasing maturity.

6.2.6 Changes in lignin content on ensilage

Table 6.2.9

LIGNIN CONTENT OF FORAGES

g kg^{-1} DM

RYEGRASS

CUT NO	GRASS	SILAGE
1	55.9	76.3
2	55.3	60.0
3	77.0	62.3
4	89.7	46.0

CLOVER

DAYS ENSILED	TREATMENT		
	WATER	FORMIC ACID	FORMALDEHYDE
0	48.9	48.9	48.9
2	46.6	44.6	42.6
20	64.0	52.9	52.8
60	42.9	47.8	43.5
150	59.1	53.6	45.2

CLOVER

CUT NO	CLOVER	SILAGE
1	35.1	37.1
2	37.4	34.7
3	30.1	34.3

LUCERNE

LUCERNE	SILAGE	
143.0	154.1	(123.3 if corrected)

Morrison (1979a) found that when ryegrass was ensiled in laboratory scale silos, the amount of core lignin remained unchanged during the ensilage period. The grass in this experiment corresponds to the second cut of grass used in the experiment ensiling ryegrass in a farm scale silo, and a similar result was found with this cut. However, loss of lignin was seen from the more mature cuts of grass on ensilage. Loss of lignin from grass on ensilage does not seem to have been reported by any other workers, probably as most use less mature cuts of grass for ensilage experiments (e.g. McDonald et al, 1968).

There was no apparent loss of lignin on ensilage in either the laboratory experiment or in the farm scale silos from clover at any stage of growth. The results for the single cut of lucerne show a slight loss of lignin if a correction for loss of dry matter is applied.

6.3 CONCLUSIONS

The results for the three forages show an increase in cell wall content as the maturity of the forage increases. There was loss of cell wall contents when the forages were ensiled, this being greater when an additive was used to aid ensilage than when no additive was used. This effect may be due to the chemical action of the additive on the forage, or to the conditions that use of the additive has created causing a change in the growth of the microbial population of the silage. The additives used reflect two of the main classes available for the

farm scale production of silage, preservative (formaldehyde) and acid (formic acid). The use of formaldehyde and other preservatives may lead to problems when the silage face is opened as secondary fermentation can take place. Coupled with the apparent effect of these additives on cell wall components this may lead to a silage of low feeding value. The acid type additive, however, gives a rapid drop in pH to form a stable silage. The loss of cellulose from the cell wall in these conditions may be due to the low pH that is achieved. The advantages of a stable, well preserved silage probably outweigh the loss of feeding value of the silage from the cell wall breakdown. Lucerne has lower losses on ensilage than clover or ryegrass and with the difficulties that can be experienced in preserving lucerne as silage due to its high buffering capacity, the use of an acid additive for the production of lucerne silage would seem to be a necessity.

CHAPTER 7

DIGESTIBILITY STUDIES

The digestibility of grass at different maturity and silage made from the grass.

7.1 INTRODUCTION

Grass is grown and preserved as silage to provide fodder for ruminants, and a knowledge of the digestibility and intake of forages is essential in order to plan feeding regimes. Any changes in digestibility between grass and grass silage will reflect changes that occur during ensilage. To study the changes in digestibility, a simple *in vitro* technique was used.

It was also decided to study the digestion of grass silages in greater detail. To allow the progress of digestion over the time scale that forage would normally be present in the rumen, RUSITEC was used. This allowed the forages to be incubated for three different incubation times under controlled conditions, which made it possible to ascertain any pattern in the digestion of the forage, especially the cell walls. The microbial population of the rumen preferentially digests soluble fractions of forage in the initial stages of digestion, and may not break down structural polysaccharides until some time after initial colonization. By removing forages from incubation in RUSITEC after 24, 48 and 72 hours digestion, any difference in the amount of cell wall broken down or components of the cell wall hydrolysed during the time scale of digestion can be determined.

7.2 IN VITRO DIGESTIBILITY OF GRASS AND GRASS SILAGES

A study was made of the grasses and grass silages from Experiment 1 using the method of Tilley and Terry (1963).

Table 7.2.1

IN VITRO DIGESTIBILITY OF GRASS AND GRASS SILAGES

CUT NO	GRASS	SILAGE	(s.d.)
1	0.73	0.70	(0.01)
2	0.76	0.70	(0.02)
3	0.52	0.53	(0.03)
4	0.54	0.52	(0.05)

The results for grass silages show that less mature cuts of grass (1 and 2) have a higher *in vitro* digestibility than the more mature cuts (3 and 4). There is a considerable drop in digestibility between the second and third cuts of grass (0.24 digestibility units), reflecting the increase in lignin content seen between these two cuts (about 20 g Kg^{-1} DM). The digestibility of the latter two cuts, at around 0.53, is very low and compares with the value of 0.48 for grass straw reported by Han, Lee and Anderson (1975).

The silages made from the first two cuts of grass have digestibilities slightly lower than those found in the original grass, although the values are still consistent with these being well preserved silages (Terry and Osbourne, 1980). The silages made from third and fourth cut grass have low digestibility, with no apparent loss of digestibility of the original grass. These two cuts

of grass had lower water soluble carbohydrate levels than the first two cuts. As it seems likely that reduction in digestibility would be associated with metabolism of water soluble carbohydrates to less readily available compounds, or to gaseous compounds subsequently lost from the silo, there would be less reduction in digestibility in the later two cuts of grass when ensiled as lower levels of these compounds were present in the grass.

7.3 DIGESTION OF GRASS SILAGE IN RUSITEC

The grass silage was incubated in Rusitec as described in Chapter 2, section 3.4. The residues after the incubation were analysed by similar methods to those used for the grass silages (Chapter 3). Output from Rusitec was also recorded during the run. Gas volume and composition, volatile fatty acid (VFA) production and pH of the liquid phase were all recorded, the latter being mainly controlled by the pH of the nutrient solution being infused into the vessel. From the measurements, the state of the fermentation in the vessels and disappearance of different fractions from the grass silages could be followed.

To assess the digestion of fractions in Rusitec, the values found from the analysis of the residue were corrected by the fraction the residue was of the original sample, to give a value for the amount of the fraction remaining relative to that put into the vessel.

e.g. Cellulose, Vessel 1, 48 hr digestion.

Cellulose content of residue = 242.19 g kg^{-1} DM

Residue as fraction of sample put into vessel = 0.36

Thus amount of cellulose recovered relative to DM put into the vessel

= 242.19 x 0.36

= 69.98 g kg^{-1} DM

In this way the losses during digestion can be followed.

7.3.1 Water solubility of grass silages

There are two processes by which forage dry matter can be removed from the sample when it is incubated in Rusitec, (i) digestion by microbial enzymes, (ii) solubilization of part of the forage affected by suspension in an aqueous solution; the latter including the effect of breakdown of the cells by endogenous enzymes. To separate these effects the grass silage was shaken with water (2.3.1) at 37°C.

Table 7.3.1

WATER SOLUBILITY OF GRASS SILAGE

TIME HOURS	RESIDUE	(s.d.)	SOLUBILITY	(s.d.)
0	1.00	(0.00)	0.00	(0.00)
3	0.67	(0.02)	0.33	(0.02)
6	0.71	(0.01)	0.29	(0.01)
24	0.71	(0.02)	0.29	(0.02)
48	0.68	(0.01)	0.32	(0.01)

Within three hours of being mixed with water,

solubilization has reached a maximum. This indicates that the water soluble fraction is released very quickly, and that subsequent mixing has no further effect on the insoluble residue. Thus, when the sample is incubated in Rusitec, any losses after this initial period can be assumed to be due to degradation by microbial enzymes.

7.3.2 Digestibility of grass silage in Rusitec

Table 7.3.2

DIGESTIBILITY OF GRASS SILAGES IN RUSITEC

INCUBATION TIME (hours)	VESSEL 1	VESSEL 2
0	0.00	0.00
24	0.51	0.45
48	0.64	0.60
72	0.70	0.65

Table 7.3.3

DIGESTIBILITY OF GRASS SILAGES IN RUSITEC

IN EXCESS OF WATER SOLUBILITY

INCUBATION TIME (hours)	VESSEL 1	VESSEL 2
0	0.00	0.00
24	0.21	0.15
48	0.34	0.30
72	0.40	0.35

The total digestibility of the grass silage is shown in Table 7.3.2. This value is the fraction of the original material put into the vessel which is removed in

solution or digested by micro-organisms. If the effect of water solubilization is removed from these figures, the extent of digestion of the non-soluble part of the forage (including cell wall material) can be seen. It is apparent that there was a steady increase in the digestion of this fraction with increased time of incubation. The digestibility characteristics of both vessels was similar, with digestion in vessel 1 slightly more efficient than in vessel 2.

7.3.3 Fermentation pattern in vessels

The fermentation characteristics of the vessels were studied throughout the run, with acetate, propionate and butyrate concentrations and total VFA concentration (including isobutyrate and valerate) being measured (Figures 7.3.1 - 7.3.4). Methane production was also recorded (Figure 7.3.5). The figures were plotted against the day on which they were recorded e.g. methane production during the first 24 hours of the run was measured on day 1 and thus plotted against day 1. The day on which the Rusitec run was started was designated day 0.

From the graphs it can be seen that VFA production drops rapidly to day 3 of the run and from then it appears to remain fairly constant. Any variation which is seen occurs simultaneously in both vessels and is therefore probably a function of the amount of forage in the vessel and the stage of digestion of that forage. The same pattern is seen in the VFAs plotted individually. Acetate is the principal VFA produced, with a

