THE NITROGENOUS CHANGES DURING ENSILAGE

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DECLARATION

I hereby declare that this thesis is my own composition and does not include work submitted for any other degree or professional qualification. The thesis reports the results of research carried out by myself except where help has been acknowledged.

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ABSTRACT

Gamma-irradiation was used to delineate the effects of plant and microbial enzymes on the nitrogenous changes occurring during ensilage, particularly in the amino acid fraction. The effect of inoculating irradiated grass with lactic acid bacteria was also studied. Results showed that proteolysis is brought about mainly by plant proteases whereas further amino acid metabolism is the result of microbial activity. The effects on individual amino acids are reported. *L. plantarum* and *S. faecalis* were shown to be non-proteolytic and to have a limited ability to catabolise amino acids.

The effect of pH on plant protease activity was investigated using gamma-irradiated aqueous grass extract. Protein breakdown appears to be largely completed within a few days. It was found that, although the overall optimum pH for ryegrass proteases is 5.5 to 6.5, activity still occurred at the pH levels found in silage.

The effect of several different types of silage additive on fermentation was studied, particularly with respect to the nitrogenous components.

A commercial inoculant ("Clampdown") was applied to grass at three levels (10^4, 10^6 and 10^8 organisms g^{-1}) before ensiling. Silages were also made after mincing both inoculated and uninoculated grass and after addition of glucose (20 gkg^{-1}), to inoculated and uninoculated grass. The homofermentative inoculant stimulated a more efficient fermentation, leading to rapid acidification and silages with high water-soluble carbohydrate and lactic acid contents. There was some reduction in proteolysis and a considerable decrease in deamination. Mincing alone did not encourage acidification nor reduce deamination, but did reduce

proteolysis. Mincing the inoculated material did not enhance the effects of inoculation. Glucose treatment was found to be ineffective.

Formalin (40% w/v formaldehyde) at 1.8 lt^{-1} and "Add-F" (85% w/w formic acid) at three levels (2.3, 4.6 and 6.9 lt^{-1}) were applied to grass before ensiling either alone or in combination. Formic acid was found to be a very effective inhibitor of fermentation. When used on its own, it had a limited effect only, in preventing proteolysis, but did reduce deamination substantially. Formaldehyde alone had little effect on fermentation, proteolysis or deamination. Combining the two additives produced a synergistic effect with respect to protein protection. The most satisfactory treatment was a combination of formic acid at 2.3 lt^{-1} with formalin.

The final experiment compared the effects of acid and alkaline treatments of grass before ensiling. Sulphuric and citric acids both brought about rapid acidification but, despite this, extensive fermentation still occurred. Both acid treatments reduced proteolysis and deamination Borax buffer and ammonia treatments were used to raise the initial pH. The pH levels of the borax treated silages remained above that of the original grass but those of the ammonia treated silages fell to between 4.2 and 4.8. Both types of alkaline treatment reduced proteolysis to a greater extent than the acid treatments.

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1. INTRODUCTION

Ensilage is an important method of conserving forage for feeding in winter, especially in countries where the climate is unsuitable for making hay. Preservation is brought about by lactic acid bacteria which ferment the water-soluble carbohydrates of the herbage, the main products being organic acids, lactic in particular. The lowering of the pH which results, prevents growth of undesirable organisms such as clostridia. Extensive changes to the nitrogenous constituents also occur. Proteolysis is considered to be chiefly the result of plant enzyme activity, with further amino acid degradation being caused mainly by microbial action (Ohshima and McDonald, 1978). A number of reviews concerning the biochemistry and microbiology of ensiling have been published (Watson and Nash, 1960; McDonald *et al*, 1966a; Whittenbury *et al*, 1967; Whittenbury, 1968; Woolford, 1972; McDonald and Whittenbury, 1973; McDonald, 1976; Beck, 1978; Edwards and McDonald, 1978; McDonald, 1981; Woolford, 1984).

Often the herbage to be ensiled is relatively unsuitable for the purpose eg it may be low in soluble carbohydrates and this may lead to an unsatisfactory fermentation. If the pH is not reduced quickly to a low level, clostridia may dominate the fermentation (Whittenbury, 1968) and saccharolytic clostridia will convert lactic acid, already formed by the lactic acid bacteria, into butyric acid. This causes an increase in the pH, making conditions more suitable for the growth of proteolytic clostridia, which will result in extensive degradation of amino acids (Barry *et al*, 1977). The feeding value of such silages is low (McDonald *et al*, 1981).

In order to increase the probability of achieving a desirable fermentation, additives are often applied before ensiling. These fall into two main groups (McDonald and Whittenbury, 1973). Stimulants work by encouraging a natural fermentation. This is achieved either by adding a source of readily available carbohydrates or by applying an inoculum of suitable lactic acid bacteria. Other additives preserve the forage by inhibiting the natural fermentation. The most common method used is direct acidification.

Although fermentation is an effective method of preserving herbage, the silage produced has a lower feeding value than the original fresh material (Barry *et al*, 1977). This results partly from a reduction in intake due to the high concentration of fermentation acids but also from a lower efficiency of nitrogen utilization resulting from the high solubility of the nitrogenous constituents (Wilkinson *et al*, 1976). In recent years attention has concentrated on finding methods which preserve herbage by methods other than acidification, and also preserve the protein of the original herbage. Formalin (40% w/v formaldehyde) has been found to fulfil both roles. Unfortunately it has many disadvantages. It is unpleasant to handle and is considered to be potentially carcinogenic in human beings (Anon., 1979). In addition, the level of application is critical. If too little is applied, a clostridial fermentation may result, and it is therefore usually used in conjunction with an acid to ensure restriction of fermentation (Crawshaw, 1977; Hinks *et al*, 1978). If too much is added, both intake and digestibility may be adversely affected (Wilkinson *et al*, 1976; McDonald, 1981). Because of these disadvantages, finding alternative methods for protecting the protein of herbage during ensilage is of considerable importance.

2. LITERATURE REVIEW

2.1 THE NITROGENOUS COMPONENTS OF PLANTS

The total nitrogen (TN) content of temperate herbages typically varies between 5 and 50 $gkg^{-1}DM$, the level in legumes being somewhat higher than in grasses (McDonald *et al*, 1981). It varies with the stage of growth, decreasing with maturity owing both to an increase in the proportion of structural material and a decrease in the protein content of the leaves (Pirie, 1959; Mowat *et al*, 1965; Lyttleton, 1973). Waite and Gorrod (1959) harvested three grass species, each at four different stages of growth. The total nitrogen content of perennial ryegrass (*Lolium perenne*) was found to decrease from 33.6 to 5.0 $gkg^{-1}DM$ between late April and mid September. Timothy (*Phleum pratense*) and cocksfoot (*Dactylis glomerata*) showed similar decreases. Although the total nitrogen content of legumes also decreases with maturity it generally remains above 24 $gkg^{-1}DM$ (Lyttleton, 1973).

Legumes do not normally benefit from nitrogenous fertilizers but grasses are very responsive. Both content and yield of total nitrogen increase, the latter resulting from stimulated plant growth (Burris, 1959; Hodgson, 1970; Lyttleton, 1973). Generally, fertilizer application early in the growing season results in a rapid rise in the total nitrogen content, but the effect diminishes thereafter unless a high nitrogen level is applied. Henderson *et al* (1962) examined the effect of adding 100 $kgNha^{-1}$ to five grasses. The total nitrogen content of

the early cuts rose from 24-30 gkg^{-1}DM (control) to 35-38 gkg^{-1}DM (treated) but both fell to 21-22 gkg^{-1}DM in later cuts. A similar effect was noted by Wilman (1970) after application of 125 $kgNha^{-1}$ to Italian ryegrass (*Lolium multiflorum*). Application of 40 $kgNha^{-1}$ had no effect (Reith and Inkson, 1964) whereas 400 $kgNha^{-1}$ produced an increase even in later cuts (Cooper *et al*, 1962). Nowakowski *et al* (1965) have shown that although most of the total nitrogen is normally present as protein, the application of nitrogenous fertilizers may lead initially to a large increase in the non-protein nitrogen (NPN) fraction. This non-protein nitrogen will contribute only to the rumen degradable protein fraction of the ruminant diet and requires the presence of readily-fermentable carbohydrate for effective utilization (McDonald and Edwards, 1976).

2.1.1 Non-protein Nitrogen

Non-protein nitrogen compounds account for 10-25% of the total nitrogen in grasses. The major components are free amino acids and amides with small quantities of peptides, amines, nitrates, nucleotides and ureides (Ohshima and McDonald, 1978).

2.1.1.1 *Nitrate and ammonia nitrogen.* Atmospheric nitrogen cannot be used directly by plant cells, but it can be changed to ammonia by nitrogen fixing bacteria and then taken up by the roots and assimilated into organic compounds. Alternatively, it may be oxidized to nitrate via nitrite by nitrifying bacteria in the soil (nitrification), absorbed by the plant in this form and converted to ammonia before assimilation.

It has been estimated that 100-200 million tons of nitrogen are turned over annually on this planet (Subba Rao, 1980). About 70% of this results from biological nitrogen fixation, most of the remainder coming from nitrogenous fertilizers (Gibson *et al*, 1977; Beevers and Hageman, 1980; Subba Rao, 1980). Some of the nitrogen is fixed by free-living bacteria eg *Azobacter* and *Clostridium* spp. but most of it results from the acitivity of symbiotic systems eg the root nodules of legumes colonised by *Rhizobium* spp. (Mishustin and Shil'nikova, 1971; Gibson *et al*, 1977; Yates, 1980). Many nodulated non-leguminous plants fix nitrogen. For example *Azospirillum* spp. can associate with a broad range of higher plants including tropical grasses and cereals (Döbreiner and De-Polli, 1980). Fixation is achieved by six-electron reduction of dinitrogen to ammonia, catalysed by the nitrogenase enzyme system (Steifel, 1977). In free-living bacteria, nitrogenase activity is low and its synthesis is inhibited by the presence of ammonia and nitrate, but nitrogenase is synthesised and active in symbiotic bacteria even in the presence of ammonia, the energy supply probably being the rate-limiting step (Burris, 1959; Gibson *et al*, 1977; Harper and Nicholas, 1978; Yates, 1980).

The absorption of ammonium and nitrate nitrogen from the soil by plants is influenced by the carbohydrate supply to the roots, especially in the case of ammonium, owing to its toxicity and thus the necessity for its immediate incorporation into organic compounds (Michael *et al*, 1970; Kirkby and Hughes, 1970). In herbage, ammonium nitrogen usually accounts for less than 10 gkg^{-1}TN (Ohshima and McDonald, 1978).

When ammonium fertilizers are applied to soils, the ammonium is rapidly converted to nitrate (nitrification) by two highly specialized groups of soil autotrophic nitrifying bacteria (DeKock, 1970) so that, in the field, nitrogen is available to the plant mainly as nitrate. Oxidation of ammonium to nitrite is carried out by *Nitrosomonas*, followed by oxidation of nitrite to nitrate by *Nitrobacter* (Thimann, 1963). Nitrification is usually so fast that there is little free ammonium in the soil (Thimann, 1963) although there may be substantial quantities bound by clay minerals, making it highly inaccessible to plants (Mishustin and Shil'nikova, 1971; Beevers, 1976). Conversion to nitrate makes the nitrogen more readily available to the plant (Mishustin and Shil'nikova, 1971) but has the disadvantage that, unlike ammonium, it is readily leached from the soil (Burris, 1959; Cooke, 1975).

Nitrate enters the root cells with the help of a nitrate-specific permease (Beevers and Hageman, 1980). Its uptake is also influenced by temperature, pH, aeration, age and morphology (Hewitt, 1970). It is relatively harmless and after its absorption, it may be accumulated in the vacuoles of root cells, reduced to ammonia in the roots or translocated unaltered to the shoot (Michael *et al*, 1970; Dalling *et al*, 1972a; Miflin, 1974).

Nitrogen assimilation occurs in both the roots and leaves and initially requires the reduction of nitrate to ammonia via nitrite (Beevers, 1976). This necessitates the donation of eight electrons, two from carbohydrate oxidation (NADH) and six from light energy capture (ferredoxin). The ammonia formed then

combines with a carbon skeleton to produce amino acids (Beevers and Hageman, 1980).

Nitrate is reduced to nitrite in the presence of nitrate reductase, which appears to be present in the cytoplasm of all plant parts (Gambourg *et al*, 1968; Hewitt, 1970), but occurs in highest concentrations in the leaves (Beevers and Hageman, 1969; Hewitt *et al*, 1976). It is not normally present in plant cells but its biosynthesis is induced by nitrate accumulation (Beevers and Hageman, 1980). Nitrate may accumulate in the plant if the external concentration is very high or if nitrate reduction is inhibited by, for example, low light intensity or a low molybdenum supply (Bonner, 1950; Hewitt *et al*, 1976; Beevers and Hageman, 1980). The nitrate nitrogen content of plants is greatly influenced by the application of nitrogenous fertilizers (Ohshima and McDonald, 1978). Henderson and McDonald (1975) found levels of 100 gkg^{-1}TN in *Lolium multiflorum* which had been fertilised two weeks prior to harvesting. It is undesirable to have nitrate levels build up in plants as it is toxic to animals in large quantities (Thimann, 1963; Bousset-Fatianoff *et al*, 1971). During ensilage nitrate is reduced to ammonia by microbial activity (Bousset-Fatianoff *et al*, 1971; Ohshima and McDonald, 1978). The nitrite intermediate formed inhibits butyrate production (Wieringa, 1966).

The reduction of nitrite to ammonia is catalysed by nitrite reductase. This also is found in all plant parts, especially the leaves, and is located in the chloroplast (Hewitt, 1970; Dalling *et al* 1972a, b; Miflin, 1974). Unlike nitrate, nitrite does not accumulate in plant tissue as nitrite reductase functions efficiently at low substrate concentrations and shows high

activity relative to nitrate reductase (Hewitt *et al*, 1976).

Once in the ammoniacal form the nitrogen may be incorporated into plant organic matter. This occurs via the glutamate synthase cycle (Rhodes *et al*, 1980). In this cycle glutamine synthetase catalyses the formation of glutamine from glutamate and ammonia. Glutamate synthase then catalyses the transfer of this ammonia to the α-carbon of 2-oxoglutarate forming two molecules of glutamate (Miflin and Lea, 1980; Stewart *et al*, 1980). Both enzymes are found mainly in the chloroplasts although the former has also been detected in the cytoplasm (Haystead, 1973; Miflin, 1974; Harel *et al*, 1977; Wallsgrove *et al*, 1979). Glutamine synthetase is widely distributed at high levels in plant tissue and has a high affinity for ammonia, so toxic levels of ammonia rarely build up (Lea and Miflin, 1980).

The organically bound nitrogen of glutamate and glutamine can be utilized for synthesis of other amino acids either at the original site of assimilation or after transport to another part of the plant.

2.1.1.2 *Free amino acids and amides* are present in plants as intermediates of protein metabolism, agents of nitrogen translocation and products of inorganic nitrogen assimilation.

All plants are able to synthesise the twenty amino acids present in their proteins (Fowden, 1965; Miflin and Lea, 1977). The carbon skeletons are derived mainly from the intermediates of glycolysis, the TCA cycle and the pentose phosphate pathway (Hegarty and Peterson, 1973; Bryan, 1976). Many amino acids can be synthesised by the transfer of the amino group from an

amino acid to a 2-keto acid eg oxaloacetate gives rise to aspartate. These reactions are catalysed by the wide variety of aminotransferases which are present in plants (Beevers, 1976; Givan, 1980). Most amino acids can act as donors of the amino group although glutamate has a prominent role (Forest and Wightman, 1972). The initial transamination reactions lead to several key amino acids each of which then functions as a precursor for the synthesis of several other amino acids. For example, aspartate gives rise to threonine, methionine, lysine and isoleucine (Beevers, 1976; Bryan, 1980; Kelly and Latzko, 1980). Some amino acids such as aspartate are also involved in purine and pyrimidine synthesis (Ross, 1980). Amino acids are capable of undergoing extensive interconversion such as occurs during germination (Beevers, 1976).