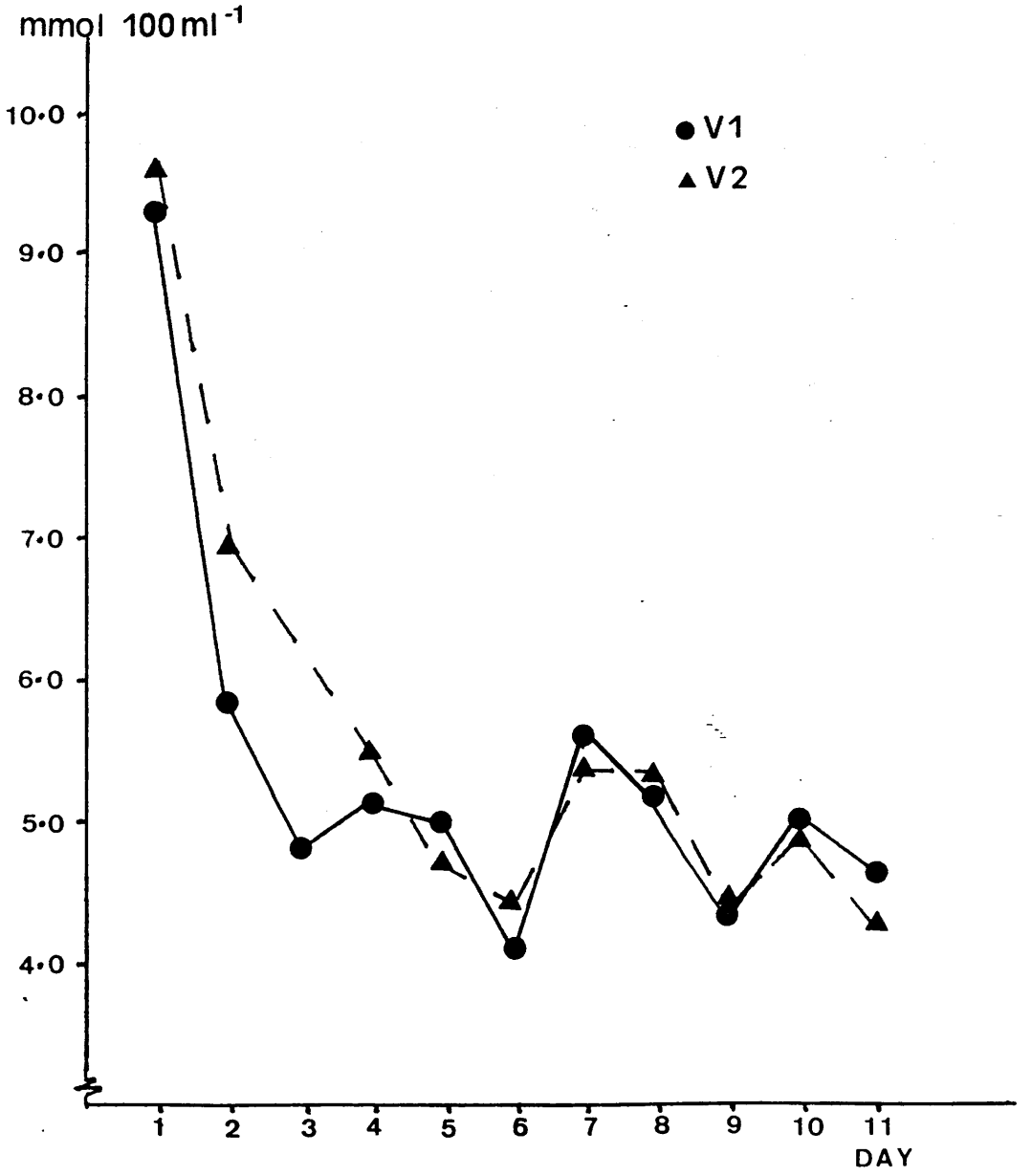


Figure 7.3.1 Total VFA concentration in vessel 1 and vessel 2

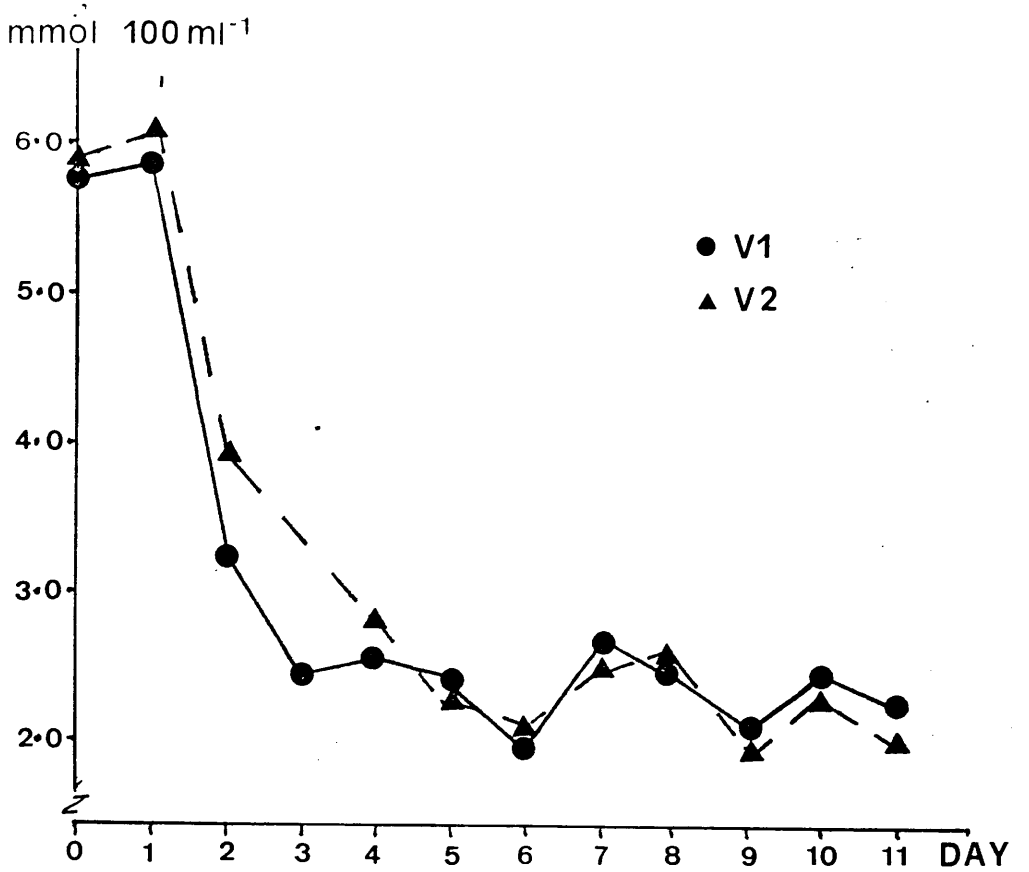


Figure 7.3.2 Acetate concentration in vessel 1 and vessel 2

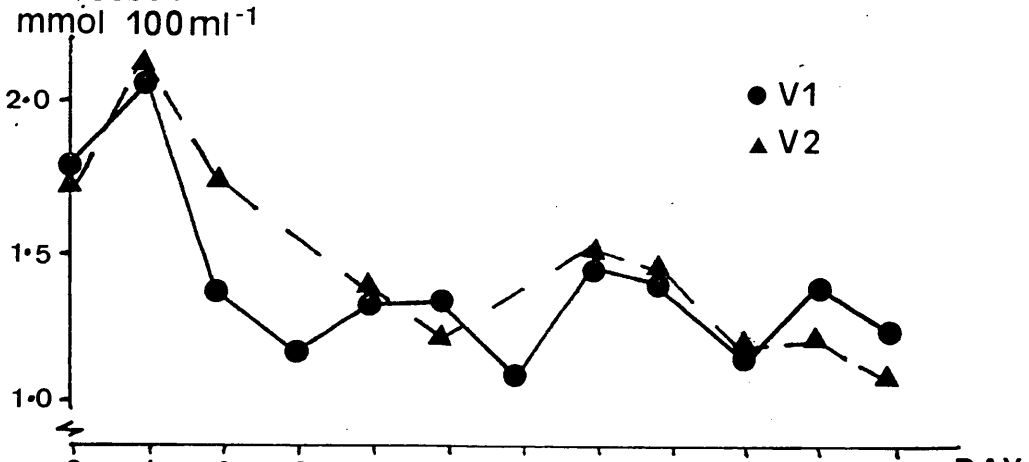


Figure 7.3.3 Propionate concentration in vessel 1 and vessel 2

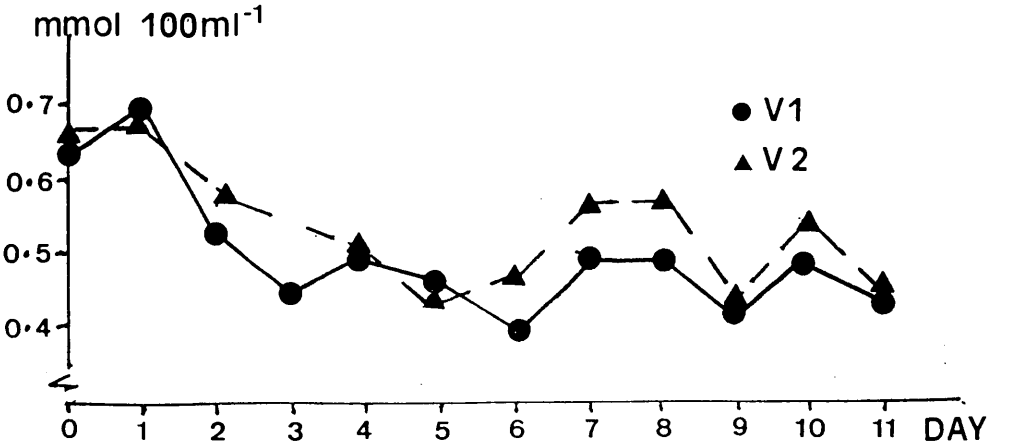


Figure 7.3.4 Butyrate concentration in vessel 1 and vessel 2

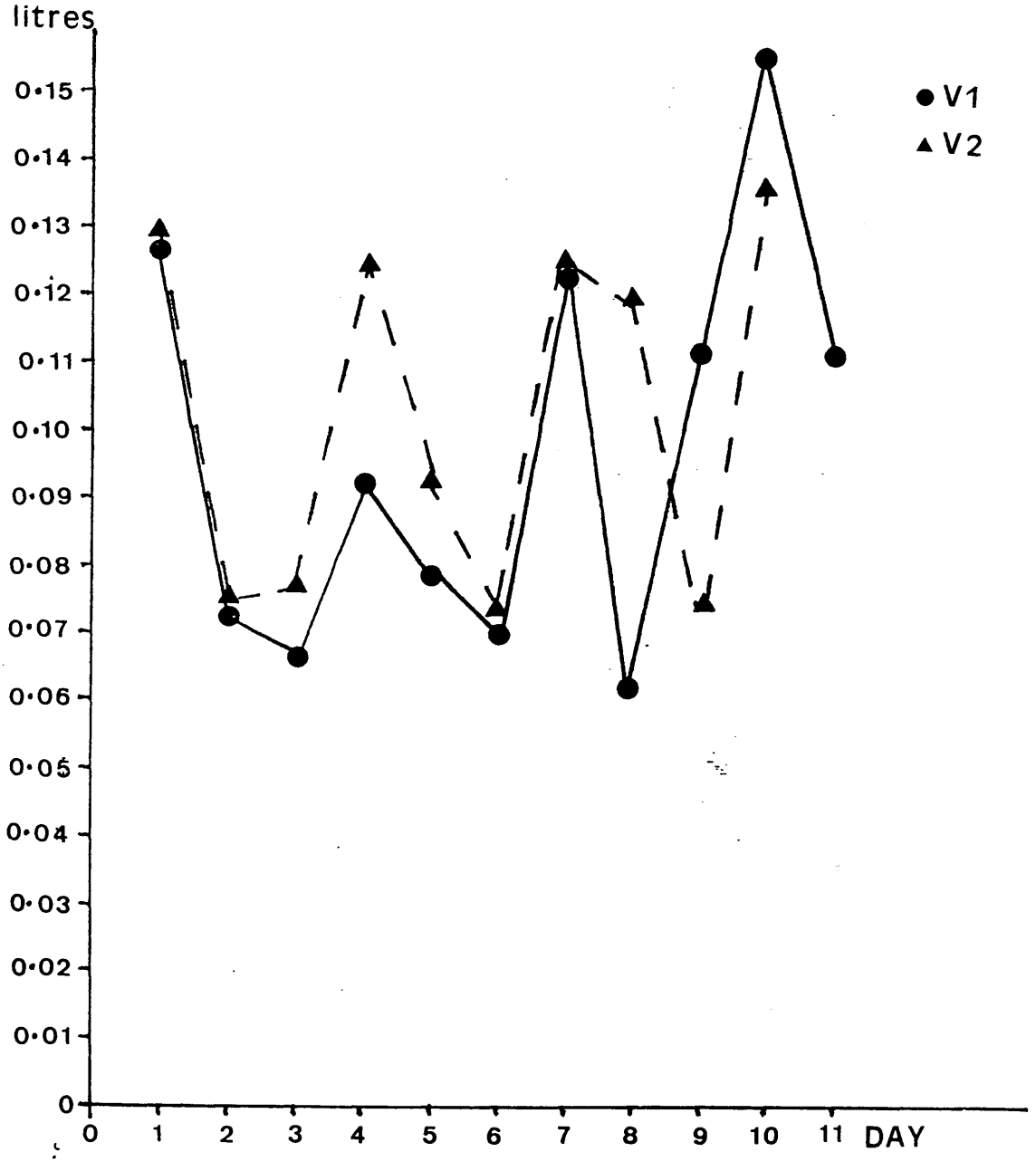


Figure 7.3.5 Methane production in vessel 1 and vessel 2

steady state level of around 2.3 (±0.5) mmol/100 ml. Propionate has a steady state level around 1.3 (±0.2) mmol/100 ml and butyrate has a steady state concentration of around 0.5 (±0.1) mmol/100 ml.

Methane production varies between 0.06 and 0.15 litres per day. This great variability appears in both vesssels, and both vessels have a similar pattern of methane production, with the exception of days 8 and 9. There are similarities between the pattern of methane production and of VFA concentration, with the highest values in both around days 4, 7 and 11. These days were all preceded by a day on which two new bags of silage were introduced into the vessels (3, 6, 7 and 10 were such days), thus it seems likely that the introduction of a fresh supply of nutrients stimulated fermentation and therefore increased VFA and methane production.

7.3.4 Changes in cell wall components during incubation

The residues were analysed for cellulose content, total hemicellulose, neutral sugar composition of the hemicellulose, lignin content and esterified phenolic acid content.

Table 7.3.4

CELLULOSE CONTENT OF RESIDUES FROM RUSITEC DIGESTION

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	215.64	(1.00)	215.64	(1.00)
24	128.33	(0.59)	153.99	(0.71)
48	87.19	(0.40)	99.07	(0.46)
72	69.98	(0.32)	72.61	(0.34)

It can be seen from Table 7.3.4 that the concentration of cellulose remaining relative to that put in the vessel decreases as the incubation time increases. There appears to be considerable cellulose digestion, with a mean for the two vessels of only 0.33 of the cellulose remaining after 72 hours digestion.

Table 7.3.5

HEMICELLULOSE CONTENT OF RESIDUES FROM RUSITEC DIGESTION

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	96.31	(1.00)	96.31	(1.00)
24	57.10	(0.59)	60.17	(0.62)
48	36.74	(0.38)	42.37	(0.44)
72	32.59	(0.34)	39.90	(0.41)

There is also considerable hemicellulose digestion in the forages, with a mean for the two vessels of 0.37

of the hemicellulose remaining after 72 hours digestion. Unlike the cellulose, most of the hemicellulose is lost in the first 48 hours of digestion, with only 0.03 of the original amount being digested between 48 hour and 72 hour digestion periods.

The amounts of the individual neutral sugars released by hydrolysis with 2M TFA were also recorded.

Table 7.3.6

ARABINOSE RELEASED FROM DIGESTED RESIDUES

BY HYDROLYSIS WITH 2M TFA

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	22.6	(1.00)	22.6	(1.00)
24	10.1	(0.45)	10.2	(0.45)
48	7.9	(0.35)	6.9	(0.31)
72	6.3	(0.28)	8.2	(0.36)

Table 7.3.7

XYLOSE RELEASED FROM DIGESTED RESIDUES

BY HYDROLYSIS WITH 2M TFA

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	59.2	(1.00)	59.2	(1.00)
24	39.2	(0.66)	38.5	(0.65)
48	23.8	(0.40)	31.4	(0.53)
72	22.5	(0.38)	23.9	(0.40)

Table 7.3.8

GLUCOSE RELEASED FROM DIGESTED RESIDUES

BY HYDROLYSIS WITH 2M TFA

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	16.6	(1.00)	16.6	(1.00)
24	11.9	(0.72)	15.4	(0.93)
48	7.8	(0.47)	6.6	(0.40)
72	6.2	(0.37)	9.7	(0.58)

Table 7.3.9

GALACTOSE RELEASED FROM DIGESTED RESIDUES

BY HYDROLYSIS WITH 2M TFA

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	7.4	(1.00)	7.4	(1.00)
24	3.4	(0.46)	3.9	(0.53)
48	2.1	(0.28)	3.0	(0.40)
72	2.0	(0.27)	3.4	(0.46)

Most of the arabinose lost disappears in the first 24 hours of the incubation, with under half (0.45) of the arabinose remaining at this stage. Some further arabinose is lost between 24 hours and 72 hours, with a mean for the two vessels of 0.32 of the original arabinose remaining after 72 hours incubation. Loss of xylose occurred more slowly than loss of arabinose, but most of

the xylose was lost in the first 48 hours of incubation in vessel 1, although there was a noticeable loss of xylose between 48 and 72 hours in vessel 2, probably due to the lower losses seen between 24 and 48 hours. Similar patterns were seen for the loss of glucose from the residues during digestion. In vessel 2 both glucose and galactose levels after 48 hours incubation seem to be low, in both cases the amount of the sugar present in the samples after 48 hours digestion is lower than after 72 hours digestion. This is probably due to slight changes in the fermentation pattern in the vessel.

Table 7.3.10

XYLOSE:ARABINOSE RATIO IN HEMICELLULOSE OF RESIDUES FROM RUSITEC DIGESTION

INCUBATION TIME (hours)	VESSEL 1	VESSEL 2
0	2.62	2.62
24	3.86	3.78
48	3.02	4.54
72	3.57	2.93

Table 7.3.11

XYLOSE:GLUCOSE RATIO IN HEMICELLULOSE OF RESIDUES FROM RUSITEC DIGESTION

INCUBATION TIME (hours)	VESSEL 1	VESSEL 2
0	3.57	3.57
24	3.30	2.50
48	3.05	4.76
72	3.65	2.46

These ratios show considerable variation and it is difficult to draw any definite conclusions from these figures. There may be a trend towards the xylose:arabinose ratio increasing as digestion time increases, indicating that more arabinose than xylose is lost during digestion. The low ratio for the Vessel 2 digestion samples is due to slightly higher concentration of arabinose found in these samples, and is a reflection of the sensitivity of these ratios to small fluctuations in the concentrations of the individual sugar components.

The xylose:glucose ratios show even more variation than the xylose:arabinose ratios. No distinct pattern can be discerned in the xylose:glucose ratios, as the concentrations of the individual sugars shows too much variation. There appears to be a decrease in the ratio as the length of digestion time increases, reflecting the loss of a larger proportion of xylose residues than glucose residues.

Lignin content of the residues was determined using the acetyl bromide method (2.2.3.1).

Table 7.3.12

LIGNIN CONTENT OF RESIDUES FROM RUSITEC DIGESTION

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	59.1	(1.00)	59.1	(1.00)
24	46.2	(0.78)	52.5	(0.89)
48	40.4	(0.68)	44.1	(0.75)
72	35.7	(0.60)	40.7	(0.69)

There is apparent loss of lignin from the silages during incubation in Rusitec. This continues throughout the incubation. The disappearance of lignin from the silages could be caused by either of two processes. There may be true digestion of the lignin by the microbial population, or more likely, there may be release of a lignin-carbohydrate complex due to breakdown of bonds in the carbohydrate. The lignin-carbohydrate complex would be released into solution in the vessel and thus apparently digested. This could not be considered as true digestion as no breakdown of the lignin would have occurred.

Table 7.3.13

ESTERIFIED PHENOLIC ACID CONTENT OF RESIDUES FROM RUSITEC DIGESTION

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	10.01	(1.00)	10.01	(1.00)
24	6.45	(0.64)	7.99	(0.80)
48	5.51	(0.55)	6.48	(0.65)
72	5.47	(0.56)	6.01	(0.60)

The phenolic acid levels show a large loss of phenolic acid during the first 24 hours of digestion, and a slight loss after that time. As the loss occurs rapidly, it seems likely that this is due to a solubilization effect rather than the digestion of this fraction by

micro-organisms. The phenolic acid may come into solution at the pH and temperature found in the vessel before any enzymatic activity by the micro-organisms affects the bonds.

7.4 DIGESTION OF CLOVER SILAGE IN RUSITEC

The series of experiments involving digestion of silage in Rusitec was continued with white clover silage. The study of clover digestion seemed to be a particularly important area to cover in this series as clover is increasingly being used as a method of reducing the need for application of large amounts of nitrogen fertilizer to grass.

In a preliminary Rusitec run, difficulty was experienced using clover silage as the forage, due mainly to the high protein content of the material (20%, Chapter 4, Table 4.2.18) and the very small mean particle size of the material. To overcome these problems the level of protein in the liquid infusion was reduced to half its previous value and the clover silage was milled to pass through a 2 mm sieve rather than to pass through a 1 mm sieve. It was also found that the system remained stable at a lower pH than was used for grass silage digestion. In the latter the vessels were maintained at around pH 6.95, whereas with the clover silage digestion it was found that the system remained stable at around pH 6.8.