More than 200 non-protein amino acids have been isolated from plants (Steward and Durzan, 1965; Fowden, 1970; Fowden *et al*, 1979). β-alanine and γ-aminobutyrate are now regarded as being present in all plants, but grasses contain few others, at least at comparable concentrations to those found in legumes (Hegarty and Peterson, 1973). They vary widely in chemical structure and distribution, and many do not seem to have an essential role in the nitrogen metabolism of plants. Some are important, however, such as citrulline which is an intermediate in arginine biosynthesis. They have a low turnover indicating they are relatively inert metabolically (Hegarty and Peterson, 1973).

Metabolites are transported around the plant in the xylem and phloem tissues. The xylem transports nitrogenous compounds

whereas the phloem transports mainly carbohydrates. In both, the bulk of the organic nitrogen is carried as nitrogen-rich compounds such as amides, ureides and arginine. The particular compound carried depends on the species and is usually the same in both transport systems. Nitrate is common in the xylem of some species but is not usually found in the phloem (Pate, 1980).

Generally the free amino acid concentration in the xylem is low compared to that in the phloem but in both a limited number of amino acids predominate, of which the amides asparagine and glutamine are the most common (Higgins and Payne, 1980). The amino acids vary quantitatively and qualitatively with species and season (Pate *et al*, 1965; Streeter, 1972; Leckstein and Llewellyn, 1975; Pate *et al*, 1975; Kirkman and Miflin, 1979) and fertilization (Kirkman and Miflin, 1979).

In the majority of seeds, nitrogen is stored as insoluble protein which is broken down to amino acids during germination. The amino acids released can be utilized in several ways (Beevers, 1976). They may be incorporated into enzymes in the reserve tissue. Alternatively they may be translocated to the growing point and used for protein synthesis there or as a source of carbon skeletons and nitrogen for synthesis of other components. During germination, soluble amino acids accumulate in the reserve tissue and developing shoot (Ingle *et al*, 1964; Beevers and Guernsey, 1966) but the ratio of amino acids is very different from that of the reserve protein owing to extensive interconversion of amino acids, large quantities of glutamine in particular being produced (Beevers, 1976; Lea and Miflin, 1980).

After exhaustion of the seed protein the plant depends on the roots for its main nitrogen supply which may be transported as nitrate or in organic form (Pate, 1973; Atkins *et al*, 1979; Lea and Miflin, 1980). Nitrate reductase activity determines the ratio of inorganic to organic nitrogen in the xylem sap. If the activity in the roots is low, more than 95% of the xylem nitrogen may be in nitrate form (Pate, 1980).

During leaf senescence there is extensive proteolysis (Peterson and Huffaker, 1975; Feller *et al*, 1977; Storey and Beevers, 1977; Thomas, 1978) followed by a series of reactions leading to amide formation (Yemm and Willis, 1956; Thomas, 1978). In attached leaves the amides are transferred from the leaf via the phloem (Lea and Miflin, 1980) and, as a result, the free amino acid concentration in the phloem may increase ten-fold (Higgins and Payne, 1980).

In forage crops the soluble amino acid composition of the whole plant is extremely variable and bears little relationship to the amino acid composition of the protein fraction which tends to be relatively stable (Beevers, 1976). The ratio of soluble amino acids is affected by plant variety, stage of growth, plant tissue and such environmental influences as nitrogen nutrition, photoperiod and mineral nutrition (Stewart and Lahrer, 1980). Generally, as plants mature, there is a decline in total nitrogen and soluble amino acids (Hegarty and Peterson, 1973). The form of nitrogen fertilizer applied affects amino acid composition as shown by Nowakowski and Cunningham (1966) who found that when ammonium nitrogen was applied to *L. multiflorum*, more amide, especially asparagine, was formed than if it was

applied as nitrate. Mineral deficiencies, with the exception of nitrogen itself and possibly molybdenum, promote an increase in the soluble amino acid content usually as a result of an increase in the amides (Hegarty and Peterson, 1973). Arginine too has been found to increase under such conditions (Adams and Sheard, 1966; Hewitt and Smith, 1975). This accumulation of amides and arginine may reflect their capacity to act as nitrogen storage compounds (Pate, 1972; Beevers, 1976).

Soluble amino acids arise as a result of biosynthesis, from protein hydrolysis or may accumulate owing to cessation of protein synthesis (Beevers, 1976). The observation that the compositions of the soluble and protein amino acid fractions differ has lead to the theory that pools of amino acids exist, some of which are directl participating in protein biosynthesis and others which act as stores of amino acids derived from both synthesis and protein breakdown (Bidwell *et al*, 1964).

2.1.1.3 *Amines*. Plant amines are almost as widespread and chemically diverse as the amino acids, but do not occur in comparable concentrations in most plants (Smith, 1971; Beevers, 1976). The amines can be divided into simple aliphatic primary amines, diamines and aromatic amines.

The aliphatic primary amines can occur free and are present in this form in relatively high concentrations in flowers where they act as insect attractants (Smith, 1971). Amine derivatives such as ethanolamine and choline are important constituents of phospholipids and have been detected in many higher plants (Smith, 1971) but only accumulate in a few species (Stein von Kamienski, 1957). The aliphatic primary amines may be formed by decarboxylation of the appropriate amino acids or by transamination of the corresponding aldehyde.

Putrescine and cadaverine are two of the main diamines present in herbage (Smith, 1971). Cadaverine is a product of lysine decarboxylation whereas putrescine is derived from agmatine, a decarboxylation product of arginine (Smith, 1971; Beevers, 1976). The physiological and metabolic roles of these amines remain to be established but agmatine and putrescine are thought to play a role in maintaining internal cellular pH (Hegarty and Peterson, 1973; Beevers, 1976) because they are known to accumulate in response to potassium deficiencies (Coleman and Richards, 1956; Smith, 1968).

Decarboxylation of the aromatic and heterocyclic amino acids phenylalanine, tyrosine, tryptophan and histidine produces their respective amines. They probably exist in trace amounts in all herbage species but are often not readily detectable and accumulate in the leaves of only a few species (Hegarty and Peterson, 1973).

The precise role of amines in plant metabolism is still unknown but some do have a definite functional role eg tryptamine is a precursor of indole acetic acid, one of the plant growth hormones (Beevers, 1976). The small amounts of amines present in the leaves of grasses and legumes probably make little contribution to the overall nutrition of livestock (Hegarty and Peterson, 1973) although the silage made from them may contain considerable quantities of amines (Macpherson, 1962; Macpherson and Violante, 1966). Their presence in silages is of considerable importance as they are potentially toxic to animals eg high concentrations of histamine can be lethal (Woolford, 1975).

2.1.1.4 *Ureides*. The ureides allantoin and allantoic acid have been found in many herbage species including grasses (Reinbothe and Mothes, 1962; Hegarty and Peterson, 1973; Beevers, 1976). Allantoin is a product of aerobic purine degradation and its hydrolysis leads to the formation of allantoic acid (Hegarty and Peterson, 1973). In most plants they are present at very low concentrations but in certain tropical legumes and some temperate legumes such as soyabean (*Glycine max*) they are the main products of assimilation in the roots (Pate, 1980). In these plants they are present in large quantities in the xylem sap (Pate, 1980) and after transportation to the shoots they may be catabolised by conversion to urea and glyoxylate (Herridge *et al*, 1978). Ureides would appear to be harmless to animals and are produced as a result of animal purine catabolism too, being excreted in the urine. They are susceptible to breakdown by the bacteria of the rumen and intestine (Hegarty and Peterson, 1973).

2.1.2 Protein Nitrogen

Herbage proteins constitute 75-90% of the total nitrogen of the plant (Ohshima and McDonald, 1978) and are similar to those from other higher plants (Lyttleton, 1973).

Plant proteins can be divided into two main categories (a) seed proteins which are mostly of a storage nature and (b) leaf proteins which are concerned with the growth and biochemical functions of the cell and are mainly enzymic.

Legumes may have a significant proportion of their protein in seed form but the seeds of most herbage plants, such as grasses, are much smaller, containing an insignificant fraction of the total plant protein (Lyttleton, 1973).

The earliest studies on the preparation of leaf protein were carried out by Chibnall (1939) and the techniques are still basically the same. The plant material is macerated to release the cell contents, the various fractions then being separated by suitable means.

The proteins of fresh forage may be considered to belong to three main groups (1) Fraction 1 leaf protein, (2) Fraction 2 proteins and (3) Chloroplast membrane proteins, since together they constitute about 90% of the total leaf protein (Mangan, 1982).

Singer *et al* (1952) used the technique of ultracentrifugation to show that although the soluble leaf protein was a heterogeneous mixture, it consisted of two main fractions, F1 and F2 which were separated by molecular weight.

Trown (1965) demonstrated that F1 protein was identical to ribulose-1, 5-diphosphate carboxylase (RuDPCase), an important enzyme in the Calvin cycle of photosynthesis (Walker and Robinson, 1980) and in photorespiration (Lorimer and Andrews, 1980). Lyttleton and Ts'o (1958) showed that it was present in the chloroplasts only and that they contain about 75% of the total leaf protein, half of which is RuDPCase (Akazawa, 1970; Steinback, 1980). Willison and Davey (1976) used freeze-etch electron microscopy to show that the chloroplast stroma consists largely of RuDPCase in a semi-crystalline state. The amino acid

composition of this protein varies only slightly with species, its universal occurrence in green plants making it the most abundant protein in existence (Kawashima and Wildman, 1970; Ellis, 1977).

RuDPCase is a large (18S) protein having a molecular weight of about 576,000, and consisting of eight large (m.wt. 56,000) and eight small (m.wt. 16,000) subunits (Baker *et al*, 1977; Johal *et al*, 1980). The large subunits contain the catalytic sites for carbon dioxide fixation and oxygenase activity (Steinback, 1980) and their amino acid composition varies little between species (Jensen and Bahr, 1977). The small subunits are thought to function in a regulatory capacity (Steinback, 1980) and vary both in size (Jensen and Bahr, 1977) and amino acid composition (Mangan, 1982) between species. Gray and Kekwick (1974) have put forward immunological evidence to support this.

Fraction 2 consists of a heterogeneous mixture of low molecular weight, soluble proteins, mainly enzymes which originate from both the cytoplasm and chloroplasts and which together constitute about 25% of the total leaf protein (Mangan, 1982). There are about 50 enzymes in chloroplasts and over 1000 in the whole plant but many of these enzymes are present only in very small amounts (Mangan, 1982). Gel electrophoresis, however, has distinguished several major peaks in Fraction 2 (De Jong, 1977). Some components of Fraction 2 have been characterized and isolated in large quantities, including ferredoxin, plastocyanin and cytochromes from the chloroplasts and tubulin, actin, ATP synthetase, carbonic anhydrase and protein elongation factors from the cytoplasm

(Mangan, 1982). The soluble fraction also contains the ribosomes which are about 50% protein and account for about 10% of the total leaf protein (Dyer and Leaver, 1980).

The insoluble plant proteins are not well characterized. Most of them are associated with the chloroplast thylakoid membranes which contain about 40% of the total chloroplast protein in an approximate 1:1 ratio with the lipid material (Park and Pon, 1963; Mangan, 1982). Treatment with sodium dodecyl sulphate (SDS) and separation by SDS-polyacrylamide gel-electrophoresis gives two main chlorophyll-protein complexes (Herrman *et al*, 1980), CPI (m.wt. 110,000) and CPII (m.wt. 23,000-25,000) which account for 28% and 49% of the thylakoid membrane proteins respectively. The other 20% consists of about five other CP complexes, whose composition and function are unknown.

A minor component of the insoluble protein fraction is the glycoprotein extensin which is firmly associated with the cell wall cellulose (Lamport and Northcote, 1960) and is believed to modify the properties of the cell wall (Lamport, 1965; Keegstra *et al*, 1973). This protein is unusual as it contains a large amount (30%) of the rare amino acid hydroxyproline (Lamport, 1967). Extensin has not been fully characterized but appears to have a molecular weight of 230,000 and contains 90% carbohydrate and only 5% protein (Mangan, 1982).

Two other minor protein components exist. Because animal mitochondria are easier to isolate, plant mitochondria have not been studied as extensively. They are smaller than chloroplasts but are present in similar numbers and probably contain less than 5% of the total leaf protein (Pirie, 1959). It is known that all

the TCA cycle enzymes are present in the mitochondria and that about 40% of the total mitochondrial protein is structural, insoluble protein (Lyttleton, 1973). Although 50% of the dry weight of the nucleus is protein, this constitutes only 1-2% of the total leaf protein. The proteins of the nucleus are mainly histones and the enzymes associated with DNA replication and protein synthesis (Lyttleton, 1973).

The amino acid composition of herbage has not been extensively studied but the information available suggests there is very little difference between species, even when the protein content is altered by fertilizer treatment, maturation or nutrient deficiencies (Lyttleton, 1973).

2.2 THE ACTIVITY OF PLANT ENZYMES

Immediately after harvesting a crop, and during the initial stages of ensilage, biochemical changes occur as a result of plant enzyme and aerobic microbial activity. The respiratory and proteolytic enzymes are the most important of the plant enzymes.

2.2.1 Respiration

Respiration can be defined as the oxidative degradation of organic compounds to yield usable energy. In higher plants, oxygen is the terminal electron acceptor (Ting, 1982). The substrate for oxidation is usually a hexose sugar which undergoes glycolysis before entering the aerobic phase. If oxygen is not present, the products of glycolysis are lactate or ethanol plus carbon dioxide and ATP (Davies, 1980). If oxygen is present, however, pyruvate from the glycolytic pathway is oxidized via the tricarboxylic acid cycle to carbon dioxide and ATP. Complete oxidation of one glucose molecule yields 38 molecules of ATP (Muller, 1979). This would suggest an efficiency of energy conservation of only about 40%, the rest of the energy released from the sugar being dissipated as heat (Muller, 1979). However, this is under standard conditions and there is evidence to suggest that under intracellular conditions the true efficiency is much higher (Lehninger, 1975).

Glucose, fructose, sucrose and fructans are the major water-soluble carbohydrates (WSC) in grasses. (Smith, 1973). Sucrose and fructans in ensiled grass are rapidly hydrolysed to glucose and fructose by plant enzymes (Macpherson *et al*, 1957; Bousset *et al*, 1972; McDonald and Whittenbury, 1973). In the growing plant, sugars are the main substrate for the respiratory enzymes, and after harvesting they continue to be metabolized until anaerobiosis is

achieved or the pH falls sufficiently (Greenhill, 1959; Pizarro and James, 1972; McDonald, 1976). This results in a loss of sugars, which are required for fermentation by lactic acid bacteria during ensilage (McDonald *et al*, 1966). It may also lead to a rise in temperature in the silo resulting in an acceleration of the activities of other enzymes, further nutrient losses, and a reduction in the digestibility of the silage (Crawshaw and Woolford, 1979).

2.2.1.1 *The wilting period.* Aerobic micro-organisms are not thought to contribute significantly to total plant respiration during wilting, the biochemical changes occurring being almost entirely plant enzyme mediated (McDonald and Whittenbury, 1973; Clark, 1974). Studies on respiration have shown that, in general, it declines with increasing dry matter content (Greenhill, 1959; Simpson, 1961; Meidner, 1967; Pizarro and James, 1972) although there may be an initial increase after harvesting in response to wounding (Roberts, 1951; Stiles and Leach, 1952; Brady, 1973; Uritani and Asahi, 1980). Thus under favourable wilting conditions, when the dry matter increase is rapid, water-soluble carbohydrate losses owing to respiration, would be expected to be low. Under moist wilting conditions, however, losses may be significant (Carpintero *et al*, 1979; Honig, 1980). Mitchell and Shepperson (1955) considered that respiration losses were directly proportional to the initial moisture content and inversely proportional to the rate of drying. The temperature during wilting affects the losses incurred as the respiration intensity of most plant material increases rapidly with temperature, especially at low dry matter levels (Greenhill, 1959; Honig, 1980). The stage of

maturity of the harvested plant is also important as younger plants have a higher rate of respiration (Greenhill, 1959, Pizarro and James, 1972). Since immature plants also have a lower content of water-soluble carbohydrates, the respiration losses are relatively more important (Pizarro and James, 1972).

Measurements of the respiratory quotients for wilting grasses and legumes have given values of around unity (Greenhill, 1959; Melvin and Simpson, 1963; Pizarro and James, 1972) indicating that the main respiratory substrate is a hexose sugar. Wylam (1953) has shown that hydrolysis of sucrose and fructans occurs after harvesting and is extremely rapid if the material is kept under moist conditions. During wilting the hexose sugars released are then rapidly catabolized (Wylam, 1953; Melvin and Simpson, 1963) and although the general trend is towards a decrease in water-soluble carbohydrates, periodic increases have been observed (Wylam, 1953; Melvin and Simpson, 1963; Clark, 1974).

It is difficult to measure accurately losses of water-soluble carbohydrates resulting from respiration as polysaccharide hydrolysis may partly replace them (Dewar *et al*, 1963; Henderson and McDonald, 1971; Clark, 1974; Morrison, 1979). It has also been suggested (Nash, 1959) that some photosynthesis may occur during wilting but, although activity has been observed in leaves during the first 24h after harvesting (MacGregor, 1966), it is unlikely to be of much significance under practical wilting conditions owing to insufficient penetration of light into the swath and the closure of the stomata (Pizarro and James, 1972; Clark, 1974).

2.2.1.2 *The ensiling period.* Respiration will continue in the silo as long as aerobic conditions persist. It is therefore necessary, in order to limit respiration losses, that every effort is made to

exclude air when the silo is being filled.

If consolidation is adequate and the silo is sealed quickly and efficiently, the amount of oxygen trapped within the herbage is small and is rapidly used up in respiration (Greenhill, 1964; Jackson, 1969; Ruxton *et al*, 1975) resulting in a loss of sugars of not more than about $10gkg^{-1}$DM (McDonald and Whittenbury, 1967). Sprague (1974) found that 90% of the oxygen initially present in large plastic silos (1-2 litre capacity) disappeared within 15 min. and virtually none was left after 30 min. It was also noted that oxygen was more rapidly utilized in silos filled with direct-cut herbage (DM-$237gkg^{-1}$) than if it had been pre-wilted for two hours, which presumably reflects a reduction in the respiration rate of the wilted herbage (DM-372 gkg^{-1}) owing to the increased dry matter content.

In silos which are inadequately consolidated and sealed, considerable losses of water-soluble carbohydrates may occur as a result of respiration (McDonald *et al*, 1960; McDonald *et al*, 1966; Henderson and McDonald, 1975). Losses may be so large that residual amounts of water-soluble carbohydrate are insufficient to allow enough acid to be produced for satisfactory preservation (Yoder *et al*, 1960; McDonald *et al*, 1966; Ruxton, 1972). Takahashi (1968) has shown this problem to be most serious with herbage of initially low water-soluble carbohydrate or dry matter contents, the effect of water-soluble carbohydrates also being noted by Henderson (1973). Laceration of the crop prior to ensiling facilitates consolidation (McDonald *et al*, 1965; Wieringa, 1960).

In the living plant, part of the energy released when sugars are oxidized is captured as ATP and the remainder is liberated as heat

(Lehninger, 1975). In the harvested plant, however, since biosynthetic reactions are limited, it is likely that nearly all the energy in the oxidized sugars is converted into heat (McDonald *et al*, 1966). In the silo this heat cannot be dissipated easily and causes a rise in temperature (Wieringa *et al*, 1961) which may adversely affect fermentation (Wieringa, 1960; McDonald *et al*, 1966; Murdoch, 1970). This increase will depend mainly on the extent and rate of respiration, but the degree of insulation of the silo and the specific heat of the ensiled material are also important (McDonald and Whittenbury, 1967).

The extent of respiration is controlled mainly by the oxygen and sugar supplies (McDonald *et al*, 1966), but in practice sugars are usually abundant and the limiting factor is usually oxygen supply. The rate of respiration is affected mainly by temperature and thus the two are interdependent. The apparent temperature optimum for respiration is the resultant of accelerated reaction rate and enzyme inactivation, with increasing temperature (Lehninger, 1975). Most enzymes are instantaneously inactivated at temperatures above 60°C and usually the inactivation is irreversible. In plants the heat stability of enzymes is influenced by the presence of other proteins, pH and substrate concentrations (James, 1963). The temperature relations of biological processes are usually expressed in terms of the temperature coefficient (Q_{10}). This is the ratio of the rate at a given temperature divided by the rate at 10°C lower. For plant respiration, irrespective of the species or part of the plant used, the Q_{10} between 5° and 15°C is usually about 2.0. As the temperature rises, the Q_{10} falls until, around 30°C, it is down to about 1.3 (James, 1963; Greenhill, 1959; Raison, 1980). The temperature optimum of a reaction is also dependent on time because at higher temperatures, although there may be a higher initial increase in rate, the enzyme is also more rapidly

denatured (Dixon and Webb, 1979).

In a well-consolidated, sealed silo a temperature rise of more than three or four degrees Centigrade would not be expected (McDonald and Whittenbury, 1967), but in a badly-consolidated silo more air is trapped and temperature rises may be high (McDonald *et al*, 1960). McDonald *et al* (1966) recorded temperatures as high as 42°C with badly-consolidated fresh grass after 3 days ensilage and 37°C with wilted grass after 7 days. The fresh herbage heated up more rapidly than wilted material, presumably as a result of greater respiratory activity in the wetter material. In practice, however, it is usually more difficult to prevent overheating in wilted material due to the problems of consolidation and preventing re-entry of air (McDonald *et al*, 1966).

Temperature can also affect bacterial activity directly. The rate of bacterial metabolism is generally increased as the temperature increases up to about 40°C but above this a selective effect occurs as very few lactic acid bacteria can grow at these temperatures. Successful fermentation at high temperature will therefore depend on these bacteria being present (McDonald *et al*, 1966; Beck, 1969).

The final temperature of ensiled herbage is mainly determined by the heat rise resulting from respiration as neither fermentation nor the ambient temperature have much influence (McDonald *et al*, 1966; Ruxton *et al*, 1975).

Wilting does not appear to result in any overall pH changes whatever the environmental wilting conditions (Carpintero *et al*, 1979). In the silo, however, lactic acid bacteria bring about a rapid drop in pH and although no direct measurements of the effect of pH on

respiration in the silo appear to have been made, there is some evidence to suggest that the respiration rate decreases as the pH falls. Virtanen (1933) demonstrated a pH effect on respiration rate in red clover and green peas, such that respiration was 20% of normal at pH 3.5 and had ceased completely at pH 3.0. Respiration rate has been measured indirectly by recording the temperature changes in the silo. Henderson *et al* (1972) ensiled wilted perennial ryegrass pre-treated with formic acid, which lowered the pH from 6.2 to 4.9, and found that, over an 8-day period, temperatures in the treated grass were consistently 4°C lower than in the ensiled, untreated material. Henderson and McDonald (1971) also found that direct acidification with formic acid prevented oxidation of water-soluble carbohydrates in the short period between harvesting and ensiling.

2.2.2 Proteolysis

Proteolysis is the cleavage of peptide bonds by hydrolysis. The enzymes which accomplish this are termed *proteases* or *peptide hydrolases*. They can be subdivided into *proteinases* or *endopeptidases*, which hydrolyse internal peptide bonds, and *peptidases* or *exopeptidases*, which hydrolyse terminal amino- or carboxy-peptide bonds (Barrett, 1980; Frith and Dalling, 1980).

In fresh herbage, 75-90% of the total nitrogen is present as protein (Ohshima and McDonald, 1978), but after harvesting rapid proteolysis takes place (Macpherson, 1952a; Macpherson and Slater, 1959; Brady, 1960; Singh, 1962; Ohyama, 1964; McDonald *et al*, 1968; Hughes, 1970; Macpherson and Wall, 1970; Ohyama, 1970; Henderson *et al*, 1972; Bergen *et al*, 1974). The extent of protein degradation

varies with plant species, rate and extent of pH changes, and temperature (Watson, 1939), but may reduce the protein content by 50-60% even in a well-preserved silage (Kemble, 1956; Whittenbury *et al*, 1967). Further breakdown of amino acids may also occur (Ohshima and McDonald, 1978).

Macpherson and Slater (1959) considered that rapid and extensive proteolysis was only halted by attainment of a high dry matter content or a low pH, as the material was dried or ensiled respectively.

2.2.2.1 *The wilting period.* Various workers have investigated the extent of proteolysis during wilting. Henderson *et al* (1972) found 20% of the initial protein was hydrolysed during a 31h wilt to a dry matter content of 323 gkg^{-1}. Kemble and Macpherson (1954a) also found a 20% decrease after wilting for 3 days to a dry matter content of 600 gkg^{-1}. Ohyama (1970) wilted unchopped cocksfoot for 5 days, to a dry matter content of about 400 gkg^{-1}, and found that as much as 50% of the protein could be hydrolysed. If the grass was chopped and incubated under aerobic conditions, however, only about 10% was hydrolysed. He concluded that the presence of oxygen inhibited proteolysis during wilting which he later confirmed (Ohyama and Masaki, 1971). This is consistent with the report of Mothes (1956), which stated that proteolytic enzymes, activated by reduced sulphydryl groups, had increased activity in plants suffering water shortage, because the closure of the stomata caused a lowering of oxygen tension. Singh (1962) suggested that plant proteases were thiol proteases, thus requiring reducing conditions for activity.

Both Macpherson (1952b) and Carpintero *et al* (1979) observed that proteolysis was more extensive under moist wilting conditions and Macpherson noted that it had ceased by the time a dry matter content of about 400 gkg^{-1} was reached. A similar result was obtained by Blagoveshensky *et al* (1978). After a series of wilting experiments on various grasses and legumes, Brady (1960) concluded that the final nitrogen distribution depended on the rapidity with which moisture was lost as little proteolysis occurred during a rapid wilt but a large increase in amino acids and amides was apparent after a slow, moist wilt. Macpherson (1952b) also found a rapid increase in amides during wilting, especially under moist conditions.

The main products of protein hydrolysis during wilting are peptides, free amino acids and amides (Kemble and Macpherson, 1954a; Brady, 1960). Kemble and Macpherson (1954a) showed that all the amino acids they measured, other than proline, were found in amounts less than calculated from the amount of protein degraded. The deficit was variable and presumably reflected different rates of subsequent amino acid breakdown as it had been shown (Kemble and Macpherson, 1952b) that protein breakdown was uniform. After 8 days wilting, free proline was 50% in excess of the total proline in the original herbage, indicating substantial proline synthesis. In a second experiment, Kemble and Macpherson (1954a) compared dry and moist wilting. Similar results to the previous experiment were obtained for dry wilting, but for moist wilting little change in dry matter was observed and no proline was synthesized. Instead there was an increase in the amide fraction. The accumulation of proline has also been noted

in growing crops suffering water shortage (Brady, 1973). The mechanism of such accumulation in leaves has been studied by Boggess and co-workers (Boggess *et al*, 1976; Stewart and Boggess, 1977; Stewart *et al*, 1977). They concluded that proline plays a role in the detoxification of ammonia in leaves during periods of water shortage, a function which is performed by amide synthesis under normal conditions of growth or moist wilting (Kemble and Macpherson, 1954a). Glutamate is first produced by reductive amination of α-ketoglutarate which is then reduced to proline via γ-glutamyl semialdehyde.

2.2.2.2 *The ensiling period.* When herbage is ensiled, either directly or after wilting, proteolysis continues and within 12 to 24h the protein content may fall from about 800 gkg^{-1}TN to about 600 gkg^{-1}TN (Kemble, 1956; Brady, 1960; Bergen *et al*, 1974; Clark, 1974). By the end of ensilage this may have decreased to 300 gkg^{-1} or less (Whittenbury *et al*, 1967; Hughes, 1970; De Vuyst *et al*, 1971; Ohshima *et al*, 1979). Wilting does not seem to have much effect on the overall decrease in protein during ensilage (Brady, 1960; Henderson *et al*, 1972; McDonald and Edwards, 1976; Marsh, 1979) but may reduce further amino acid metabolism, especially deamination (McDonald *et al*, 1965; McDonald and Edwards, 1976).

The main products of protein breakdown during ensilage are amino acids and ammonia (McDonald and Edwards, 1976), the proportion of each depending on whether there has been further amino acid breakdown by clostridia (McDonald and Whittenbury, 1973; Ohshima and Mc Donald, 1978).

2.2.2.3 *The contribution of plant proteases.* The importance of the endogenous plant enzymes during proteolysis, especially during the initial post-harvest stage, has been recognised for some time (Kemble, 1956; Macpherson *et al*, 1957; Brady, 1960; Watson and Nash, 1960; Ohyama and Masaki, 1969; Gouet *et al*, 1970; Bousset *et al*, 1972). Early evidence was obtained by Mabbitt (1951) and Kemble (1956) using aseptically-grown grass. Kemble found that after 16 days incubation the non-protein nitrogen content had increased to 600 gkg^{-1}TN. There was some doubt, however, as to how closely this grass resembled 'natural' grass (Brady, 1966; Gouet *et al*, 1970; Woolford, 1972). Other methods, such as treatment with toluene, chloroform and antibiotics, have been tried in an attempt to separate the effects of plant and microbial enzymes (Macpherson, 1952a; Brady, 1960; Gouet and Fatianoff, 1964; Woolford, 1972), but they are unreliable and the plant material is no longer suitable for inoculation experiments (Gouet *et al*, 1970). By far the most successful method has been sterilization using gamma-irradiation, initially developed in France by Gouet and his co-workers, who have shown that it can successfully inactivate micro-organisms without harming the plant enzymes (Gouet *et al*, 1970, 1972). The sterilized herbage can also be used for subsequent inoculation studies. Using this method, Gouet *et al* (1970) ensiled lucerne (*Medicago sativa*) directly and after irradiation and found that protein degradation in both followed parallel courses and that activity in the irradiated material was only about 10% less than in the control. Clark (1974) showed that the pattern of protein changes over a 48h period was the same for both direct cut and sterile ensiled ryegrass, the extent of protein hydrolysis being slightly less for the sterile material throughout. Bergen *et al*

(1974) found that immediately after ensiling corn plant (*Zea mays*), proteolysis was rapid and was almost complete within 12h. They also made a crude extract of the proteolytic enzymes and assayed them against casein. The results showed that proteolytic activity fell from a maximum in the fresh material to less than 25% of maximum after 5 days of ensiling.

Virtanen (1933) stated that neither plants nor aerobic micro-organisms contained proteolytic enzymes capable of activity below pH 4.0 and, from studies with grass juice, Macpherson (1952a) concluded that the achievement of pH 4.3 during ensilage was sufficient to prevent any further significant proteolysis. In studies with extracted leaf proteases of tobacco (Tracey, 1948), white clover (Brady, 1961) and wheat (Singh, 1962), pH optima were found to lie within the range 5.0 to 6.0. The tobacco and wheat proteases were found to have significant activity even at pH 4.0. Carpintero *et al* (1979) acidified a ryegrass-clover mixture with formic acid, achieving an initial pH of less than 4.0. The protein nitrogen in the control fell from 819 to 265 $gkg^{-1}TN$ after 50 days, whereas in the treated herbage it decreased to 462 $gkg^{-1}TN$. Therefore it would seem that, although proteolysis may be inhibited by reducing the pH to about 4.0, it is not prevented.

2.2.2.4 *Water deprivation.* Harvesting results in physical, physiological and biochemical changes in the plant as it responds to injury, water deficiency during the initial stages of wilting, and the onset of senescence during the later stages (Clark, 1974).

During periods of water deficiency the macromolecular content of tissues usually declines as a result of increased hydrolysis and decreased synthesis (Brady, 1973). There is evidence of inhibition

of nucleic acid and protein synthesis and also of their increased catabolism under such conditions (Crafts, 1968; Naylor, 1972; Brady, 1973; Clark, 1974; Beevers, 1976). Barnett and Naylor (1966) found that the soluble protein content declined with increasing water shortage. Using ^{14}C-labelling techniques they showed that amino acids, especially proline and the amides, were synthesized continuously during periods of water deficiency, but protein synthesis was inhibited, thus causing an accumulation of free amino acids. Todd and Basler (1965) found that the breakdown of wheat cell constituents during drought was similar in intact and detached leaves.