7.4.1 Water solubility of clover silage

The processes acting on the silage samples in the

Rusitec vessel include digestion by microbial enzymes and solubilization of part of the forage whilst in suspension in the aqueous phase of the culture fluid, the latter including the effect of breakdown of the cell walls by endogenous enzymes. To separate these effects the clover silage was shaken with water at 37°C (2.3.1) The results are shown in Table 7.4.1.

Table 7.4.1

WATER SOLUBILITY OF CLOVER SILAGE

TIME (HOURS)	RESIDUE	(s.d.)	SOLUBILITY	(S.D.)
0	1.00	(0.00)	0.00	(0.00)
3	0.50	(0.01)	0.41	(0.01)
6	0.60	(0.03)	0.39	(0.03)
24	0.62	(0.03)	0.37	(0.03)
48	0.59	(0.01)	0.42	(0.01)

It seems that the water soluble material is removed from the clover silage in the first three hours of suspension in water, with no loss of material being seen after this time. A large fraction of the clover silage (0.40) is solubilized in water, indicating that these silages probably contain a low ratio of cell wall to cell contents.

7.4.2 Digestibility of clover silage in Rusitec

The digestibility of the silage in Rusitec is shown in Table 7.4.2, with the digestibility in excess of mean water solubility (0.40) shown in Table 7.4.3.

Table 7.4.2

DIGESTIBILITY OF CLOVER SILAGES IN RUSITEC

INCUBATION TIME (hours)	VESSEL 1	VESSEL 2
0	0.00	0.00
24	0.49	0.49
48	0.60	0.60
72	0.69	0.68

Table 7.4.3

DIGESTIBILITY OF CLOVER SILAGES IN RUSITEC

IN EXCESS OF WATER SOLUBILITY

INCUBATION TIME (hours)	VESSEL 1	VESSEL 2
0	0.00	0.00
24	0.09	0.09
48	0.20	0.20
72	0.29	0.28

As water solubility reaches a maximum after three hours, the figures in Table 7.4.3 can be taken to represent the breakdown of silage fibre by enzymes derived from the microbial population of the vessel. It can be seen that the digestion of the silages progresses over the time scale that they are incubated in the vessel, with approximately 0.1 of the material being digested every 24 hours. This gives a digestibility of about 0.30 for the non-water soluble fraction of the clover silage after 72 hours.

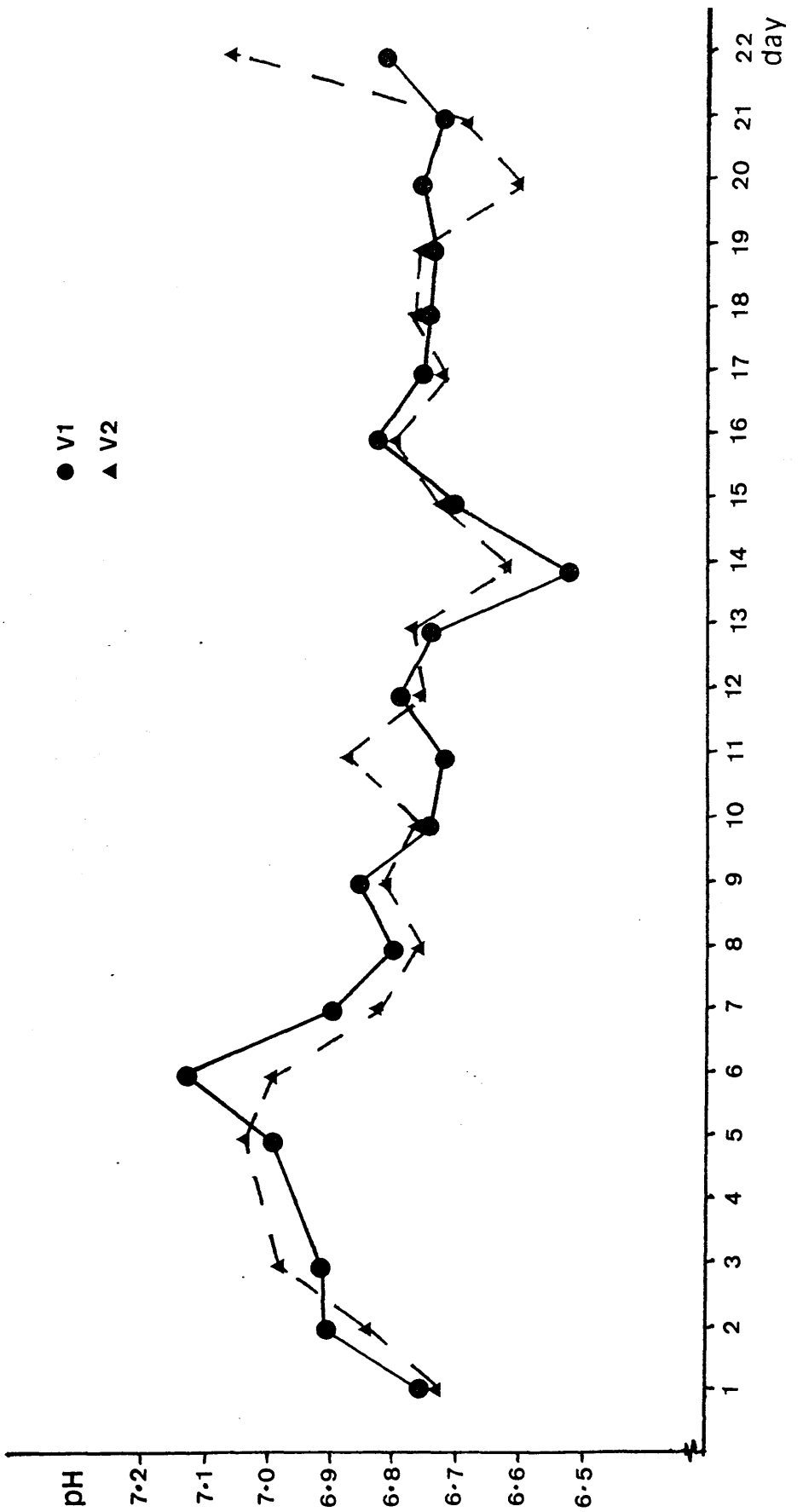


Figure 7.4.1 pH for vessel 1 and vessel 2 during clover silage Rusitec run.

Figure 7.4.2 Total VFA concentration in vessel 1 and vessel 2

7.4.3 Fermentation pattern in the vessels

As was discussed in the introduction to this section, it was found that the optimum pH at which to run the system was around 6.8. Figure 7.4.1 shows the pH recorded in the vessels for each day during the Rusitec run, and apart from day 14, the pH was maintained at this level from day 7 onwards. The pattern of change in pH is the same for both vessels. This would be expected as the fermentation pattern was similar in both vessels and a joint reservoir of infusion medium was used for both vessels.

Total VFA concentration (Figure 7.4.2) during the run showed a similar pattern in both vessels, with a tendency for the second vessel to have slightly higher VFA concentrations than the first vessel. The variation in VFA concentration could be due to the pattern of bag changing (Chapter 2 Table 2.3.4) and the resultant variation in nutrient supply to the microbial population.

The patterns of individual acid concentrations (Figures 7.4.3, 7.4.4 and 7.4.5) were similar to that of total VFA concentration, all showing an initial drop in concentration over the first three days of the run, followed by a peak at day 8, and a subsequent levelling out of the concentration to a steadier level after this peak. Towards the end of the run (day 20 onwards) the levels of VFA concentrations started to rise, this being most noticeable in acetate. A rise in pH was also noted on days 21 and 22 in vessel 2, which would seem to run contrary to an increase in VFA concentration. It is