2.2.2.5 *Senescence*. Senescence may be defined as the deteriorative events which precede the death of a mature cell (Beevers, 1976). Most studies on senescence have been conducted on detached leaves which show the same characteristic changes as the whole plant although these occur at an accelerated rate in the detached leaves (Beevers, 1976; Thimann, 1980). Water deficiency induces premature senescence in many tissues (Brady, 1973) and numerous studies of senescence in detached leaves are relevant to the situation within the swath during wilting (Clark, 1974). Many of the initial changes occurring during ensilage can also be explained in terms of senescence.

As in the case of water shortage, senescence brings about a change in the balance of anabolic and catabolic processes such that the latter predominate (Varner, 1965; Carr and Pate, 1967). These changes are reflected in the leaf ultrastructure which shows a rapid degradation of the cell's organelles (Shaw and Manocha, 1965; Barton, 1966; Butler and Simon, 1971). The first detectable changes are a

decline in the population of ribosomes and the initiation of chloroplast disorganization. The chloroplast thylakoids, endoplasmic reticulum and Golgi apparatus all disintegrate but the mitochondria and nucleus remain structurally intact until the final stages of senescence. The tonoplast membrane disappears before the other organelles completely disintegrate but the plasmalemma remains intact until cell death.

Chlorophyll loss is a visible sign of senescence but degradation of ribonucleic acid, protein, lipid and carbohydrate also occurs and net losses of ribonucleic acid and protein may occur before the chlorophyll content declines (Brady, 1973; Beevers, 1976; Tetley and Thimann, 1974). Proteolysis is very rapid (Peterson and Huffaker, 1975; Feller *et al*, 1977) especially in grasses and other monocotyledons (Clark, 1974). It is not possible yet to say definitely whether the protein breakdown results from increased degradation, decreased synthesis, or a combination of both but evidence to date seems to indicate that increased degradation is mainly responsible (Brady, 1973; Frith and Dalling, 1980). This increase could be the result of increased synthesis of proteases, activation of existing proteases, removal of inhibitors or increased access to substrate.

Proteolytic enzymes are always present in leaves (Thimann, 1980), being active during germination, tissue differentiation and senescence as well as in the continuous turnover of cellular proteins (Ryan, 1973; Frith and Dalling, 1980). In detached leaves there is an increase in proteolytic activity during senescence (Beevers, 1976; Frith and Dalling, 1980). According to Brady (1973), the potential for protein breakdown in the plant is

always high and it is the regulation of these existing proteases which is important in determining the extent of proteolysis. Nevertheless, some proteases would appear to be synthesized during senescence especially in the initial stages (Peterson and Huffaker, 1975).

Ribulose diphosphate carboxylase (RuDPCase), which is found only in the chloroplast stroma, is broken down rapidly in senescing leaves and may account for most of the initial protein loss (Peterson and Huffaker, 1975; Wittenbach, 1978). Choe and Thimann (1974) found that isolated chloroplasts retained more of their protein than those in intact leaves, which suggests that the enzymes responsible for hydrolysis originate in the cytoplasm. This was substantiated by experiments which showed that senescence was inhibited by cycloheximide, an inhibitor of cytoplasmic protein synthesis, but not by chloramphenicol, an inhibitor of chloroplastic protein synthesis (Martin and Thimann, 1972a; Choe and Thimann, 1974; Peterson and Huffaker, 1975). These experiments also show that some protein synthesis is necessary for senescence to occur.

In the later stages of senescence, when the chloroplast membranes have to a large extent been broken down and dispersed, the tonoplast membrane is ruptured (Barton, 1966). There is evidence that the cell vacuoles of some plants contain a variety of hydrolases, including proteases, which would then be released (Matile, 1975).

2.2.2.6 *Proteolytic enzymes.* The earliest studies of the proteolytic enzymes of leaves were carried out at the beginning of the century (Vines, 1903, 1909) but, other than investigations by Tracey (1948), Brady (1961) and Singh (1962) on tobacco, (*Nicotania tabacum*), white clover (*Trifolium repens*) and wheat (*Triticum*

aestivum) leaves respectively, very little further interest was shown until fairly recently.

Tracey (1948) assayed a partly purified proteolytic fraction against gelatin and found activity over a wide pH range with an optimum between 5.5 and 6.5 At pH 4.0 activity was still more than 50% of maximum. Incubation at temperatures between 5°C and 40°C showed that the greatest activity was at the highest temperature. Thioglycollate stimulated and iodoacetate partly inhibited proteolysis indicating that a thiol protease may be present. Tracey suggested that more than one enzyme was involved but was unable to demonstrate this.

Brady (1961) examined a crude extract of proteases using both gelatin and endogenous substrates. Generally, activity was similar with both but the response to some activators and inhibitors differed. The optimum pH was about 6.0, no activity being found below pH 3.9. If the extract was stored above pH 5.5, 60 to 70% of the original activity was retained but below this pH, activity rapidly declined. The temperature optimum was found to be 45°C and reducing agents increased activity with the endogenous substrate but not gelatin.

Singh (1962) investigated crude protease extracts from various species for autolysis and found that, at 37°C, up to 40% of the endogenous protein could be hydrolysed within two hours. The protease activity of wheat was investigated further using casein as substrate. Unlike gelatin, casein contains sulphydryl groups which many plant proteases require. The pH optimum was between 5.0 and 6.0 with 35% of the maximum activity remaining at pH 4.0. Incubation temperatures of 25°C and 37°C were compared and activity at the higher temperature was double that at 25°C. There was

evidence that sulphydryl groups were required for activation.

Most recent studies on leaf proteases have been related to senescence, and the greatest interest has been shown in cereals, especially wheat and oats (*Avena sativa*). Indications are that there are several different proteases in leaves and it is conceivable that each may have specific and possibly interdependent roles in both protein turnover and terminal degradation (Frith and Dalling, 1980). Drivdahl and Thimann (1977) suggested that the presence of several proteases would be an advantage to the senescing plant, given the range and structural complexity of the proteins to be degraded. Many proteases have specificities for certain bonds and substrates (Huffaker and Peterson, 1974).

Proteases can be classified into four main types based on their reaction mechanisms, ie carboxyl, thiol, serine and metal proteases (Barrett, 1980). In the plant leaves most proteases have acid pH optima and belong to the thiol and carboxyl groups (Frith and Dalling, 1980; Ryan and Walker-Simmons, 1980). It is of interest to note that, in contrast to plants, animals and microorganisms utilize mainly serine proteases (Ryan and Walker-Simmons, 1980).

Wheat leaves have been shown to contain proteases which are active in acidic (Frith *et al*, 1975, 1978; Peoples and Dalling, 1978) and neutral (Feller, 1978) conditions. Frith *et al* (1978) detected six different proteases with acid pH optima, at least three of which were carboxyl proteinases which degraded haemoglobin at pH 4.5. One of the proteinases was also able to degrade RuDPCase but had a slightly higher pH optimum of about 5.0 (Frith *et al*, 1978; Peoples and Dalling, 1978). A wheat protease with an acid pH optimum has

been shown to be a thiol proteinase (Wittenbach, 1978).

Oat leaves also contain proteases which are active under acidic and neutral conditions (Drivdahl and Thimann, 1977, 1978; Martin and Thimann, 1972, a, b). Both types showed broad pH, and high temperature, optima. The activity at acid pH seemed to be mainly due to a proteinase with a serine residue at the active site, whereas at neutral pH the active enzyme appeared to be a thiol peptidase.

Both amino-peptidase (pH optimum 6.5-7.0) and carboxy-peptidase activities (pH optima 3.5 and 5.0), as well as proteinases with acid and neutral pH optima, have been detected in maize (*Zea mays*) leaves (Feller *et al*, 1977).

It is apparent from a number of studies that the pH optima for proteases may be dependent on the substrate as well as the incubation temperature (Martin and Thimann, 1972a; Mikola and Kohlemainen, 1972; Drivdahl and Thimann, 1978) so that the pH optimum on an artificial substrate may not reflect the situation in the plant (Frith *et al*, 1978; Frith and Dalling, 1980). However, it appears that proteases active at acid pH predominate, and hence the widely held opinion, that the achievement of pH 4.0 prevents further proteolysis during ensilage, is unlikely to hold true, as the results of Carpintero *et al* (1979) have already shown.

In general, proteases seem to have high temperature optima, ie in the region of 45-55°C (Brady, 1961; Feller *et al*, 1977; Drivdahl and Thimann, 1977; Frith *et al*, 1978; Peoples and Dalling, 1978), but if incubated in the absence of substrate they are much more temperature sensitive (Drivdahl and Thimann, 1977; Peoples and

Dalling, 1978), and may be rapidly inactivated at temperatures above 30°C (Peoples and Dalling, 1978). Tracey (1948) and Brady (1961) determined the thermal stability of proteases, by measuring residual activity after pre-incubation at various temperatures in the absence of substrate. In both cases, inactivation was rapid above about 50°C and complete by about 80°C. Brady concluded that inactivation at high temperature was unlikely during ensilage. Macpherson (1952a) compared incubation of grass juice at 18°C and 30°C. At the higher temperature the pH dropped faster, but the rate of proteolysis was increased, so that the final outcome after 14 days was very similar. Ohyama *et al* (1973) ensiled grass at 15°C and 30°C and concluded that proteolysis was affected by temperature during the early stages of ensilage and by pH during the later stages.

2.2.2.7 *The regulation of protease activity*. Since appreciable protease activity is found even in young leaves (Frith *et al*, 1975; Peoples and Dalling, 1978) there must be some mechanism by which the activity in non-senescing cells is controlled eg by compartmentalization or inhibition.

Most plant protease inhibitors have been found to be active against proteases from animals and micro-organisms and presumably function in a protective role against such organisms (Ryan, 1980). Inhibitors against endogenous proteases have been found but in most cases have been associated with the seeds where they presumably play a role in protein mobilization during germination (Huffaker and Peterson, 1974). They are usually found in the protein bodies although some may be found in the seed cytoplasm where they may inhibit any proteases which escape from ruptured protein bodies (Ryan, 1973, 1980).

Hydrolases, including proteases, are present in many plant vacuoles (Ryan, 1973; Matile, 1975; Bollen and Kende, 1979) but little is known about the mechanism of intracellular protein degradation, especially with regard to protein turnover. It is possible that protein degradation occurs, not in the cytoplasm but in the vacuole itself or in lysosome-like structures in the cytoplasm as has been demonstrated for some animal cells (Matile, 1978). The protein bodies in seeds have been likened to lysosome-like organelles (Beevers, 1976). Also in support of this theory, vesicles of cytoplasmic material have been observed in plant vacuoles, presumably formed by autophagy (Marty *et al*, 1980). Studies on yeast cells have shown that the proteases are compartmentalized in the vacuole and in addition a system of protease inhibitors exists in the cytoplasm, which strongly suggests that proteolysis in yeast cells is restricted to the vacuole (Matile, 1978).

The breakdown of proteins during protein turnover and senescence are regulated events and not due to uncontrolled autolysis (Huffaker and Peterson, 1974; Beevers, 1976), thus some system must exist which allows for the selectivity of protein breakdown which occurs. Holzer (1976) suggested that specificity could be provided by conformational changes occurring in a protein molecule making it vulnerable and recognisable by the cell's protein degradation system.

In most plants the vacuole pH is maintained at a lower level (5.0-5.5) than that of the cytoplasm (7.0-7.5) by active transport across the tonoplast (Marty *et al*, 1980; Smith and Raven, 1979) which could explain why so many plant proteases have acidic pH optima. In wheat leaves the vacuole appears to be devoid of proteases but some proteases with acid pH optima, which degrade RuDPCase, have been

found associated with the chloroplast (Peoples and Dalling, 1979). Such proteases have also been found in oat chloroplasts (Choe and Thimann, 1975). Although the pH of the chloroplast stroma ranges from 7 (dark) to 8 (light), it is possible that the proteolytic activity is associated with localized zones of low pH within the chloroplast. For instance, the pH of the thylakoid membrane and intra-thylakoid spaces may be as low as 4.5 during chloroplast illumination (Peoples and Dalling, 1978).

If compartmentalization of proteases does occur, it is conceivable that they will eventually be released during ensilage, either as a result of mechanical disruption during harvesting or cellular disintegration under anaerobic conditions.

There is also some evidence for the presence of zymogen-type pro-enzymes in plants (Huffaker and Peterson, 1974) which could account for the rapid increase in protease activity often observed (Frith and Dalling, 1980; Thimann, 1980).

Certain amino acids may be involved in the regulation of proteolysis (Martin and Thimann, 1972a, b). Detached leaves exhibit higher levels of proteolytic activity than attached leaves, and during senescence amino acids accumulate in the former but not the latter (Beevers, 1976). In addition, protease activity is initially high in young leaves which have a high soluble amino acid content (Beevers, 1976). Shibaoka and Thimann (1970) found that L-serine, one of the most common amino acids in many plants, promoted proteolysis during senescence in oat leaves. They attributed this to an increase in the formation of serine proteases. Similar, but weaker, effects were exerted by cysteine and glycine (Martin and

Thimann, 1972b). Serine and thiol proteases are two of the main classes of protease and it is likely that these amino acids promote their synthesis (Thimann, 1980). Glycine is a precursor in both cysteine and serine biosynthesis. Arginine has been found to have the opposite effect to serine in oat leaves (Martin and Thimann, 1972b). Martin and Thimann (1972b) suggested that the oat proteases with pH optima of 3.0 and 7.5 may contain both thiol and hydroxyl groups at their active sites, a situation which occurs in proteases from papaya (Arnon, 1970).

2.2.2.8 *Limitations to the extent of proteolysis.* Proteolysis during ensilage does not proceed to completion, even when the pH is not inhibiting (Macpherson *et al*, 1957; Gouet *et al* 1970). In fact, the protein content does not usually fall below 250 gkg^{-1} TN. Therefore it would appear that not all the proteins are readily available, nor are all proteins degraded to the same extent. Tracey (1948) found that tobacco proteases were more active on gelatin than on egg albumin. Martin and Thimann (1972a) found that oat leaf proteases were very active on haemoglobin but much less so on bovine serum albumin.

It would appear that the water-soluble proteins of fresh herbage are preferentially degraded during ensilage, since they are absent from silage (Ohyama, 1964; Hughes, 1970; Ohshima, 1971). According to Brady (1960) it is also the water-soluble fraction which is preferentially degraded during wilting. Ohshima (1971) found that peptides were degraded during ensilage and concluded that their low molecular weight made them more accessible. The water-insoluble protein, zein, of corn is not readily degraded (Bergen *et al*, 1974) and may even by-pass the rumen (McDonald, 1954).

There is some evidence that end-product inhibition may partly control the extent of proteolysis as various studies (reviewed by Coppock and Stone, 1968) indicate that the addition of non-protein nitrogen (NPN) to silage spares the plant proteins from proteolysis. In the studies by Bergen *et al* (1974) proteolysis decreased to less than 25% of maximum after 5 days with or without NPN, but the activity was higher in the untreated material throughout, especially in the earlier days.

It is possible that phenolic substances, especially tannins, may play a role in protecting some proteins. Tannins are widespread in nature (Swain, 1965; Loomis and Battaile, 1966) and, although they are of little importance in grasses, they may accumulate in the tissues of many dicotyledons (Haslam, 1979). They are more abundant in dead or dying cells (Swain, 1965) and are frequently synthesized when injury causes cellular disruption, bringing together the polyphenol oxidases and their substrates (Haslam, 1979). Tannins are high molecular weight compounds containing a high concentration of phenolic hydroxyl groups, (1-2 per 100 molecular weight), which can form cross-links with proteins and other macromolecules to produce almost undissociable complexes which cannot be readily degraded enzymatically (Swain, 1979). In intact cells proteins are usually protected from them as the tannins are located in organelles within the cytoplasm (Ferguson, 1975). There are two types of tannins, hydrolysable and condensed. Both bind reversibly via hydrogen bonding to the peptide linkages in the protein but react very differently to changes in pH. The former bind strongly at pH 3-4 but binding decreases above pH 5, whereas condensed tannins are bound almost independently of pH

below pH 7-8, although binding rapidly decreases above pH 8 (Loomis and Battaile, 1966). Under aerobic conditions covalent bonding also occurs between tannins and proteins because the phenolic groups of the tannins may be oxidized to form quinones, either non-enzymatically, or enzymatically by phenoloxidases or peroxidases which are widely distributed in plants (Loomis and Battaile, 1966). The quinone groups produced are very reactive and can form strong covalent bonds with various amino acids (Van Sumere *et al*, 1975).

2.2.3 Amino Acid Metabolism

In addition to the hydrolysis of proteins to amino acids, plant enzymes catabolise certain amino acids (Ohshima and McDonald, 1978). Kemble (1956) studied the neutral amino acids in incubated sterile grass and found that for most the amounts found agreed well with the calculated values expected from proteolysis, but glycine was appreciably lower and alanine higher. Since then, however, Ohshima (1971) has shown that glycine is one of the most stable amino acids during the early stages of ensilage. Plant enzymes can decarboxylate aspartate and glutamate to α-alanine and γ-aminobutyrate (GABA) respectively (Ohshima and McDonald, 1978). Macpherson and Slater (1959) reported the appearance of considerable quantities of GABA in incubated microbe-free grass. In a study using ^{14}C-labelled amino acids, Ohshima (1971) found that 50% of the added glutamate was decarboxylated within 4h of incubation of red clover (*Trifolium pratense*) juice but, if labelled glutamate was added to 3-day old silage (pH 4.2), it was not significantly decarboxylated. In a similar study with labelled aspartate, decarboxylation did not proceed until after 4h and only 6% and 13% was degraded after 8h and 48h respectively, therefore it would seem that plant

enzymes decarboxylate glutamate more readily than aspartate. Ohshima (1971) suggested that plant enzymes were mainly responsible for the decarboxylation of glutamate and micro-organisms for aspartate decarboxylation. Voss (1966) reported that plant decarboxylases were active during early ensilage and bacterial decarboxylases later.

Brady (1960) and Gouet and Fatianoff (1964) both reported some deamination of amino acids by plant enzymes, but Kemble (1956) could find no evidence of this with ensiled microbe-free Timothy and Gouet *et al* (1970) found no ammonia production in sterile ensiled lucerne. However, Mabbitt (1951) also ensiled microbe-free Timothy and, unlike Kemble, found substantial quantities of ammonia produced. Macpherson (1952a) also reported ammonia production if grass sap was incubated with toluene and Ohshima (1971) demonstrated deamination of histidine in red clover juice within 4h of incubation, using ^{14}C-labelling techniques. In many studies the absence of ammonia has been taken to mean that deamination has not occurred. However, this may not be the case as ammonia formed could combine with α-ketoglutarate to form glutamate or with glutamate or aspartate to form their respective amides, and thus would not be detected as free ammonia. Increases in amide concentration may take place during wilting (Kemble and Macpherson, 1952a; Brady, 1960), but during ensilage the amide concentration generally decreases (Kemble, 1956; Hughes, 1970; Ohshima, 1971) and it is difficult to detect if transient amide formation has occurred.

In their review, Ohshima and McDonald (1978) concluded that plant enzymes were mainly responsible for protein hydrolysis but did not participate to any significant extent in further amino acid catabolism, a task which was performed by microbial enzymes.

The activity of plant enzymes declines rapidly within 2 to 5 days of ensiling when vigorous microbial fermentation occurs (Kemble, 1956; Ohyama and Masaki, 1971; Bergen *et al*, 1974).

2.3 THE ACTIVITY OF MICROBIAL ENZYMES

The groups of micro-organisms which are important during ensilage are the lactic acid bacteria (lactobacilli, streptococci, leuconostocs and pediococci), coliform bacteria, clostridia, fungi (yeasts and moulds) and *Bacillus* sp.

2.3.1 The Epiphytic Microflora

The changes brought about in ensiled herbage are mainly caused by bacteria (McDonald *et al*, 1966a). The total number on fresh grass has been shown to vary between 10^6 and 10^9 g^{-1}DM (Stirling, 1951). Similar numbers have been observed by Moon and Henk (1980) using a scanning electron microscope. They noted also that the bacteria were located primarily on the outer surfaces of the leaves, rather than on the internal surfaces.

The great majority of the bacteria are strict aerobes which contribute little or nothing to silage preservation (Kroulik *et al*, 1955a; Gibson *et al*, 1958, 1961; McDonald *et al*, 1966a; Dickinson *et al*, 1975; Gouet *et al*, 1979a) and, since anaerobiosis is achieved rapidly after the silo is sealed, their growth is soon inhibited (Whittenbury, 1968). Of those organisms capable of anaerobic growth the coliforms (enterobacteriaceae) are the most abundant (Gibson *et al*, 1958; McDonald *et al*, 1964, 1965) but are not particularly important in the preservation process (McDonald *et al*, 1966a). Gibson *et al* (1958) identified *Klebsiella* sp. and *Bacterium herbicola (Erwinia herbicola)* as the dominant types of coliform present on fresh perennial ryegrass whereas Gouet and Chevalier (1966) identified *Enterobacter* on lucerne.

Species of *Bacillus* and *Clostridium* are also found in small numbers on fresh material (Nilsson and Nilsson, 1956; Gibson *et al*, 1958; Langston *et al*, 1962; Gibson, 1965; Whittenbury, 1968). The latter, being obligate anaerobes, occur only in endospore form and probably result from soil contamination (Whittenbury, 1968; Gouet *et al*, 1979a). Their numbers may be increased considerably by the presence of animal faeces (Gouet *et al*, 1979a).

The lactic acid bacteria (lactobacilli, streptococci, leuconostocs and pediococci) are the organisms primarily responsible for silage preservation but are generally very scarce on fresh foliage (Stirling, 1953; Kroulik *et al*, 1955a, b; Gibson *et al*, 1958; Keddie, 1959; Langston *et al*, 1962; McDonald *et al*, 1962; Stirling and Whittenbury, 1963; McDonald *et al*, 1964, 1965). Stirling and Whittenbury (1963) carried out an extensive survey into the numbers of lactobacilli, leuconostocs and pediococci occurring on growing plants. They were all scarce and, when present, were usually found on the sheath material at the base of grasses or on partially decayed material. Of 400 colonies isolated, 80% were found to be leuconostocs, the remainder being lactobacilli and pediococci. Leuconostocs were also the most widely distributed. The lactobacilli were least common and were found only on grasses. Langston *et al* (1962) found that the dominant lactic acid bacteria on cocksfoot were streptococci and Mundt (1961) found that streptococci occurred mainly on the flowering parts of plants.

Yeasts and moulds are always present on the growing crop and, although of little significance during the ensilage process itself (Woolford, 1976), become very important after the silo is opened as they are the main agents of aerobic deterioration (Beck, 1978). The counts on fresh foliage are sometimes high (Kroulik *et al*, 1955a) especially with corn crops (Beck, 1978).

2.3.2 Changes After Harvesting

Once the crop has been cut, and chopped or bruised, the bacteria spread throughout the material in the plant sap released by such treatment (Stirling, 1951; Wieringa, 1959a, b; Gibson *et al*,1961; McDonald *et al*, 1965; Whittenbury, 1968). Growth of lactic acid bacteria under these conditions is rapid and high numbers can be detected on all the equipment used during harvesting. This serves as a natural inoculant which considerably increases the count of lactic acid bacteria by the time the material reaches the silo (Stirling and Whittenbury, 1963; Henderson *et al*, 1972).

2.3.3 Changes During Wilting

Many crops are wilted before ensiling. The effect of this on the microbial population is important as it may affect the subsequent fermentation. Wilting delays bacterial multiplication (Stirling, 1951; Keddie, 1954) and generally results in a restricted fermentation (McDonald *et al*, 1965, 1968; Anderson and Jackson, 1970a, b; Jackson and Forbes, 1970; Marsh, 1979). Gibson and Stirling (1959) stated that bacterial activity in the silo decreased as the dry matter content of the ensiled herbage increased and that the role of the fermentation acids in

preservation decreased. The latter effect was demonstrated by Morgan *et al* (1980) who found that both direct-cut (175 gkg^{-1}) and wilted (360 gkg^{-1}) ryegrass-clover silages preserved well although the pH of the latter was only 5.09 compared with 4.00 for the former.

Weise (1969) found that all organisms, except coliforms, decreased during wilting but Budzier (1967) found that wilting had no significant effect on the microflora, except for slight increases in lactic acid bacteria and yeasts. Others have also found an increase in yeasts during wilting (Honig and Woolford, 1980) and that there are more yeasts in high dry matter (McDonald *et al*, 1968; Ohyama *et al*, 1980) and pre-wilted silages (Weise, 1967a; Henderson *et al*, 1972). Such silages often have high residual water-soluble carbohydrates (McDonald *et al*, 1968; McDonald and Whittenbury, 1973) and there may be problems with aerobic deterioration after the silo is opened (Honig and Woolford, 1980).

Wilting to a dry matter above 300 gkg^{-1} has an inhibitory effect on clostridia which are very sensitive to osmotic pressure (Wieringa, 1958), and their activity is inhibited at higher pH in silages of high dry matter (Whittenbury, 1968). It has also been suggested that saccharolytic clostridia are more sensitive to osmotic pressure than proteolytic clostridia (Wieringa, 1960).

Lactic acid bacteria are tolerant of dry matter contents up to 700 gkg^{-1} and may even produce a slight fermentation at this level although it is unnecessary for preservation of such dry material as long as anaerobiosis is maintained (Whittenbury, 1968).

Beck (1972) found that wilting could influence the relative growth of homo- and heterofermentative lactic acid bacteria. At the end of a 142 day ensiling period, heterofermentative species constituted 75 and 98% of the total lactobacilli in low and high dry matter silages respectively. Weise (1967) also noted a decrease in homolactic bacteria with an increase in dry matter content.

Henderson *et al* (1972) followed the microbial changes occurring after harvesting and during wilting of perennial ryegrass. They found that immediately after forage harvesting the total viable count increased from 10^3 to 10^6 g^{-1}. The counts of lactic acid bacteria increased from 100 to 400 g^{-1} during this period. These results confirmed those of a previous experiment (Stirling and Whittenbury, 1963) which showed that the grass was inoculated as it passed through the forage harvester. After wilting for 24h, the total count was similar but the lactic acid bacteria increased to 10^4 g^{-1} reaching 10^6 g^{-1} after a further 7h of wilting.

2.3.4 Changes After Ensiling

When forage is ensiled the trapped oxygen is quickly respired, mainly by plant enzymes (Gibson *et al*, 1958; Whittenbury, 1968). The obligate aerobes disappear rapidly (Stirling, 1951; Kroulik *et al*, 1955b; Gibson *et al*, 1958, 1961) and those organisms capable of anaerobic growth (lactic acid bacteria, coliforms, clostridia and *Bacillus* sp.) multiply (Gibson *et al*, 1958). There is rapid proliferation for a few days before the period of decreasing viable count begins (Stirling, 1951; Gibson and Stirling,

1959). There may be an initial decrease in the total count because several of the bacteria now capable of growth were present originally in very small numbers (Gibson *et al*, 1958). The rates of growth and final density of the microbial population vary with the herbage, whether or not it is bruised or lacerated, and the temperature (Stirling, 1951; Gibson *et al*, 1958, 1961; Whittenbury, 1968). Moon and Henk (1980) demonstrated the importance of plant cell plasmolysis in this process. They followed bacterial development on wheat and lucerne leaves during ensilage, using a scanning electron microscope. Initially the bacteria were found almost exclusively on the leaf surfaces where they increased rapidly after 24h to a maximum by day 4. After this period, lactic acid bacteria were detected in large numbers on, or near, the stomata and in the mesophyll air spaces. By the eighth day, the mesophyll cells had deteriorated sufficiently to allow the lactic acid bacteria to colonise their inner surfaces.

In a lactate silage (low pH and volatile-nitrogen, high lactic acid), coliforms dominate the microflora at first but are soon replaced by leuconostocs and streptococci. They in turn are superseded by lactobacilli and pediococci, which reduce the pH to about 4.0, and this protects the silage against subsequent clostridial fermentations (Whittenbury, 1968).

Gibson *et al* (1958) examined grass silages incubated at 22, 30 and 40°C. They found that at 22 and 30°C coliforms dominated the microflora during the phase of active growth but at 40°C they competed less successfully, probably because the maximum temperature for some strains is below 37°C. *Kebsiella* sp. were found to dominate although some *B. herbicola* and *Escherichia coli* were also

detected. The coliforms were usually the first of the main bacterial groups to show loss of viability after active multiplication ceased. Similar results for lactate silages have been observed by other workers (Kroulik *et al*, 1955b; Langston and Bouma, 1960; Gibson *et al*, 1961; Langston *et al*, 1962; McDonald *et al*, 1962, 1964; Gouet and Chevalier, 1966; Gouet *et al*, 1972; Pedersen *et al*, 1973). Langston and Bouma (1960) noted that coliforms persisted longer in poor quality silages.

If the development of lactic acid bacteria is rapid then the growth of coliforms is likely to be slight (McDonald *et al*, 1960; Ohyama *et al*, 1970). The lactic acid bacteria usually reach a peak after 2 to 4 days, declining slowly thereafter (Gibson *et al*, 1958, 1961; Ohyama *et al*, 1971; Gouet *et al*, 1972).

Heterofermentative lactic acid bacteria are less desirable in silage as they are less efficient at producing the acid essential for preservation (Whittenbury, 1968; McDonald *et al*, 1973). Nevertheless, heterofermentative leuconostocs have been found to be the dominant lactic acid bacteria on most fresh herbage (Stirling and Whittenbury, 1963) and they along with the homofermentative streptococci, initiate the lactate fermentation process (Whittenbury, 1968). They become inactive later, when conditions become more acidic, and the acid-tolerant lactobacilli and pediococci take over (Gibson *et al*, 1958; McDonald *et al*, 1964, 1966a; Beck, 1978). This was substantiated by Langston's group (Langston and Bouma, 1960; Langston *et al*, 1962) who noted that there was a progression from cocci to rods as fermentation proceeded.

Orla-Jensen (1949) reported that homofermentative organisms became dominant as fermentation proceeds. More recently, however, Beck (1972) studied the quantitative changes in lactobacilli occurring during the fermentation of grass and red clover. In well-preserved silage, acidification was initiated, in both fresh and wilted herbage, by homofermentative bacteria, the predominant species being *Lactobacillus curvatus* and *Lactobacillus plantarum*. After 4 days, 85% of the lactobacilli were homofermentative but, as fermentation proceeded, heterofermentative bacteria, especially *Lactobacillus buchneri* and *Lactobacillus brevis*, became dominant. After 142 days, heterofermentative lactobacilli accounted for 75 and 98% of the total lactobacilli in direct-cut and wilted silage respectively. Beck suggested that the shift may be due to differences in acetate tolerance since previously (Beck, 1969) *L. buchneri* and *L. brevis* had been found to be two to three times more tolerant than species prevalent during the early stages of ensilage. Langston and Bouma (1960) found that *L. brevis*, *L. plantarum* and *Pediococcus* sp. were the dominant species in well-preserved silages, *L. brevis* usually being more common in the later stages of fermentation. Gouet *et al* (1972) found that *L. brevis* and *L. plantarum* persisted longest in silage, followed by other lactobacilli and pediococci. *Streptococcus faecalis* had the most limited growth and the shortest life span.

Under ideal conditions lactic acid bacteria will dominate the fermentation, the pH will decrease to about 4.0 and subsequent microbial activity will be negligible. (McDonald *et al*, 1966a).

Clostridia are the main species responsible for anaerobic deterioration of silage. They multiply in silage almost as early as

other silage bacteria (Gibson *et al*, 1958; Gouet *et al*, 1972) unless the material has been pre-wilted to a dry matter above 300 gkg^{-1} (Thomas, 1978a). Generally the lactic acid bacteria quickly produce enough acid to lower the pH sufficiently to inhibit further clostridial activity but, if the lactic acid bacteria are not very active, or if the crop has a low dry matter or water-soluble carbohydrate content, or a high buffering capacity, clostridia may become dominant, leading to secondary fermentations (Langston *et al*, 1962; Whittenbury, 1968; Daniel *et al*, 1970). High temperatures in the silo also favour clostridial growth as the optimum temperature for most species in 37°C (Wieringa, 1960; Ohyama *et al*, 1973a). This was clearly shown by McDonald *et al* (1966) who ensiled a grass of low dry matter content (152 gkg^{-1}) at 20 and 42°C. In the former case a lactate fermentation ensued but in the latter case clostridia dominated. In farm silos high temperatures usually occur as a result of excessive respiration which also depletes the supply of fermentable sugars (McDonald *et al*, 1966a). Both of these factors favour clostridial development and the subsequent fermentation leads to a rise in the pH of the silage owing to the production of butyrate from lactate by the saccharolytic clostridia (McDonald *et al*, 1966a). The higher pH then encourages the development of proteolytic clostridia (Anderson and Jackson, 1970a) which degrade amino acids to form amines, fatty acids, carbon dioxide and ammonia and there are considerable losses of dry matter. (Ohshima and McDonald, 1978). The temperature in the silo does not seem to affect the species of clostridia which develop (Gibson *et al*, 1958).