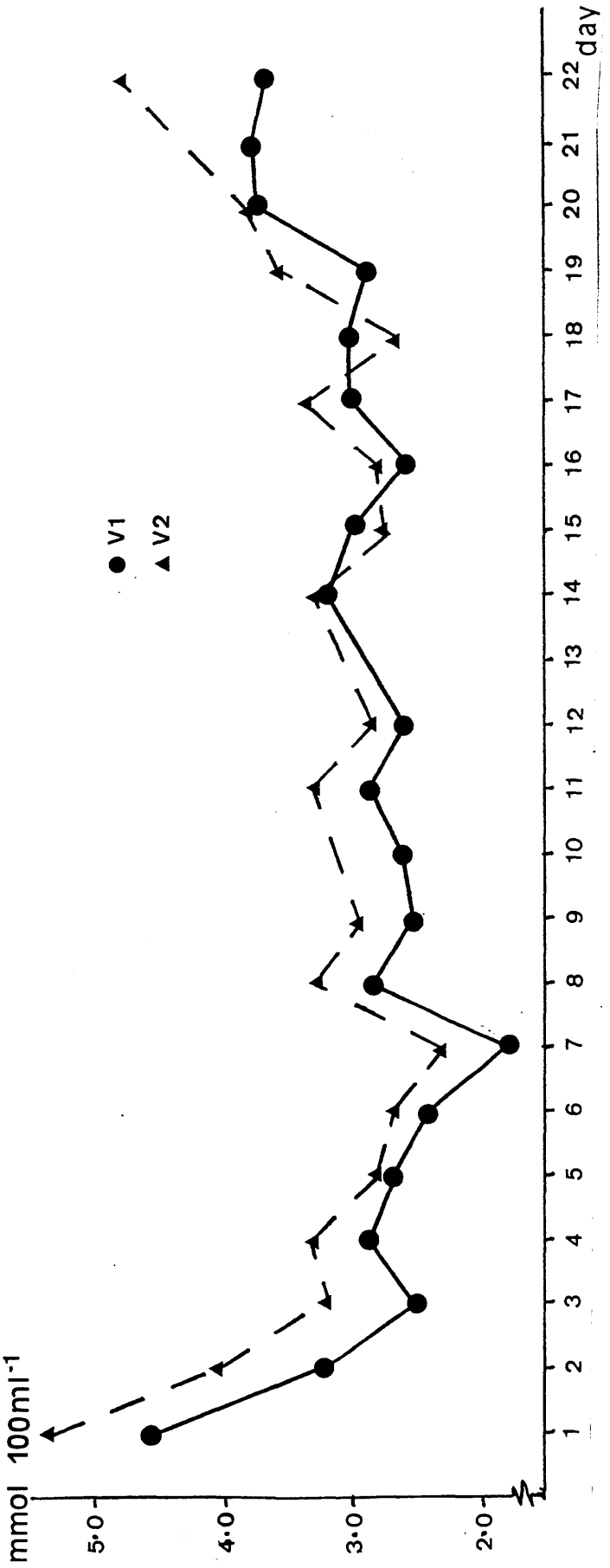


Figure 7.4.3 Acetate concentration in vessel 1 and vessel 2

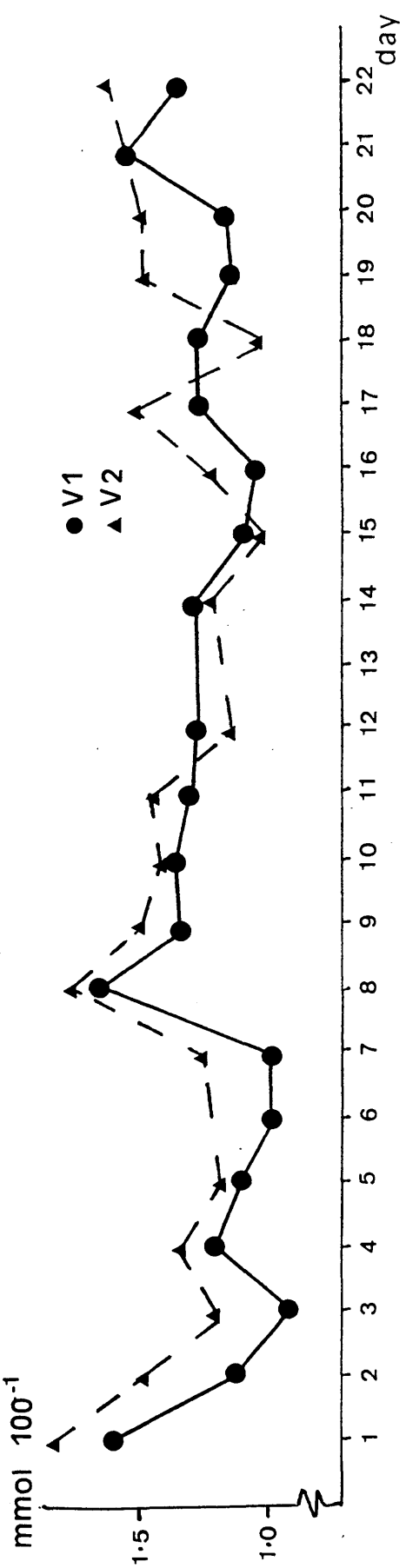


Figure 7.4.4 Propionate concentration in vessel 1 and vessel 2

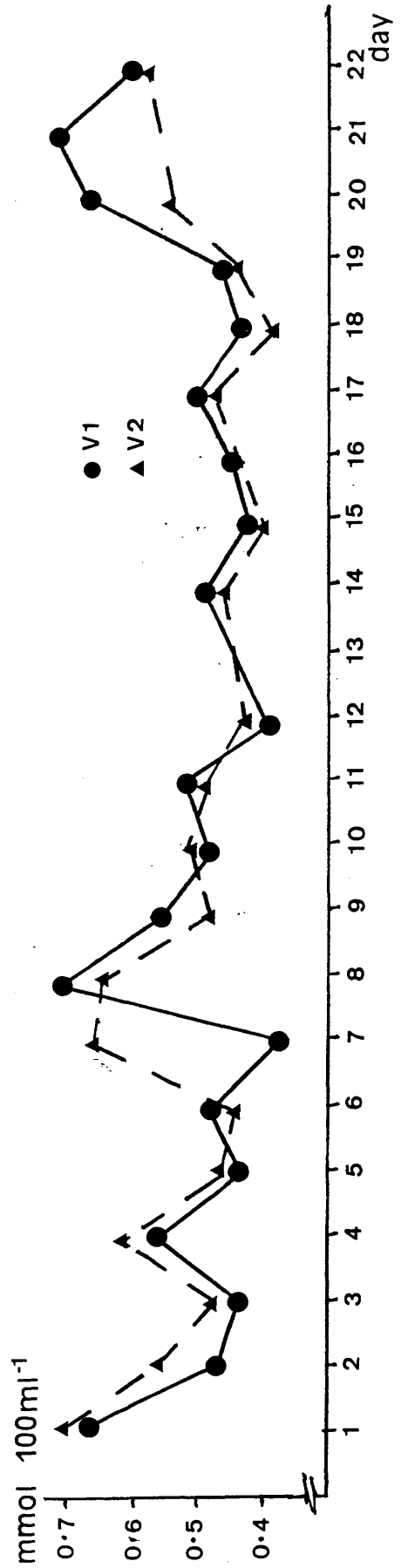


Figure 7.4.5 Butyrate concentration in vessel 1 and vessel 2

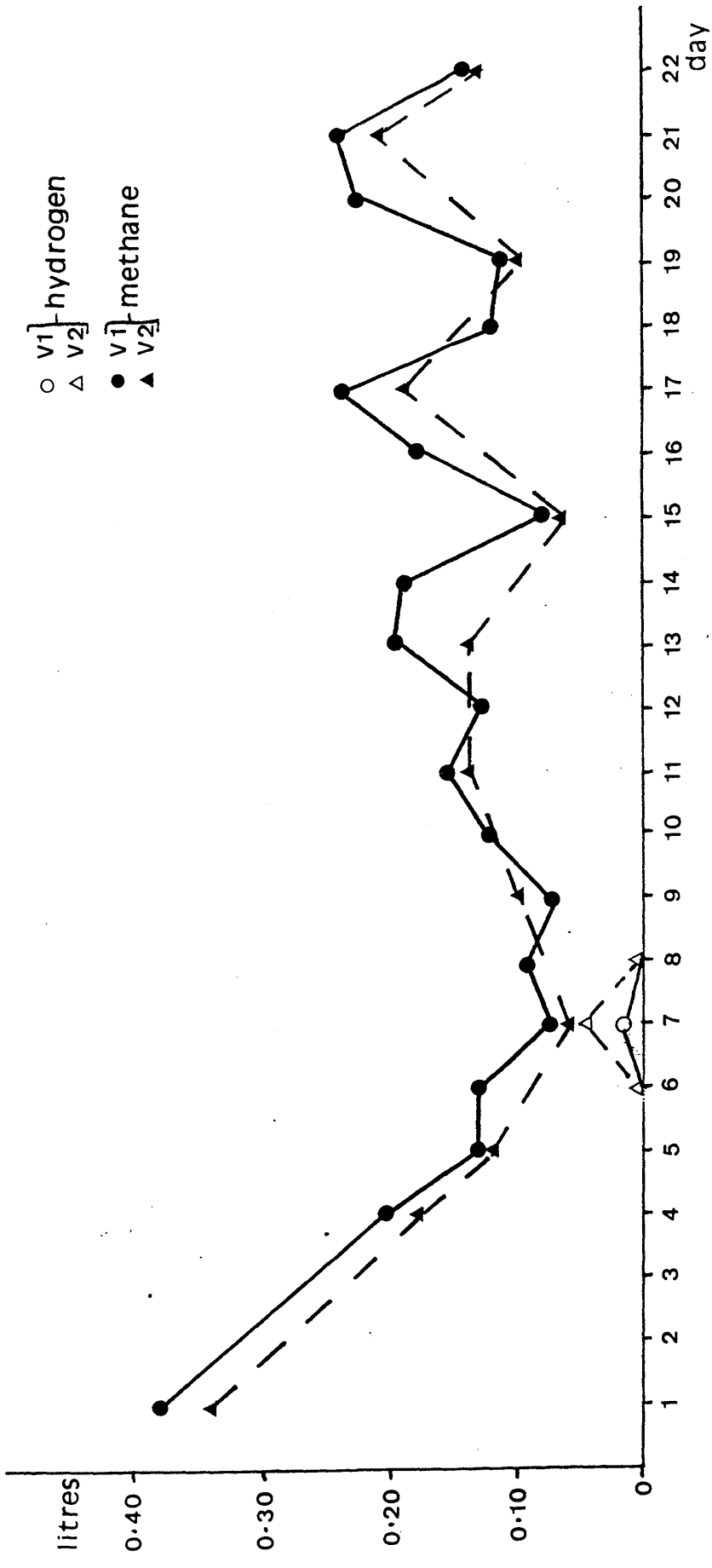


Figure 7.4.6 Gas production in vessel 1 and vessel 2

possible the system was becoming unstable at this stage and these rather odd results reflect this.

The methane production of the vessels decreased over the first six days, and on day 7 some hydrogen production was recorded (Figure 7.4.6). This corresponds to the days on which pH was found to be highest (days 6 and 7, pH 6.9 - 7.15), and it was thought that the methanogenic bacteria were unable to operate properly at pH 6.8 in this system. As pH was reduced methane production increased again to around 0.15 litres per day. As with VFA concentration, there was variation in the level of methane produced between the days, with peaks on days 13, 17 and 21. A certain amount of difficulty was encountered in measuring the methane production in vessel 2 from day 12 onwards as one gas collection bag developed a slow puncture. The effect of this on the results was not detected until a series of low values had been obtained. Thus, in vessel 2, the results for every second day are plotted, these being the days on which an unpunctured gas bag was connected to the vessel.

In an attempt to discover whether pH had any effect on VFA production or VFA concentration, both of these parameters were plotted against the pH of the vessel. As pH changed during the day when the buffered infusion was pumped into the vessel, methane production and VFA concentration were first plotted against the pH recorded on the previous day (i.e. at the beginning of the 24 hour period in which the methane or VFA were produced). The resulting graphs are seen in Figures 7.4.7 and 7.4.8. It

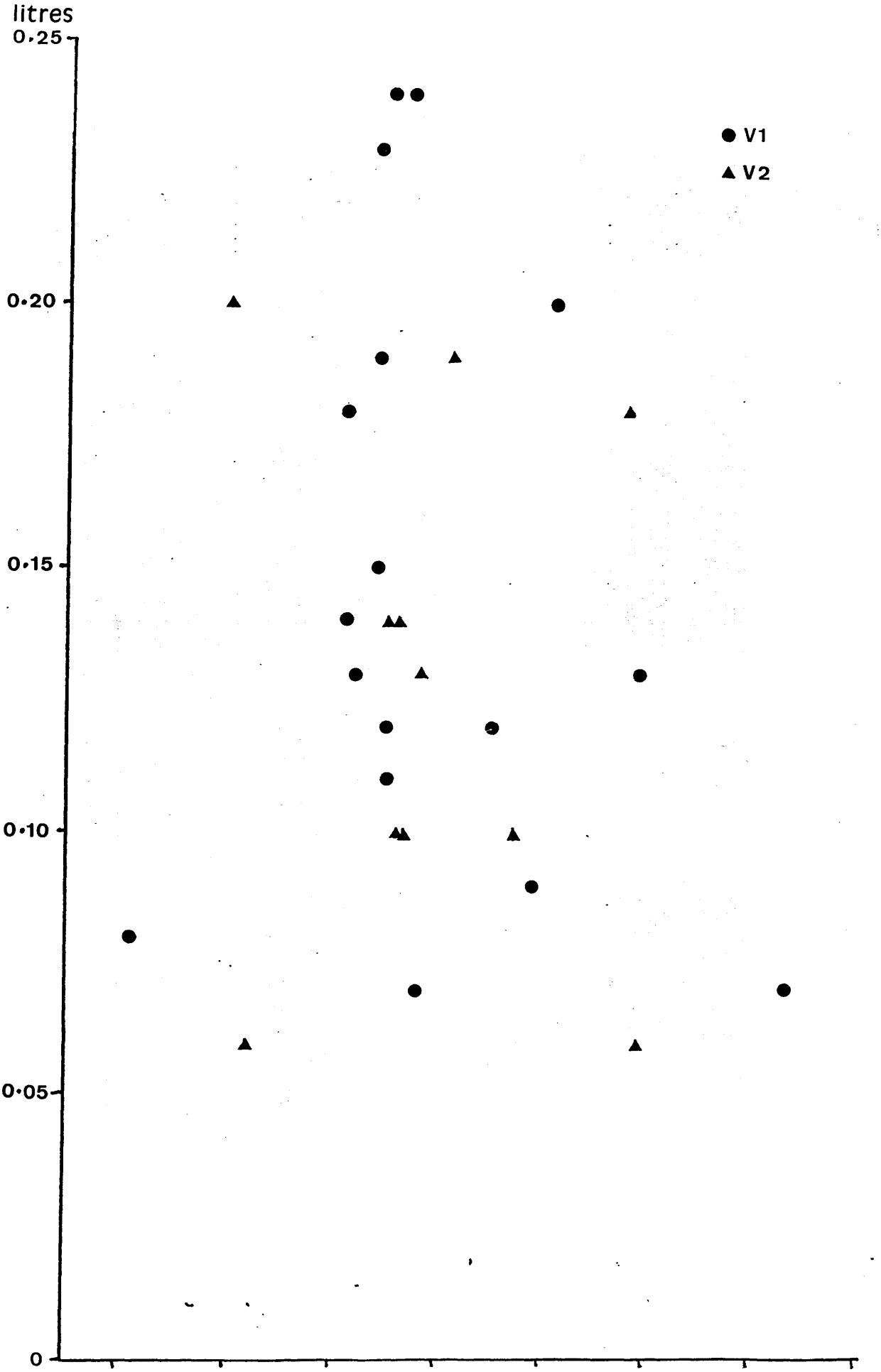


Figure 7.4.7 Methane production against previous day pH

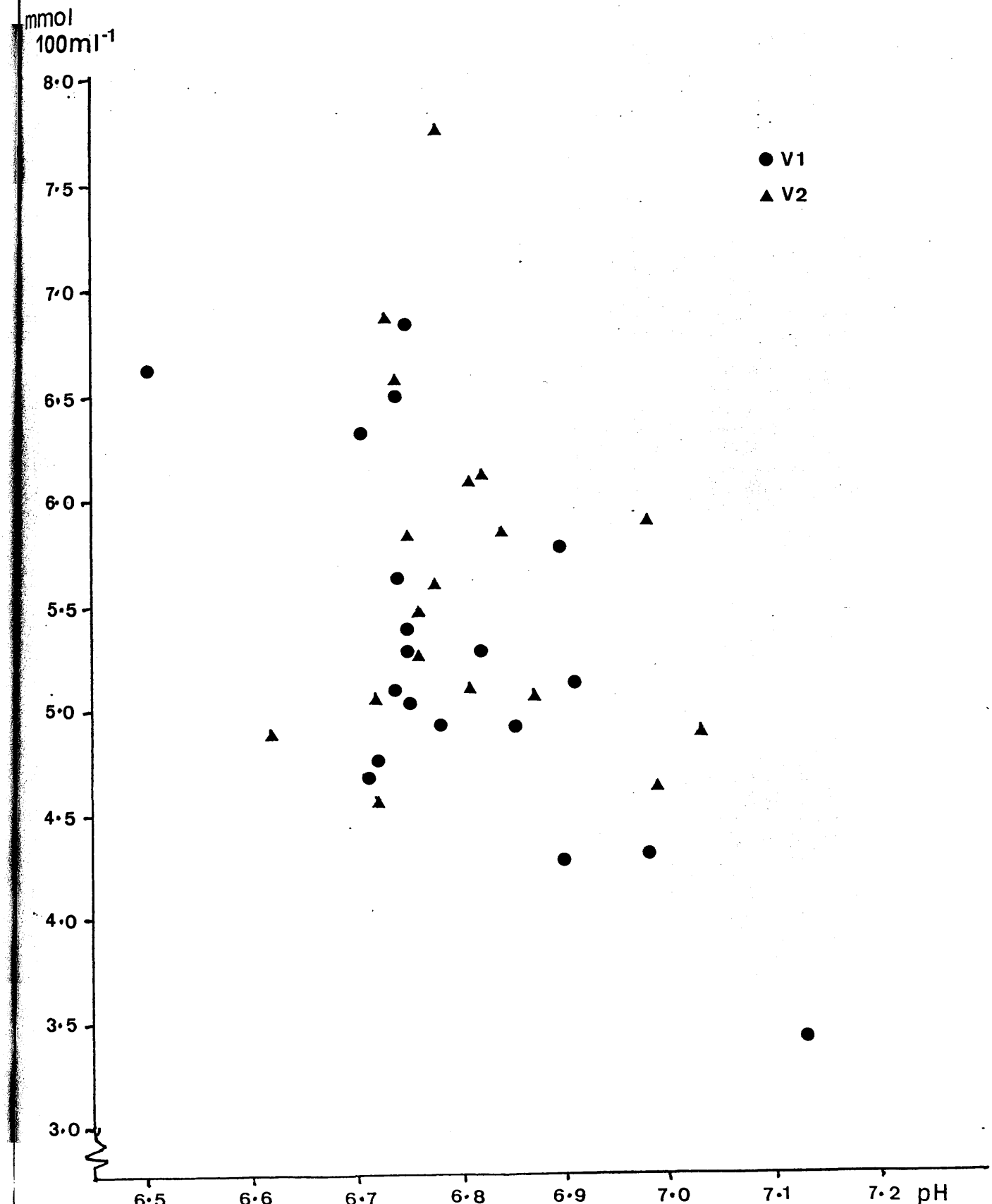


Figure 7.4.8 VFA concentration against previous day pH

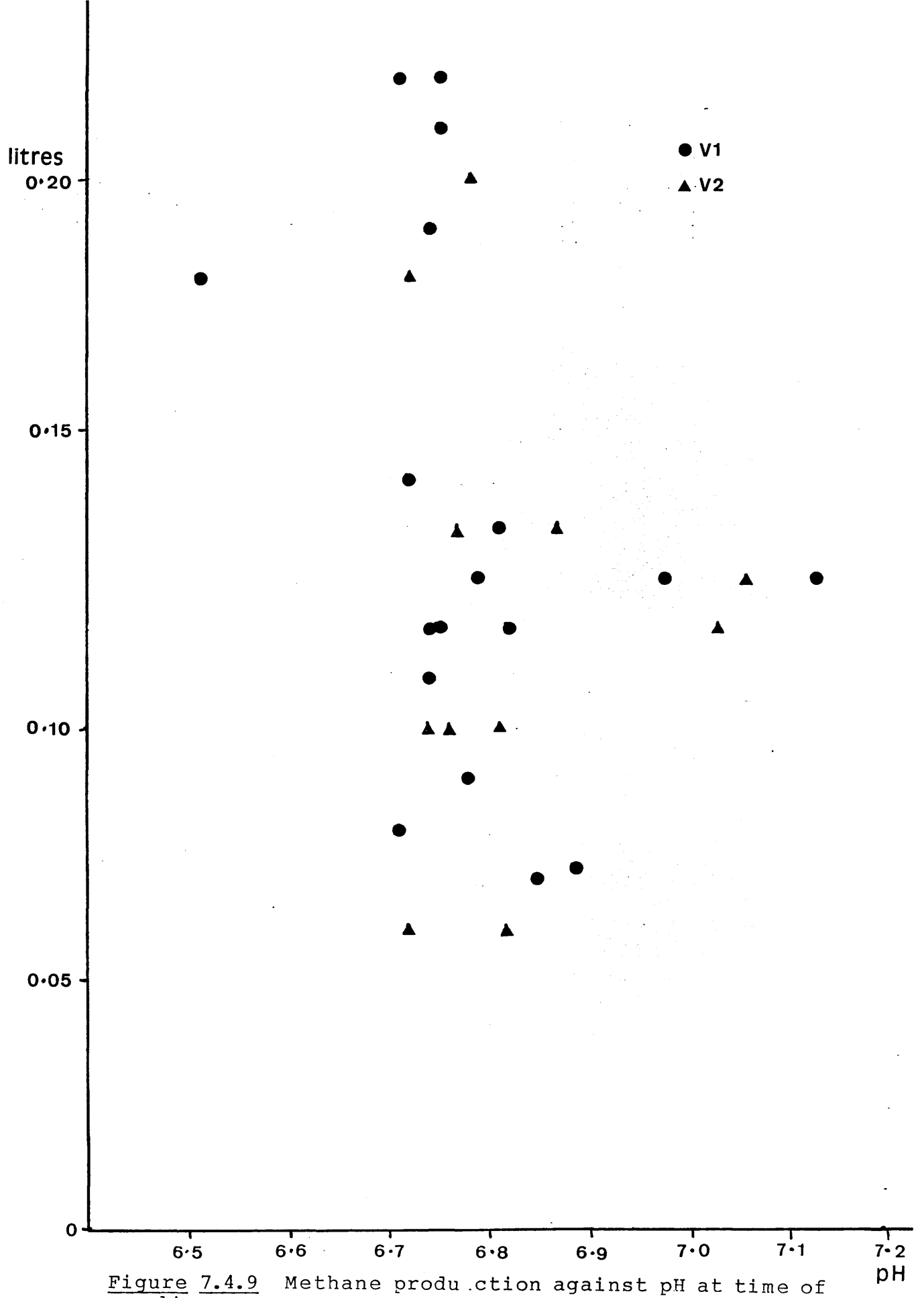


Figure 7.4.9 Methane produ .ction against pH at time of sampling.