Yeasts are normal components of fresh herbage and, being acid-tolerant, can survive well, though not necessarily proliferate,

in silage (Woolford, 1972). Studies indicate that yeasts have a minor role in silage fermentation as they are generally a minority group (Henderson *et al*, 1972; Woolford, 1976; Kibe *et al*, 1977). This is probably partly because of the rapid proliferation of lactic acid bacteria under normal circumstances, which reduces the content of available sugars (Weise, 1968; Woolford, 1976). Another contributing factor to the relative scarcity of yeasts in grass silages may be their apparent preference for drier conditions (McDonald *et al*, 1968; Henderson *et al*, 1972; Ohyama *et al*, 1980). They are present in much higher numbers in maize silages which have high dry matter and sugar contents (Gross and Beck, 1970; Woolford, 1976; Hara and Ohyama, 1979). Henderson *et al* (1972) found that yeasts were encouraged in formic acid-treated grass silages.

Yeasts compete with lactic acid bacteria for sugars which they ferment mainly to ethanol (McDonald and Whittenbury, 1973). This makes little contribution to preservation in silages (Byers *et al*, 1969). Small quantities of acetic and lactic acids are also produced (Wilkins, 1975) but this small contribution to fermentation must be set against the detrimental effects of yeasts after the silo is opened (Woolford, 1976).

The importance of yeasts in aerobic deterioration of silage was first noted by Beck and Gross (1964) who showed that silages with high yeasts counts were less stable after exposure to air. Since then, several other workers have confirmed their importance (Daniel *et al*, 1970; Gross and Beck, 1970; Ohyama and Hara, 1975; Ohyama *et al*, 1980). The yeasts implicated are members of the genera *Candida*, *Hensenula*, *Pichia* and *Torulopsis* (Beck and Gross, 1964; Ohyama and Hara, 1975; Woolford, 1978). It has been

suggested that it is the number of yeasts present, rather than the composition of the silage, which determines aerobic stability (Beck and Gross, 1964). Daniel *et al* (1970) showed that if the yeast count was higher than 10^5 g^{-1}DM, the silages had low stability, but others have demonstrated aerobic deterioration when yeast populations have been lower (Ohyama and McDonald, 1975; Henderson *et al*, 1979). Aerobic yeast activity results in the oxidation of water-soluble carbohydrates and organic acids with corresponding large dry matter losses as carbon dioxide (Zimmer, 1969; Honig and Woolford, 1980).

Other micro-organisms are also important in silage deterioration. Beck (1975) found that after the initiation of deterioration by yeasts, a second microflora was built up, consisting of proteolytic bacteria, streptomycetes and moulds. Yamashita and Yamazaki, (1975) found that, in aerobically-deteriorating, wilted grass silages, there were two high temperature peaks, the first resulting from yeast activity and the second from that of moulds. A large number of the latter have been identified in deteriorating silages (Pelhate, 1977; Hara and Ohyama, 1979; Barry *et al*, 1980), and some produce mycotoxins which are potentially dangerous to livestock (Vetter and Von Glan, 1978). Bacteria may also be important in deteriorating silages, especially maize (Honig and Woolford, 1980). The main bacteria involved seem to be spore-forming *Bacillus* sp. which, in addition to being saccharolytic, are also proteolytic (Woolford, 1978).

Silages particularly prone to aerobic deterioration are those with a high residual sugar content such as maize (Gross and Beck, 1970) or silages in which fermentation has been restricted by the use

TABLE 2.1

Species of lactic acid bacteria commonly found in silage

Homofermentative	Heterofermentative
Rod:	Rod:
Lactobacillus acidophilus	*Lactobacillus brevis*
Lactobacillus casei	*Lactobacillus buchneri*
Lactobacillus coryneformis	*Lactobacillus fermentum*
Lactobacillus curvatus	*Lactobacillus viridescens*
Lactobacillus plantarum	
	Coccus:
Coccus:	*Leuconostoc cremoris*
Pediococcus acidilactici	*Leuconostoc mesenteroides*
Pediococcus cerevisiae	
Streptococcus faecalis	
Streptococcus faecium	

of chemical additives (Henderson *et al*, 1972) although this in itself does not necessarily lead to aerobic deterioration (Ohyama *et al*, 1975 Ohyama and McDonald, 1975). On the other hand, silages which have undergone a clostridial fermentation are particularly stable (Ohyama *et al*, 1975). This is the result of the antimicrobial properties of the higher volatile fatty acids produced (Woolford, 1975). Thus well-preserved silages are particularly prone to aerobic deterioration.

2.3.5 Lactic Acid Bacteria

Orla-Jensen (1919) originally defined lactic acid bacteria as micro-aerophilic, Gram-positive, non-sporeforming, and usually non-mobile bacteria which ferment sugars. He subdivided them on the basis of whether they had a homo- or heterofermentative metabolism, the optical properties of the lactate product, and their temperature characteristics. Since then, considerable information has been gathered concerning their identification (Beck, 1978). Some of the species commonly found in silage are shown in Table 2.1.

The lactic acid bacteria have been described as facultative anaerobes (Brock, 1974; Buchanan and Gibbons, 1974; Stanier *et al*, 1976), micro-aerophilic (Thimann, 1963; Wilson and Miles, 1975) and aero-tolerant (Davis *et al*, 1980). However, they are probably best described as facultative anaerobes in that they can grow under aerobic or anaerobic conditions (Stanier *et al*, 1976) but, unlike other such organisms which change from fermentative to respiratory pathways in the presence of oxygen, their metabolism remains fermentative (Stanier *et al*, 1976; Davis *et al*, 1980).

TABLE 2.2

Examples of sugar and organic acid fermentations by lactic acid bacteria

Homofermentative

Glucose (or fructose) ⟶ 2 Lactate

Pentose* ⟶ Lactate + Acetate

2 Citrate* ⟶ Lactate + Acetate + 3 CO_2

Malate* ⟶ Lactate + CO_2

Heterofermentative

Glucose ⟶ Lactate + Ethanol + CO_2

3 Fructose ⟶ Lactate + Acetate + 2 Mannitol + CO_2

Glucose + 2 Fructose ⟶ Lactate + Acetate + 2 Mannitol + CO_2

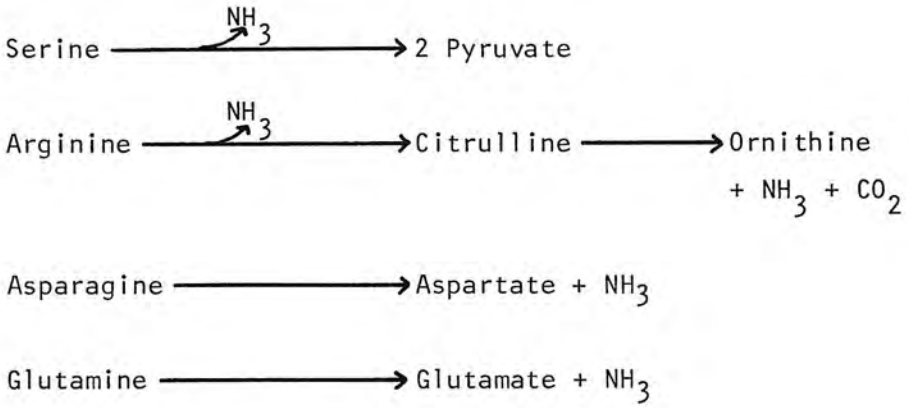
* Pathways are similar for heterofermentative lactic acid bacteria.

Under anaerobic conditions hexoses are fermented by either a homo- or a heterofermentative mechanism. The former proceeds via the glycolytic pathway (Dawes and Sutherland, 1976) yielding two moles of lactate per mole of glucose or fructose oxidized (Table 2.2), with no dry matter, and negligible energy, losses (McDonald *et al*, 1973). The heterolactic type of fermentation follows the phosphoketolase pathway (Dawes and Sutherland, 1976), the products and losses depending on the substrates (Table 2.2). The energy losses are generally small but the dry matter losses vary, being 5% and 24% for glucose and fructose fermentation respectively (McDonald *et al*, 1973). Homolactic fermentation yields two moles of ATP per mole of glucose oxidized but heterolactic fermentation yields only one (Brock, 1974). The heterolactics are also less efficient producers of acid, especially if fructose is the substrate. This was clearly demonstrated by Bousset *et al* (1972) who inoculated gamma-irradiated grass with pure strains of lactobacilli and found that the pH was consistently higher with heterolactic organisms. The acetate concentration was also 3 to 4 times higher and the dry matter losses 5 to 10 times greater. From this it follows that the glucose/fructose ratio in the material to be ensiled is of great importance, especially if the water-soluble carbohydrates content is low (Whittenbury, 1968; Woolford, 1972). There are few pentoses in freshly harvested herbage but they may be formed later by the action of hemicellulases and acid hydrolysis (Dewar *et al*, 1963; McDonald and Whittenbury, 1973; Morrison, 1979). Homo- and heterolactic organisms ferment pentoses by the same pathway, producing lactate and acetate (Table 2.2).

Some lactic acid bacteria can ferment organic acids (Whittenbury, 1961; Bryan-Jones, 1969), lactate and acetate being

TABLE 2.3

Deamination of amino acids by lactic acid bacteria



the main products (McDonald and Whittenbury, 1967). The plant organic acids citrate, malate and glycerate play a major role as buffering substances (Playne and McDonald, 1966). Their breakdown during ensilage does not lead to much change in the buffering capacity as most exist as salts and thus their degradation results in the liberation of an excess of cations which must be neutralized by fermentation acids, such as lactic and acetic. There is a large increase in buffering capacity during ensilage, mainly due to the formation of acetate and lactate (Playne and McDonald, 1966).

Lactic acid bacteria are non-proteolytic (Wilson and Miles, 1975; Beck, 1978), but may be involved to a limited extent in amino acid catabolism (Table 2.3), notably that of serine and arginine (Whittenbury *et al*, 1967). Brady (1966) showed that *L. plantarum* could deaminate serine to pyruvate and *L. brevis* and *Pediococcus* sp. could deaminate arginine to ornithine, but no other amino acids were attacked. Both *S. faecalis* and *Streptococcus faecium* can deaminate arginine but only the former uses it as an energy source (Slade, 1953; Deibel, 1960; Buchanan and Gibbons, 1974). *Streptococcus* sp. also deaminate the amides asparagine and glutamine (McIlwaine *et al*, 1948).

Most strains of *S. faecalis* can decarboxylate tyrosine to tyramine (Gale, 1940; Buchanan and Gibbons, 1974). Although significant increases in cadaverine and putrescine have been reported in well-preserved silages there is no evidence to implicate the involvement of lactic acid bacteria (Macpherson and Violante, 1966; Hughes, 1970).

Some lactic acid bacteria are able to reduce nitrate (Buchanan and Gibbons, 1974). Bousset-Fatianoff *et al* (1971) showed that *L. plantarum* could do so but not *L. brevis*, *S. faecalis* and *Pediococcus* sp.

Lactic acid bacteria have very limited synthetic abilities and therefore have complex nutrient requirements, especially with respect to amino acids and vitamins (Thimann, 1963; Wieringa, 1969; Buchanan and Gibbons, 1974; Wilson and Miles, 1975; Stanier *et al*, 1976). Another distinctive feature is their high acid tolerance. Although most of the coccus forms can initiate growth on a neutral or alkaline medium, the rod-shaped lactobacilli cannot usually grow on a media with an initial pH above 6.0 (Brock, 1974; Stanier *et al*, 1976). The pH range for growth of lactic acid bacteria is about 4.0 to 6.8, although some species will grow at pH 3.5 (Buchanan and Gibbons, 1974; Wilson and Miles, 1975). The temperature range is very variable, growth occurring within the range 5-50°C, but the optimum for most strains is about 30°C. (Buchanan and Gibbons, 1974).

2.3.6 Clostridia

According to Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974), clostridia are Gram-positive, sporulating, usually mobile, rod-shaped bacteria, which grow under strict anaerobic conditions and ferment sugars, organic acids or proteins. They can be divided into two major physiological groups based on their substrates (Beck, 1978). Saccharolytic clostridia eg *Clostridium butyricum* ferment mainly carbohydrates and organic acids and possess little activity against proteins or amino acids.

TABLE 2.4

Clostridia important in silage

Lactate fermenters:

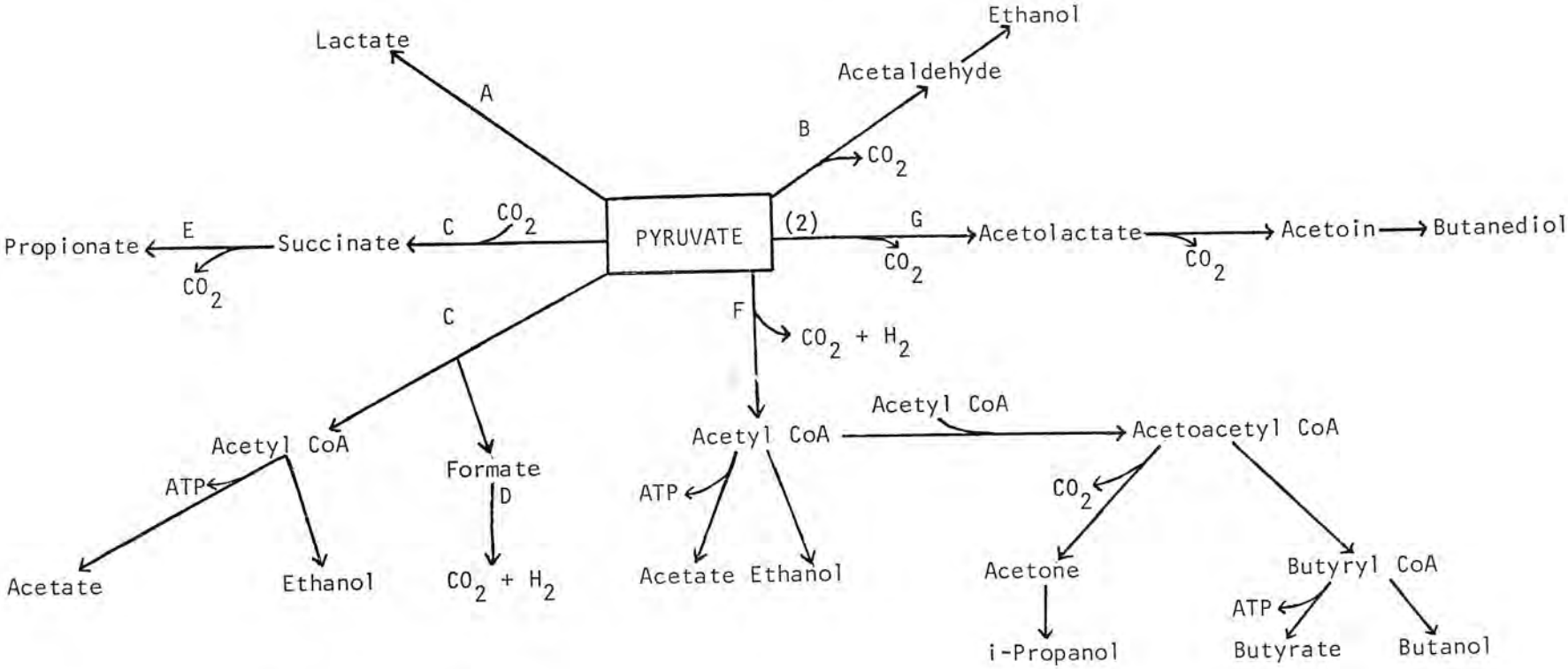
C. butyricum
C. paraputrificum
C. tyrobutyricum

Amino acid fermenters:

C. bifermentans
C. sporogenes

Others:

C. perfringens
C. sphenoides



A - *Streptococcus* sp., *Lactobacillus* sp.

B - Yeasts

C - Coliform bacteria

D - *E. coli*

E - Propionic acid bacteria

F - *Clostridium* sp.

G - *Klebsiella* sp.

Figure 2.1. Fermentation of pyruvate by bacteria

TABLE 2.5

Catabolism of amino acids by clostridia

Deamination

Arginine $\xrightarrow{NH_3}$ Citrulline $\longrightarrow$ Ornithine + NH_3 + CO_2

Aspartate $\xrightarrow{NH_3}$ Fumarate $\longrightarrow$ Acetate + Pyruvate

Glutamate $\xrightarrow{NH_3}$ Mescanoate $\longrightarrow$ Acetate + Pyruvate

Lysine $\longrightarrow$ Acetate + Butyrate + NH_3

Serine $\longrightarrow$ Pyruvate + NH_3

Asparagine $\longrightarrow$ Aspartate + NH_3

Glutamine $\longrightarrow$ Glutamate + NH_3

Decarboxylation

Arginine $\longrightarrow$ Ornithine $\longrightarrow$ Putrescine + CO_2

Aspartate $\longrightarrow$ Alanine + CO_2

Glutamate $\longrightarrow$ γ-aminobutyrate + CO_2

Histidine $\longrightarrow$ Histamine + CO_2

Lysine $\longrightarrow$ Cadaverine + CO_2

Tyrosine $\longrightarrow$ Tyramine + CO_2

Oxidation/Reduction (Stickland)

Alanine + 2 Glycine $\longrightarrow$ 3 Acetate + 3 NH_3 + CO_2

Valine + 2 Proline $\longrightarrow$ Isobutyrate + δ-aminovalerate + NH_3 + CO_2

Leucine + 2 Ornithine $\longrightarrow$ Isovalerate + δ-aminovalerate + 2 NH_3 + CO_2

Proteolytic* clostridia eg *Clostridium sporogenes* ferment mainly amino acids. Some clostridia such as *Clostridium perfringens (welchii)* have both types of activity. Species of clostridia which ferment lactate are of particular importance in ensilage, and Gibson (1965) divided silage clostridia into lactate and amino fermenters (Table 2.4). Some species such as *C. butyricum* and *C. tyrobutyricum* can only ferment lactate in the presence of acetate (Bryant and Burkey, 1956; Gibson, 1965; Beck, 1978).

The initial stage of carbohydrate fermentation is the formation of acetyl CoA, carbon dioxide and hydrogen (Fig. 2.1). The acetyl CoA is then reduced mainly to butyrate and acetate (Edwards and McDonald, 1978). According to Wood (1961), *C. butyricum* ferments glucose in the following manner:

$$4 \text{ glucose} \longrightarrow 2 \text{ acetate} + 3 \text{ butyrate} + 8\ CO_2 + 8\ H_2$$

Some clostridia also form butanol, acetone, propanol and a little ethanol but these are usually only produced in the later stages of fermentation after the pH has decreased (Wood, 1961; Brock, 1974; Stanier *et al*, 1976).

The fermentation of lactate to butyrate results in a rise in pH (Whittenbury, 1968) and provides conditions more suitable for the growth of the proteolytic clostridia (Whittenbury, 1968; Anderson and Jackson, 1970a), which can selectively degrade many amino acids (Mead, 1971). Three types of reaction occur, examples of which are given in Table 2.5. The most widespread mechanism of amino acid catabolism is the fermentation of pairs of amino acids (Stickland

*The term proteolytic, when used in reference to clostridia, includes the ability to catabolise amino acids.

reaction) one of which acts as electron donor, and the other as electron acceptor (Barker, 1961; Brock, 1974; Stanier *et al*, 1976; Ohshima and McDonald, 1978). Most amino acids serve as either donor or acceptor, only tyrosine and tryptophan playing both roles (Stanier *et al*, 1976). The oxidized amino acids are converted into fatty acids with one less carbon atom than in the original compound, while the reduced amino acid forms a fatty acid with the same number of carbon atoms (Ohshima and McDonald, 1978). Some amino acids can be fermented singly by certain species of clostridia eg *C. sporogenes* ferments arginine, cysteine, leucine, methionine, phenylalanine, threonine and serine (Barker, 1961; Stanier *et al*, 1976). Deamination produces an organic acid residue and decarboxylation leads to the formation of an amine. Many of the clostridia which ferment amino acids produce a wide variety of proteases with different specificities (Stanier *et al*, 1976). Mead (1971) examined the ability of various pure cultures of clostridia to degrade amino acids and found that the amino acids were degraded to different extents by different species. Ohshima *et al* (1979) noted an increase in alanine, the decarboxylation product of aspartate, and little change in isoleucine, leucine and valine in badly-preserved lucerne silages. Most other amino acids decreased significantly, especially aspartate and arginine. Various amines, including tyramine, histamine, cadaverine and putrescine, as well as large amounts of γ-aminobutyrate, also appeared, presumably from decarboxylation of amino acids.

The optimum pH for clostridia is 7.0-7.4 and they cannot tolerate acid conditions (Pelczar and Reid, 1972), a pH of 4.0

usually being sufficient to inhibit their growth (Whittenbury, 1968). Their growth is also restricted at dry matter levels above about 300 gkg^{-1} (Wieringa, 1958). The critical pH, below which they cannot grow, depends on the dry matter content of the material (Wieringa, 1958; Whittenbury, *et al*, 1967; Whittenbury, 1968; Weissbach *et al*, 1974). The optimum temperature for growth for most species is 37°C but *Clostridium sphenoides* is thermophilic, growing at temperatures as high as 50°C, with an optimum at 45°C (Wieringa, 1960; Buchanan and Gibbons, 1974).

Many species of clostridia can reduce nitrates (Buchanan and Gibbons, 1974). Bousset-Fatianoff *et al* (1971) used ensiled gamma-irradiated grass to show that *C. tyrobutyricum* can reduce nitrate but *C. butyricum* cannot. *C. sporogenes* and *C. perfringens* are also known to reduce nitrate (Ohshima and McDonald, 1978). The nitrate content of silages can have a marked influence on the production of butyrate by saccharolytic clostridia. Wieringa (1966) found that nitrate levels of 6-10 gkg^{-1}DM inhibited butyrate formation, although higher levels were not so effective. He considered that the effect was due to nitrite, an intermediary product of the reduction of nitrate to ammonia. Hein and Weissbach (1977) stated that in the presence of nitrate, lactate was decomposed to give only acetate plus a little propionate, and concluded that nitrate functioned as a hydrogen acceptor. The effect is only temporary, and may lead to increased clostridial development once the nitrate has been completely reduced. This is because herbage with a high nitrate content tends to be low in water-soluble carbohydrates, and the decomposition of nitrate to ammonia leads to a rise in buffering capacity (Wieringa, 1966; Hein and Weissbach, 1977).

Some species of clostridia can also degrade purines and pyrimidines giving a range of products (Ohshima and McDonald, 1978).

2.3.7 Coliform Bacteria (Enterobacteriaceae)

The enterobacteriaceae occurring in silage are Gram-negative, non-sporulating, rod-shaped, usually mobile, non-pathogenic, facultative anaerobes which ferment carbohydrates, and are commonly known as 'coliforms' (Beck, 1978). A major characteristic is their ability to ferment lactose, producing carbon dioxide (Beck, 1978). They form a diverse group and one of the key taxanomic characteristics is the range and proportion of fermentation products (Brock, 1974). There are two main types of fermentation (Brock, 1974; Dawes and Sutherland, 1976; Stanier *et al*, 1976; Davis *et al*, 1980). In the mixed acid fermentation, characterized by *Escherichia*, lactate, acetate, succinate and formate are formed in large amounts together with smaller amounts of ethanol (Fig. 2.1). Formate either accumulates or, under acid conditions, may be converted to carbon dioxide and hydrogen by certain bacteria eg *Escherichia coli* (Wood, 1961; Davis *et al*, 1980).

In other coliforms, including *Klebsiella*, *Enterobacter*, *Erwinia* and *Serratia*, a butanediol fermentation occurs (Davis *et al*, 1980). In this case smaller amounts of acids are formed and the major products are butanediol and ethanol (Brock, 1974). More carbon dioxide is produced by this type of fermentation as it is a product of the butanediol reaction as well as formate degradation (Fig. 2.1.).

Although each genus carries out a particular type of fermentation, the ratio of products formed may vary considerably, both from strain to strain and within strains grown under different environmental conditions (Brock, 1974; Beck, 1978). The pH has a marked effect on butanediol fermentation. Above pH 6.3, acetate and formate accumulate and production of butanediol, carbon dioxide and hydrogen does not occur but below 6.3, acetate is converted to acetoin and butanediol (Wood, 1961). During ensilage, coliforms tend to produce significant amounts of acetate hence they are often referred to as "acetic acid bacteria".

Coliforms have weak proteolytic activity but can deaminate and decarboxylate some amino acids (Beck, 1978) and most species can reduce nitrate (Bousset-Fatianoff *et al*, 1971, Buchanan and Gibbons, 1974).

Their optimum pH is about 7.0 and they usually disappear rapidly as conditions become acidic (Whittenbury, 1968). Under certain circumstances when lactic acid bacteria are scarce, as when ensiling hand-harvested grass in laboratory silos, coliforms may dominate the fermentation resulting in silages containing high levels of acetate (McDonald and Whittenbury, 1973).

2.3.8 Fungi

Fungi are eukaryotic, heterotrophic and generally strictly aerobic, although most yeasts are facultative anaerobes (Davis *et al*, 1980; Deacon, 1980). They usually exist either as single cells (yeasts) or as multicellular, filamentous colonies (moulds), although a few can occur in both forms (Deacon, 1980). They are

abundant in soil, on vegetation, and in water, where they live largely on decaying plant material (Davis *et al*, 1980), obtaining their nutrients by secreting extracellular hydrolases (lipases, proteases, amylases, cellulases) and absorbing the products of digestion (Deacon, 1980).

The yeasts and moulds are the most acid-tolerant components of the silage microflora, (Woolford, 1972). They have a broad pH range for growth of about 4.5 to 8.0 with an optimum in the region of 5.5-7.5, but some yeasts can tolerate a pH of less than 2.0 (Deacon, 1980).

Beck (1978) classified yeasts in silage into two physiological groups. The ground-growing (sediment) yeasts eg *Torulopsis* which preferentially ferment sugars, and the top-growing (pellicle) yeasts eg *Hansenula*, *Pichia*, *Candida* and a few *Saccharomycetes* which have a weak fermentation capacity but a high respiration capacity for lactate.

Most yeasts can grow anaerobically, usually carrying out an alcoholic fermentation although some lactate may also be formed (Wilkins, 1975; Deacon, 1980). Some other products of anaerobic fermentation have also been detected eg n-propanol, i-butanol, i-pentanol, acetate, propionate, butyrate and isobutyrate (Kibe and Kagura, 1976; Woolford, 1976; Kibe *et al*, 1977).

Under aerobic conditions yeasts grow much faster, producing energy from glucose via glycolysis and the TCA cycle in the same manner as higher plants (Deacon, 1980).

In addition to hexose sugars, some yeasts can use pentoses, starch, sugar alcohols and organic acids including lactate (Pelczar

and Reid, 1972). They can obtain nitrogen from ammonia as well as from amino acids, amines, amides and even proteins (Pelczar and Reid, 1972) but their ability to break down proteins is limited (Harris, 1958).

The vast majority of the moulds are strict aerobes and are therefore only found on the exposed areas of silage (McDonald and Whittenbury, 1973). They are thought to be primarily responsible for proteolysis and amino acid degradation during aerobic deterioration (Vetter and Von Glan, 1978).

Yeasts and moulds are inhibited by the higher volatile fatty acids (Woolford, 1975; Ohyama and McDonald, 1975; Ohyama *et al*, 1977).

2.4 ADDITIVES

Ensilage by natural fermentation is dependent on a number of factors summarised by McCullough (1977) as follows:- (1) moisture content of the crop; (2) buffering capacity of the crop; (3) availability of water-soluble carbohydrates; (4) type of bacteria present and (5) rate of fermentation. Undesirable fermentations often occur, leading to high nutrient losses and a product which may be inedible (Smith, 1961; McCullough, 1975).

The feeding value of silage depends on its intake and digestibility and the efficiency of utilization of the products of digestion (McDonald, 1976). Even when a desirable fermentation has been achieved, the products of fermentation may reduce intake and the nitrogenous constituents are often utilized with low efficiency. This is due partly to their high solubility and to a shortage of dietary energy (Wilkins, 1974; McDonald, 1976; Wilkinson *et al*, 1976; Wilkins, 1980).

Additives are used in silage making in an attempt to increase the chances of achieving a satisfactory preservation with minimum loss of dry matter and feeding value. Silage additives have been classified in several ways but in this treatment the simple division into stimulants and inhibitors proposed by McDonald and Whittenbury (1973) has been adopted. In this review, only those additives pertinent to this study will be discussed.

2.4.1 Stimulants

Stimulation of a lactate fermentation is the natural way to ensure silage preservation and may be achieved by the provision of an additional source of readily available substrate for the indigenous lactic acid bacteria, or by addition of an inoculum of lactic acid bacteria to ensure efficient use of the fermentable carbohydrates already present.

2.4.1.1 *Carbohydrates*. When relatively undamaged herbage is ensiled, the supply of nutrients may limit the ability of the lactic acid bacteria to compete successfully and to dominate the fermentation (Gibson *et al*, 1961). Some crops such as legumes, immature herbage and certain grass species eg cocksfoot, are inherently low in soluble carbohydrates and others may be made so by heavy nitrogen fertilization. In addition legumes have a high buffering capacity (Smith, 1961; McDonald and Whittenbury, 1973). The addition of a readily available source of carbohydrate before ensiling such crops should prove beneficial (McDonald and Whittenbury, 1973; Thomas, 1978a).

Laceration of the crop during harvesting releases the cell sap and increases the availability of fermentable carbohydrate to the bacteria (Thomas, 1978a). The importance of physical damage to the crop during harvesting has been stressed by several workers (Wieringa, 1959a, 1959b, 1960; Gibson *et al*, 1961; McDonald *et al*, 1964, 1965) but it will only bring about successful preservation if there is enough suitable carbohydrate present in the first place (Gibson *et al*, 1961). Wieringa (1959a) found that even severe crushing of grass containing low dry matter and

high protein contents, did not result in successful preservation unless molasses was added.

The advantages of adding glucose and sucrose have been demonstrated by several workers (Zelter, 1960; Catchpoole, 1965; Weise, 1967 ; Ohyama *et al*, 1971a, 1973, 1973a, 1975a; Gouet *et al*, 1979b). Weise (1967a) added sucrose ($10gkg^{-1}$) before ensiling grass which had a low dry matter content ($150gkg^{-1}$) and a water-soluble carbohydrate of only $100gkg^{-1}$DM. There was a rapid increase in lactic acid bacteria in the treated material, especially homolactic, whereas in the untreated grass they did not develop until the second week of ensilage. Ohyama *et al* (1971a) added various levels of glucose (5, 10 and $20gkg^{-1}$) to Italian ryegrass and cocksfoot, both of which had low water-soluble carbohydrate contents. In most cases the addition of sugar, especially at the higher levels, helped to reduce the pH more rapidly. The addition of glucose to lucerne (De Vuyst *et al*, 1968a, b) and cocksfoot (Ohyama *et al* 1973 1973a) has been shown to reduce proteolysis and amino acid catabolism, presumably because of the rapid reduction in pH caused by the treatment.

Glucose addition is preferable to sucrose because the latter contain fructose which is not used as efficiently for acid production by the heterofermentative bacteria (Whittenbury, 1968). The ideal sugar would be a pentose as it would be used in the same way by both types of lactic acid bacteria, yielding equal quantities of lactate and acetate (Whittenbury, 1968). In practice the cheaper product, molasses, is usually used (McDonald and Whittenbury, 1973).