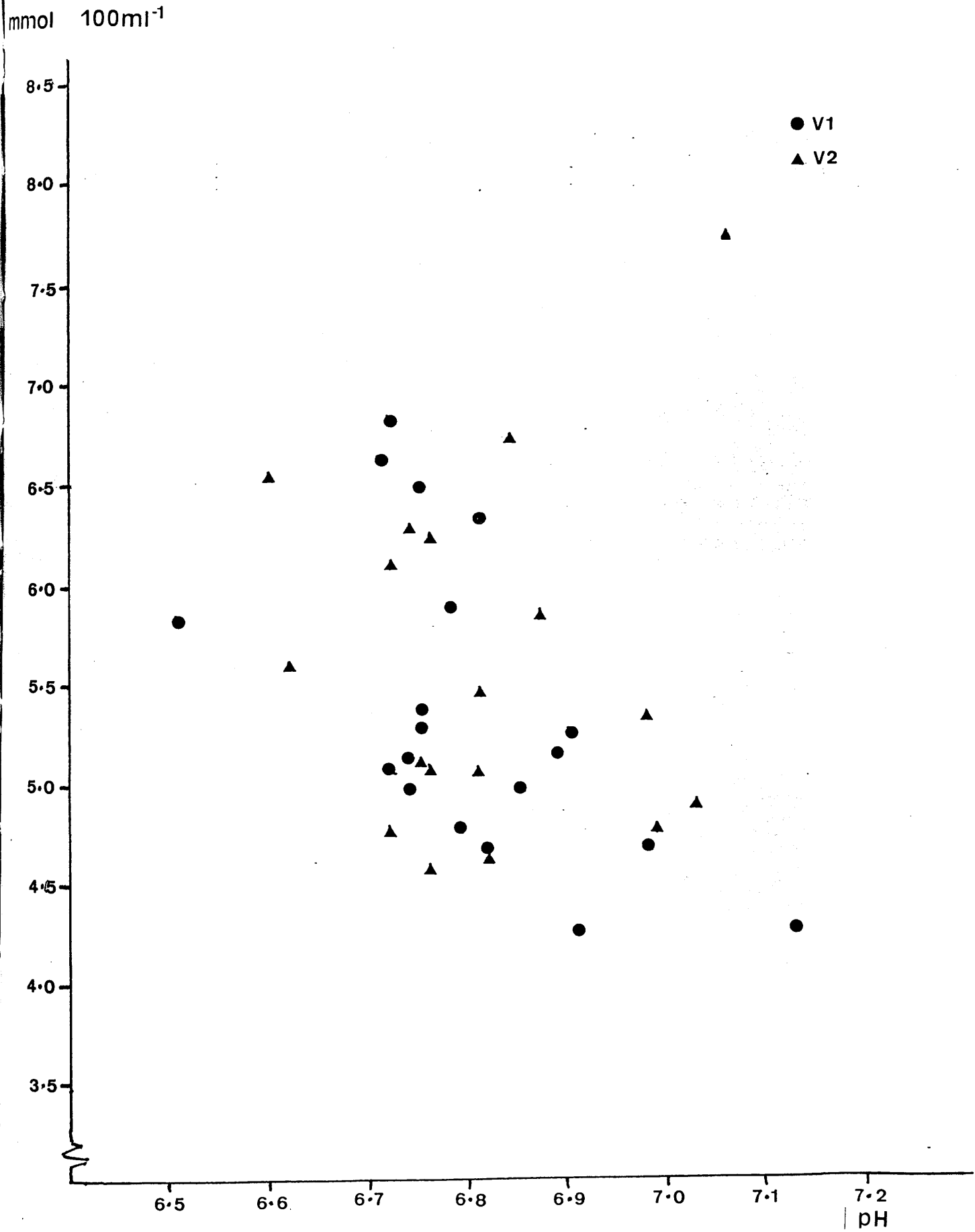


Figure 7.4.10 VFA concentration against pH at time of sampling.

can be seen that there is no significant correlation between methane production and the pH at the beginning of the 24 hour period in which it is produced. A trend towards higher VFA concentration when initial pH is low can be seen, but again there is no definite correlation.

The methane production and VFA concentration were then plotted against pH at the end of the 24 hour period in which the methane of VFA was produced, giving Figures 7.4.9 and 7.4.10. There was no correlation between methane production and pH at the end of the twenty four hour period in which it was produced. However, when the graph of VFA concentration against pH at the end of the twenty four hour period was studied, a correlation coefficient of -0.54 was obtained for vessel 1, although a correlation coefficient of 0.05 was found for vessel 2. It seems likely that VFA concentration would be related to pH as presence of a high VFA concentration will lead to a lowered pH. This effect is partially obscured by the infusion of a highly buffered solution, giving a reduced correlation coefficient between pH and VFA concentration.

7.4.4 Changes in cell wall composition during incubation

For any of the constituents measured, content of the residues was corrected for losses to give the amount of the constituent removed from the vessel relative to the amount put into the vessel.

The cell wall constituents of the residues from digestion were determined by the same methods as those

used for the clover silage.

Table 7.4.4

CELLULOSE CONTENT OF RESIDUES FROM RUSITEC DIGESTION

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	73.88	(1.00)	73.88	(1.00)
24	76.76	(1.03)	76.61	(1.04)
48	66.89	(0.91)	62.23	(0.84)
72	49.13	(0.66)	45.62	(0.62)

There appears to be no cellulose digestion in the first twenty four hours of incubation in Rusitec, the slight increase in cellulose content probably being due to contamination of the sample by microbes or products of microbial breakdown of the forage. Cellulose breakdown seems to occur after twenty four hours digestion, with breakdown continuing at a faster rate between forty eight and seventy two hours digestion than between twenty four and forty eight hours digestion. This would tend to indicate that microbial colonization of clover cellulose takes a little longer than twenty four hours, and continues to increase after forty eight hours incubation.

No such delay is seen in the digestion of hemicellulose.

Table 7.4.5

HEMICELLULOSE CONTENT OF RESIDUES FROM RUSITEC DIGESTION

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	40.06	(1.00)	40.06	(1.00)
24	29.97	(0.75)	14.82	(0.37)
48	18.84	(0.47)	19.52	(0.49)
72	16.48	(0.41)	18.99	(0.47)

The value for vessel 2 twenty four hours digestion seems anomalous, but disregarding this figure, most of the digestion of hemicellulose occurs in the first twenty four hours, with some continuing between twenty four and forty eight hours incubation. There appears to be very little digestion of the hemicellulose after forty eight hours incubation.

When the individual sugars are considered the same pattern can be seen as was noted in the overall hemicellulose figures.

Table 7.4.6

ARABINOSE FROM HEMICELLULOSE IN RESIDUES FROM RUSITEC DIGESTION

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	9.54	(1.00)	9.54	(1.00)
24	6.27	(0.66)	3.62	(0.38)
48	3.54	(0.37)	3.59	(0.38)
72	3.05	(0.32)	1.76	(0.18)

Table 7.4.7

XYLOSE FROM HEMICELLULOSE IN RESIDUES FROM RUSITEC DIGESTION

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	11.27	(1.00)	11.27	(1.00)
24	9.31	(0.83)	5.28	(0.47)
48	5.76	(0.51)	5.74	(0.51)
72	6.65	(0.60)	7.44	(0.66)

Table 7.4.8

GLUCOSE FROM HEMICELLULOSE IN RESIDUES FROM RUSITEC DIGESTION

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	15.42	(1.00)	15.42	(1.00)
24	12.54	(0.81)	8.69	(0.56)
48	8.74	(0.57)	9.11	(0.59)
72	6.48	(0.42)	8.63	(0.56)

Table 7.4.9

GALACTOSE CONTENT OF RESIDUES FROM RUSITEC DIGESTION

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	8.94	(1.00)	8.94	(1.00)
24	5.57	(0.62)	3.74	(0.42)
48	3.11	(0.35)	3.62	(0.40)
72	2.53	(0.28)	3.59	(0.40)

There is greatest digestion of the arabinose and galactose, with over 0.6 of these sugars being digested. Lower digestion of xylose and glucose was seen. There is an indication that glucose and arabinose digestion continues after 48 hours. This is reflected in the xylose:arabinose and xylose:glucose ratios, both of which increase between 48 and 72 hours digestion, suggesting a decrease in the amounts of arabinose and glucose relative to xylose.

Table 7.4.10

XYLOSE:ARABINOSE RATIO IN HEMICELLULOSES OF RESIDUES FROM RUSITEC DIGESTION

INCUBATION TIME (hours)	VESSEL 1	VESSEL 2
0	1.2	1.2
24	1.5	1.5
48	1.7	1.6
72	2.2	4.1

Table 7.4.11

XYLOSE:GLUCOSE RATIO IN HEMICELLULOSES

OF RESIDUES FROM RUSITEC DIGESTION

INCUBATION TIME (hours)	VESSEL 1	VESSEL 2
0	0.73	0.73
24	0.74	0.61
48	0.67	0.62
72	1.01	0.86

Lignin was measured in these samples using the acetyl bromide method.

Table 7.4.13

LIGNIN CONTENT OF RESIDUES FROM RUSITEC DIGESTION

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	61.4	(1.00)	61.4	(1.00)
24	59.1	(0.96)	58.1	(0.95)
48	47.5	(0.77)	48.4	(0.79)
72	40.6	(0.66)	43.1	(0.70)

There is evidence of loss of lignin from the silages in the period between 24 and 48 hours incubation, with some slight loss after 48 hours. This could be due to solubilization of the lignin-carbohydrate complex and would thus correspond with the losses of hemicellulose during this period.

Table 7.4.14

ESTERIFIED PHENOLIC ACID CONTENT OF RESIDUES FROM RUSITEC DIGESTION

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	1.15	(1.00)	1.15	(1.00)
24	1.94	(1.69)	1.82	(1.58)
48	1.50	(1.30)	1.42	(1.23)
72	1.06	(0.92)	0.93	(0.81)

The phenolic acid levels in the clover silage were very low. There appeared to be a slight increase in phenolic acid levels after 24 and 48 hours incubation, this possibly being due to contamination of the sample with fluid from the incubation vessel containing products of microbial metabolism which would be measured as phenolic acids.

Clover silage contains starch as a storage polymer and it was of interest whether this was being used as an energy source by the microbial population, as this could possibly interfere with cellulose digestion.

Table 7.4.15

GLUCOSE FROM STARCH IN RESIDUES FROM RUSITEC DIGESTION

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	8.22	(1.00)	8.22	(1.00)
24	0.15	(0.02)	0.11	(0.01)
48	0.16	(0.02)	0.08	(0.01)
72	0.10	(0.01)	0.03	(.01)

From Table 7.4.15 it can be seen that more than 98% of the starch was digested in the first 24 hours of incubation. It therefore seems unlikely that starch will interfere with the digestion of the cell wall as it is such a rapidly utilised energy source.

Table 7.4.16

WATER SOLUBLE GLUCOSE IN RESIDUES FROM RUSITEC DIGESTION

g kg^{-1} DM of forage into the vessel

INCUBATION TIME (hours)	VESSEL 1	(fraction of original)	VESSEL 2	(fraction of original)
0	3.62	(1.00)	3.62	(1.00)
24	0.45		0.42	
48	0.56		0.68	
72	0.31		0.47	

The low levels of water soluble carbohydrate found in the silage were rapidly metabolised to give very low levels in the digesta. This is as expected, since the micro-organisms in the RUSITEC system will preferentially

metabolise the soluble sugar.

7.5 DIGESTION OF LUCERNE SILAGE IN RUSITEC

The third forage in the series studied using Rusitec digestion was lucerne silage. The silage was obtained from a farm scale silo which had been ensiled using formic acid as a perservative. This silage was taken as another example of a leguminous crop. The use of lucerne as a forage for ruminants in Britain is limited, but extensive use has been made of it in the USA, where it is called alfalfa. However, little work appears to have been done on the breakdown of lucerne cell walls in the rumen. The lucerne silage was found to be high in protein (similar to the clover) and the conditions maintained in the vessel were similar to those maintained during the incubation of the clover silage.

The milling of the lucerne was not such a problem as milling of the clover silage had been as the lucerne was a stemmier material, however it was decided to maintain a 2 mm sieve size for milling to prevent too rapid solubilization of the water soluble carbohydrate components as this could cause destabilization in the Rusitec vessels.

7.5.1 Water solubility of lucerne silage

The processes acting on the silage samples in the Rusitec vessel include both digestion by microbial enzymes and solubilization of part of the forage whilst in suspension in the aqueous phase of the culture fluid. The latter effect includes the breakdown of cell walls by

endogenous enzymes. To separate the two types of breakdown, the lucerne silage was suspended in distilled water at 37°c and shaken for varying periods (2.3.1). The results are shown in Table 7.5.1.

Table 7.5.1

WATER SOLUBILITY OF LUCERNE SILAGE

TIME (hours)	SOLUBILITY	(s.d.)	RESIDUE
3	0.34	(0.01)	0.66
6	0.35	(0.04)	0.65
24	0.31	(0.01)	0.69
48	0.38	(0.02)	0.65
72	0.35	(0.03)	0.65
Mean	0.35		0.65

From the table it can be seen that the water soluble components of the lucerne silage dissolve into the aqueous phase in the first three hours of incubation in water at 37°C. About one-third of the lucerne silage is solubilized in water and this probably represents the cell contents of the silage. These figures can be used to adjust the values obtained for apparent digestion of lucerne silage in the Rusitec vessels, to give figures showing the loss of material due to the action of microbial enzymes.

7.5.2 Digestibility of lucerne silage in Rusitec

The lucerne silage was incubated in Rusitec as

described in 2.3.4. The residues after the incubation were analysed by similar methods to those used for grass and clover silages. Output from Rusitec was also recorded during the run. Gas volume, composition, volatile fatty acid (VFA) production and pH of the liquid phase were all recorded, the latter being mainly controlled by the pH of the nutrient solution being infused into the vessel. From the measurements, the state of fermentation in the vessels and the disappearance of different fractions from the lucerne silage could be followed.

To assess the digestion of fractions in Rusitec, the values found from the analysis of the residue was adjusted to give a value for the amount of the fraction remaining relative to that put in the vessel, as described in section 3.

7.5.3 Fermentation patterns in vessels

The digestibility of lucerne silage increases with time. The figures for total digestion show that the increase in digestibility is steady over the time scale of the digestion, with no apparent plateau of digestibility being reached by 72 hours. Both vessels had similar digestibilities. Losses due to water solubility are subtracted from the apparent digestibility to give a value for digestibility due to microbial enzyme action.

Table 7.5.2

LUCERNE SILAGE TOTAL DIGESTIBILITY

DIGESTION TIME hours	V1	(s.d.)	V2	(s.d.)
0	0.00		0.00	
24	0.44	(0.08)	0.45	(0.07)
48	0.50	(0.07)	0.53	(0.06)
72	0.56	(0.06)	0.58	(0.07)

Table 7.5.3

LUCERNE SILAGE DIGESTIBILITY

(in excess of water solubility)

DIGESTION TIME hours	V1	(s.d.)	V2	(s.d.)
0	0.00		0.00	
24	0.09	(0.08)	0.10	(0.07)
48	0.15	(0.07)	0.18	(0.06)
72	0.21	(0.06)	0.23	(0.07)

A steady increase in digestibility of the non-water soluble components was seen, with a lag phase being shorter than 24 hours. The digestion of non-water soluble components seemed to be slightly higher in the first 24 hours of digestion than in subsequent 24 hour periods, which may reflect increased availability of easily digested components in the first 24 hours of digestion.

The vessel pH, VFA production and gas production were measured throughout the run. To maintain the pH of

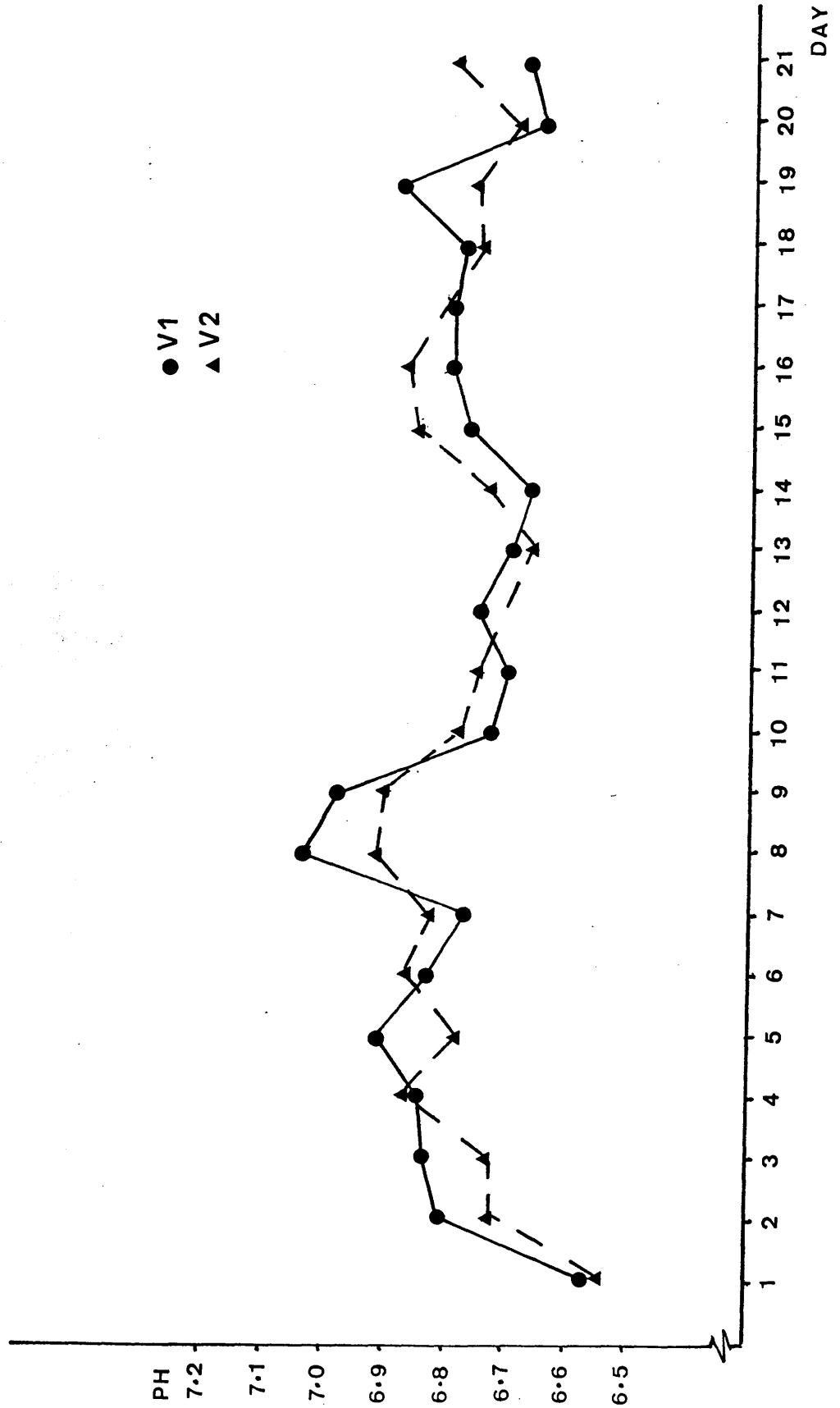


Figure 7.5.1 pH for vessel 1 and vessel 2 during lucerne silage Rusitec run.

the vessels at a constant level the pH of the infusion was adjusted. A pH of around 6.8 was considered to be optimum for the survival of the micro-organisms which break down the cell wall without encouraging the growth of hydrogen-producing bacteria at the expense of methanogens. From the graph (Figure 7.5.1) it can be seen that the pH was maintained at around this level, except for two days when it rose to around 7.00. The pH of both vessels had the same pattern throughout the run.

The production of VFAs during the experiment is shown in Figures 7.5.2 to 7.5.5. It can be seen that VFA production reached a plateau around day 7. The three main VFAs produced were acetate, propionate and butyrate. The pattern of VFA production for all three acids follows the same pattern as the total VFA production. The dip in VFA production on day 19 appears to be a reduction in acetate and propionate production rather than butyrate production.

Methane production drops rapidly after the first day with a fairly steady level of production after day 3. (Figure 7.5.6) Some difficulty was experienced in measuring methane production as technical problems were encountered. The apparent high value on day 10 could have been due to machine error. As the pH was high on days 9 and 10, which would usually lead to a depression in the activity of the methanogens, this seems the most likely explanation.

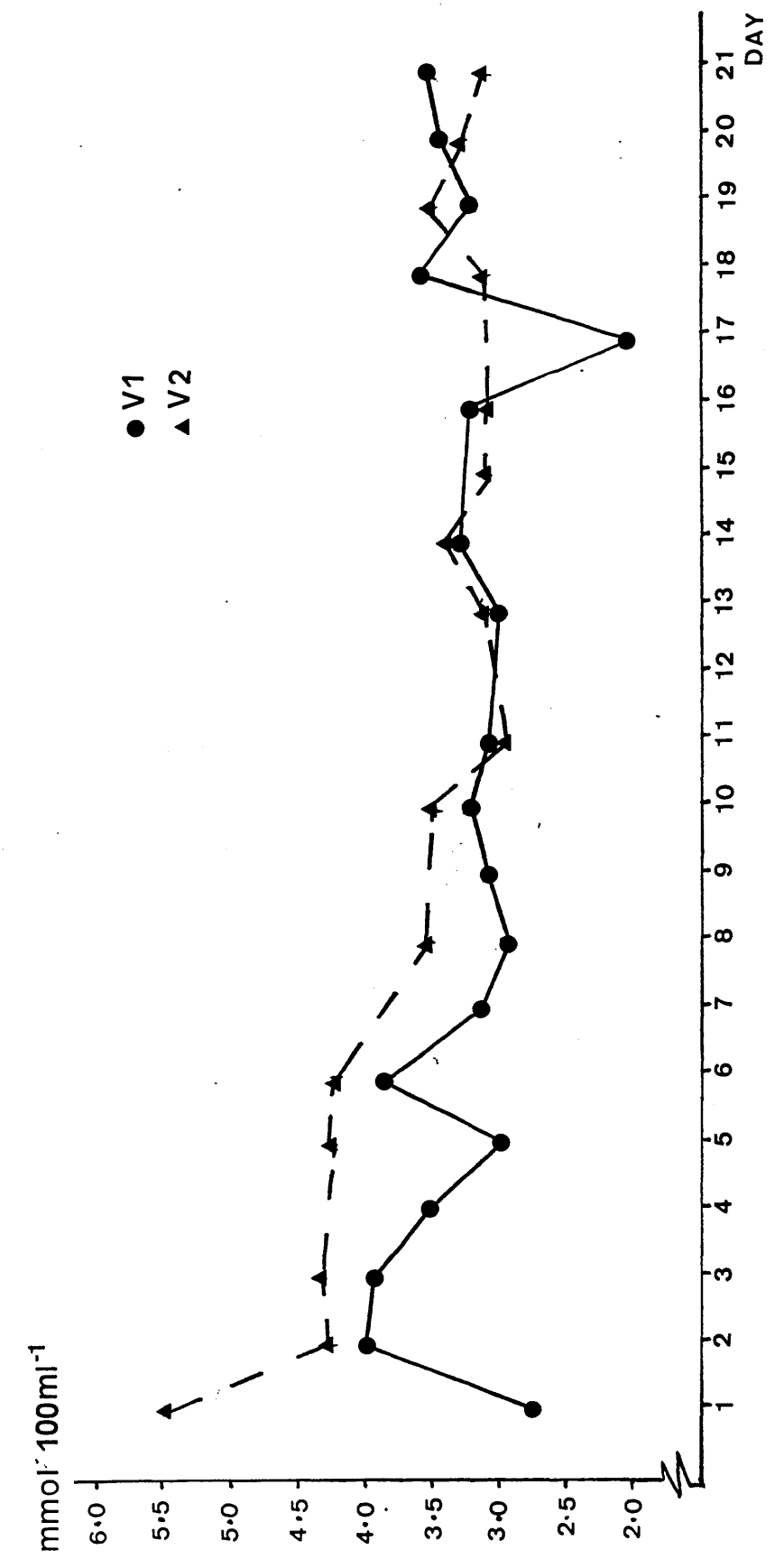


Figure 7.5.2 Total VFA concentration in vessel 1 and vessel 2

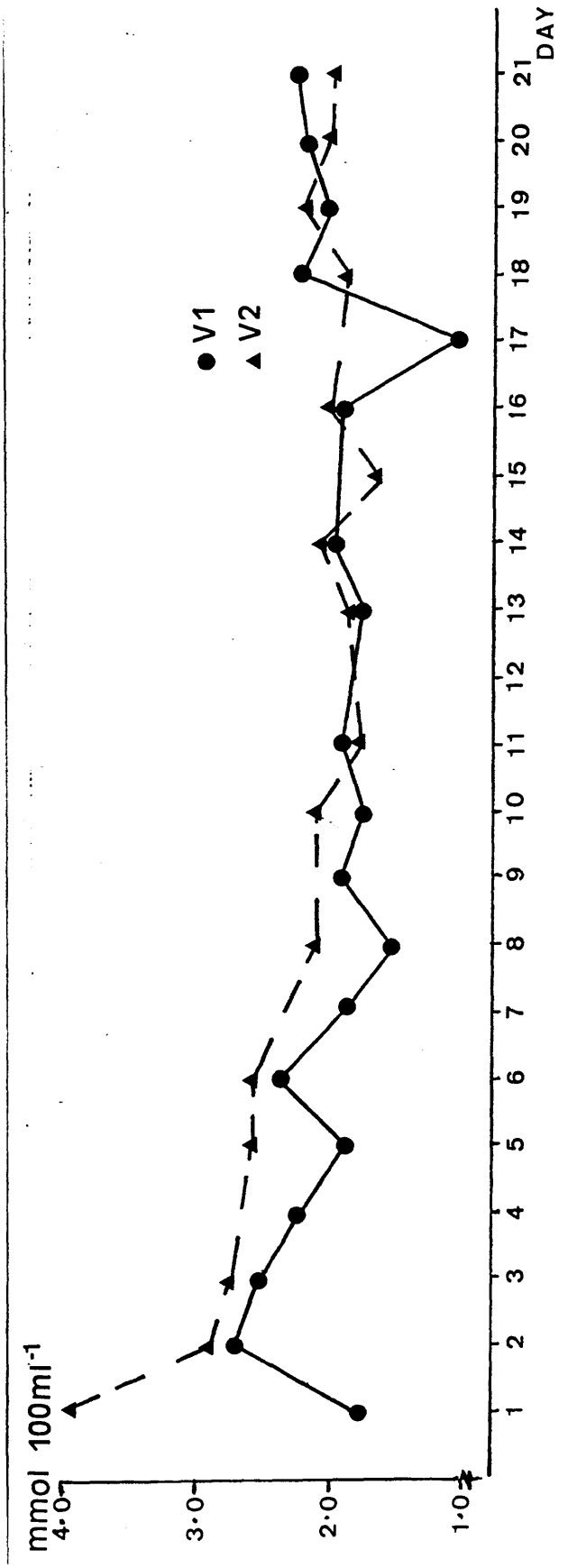


Figure 7.5.3 Acetate concentration in vessel 1 and vessel 2

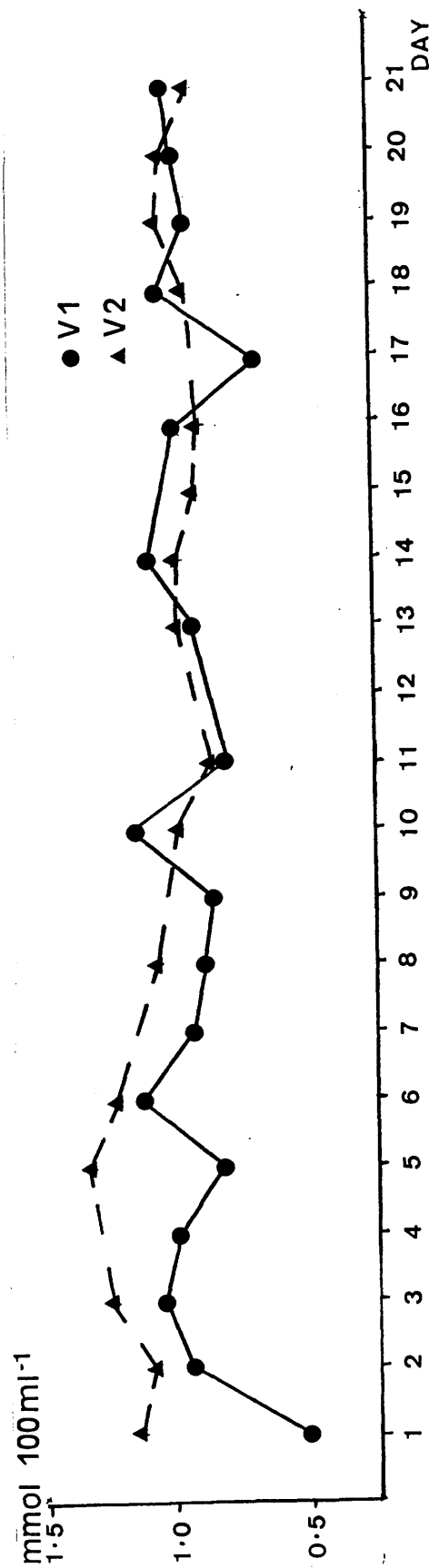


Figure 7.5.4 Propionate concentration in vessel 1 and vessel 2

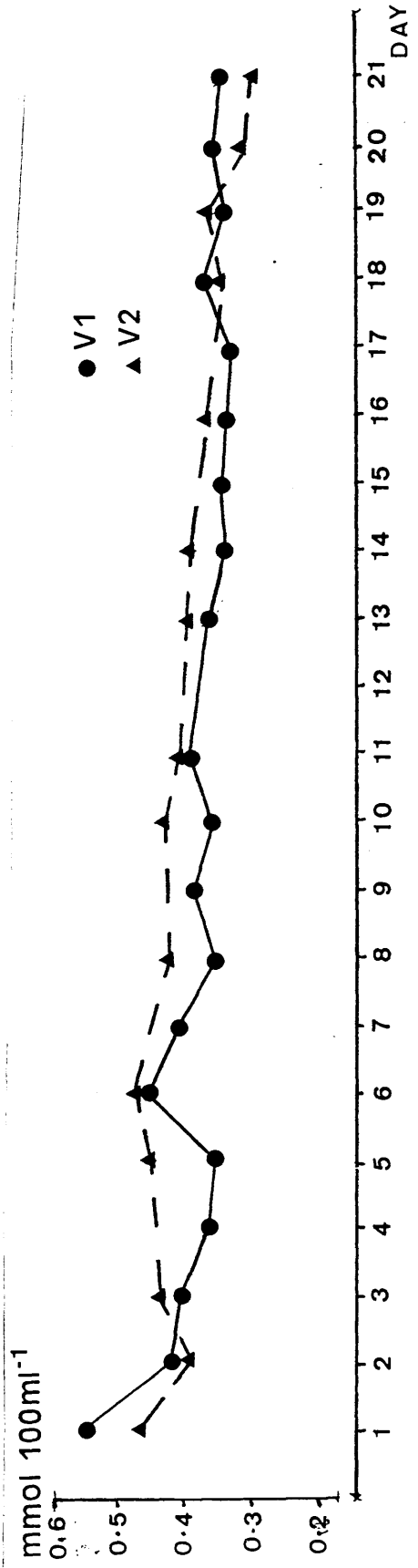


Figure 7.5.5 Butyrate concentration in vessel 1 and vessel 2

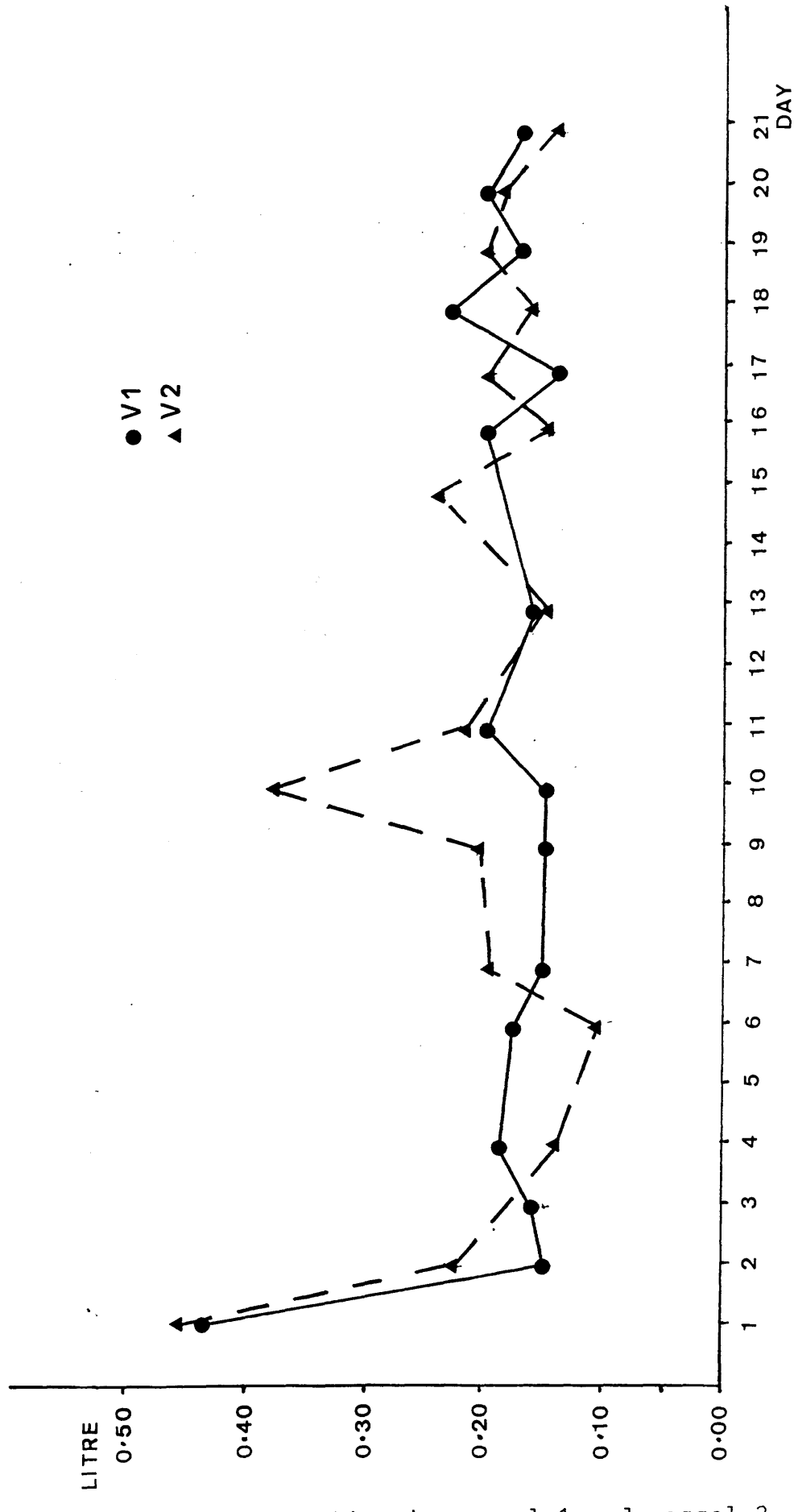


Figure 7.5.6 Methane production in vessel 1 and vessel 2

7.5.4 Changes in cell wall composition during incubation

The residues were analysed for cellulose content, total hemicellulose, neutral sugar composition of the hemicellulose, lignin content and esterified phenolic acid content.

Table 7.5.4

CELLULOSE CONTENT OF LUCERNE SILAGE RESIDUES AFTER DIGESTION IN RUSITEC

Digestion period	V1 (fraction of original)		V2 (fraction of original)	
0	120.81	(1.00)	120.81	(1.00)
24	85.05	(0.70)	97.69	(0.81)
48	75.16	(0.62)	71.24	(0.59)
72	56.68	(0.50)	56.45	(0.47)

The pattern of digestion of the cellulose shows a loss of cellulose over the whole of the digestion period. The two vessels do not seem to behave identically in the first 48 hours of digestion, with vessel 1 showing a greater loss over the first 24 hours compared with the period 24-48 hours, but vessel 2 showing an almost even rate of digestion throughout the first 48 hours in the vessel. Both vessels show a slowing down of rate of digestion of the cellulose as the time in the vessel increases.

Table 7.5.5

HEMICELLULOSE CONTENT OF LUCERNE SILAGE RESIDUES

AFTER DIGESTION IN RUSITEC

Digestion period (hrs)	V1	(fraction of original)	V2	(fraction of original)
0	80.99	(1.00)	80.99	(1.00)
24	41.21	(0.51)	30.62	(0.38)
48	35.09	(0.43)	35.81	(0.44)
72	30.51	(0.38)	25.03	(0.31)

Table 7.5.6

ARABINOSE CONTENT OF LUCERNE SILAGE RESIDUES

AFTER DIGESTION IN RUSITEC

Digestion period (hrs)	V1	(s.d.)	(fraction of original)	V2	(s.d.)	(fraction of original)
0	15.62	(1.93)	(1.00)	15.62	(1.93)	(1.00)
24	6.22	(0.79)	(0.40)	4.59	(0.38)	(0.29)
48	3.42	(0.41)	(0.22)	4.53	(0.85)	(0.29)
72	3.18	(0.18)	(0.20)	2.81	(0.66)	(0.18)

Table 7.5.7

XYLOSE CONTENT OF LUCERNE SILAGE RESIDUES

AFTER DIGESTION IN RUSITEC

Digestion period (hrs)	V1	(s.d.)	(fraction of original)	V2	(s.d.)	(fraction of original)
0	27.88	(6.78)	(1.00)	27.88	(6.78)	(1.00)
24	15.50	(4.05)	(0.56)	11.02	(3.71)	(0.40)
48	16.60	(1.52)	(0.60)	16.05	(1.18)	(0.43)
72	13.76	(0.64)	(0.49)	12.05	(0.71)	(0.43)

Table 7.5.8

GLUCOSE CONTENT OF LUCERNE SILAGE RESIDUES

AFTER DIGESTION IN RUSITEC

Digestion period (hrs)	V1	(s.d.)	(fraction of original)	V2	(s.d.)	(fraction of original)
0	24.51	(1.96)	(1.00)	24.51	(1.96)	(1.00)
24	18.55	(4.96)	(0.76)	12.64	(2.13)	(0.51)
48	15.01	(2.00)	(0.61)	16.09	(1.06)	(0.66)
72	14.34	(0.73)	(0.59)	10.54	(2.20)	(0.43)

Table 7.5.9

GALACTOSE CONTENT OF LUCERNE SILAGE RESIDUES

AFTER DIGESTION IN RUSITEC

Digestion period (hrs)	V1	(s.d.)	(fraction of original)	V2	(s.d.)	(fraction of original)
0	11.75	(3.22)	(1.00)	11.75	(3.22)	(1.00)
24	6.01	(1.08)	(0.51)	3.96	(1.03)	(0.34)
48	4.40	(0.33)	(0.37)	3.59	(1.03)	(0.31)
72	2.99	(0.77)	(0.25)	2.73	(1.13)	(0.23)

Hemicellulose is also lost during digestion of lucerne silage in Rusitec. The loss is greatest in the first twenty four hours of digestion, with a steady but small loss ocurring after this time to give digestion of between 0.60 and 0.70 of the hemicellulose after 72 hours incubation. When the figures for the neutral sugars are studied, it can be seen that there is a greater loss of arabinose and galactose than xylose or glucose. The large losses of arabinose seem to occur mainly in the

first 24 hours of digestion, with smaller losses after this time. The figures for losses of xylose are very erratic, but seem to suggest that greatest loss is in the first twenty four hours, but some smaller losses occur up to 72 hours digestion. A similar pattern is seen in the glucose component of the hemicellulose as in the xylose, although the losses in the first 24 hours are not so large.

The pattern of loss of galactose follows that of the arabinose, with most of the loss occurring in the first 24 hours, and smaller losses occurring after this time.

Table 7.5.10

LIGNIN CONTENT OF LUCERNE SILAGE RESIDUES

AFTER DIGESTION IN RUSITEC

Digestion period (hrs)	V1	(s.d.)	(fraction of original)	V2	(s.d.)	(fraction of original)
0	154.1	(0.73)	(1.00)	154.1	(0.73)	(1.00)
24	72.2	(0.16)	(0.46)	75.1	(0.25)	(0.49)
48	71.0	(0.63)	(0.46)	74.5	(0.37)	(0.48)
72	74.9	(0.52)	(0.49)	71.9	(0.40)	(0.47)

Table 7.5.11

PHENOLIC ACID CONTENT OF LUCERNE SILAGE RESIDUES AFTER DIGESTION IN RUSITEC

Digestion period (hrs)	V1	(s.d.)	(fraction of original)	V2	(s.d.)	(fraction of original)
0	3.00	(0.27)	(1.00)	3.00	(0.27)	(1.00)
24	1.94	(0.40)	(0.65)	1.76	(0.07)	(0.59)
48	1.13	(0.13)	(0.38)	0.94	(0.13)	(0.31)
72	0.69	(9.12)	(0.23)	0.75	(0.05)	(0.25)

There is a large apparent loss of lignin from the lucerne silage on digestion, most of which occurs in the first 24 hours of digestion. This is not related to phenolic acid losses as the amounts of phenolic acid relative to the lignin are very small. It is probable that the apparent loss of lignin is due to solubilization of a lignin-carbohydrate complex, which is then lost from the Rusitec system in the effluent. The steady level to which the lignin is digested suggests that there are two different lignin fractions present in the lucerne silage, one of which is soluble as a lignin-carbohydrate complex, and which is solubilized in the first 24 hours of digestion, and a second fraction which is not soluble as a lignin-carbohydrate complex, even after 72 hours digestion.

Table 7.5.12

STARCH CONTENT OF LUCERNE SILAGE RESIDUES

AFTER DIGESTION IN RUSITEC

Digestion period (hrs)	V1	(fraction of original)	V2	(fraction of original)
0	9.94	(1.00)	9.94	(1.00)
24	0.60	(0.06)	1.12	(0.10)
48	0.32	(0.03)	0.05	(0.00)
72	0..5	(0.01)	0.06	(0.01)

The starch present in the lucerne silage is digested within the first 24 hours of digestion to trace levels. The starch found in the digesta is probably due to contamination of the sample with micro-organisms containing starch-like polymers.

CHAPTER 8

COMPARISON OF CHANGES IN CELL WALL COMPOSITION OF GRASS, CLOVER AND LUCERNE DURING DIGESTION

8.1 INTRODUCTION

The results for digestion of each of the forages in Rusitec were discussed individually. In this comparison of the major components of all three sets of results it is hoped that any differences and similarities between the three forages will be highlighted and the results can be compared with those found by previous workers in the same area.

8.2 CELL WALL DIGESTIBILITY

8.2.1 Digestibility of silages

Table 8.2.1

WATER SOLUBILITY OF SILAGES

Grass silage	0.30
Clover silage	0.40
Lucerne silage	0.35

Table 8.2.2

DIGESTIBILITY IN EXCESS OF WATER SOLUBILITY
(mean of two vessels)

TIME IN VESSEL (hrs)	GRASS SILAGE	CLOVER SILAGE	LUCERNE SILAGE
0	0.00	0.00	0.00
24	0.18	0.09	0.09
48	0.32	0.20	0.17
72	0.37	0.28	0.22
OVERALL DIGESTIBILITY	0.67	0.68	0.57

The greater water solubility of the clover silage suggests that this forage had the highest ratio of cell contents to cell wall. It was found that it was difficult to maintain a stable RUSITEC system for this forage, and in feeding trials with dairy cattle a high level of milk production was attained (Castle, Gill and Watson, 1983). These two factors can probably both be attributed to the high level of soluble nutrients in the clover silage. Lucerne has a slightly lower water solubility than the clover, and grass has the lowest water solubility.

The figures for digestibility in excess of water solubility show the digestibility of the cell wall of each of the silages. The grass silage cell walls are the most digestible of the three silages with clover and lucerne silage being 0.09 and 0.15 units less digestible than grass silage respectively.

There are very few published figures on the cell wall digestibility of silages. When compared with the published results of the digestibility of lucerne and lucerne hay, the results for cell wall digestibility of the lucerne silage seem very low.

	Immature	Mature	
Lucerne stem digestibility	0.82	0.74	(1)
Lucerne leaf digestibility	0.76	0.39	(1)
Lucerne Hay	0.50	0.42	(2)

(1) Wilman and Altimimi, 1982

(2) Jung, Fahey and Merchen, 1983

It is possible, however, that the changes that take place to the cell wall during ensilage remove some of the components that are most digestible in the rumen, thus giving a lower digestibility for the remaining cell wall. The growing environment of the lucerne may also affect cell wall composition, and thus digestibility.

When the water solubility and digestibility of the cell wall are considered together to give the overall digestibility *in vitro*, the grass and clover silages have very similar *in vitro* digestibility but that of the lucerne is much lower. This may influence the usefulness of lucerne as a forage crop grown for conservation.

8.2.2 Digestion of cellulose

Table 8.2.3

CELLULOSE CONTENT OF SILAGES (g kg^{-1} DM into vessel)

(mean of two vessels)

TIME IN VESSEL (hrs)	GRASS SILAGE	CLOVER SILAGE	LUCERNE SILAGE
0	215.64	73.88	120.81
24	141.16	76.69	91.37
48	93.13	64.56	73.20
72	71.30	47.38	56.56
DIGESTION AFTER 72 HOURS	0.67	0.36	0.53

The digestion of cellulose was greatest from the grass silage and lowest from the clover silage. The

clover silage seemed to have a longer lag phase before digestion started than was seen in the other two silages, with no digestion being seen in the clover silage cellulose between 0 and 24 hours of incubation. The values for cellulose digestion compare favourably with those found by other authors. Beever, Thompson, Pfeffer and Armstrong (1971) found that wilted perennial ryegrass silage cellulose had a digestibility of 76.5% compared with 80.6% for unwilted ryegrass cellulose and 75.2% for fresh ryegrass cellulose. Matthews and McManus (1976) found the digestibility of cellulose from fresh lucerne to be 49.9%, which would suggest that although there are losses of cellulose from lucerne on ensilage, these do not affect the digestibility of the cellulose.

8.2.3 Digestion of Hemicellulose

Table 8.2.4

HEMICELLULOSE CONTENT OF SILAGES (g kg^{-1} DM into vessel)
(mean of two vessels)

TIME IN VESSEL (hrs)	GRASS SILAGE	CLOVER SILAGE	LUCERNE SILAGE
0	96.31	40.06	80.99
24	58.64	22.40	35.92
48	39.56	19.18	35.45
72	36.25	17.74	27.77
DIGESTION AFTER 72 HOURS	0.62	0.55	0.66

The clover silage has the lowest digestibility of hemicellulose during *in vitro* digestion. The results from the experiments on changes during ensilage showed that there was no loss of lignin from the clover during ensilage, whereas loss of lignin was seen from both the grass and lucerne samples at the maturity of forage used to produce the silage for the digestibility experiments. This may account for the lower digestibility of the clover hemicellulose, with the lignin present preventing microbial breakdown of the hemicellulose to as large an extent as is seen in the other two silages. Bailey, Monro, Pickmere and Chesson, (1976) commented on results which indicate the protective effect of lignin on hemicellulose digestion. The lucerne silage hemicellulose in this experiment has a higher digestibility than reported by Matthews and McManus (1976) for lucerne hemicellulose (digestibility 0.60). The value for grass silage hemicellulose corresponds to that found by Beever *et al* (1971), who reported digestibility of wilted ryegrass silage hemicellulose to be 0.60 and unwilted ryegrass silage hemicellulose to be 0.63.

Table 8.2.5

DIGESTIBILITY OF CONSTITUENT SUGARS OF HEMICELLULOSE

AFTER 72 HOURS
(mean of two vessels)

	GRASS SILAGE	CLOVER SILAGE	LUCERNE SILAGE
ARABINOSE	0.68	0.75	0.81
XYLOSE	0.61	0.38	0.54
GLUCOSE	0.52	0.51	0.49

These results show a greater loss of arabinose than xylose in all the hemicelluloses, this being most noticeable in the legume hemicelluloses. This is in accord with reported results (Bailey et al, 1976). The loss of xylose seen from the legume silage is less than that seen from the grass silage, suggesting that the main β-D-glycan chain of the legume hemicellulose is resistant to breakdown, this being especially noticeable in the clover silage hemicellulose.

8.2.4 Digestion of Lignin

Table 8.2.6

LIGNIN CONTENT OF SILAGES (g kg^{-1} DM into vessel)
(mean of two vessels)

TIME IN VESSEL (hrs)	GRASS SILAGE	CLOVER SILAGE	LUCERNE SILAGE
0	59.1	61.4	154.1
24	49.3	58.6	73.6
48	42.5	48.9	72.7
72	38.3	41.8	73.4
DIGESTION AFTER 72 HOURS	0.35	0.32	0.52

There are differences in the pattern of loss of lignin between the silages. The lucerne silage shows a rapid loss of lignin in the first 24 hours, with no further loss after this, whereas in the grass and clover silage a steady loss of lignin over the entire period of digestion is seen. In the clover silage this loss seems to be greater between 48 and 72 hours than between 24 and

48 hours. In the grass silage the loss in both periods is about the same. From previous work (Gaillard and Richards, 1975), it seems likely that the loss of lignin is not true digestion, but from the formation of a soluble lignin-carbohydrate complex, which moves into the rumen liquor and is lost from the system in this way. The lignin is not truly digested as there are no break-down products available to the rumen micro-organisms for metabolism. Gaillard and Richards (1975) suggested that the liberation of the soluble lignin-carbohydrate complex follows the enzymic scission of cellulose and hemicellulose chains of the cell wall by rumen micro-organisms, although Akin (1983) states that the lignin-carbohydrate complex involves only lignin and hemicellulose as no evidence has been reported to suggest cellulose is covalently linked to lignin.

8.3 *CONCLUSIONS*

These results show the differences in the digestion of the different stages and some reasons for these differences have been suggested. There are a number of different points that could be followed up by further work, including a study of the effluent liquor from the Rusitec digestion, to give a clearer picture of the fate of the lignin apparently digested.

CHAPTER 9

PHYSICAL PROPERTIES OF CELL WALLS

9.1 INTRODUCTION

The cell wall of forages contains a fibre matrix consisting of cellulose, hemicelluloses, pectins, lignin and probably small amounts of protein. The constituents of these polymers contain functional groups such as carboxy and amino groups which can become charged depending on the pH of the material surrounding them. As the proportion of the consitituents is different in different cell walls, so is the proportion of the various charged groups. The composition of the fibre matrix, and the charge of the functional groups will affect various physical properties of the forage, including water holding capacity and buffering capacity. The measurement of cation-exchange capacity will reflect the buffering capacity of the fibres. This affects the fermentation of the silage as the achievement of a low pH is necessary to produce a well-preserved silage. Any change in buffering capacity on ensilage will reflect the change in groups that are present in the silage and their bonding to other constituents in the fibre matrix.

The cation exchange capacity was estimated as described in 2.2.5.2. The results were calculated as mg $Cu(NO_3)_2$ equivalents per g DM of the freeze dried milled forage.

There is considerable importance placed on the reduction of effluent from the silo during ensilage. Many practical solutions are being offered for the

prevention, collection and use of this effluent, but little is known about the effect of the cell wall structure on the amount of effluent released. The water holding capacity of the walls may be altered by the conditions in the silo, with different conditions affecting the amount of water held. Any relationship between cell wall structure and water holding capacity would provide a method of predicting effluent volume. It may be desirable to select a forage for ensilage not just for its nutritive value but also for its ability to retain bound water.

9.2 CATION EXCHANGE CAPACITY (C.E.C.)

9.2.1 Results for forages and their silages

As this was a preliminary study using this technique for forages and their silages, it was decided to estimate CEC in only one of the replicates of the silages and take this as a representative of the silages as a whole. This assumption was made for both the grass and the clover silages. In the grass silages, all the site A bags were used, and for the clover silages Bags 1, 5 and 7 were used, these three bags having been tied together when they were ensiled, thus representing the same site in the silo for all three cuts of clover.

Table 9.2.1

CATION EXCHANGE CAPACITY OF GRASS AND GRASS SILAGE
mg $Cu(NO_3)_2$ equivalents per g DM

	GRASS	(s.d.)	SILAGE (Site A)	(s.d.)
1st Cut	45.5	(5.8)	45.7	(2.7)
2nd Cut	31.4	(4.6)	20.3	(0.6)
3rd Cut	30.2	(3.9)	14.0	(2.0)
4th Cut	52.5	(0.9)	17.6	(0.1)

The results for grass and grass silage show some interesting changes in cation exchange capacity during the growth of grass and on ensilage of grasses of different maturities. If the first three cuts of grass are considered, there appears to be a decrease in cation exchange capacity with maturity. This probably reflects the proportion of ionisable groups within the cell wall with fewer groups free for bonding with the copper ions. However, the fourth cut grass, which was a very mature, almost straw like sample, has a much higher cation exchange capacity than the other cuts of grass.

On ensilage a pattern of increasing loss of cation exchange capacity with increasing maturity of the grass ensiled is seen. There is no loss of cation exchange capacity when first cut grass is ensiled. The cation exchange capacity of the second cut grass is reduced by about 0.3, the third cut grass by 0.5, and the fourth cut grass by 0.6. This may reflect changes in the chemical composition and binding in the grasses during ensilage.

Table 9.2.2

CATION EXCHANGE CAPACITY OF CLOVER AND CLOVER SILAGE
mg $Cu(NO_3)_2$ equivalents per g DM

	CLOVER	(s.d.)	SILAGE (Site A)	(s.d.)
1st Cut	41.8	(4.2)	73.3	(8.7)
2nd Cut	40.3	(0.3)	70.0	(2.0)
3rd Cut	53.8	(1.6)	71.4	(0.8)

Table 9.2.3

CATION EXCHANGE CAPACITY OF CLOVER AND CLOVER SILAGE

PREPARED UNDER FIELD CONDITIONS
mg $Cu(NO_3)_2$ equivalents per g DM

		(s.d.)
Unwilted clover	53.8	(1.6)
Wilted clover	50.8	(0.7)
Clover silage	76.6	(1.4)

The clover samples and silages show an entirely different pattern of cation exchange capacity changes with changing maturity and on ensilage. There is an increase in cation exchange capacity between the second and third cuts of clover and an increase in all three cuts on ensilage. This increase on ensilage gives all three clover silages about the same cation exchange capacity.

The cation exchange capacity of clover under field conditions was also measured. It was found that there was no difference in the cation exchange capacity of wilted and unwilted clover. The same increase in cation exchange capacity was seen between wilted clover and

clover silage as was seen between the clover cuts and the bag silages. The cation exchange capacity of the silage from the farm scale silo was similar to that of the bag silages.

Table 9.2.4

CATION EXCHANGE CAPACITY OF LUCERNE AND LUCERNE SILAGE
mg $Cu(NO_3)_2$ equivalents per g DM

		(s.d.)
Lucerne	43.51	(12.1)
Lucerne silage	43.75	(0.4)

The lucerne and lucerne silage figures show little difference between the lucerne and its silage. The lucerne silage had been treated with a formic acid based additive, but it is unlikely that this made any appreciable difference to the cation exchange capacity.

9.2.2 Cation exchange capacity of forages and their digesta from Rusitec

The changes in cation exchange capacity on digestion are useful as they may reflect changes in the cell wall, and may have some relevance to the digestibility of the silage.

Table 9.2.5

CATION EXCHANGE CAPACITY OF GRASS SILAGE AND DIGESTA SAMPLES

mg $Cu(NO_3)_2$ equivalents per g DM

DIGESTION PERIOD (hours)	V1	(s.d.)	V2	(s.d.)
0	23.9	(0.8)	23.9	(0.8)
24	63.1	(4.7)	67.6	(1.3)
48	60.9	(3.6)	65.6	(2.5)
72	64.2	(7.4)	59.1	(6.0)

There is a large increase in the cation exchange capacity of the grass silage after digestion. This appears to occur in the first 24 hours of digestion. There was no further change in the cation exchange capacity when the digestion period increased beyond 24 hours.

Table 9.2.6

CATION EXCHANGE CAPACITY OF CLOVER SILAGE AND DIGESTA SAMPLES

mg $Cu(NO_3)_2$ equivalents per g DM

DIGESTION PERIOD (hours)	V1	(s.d.)	V2	(s.d.)
0	76.6	(1.4)	76.6	(1.4)
24	116.9	(6.4)	133.4	(5.4)
48	124.2	(13.7)	108.9	(5.2)
72	110.9	(28.7)	105.9	(11.7)

An increase in cation exchange capacity after digestion is also seen for clover silage. A large amount of

variation is seen in the results for clover silage, but it seems that most of the change occurs in the first 24 hours of digestion, as in the grass silage. The same pattern is also seen for lucerne silage, although these results are not as variable as those found for clover silage.

Table 9.2.7

CATION EXCHANGE CAPACITY OF LUCERNE SILAGE AND DIGESTA SAMPLES

mg $Cu(NO_3)_2$ equivalents per g DM

DIGESTION PERIOD (hours)	V1	(s.d.)	V2	(s.d.)
0	43.7	(0.4)	43.7	(0.4)
24	74.4	(0.7)	77.3	(7.1)
48	67.8	(0.1)	72.6	(1.8)
72	72.8	(4.8)	68.7	(6.8)

9.2.3 Discussion of change in cation exchange capacity on ensilage

The different patterns of change for the three forage samples reflect the different problems that can be encountered when making silage from these crops.

The low cation exchange capacity of the first three cuts of the grass and grass silage indicate that buffering capacity would not be the main obstacle to achieving a low pH during the ensilage of grass. This is what is generally found in field conditions. The higher cation exchange capacity of the first cut does not present problems as this cut also has a high water soluble carbo-

hydrate content, giving a rapid and large production of acid on fermentation by lactic acid bacteria, overcoming the increased buffering capacity.

The high buffering capacity of the fourth cut grass could lead to problems on ensilage, especially as this is accompanied by a lower water-soluble carbohydrate content. However, if a satisfactory fermentation was established (possibly with the use of additives), formation of a stable silage should result, as the cation exchange capacity falls sharply during ensilage.

One of the major problems in making legume silage is reducing the pH of the forage quickly, and achieving a low, stable pH. The cation exchange capacity of both the clover and the lucerne silages is greater than that of the forages from which the silage was made, indicating that during the ensilage period this problem will worsen, with buffering capacity increasing as the silage matures. Thus even if a satisfactory primary fermentation is established, the production of acid may not be great enough to overcome the buffering capacity of the forage. This may lead to problems with secondary fermentation by undesirable micro-organisms and aerobic breakdown of the silage when the face is exposed to the air during emptying of the silo. The cation exchange capacity of clover silage made from all three cuts of clover was practically the same, suggesting that there is a maximum value which can be obtained for different ages of clover. This could relate to some constituents, such as pectin, which are in all three cuts of clover, and which has its chemical

bonding affected by the conditions during ensilage.

9.3 WATER HOLDING CAPACITY

The ability of forage to hold water is of great importance to practical silage making. The storage and disposal of silage effluent can be a problem for some farms. As the effluent has a high biological oxygen demand its disposal requires care, with pollution of water courses carrying heavy fines. The water holding capacity of the samples was measured using freeze-dried samples. This is a false situation, as it is generally the fresh material which is ensiled, but it was hoped that this would give an indication of any changes in water holding capacity (WHC).

Table 9.3.1

WATER HOLDING CAPACITY OF GRASS AND ITS SILAGES
g kg^{-1} DM

	GRASS	(s.d.)	SILAGE	(s.d.)
1st Cut	9.29	(0.95)	6.46	(0.23)
2nd Cut	9.35	(0.07)	8.64	(0.09)
3rd Cut	8.81	(0.23)	5.95	(0.18)
4th Cut	6.90	(0.08)	7.82	(0.40)

There is a reduction of WHC in the first three cuts of grass on ensilage, but a slight increase is seen in the fourth cut. The fourth cut was a very mature cut of grass, and it is possible that the changes that take place as the grass reaches maturity will affect the WHC.

Table 9.3.2

WATER HOLDING CAPACITY OF LEGUMES AND THEIR SILAGES
(Figures corrected for DM recovery) g kg^{-1} DM

	CLOVER	(s.d.)	SILAGE	(s.d.)
1st Cut	7.37	(0.82)	8.95	(0.69)
2nd Cut	7.99	(1.21)	8.20	(1.20)
3rd Cut	8.98	(0.83)	7.39	(0.53)
Wilted 3rd cut clover			7.50	(0.80)
Clover silage from pit			8.69	(0.56)
Lucerne			9.70	(0.93)
Lucerne silage			5.66	(0.47)

In the clover silages a slight increase in WHC is seen after ensilage, and similar results were seen when wilted clover and clover from a farm scale silo were compared. Lucerne silage had a lower WHC than lucerne.

The usefulness of this method for estimating the role of the cell wall in water retention, and the effect of this on the amount of effluent produced from the silo seems limited. The method lacks the sensitivity to show any subtle changes in water retention capacity. It is suggested that future experiments should consider fresh forage.

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ADDENDUM

There have been very few further advances in understanding the structure and composition of the carbohydrate fraction of secondary cell walls. The most exciting advance has been in the area of non-carbohydrate components. The occurrence of p-coumaric and ferulic acids has been documented earlier but recent investigations have identified a number of dimers of these acids which may be responsible for cross-linking wall components and also influencing digestibility. 4,4' Dihydroxytruxillic acid has been identified as a component of the cell walls of Lolium multiflorum (Hartley, Whatley and Harris, 1988) but other dimers have since been identified by capillary GC (Ford and Hartley, 1988) and their structures have been confirmed by GC/MS (Ford and Hartley, 1989).

Considerable use is still made of the Rusitec system and a comprehensive review has been published (Czerkawski, 1986). Other workers have investigated more complex systems which have the advantage of continuous feeding. The prototype was developed some time ago (Hoover, Crooker and Sniffen, 1976) and the design has recently been improved by Merry, Smith and McAllen (1987). Although it is probably an improved model for rumen digestion, the technical complexity causes severe restriction on replication.

Some of the results in this thesis have already been published (Barwick and Morrison, 1984; Morrison and Barwick,

1987). Work on the influence of some chemical and biological additives on the fibre fraction of lucerne on ensilage in laboratory silos has been reported by Morrison (1988). Most of the recent work on silages has, however, been concerned with inoculants and enzymes as silage additives. Recent reviews on both inoculants (Henderson, Neilson and Anderson, 1988) and enzymes (Chamberlain and Robertson, 1989) have been published; the enzyme treatments do cause changes in the fibre fraction.

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