Molasses contains over 500gkg^{-1} sucrose and in order to obtain maximum benefit relatively high levels of addition are recommended eg Lanigan (1961) recommended 40-50gkg^{-1}. Carpintero *et al* (1969) added molasses (40gkg^{-1}) to lucerne, raising its water-soluble carbohydrate content from 70 to 190gkg^{-1}DM. The treated silage had a lower pH as well as lower acetate and ammonia contents, and higher lactate and sugars. Thomas (1978a) considered that the addition of molasses reduced the pH and the ammonia and butyrate contents while increasing lactate production. One disadvantage of this treatment is increased effluent production which makes it somewhat wasteful (Smith, 1961).

Other sources of carbohydrates which have also been used in silage making are whey, beet pulp, potatoes and cereals (Smith, 1961; Ely, 1978; Thomas, 1978a). The lactic acid bacteria found in silage cannot usually use starch (Gibson and Stirling, 1959; McDonald and Whittenbury, 1973) so when an additive such as cereal is used, a suitable hydrolase must be included (McDonald and Whittenbury, 1973; Wilson, 1980).

Increasing fermentable carbohydrate levels indirectly by the addition of cellulases has also been investigated (Gross, 1969; Henderson and McDonald, 1977; Whittemore and Henderson, 1977). Most commercially-produced preparations also contain hemicellulases and a mixture of hexoses and pentoses results (Henderson and McDonald, 1977).

Research results generally indicate that the addition of a readily fermentable carbohydrate produces a well-preserved silage and may improve animal performance (Wilkins, 1974; Thomas, 1978a).

McDonald and Whittenbury (1973) pointed out that the potential benefits of carbohydrate addition may be reduced if the initial respiratory phase in the silo is prolonged either because of inadequate compaction or delayed sealing. Laceration of the crop helps to reduce respiratory losses as such material is easier to compact (Wieringa, 1960; McDonald *et al*, 1965).

2.4.1.2 *Inoculants*. Another technique for stimulating lactate fermentation is to inoculate the starting material with lactic acid bacteria. Early attempts using this method gave disappointing results (Watson and Nash, 1960), but recently there has been renewed interest and several inoculant-based commercial additives have been introduced.

Most crops have a very low population of lactic acid bacteria (Gibson *et al*, 1958; Stirling and Whittenbury, 1963) and those present may not be the most desirable types (Wieringa, 1961). Provided there is enough fermentable carbohydrate present, inoculation with suitable lactic acid bacteria should overwhelm the resident bacterial population and ensure a lactate fermentation (Whittenbury, 1968). Inoculation with homofermentative bacteria should be particularly effective when the content of soluble carbohydrate is just sufficient for preservation as it will ensure that the maximum amount of acid is formed from the available sugars (McDonald and Whittenbury, 1973).

Whittenbury (1961) suggested several criteria to which a potential organism should conform:

1. It must grow vigorously and be competitive.
2. It must be homofermentative.
3. It must be acid-tolerant
4. It must ferment glucose, fructose and sucrose, and preferably fructans and pentoses.
5. It must not produce dextran from sucrose or mannitol from fructose.
6. It should not ferment organic acids.
7. It should be active up to 50°C.
8. It should be capable of growth at high dry matter levels.

Wieringa and Beck (1964) recommended similar properties and in addition suggested it should be non-proteolytic.

On the basis of these criteria, leuconostocs and heterofermentative lactobacilli were excluded on the basis of metabolism, and streptococci on the grounds of acid-tolerance (Whittenbury, 1961). Of the remainder, *L. plantarum* has proved to be the most satisfactory single organism (Wieringa and Beck, 1964; Bryan-Jones, 1969; Whittenbury *et al*, 1967; Woolford, 1972; Ohyama *et al*, 1973a). Wieringa (1961) reported that grass of low dry matter content produced silage of high quality when inoculated with *L.plantarum* and Wieringa and Beck (1964) showed that if lacerated grass was inoculated with *L. plantarum*, they dominated within 3 days. Not all inoculation experiments using this organism have been successful. Ohyama *et al* (1973a) inoculated Italian ryegrass and cocksfoot at the rate of $10^6 g^{-1}$ and found no benefit unless glucose ($10 gkg^{-1}$) was added too. In a previous study Ohyama *et al*

(1971a) showed that inoculation sometimes resulted in silages with low lactate and high butyrate contents, but if glucose was added too they were all well-preserved.

Unfortunately *L. plantarum* is slow to produce acid until the pH falls below 5 (Woolford, 1972), and it has been found to be beneficial to add another species of lactic acid bacteria which is active over the pH range 6.5-5.0. According to Woolford (1972) the most suitable is *S. faecalis* which exhibits extremely rapid growth, especially under aerobic conditions (Wieringa and Beck, 1964). Bryan-Jones (1969) has demonstrated the effectiveness of inoculation with a mixed culture of *L. plantarum* and *S. faecalis* in laboratory studies. He inoculated ryegrass (DM-157gkg^{-1}; WSC-138gkg^{-1}DM) at the rate of 10^5g^{-1}DM. The control was badly-preserved (pH 5.1) but the pH in the inoculated material rapidly fell to 4.1. Similar results were obtained with directly-ensiled cocksfoot (DM-182gkg^{-1}; WSC-123gkg^{-1}DM) but when wilted cocksfoot (DM-272gkg^{-1}) was ensiled all the silages were well-preserved, no further improvement being obtained from inoculation. In a second experiment, a timothy (*Phleum pratense*)/meadow fescue (*Festuca pratensis*) mixture was ensiled in 1 tonne tower silos and also in laboratory silos. In both cases, however, no great advantage could be detected from the use of an inoculum, possibly because the lactic acid bacteria count on the fresh herbage immediately prior to ensiling was quite high (3×10^3g^{-1}DM) as was the water-soluble carbohydrate content.

The beneficial effects of inoculation are explained by the fact that a large number of exponentially growing lactic acid bacteria are being added to the crop, whereas it would take the small

numbers normally present on the standing crop many hours to reach significant numbers (Gouet *et al*, 1979b). The effectiveness of inoculation is related to the characteristics of the species used as well as their numbers and activity at the time of inoculation (Gouet *et al*, 1979b).

If the crop is low in water-soluble carbohydrates, it may be necessary to add a carbohydrate source too (Ohyama *et al*, 1971a). Carpintero *et al* (1979) ensiled a ryegrass-clover mixture with glucose ($43 gkg^{-1}$ DM) and a mixed culture of *L. plantarum*, *Lactobacillus mesenteroides* and *S. faecalis* at the rate of $10^3 g^{-1}$ DM. After 50 days, both the control and treated silages were well-preserved (pH 3.87 and 3.64 respectively) but the pH decreased more rapidly in the inoculated material resulting in significantly less proteolysis and amino acid breakdown as well as higher lactate and residual sugar contents. Several other workers have demonstrated that a combination of inoculum and carbohydrate source is more effective than an inoculum alone (Wieringa, 1960; Gross, 1969; Ohyama *et al*, 1971a, 1973a).

The results of inoculation experiments indicate that if the crop contains adequate fermentable carbohydrates, inoculation is unlikely to bring about much improvement in preservation provided the crop is lacerated during harvesting (Wieringa, 1960; McDonald *et al*, 1964, 1965; Woolford, 1972). Inoculation may, however, inhibit proteolysis as a result of the more rapid decrease in pH usually obtained (McDonald *et al*, 1965; Carpintero *et al*, 1979; Gouet *et al*, 1979b). With crops having a low soluble carbohydrate content it is especially important

that the available carbohydrates are used efficiently and they will probably benefit from inoculation, especially if a soluble carbohydrate supply is included (McDonald *et al*, 1964; Whittenbury, 1968; Woolford, 1972; Thomas, 1978a).

Laboratory-scale inoculation experiments have been much more successful than those carried out on a farm scale. This is probably because the hand-harvested grass, which is usually used for the former, tends to have a very low count of lactic acid bacteria (McDonald and Whittenbury, 1973), whereas forage-harvested grass is inoculated to a certain extent by the harvesting equipment (Stirling and Whittenbury, 1963; Henderson *et al*, 1972). These observations demonstrate the importance of using forage-harvested material for laboratory studies. Wilson and Wilkins (1972) suggested that forage-harvested grass should be used for laboratory studies. On the other hand, Woolford and Hall (1974) demonstrated that, although hand-harvested material did have lower numbers of lactic acid bacteria present, if the material was extensively lacerated the fermentation produced was identical to that from forage-harvested material. This indicates that it is the substrate availability rather than the initial microbial population which is of most importance.

2.4.2 Inhibitors

Inhibitors preserve the silage by methods other than lactate fermentation, the extent of the inhibition varying for different additives.

Additives which inhibit fermentation fall into two categories, those which prevent microbial activity within silage by a direct

sterilizing action and those which acidify the crop to such an extent that both microbial and plant enzyme activities are inhibited (Mann, 1975).

2.4.2.1 *Direct acidification*. In untreated silage, the controlling factor is the acidity which develops in the mass. If the pH decreases rapidly there is little chance for undesirable organisms, such as clostridia, to develop (Smith, 1961). One way of reducing the pH rapidly is by direct acidification. This also inhibits respiration which reduces heating as well as preserving the water-soluble carbohydrates (Virtanen, 1933; Saue and Brierem, 1969; Castle and Watson, 1970a, b; Henderson and McDonald, 1971; Henderson *et al*, 1972; Wilkinson *et al*, 1976.

The AIV process developed in Finland by Virtanen (1933) was designed to inhibit fermentation completely by lowering the pH to below 4.0 using a mixture of hydrochloric and sulphuric acids. It was a popular system in Scandinavia but not in the United Kingdom, mainly because of the difficulties in handling such corrosive acids. These acids also caused a large decrease in intake as well as giving rise to acidosis unless neutralized before feeding (Wilkinson *et al*, 1976). They have now been almost exclusively replaced by organic acids, especially formic (McDonald and Whittenbury, 1973). In early work formic acid did not compare well with mineral acids (Watson and Nash, 1960) and it was not until the development of forage-harvesters for lacerating the herbage, and the use of efficient acid applicators, that its full potential was realized (Saue and Brierem, 1969; Crawshaw, 1977). Unlike the mineral acids, formic is degraded in

the rumen, and has some nutritive value (Hungate, 1966; McCullough, 1975).

Although formic acid is the strongest of the fatty acid series, it is considerably weaker than the mineral acids used in the AIV process and no attempt is made to use it commercially at a rate which would reduce the pH to less than 4.0. The recommended rate for grass silage is $2.3gkg^{-1}$, which reduces the pH to about 4.6-4.8. At this level coliforms and clostridia are inhibited but lactate fermentation continues, leading to a lowering of pH to levels which ensure satisfactory preservation (McDonald and Whittenbury, 1973; Wilkinson *et al*, 1976). Legumes, which are more highly buffered than grasses, require a higher dose to achieve a satisfactory pH (Lancaster and Brunswick, 1977). Complete inhibition of fermentation would require two to three times the amount used commercially, depending on the crop and its dry matter content (Carpintero *et al*, 1969; Henderson and McDonald, 1971; Wilkins and Wilson, 1971).

Unlike the mineral acids, the fatty acids are also bacteriostatic, especially with respect to coliforms and clostridia (Beck, 1968; Saue and Brierem, 1969; Henderson *et al*, 1972; McDonald and Henderson, 1974; Beck, 1978; Woolford, 1978; Carpintero *et al*, 1979; Wilkins, 1980). Yeasts, however, do not seem to be inhibited to the same extent (Henderson and McDonald, 1971; Henderson *et al*, 1972). Within the homologous series of fatty acids, the anti-microbial activity increases with increasing chain length while the acidic properties decrease (Galbraith *et al*, 1971; Woolford, 1975; Crawshaw, 1977). The exception

to the above rule is butyric acid, which is tolerated well by bacteria, especially clostridia (Woolford, 1975; Crawshaw, 1977).

Waldo *et al* (1973) stated that formic acid addition, to direct-cut or wilted herbage, increases energy recovery, reduces acid production and protein degradation during fermentation and improves acceptability and animal performance.

The effect on silage composition depends on the dose applied as well as on the species and dry matter content of the crop. When it is applied to crops such as *Lolium* sp. which are rich in water-soluble carbohydrates and normally preserve well by lactate fermentation, formic acid results in a reduction in the amount of fermentation with concomitant conservation of sugars. This was well illustrated by Carpintero *et al* (1979) who ensiled a ryegrass-clover crop (WSC-203gkg^{-1}DM) with different levels of formic acid (0.4-7.7gkg^{-1}). At the higher levels, the residual sugars were higher than in the original grass and there was much less lactate, acetate and butyrate produced. A decrease in proteolysis and deamination was also noted with increasing formic acid addition.

Barry *et al* (1978a) applied formic acid at low, medium and high levels (1.5, 3.0 and 6.0 lt^{-1}) to flail-harvested and precision-chopped lucerne. In the former case, the low level of acid had no effect in preventing a proteolytic clostridial fermentation and it was only at the high level that satisfactory preservation occurred. With the precision-chopped material, however, all treatments produced well-preserved silages. Increasing the formic acid rate of application resulted in a decrease in the production

of fermentation acids and an increase in residual sugars and protein in the silages. Further studies (Barry *et al*, 1978b) showed that formic acid treatment considerably reduced amino acid catabolism, especially in precision-chopped material. Similar results to those of Barry and co-workers have been obtained by others when treating crops of low soluble carbohydrate content, with formic acid (Henderson and McDonald, 1971; Wilson and Wilkins, 1973; Lancaster and Brunswick, 1977; Lancaster *et al*, 1977).

The preserving effect of formic acid is enhanced with increasing dry matter levels (Henderson and McDonald, 1976; Hinks *et al*, 1976). Henderson and McDonald (1976) applied formic acid at 11.5gkg^{-1}DM to grass cut at three different stages of growth and wilted to different dry matter levels. At low dry matter contents, formic acid improved preservation by encouraging a lactate fermentation but as the dry matter content increased, it restricted fermentation and conserved the sugars. The restriction of fermentation was more apparent with more mature grass.

The beneficial effects of formic acid on improving voluntary dry matter intake and animal performance are well established (Brierem and Ulvesi, 1960; Castle and Watson, 1970a, b; Waldo *et al*, 1971; Waldo, 1978; McIlmoyle and Murdoch, 1979). When added to difficult crops such as cocksfoot and lucerne, the effect on animal performance may be quite striking (Lancaster *et al*, 1977) but when applied to crops of moderate dry matter and high carbohydrate content, the effects are not so apparent (Hinks *et al*, 1976).

The addition of formic acid to direct cut herbage can have a beneficial effect in protecting forage protein, although at the commercial application rate proteolysis is still extensive (McDonald and Edwards, 1976; Carpintero *et al*, 1979). It does, however, protect the liberated amino acids from further degradation (De Vuyst *et al*, 1972; Wilson and Wilkins, 1973; Carpintero *et al*, 1979). Demarquilly and Dulphy (1977) have demonstrated that formic acid treatment improves utilization of silage nitrogen in grasses and lucerne.

2.4.2.2 *Sterilizing agents*. Formic acid has some associated handling problems owing to its volatile, acidic nature (Wilkins, 1980) and in addition, the emphasis on silage making procedures is now changing. The reduction in intake and nitrogen utilization by animals associated with lactate silages, is caused by excessive carbohydrate fermentation and nitrogen breakdown in the silo (Wilkinson *et al*, 1976). The accumulation of experimental evidence that the voluntary intake of silages is negatively correlated with high concentrations of fermentation acids has now turned attention to additives which preserve herbage by methods other than acidification ie sterilizing agents (Wilkinson *et al*, 1976; Crawshaw, 1977).

The greatest success has been with formalin (40% w/v solution of formaldehyde), which was first used successfully as a bacteriostat for silage making in the early 1930's although little further research was carried out until the early 1970's (Mann, 1975), when Brown and Valentine (1972) investigated its effect on lucerne. They

applied formalin at 22.6, 33.9 and 45.0gkg^{-1} (166, 249 and 331 g formaldehyde per kg crude protein) and found that, although fermentation and proteolysis were inhibited, the intakes and digestibilities of the silages were markedly reduced. A second experiment (Valentine and Brown, 1973) used a lower level of formalin (6.7gkg^{-1}) on the same crop and produced a silage in which fermentation had been restricted but, although digestibility of the nitrogen was significantly reduced, intake was slightly increased and animal performance was significantly improved.

Formaldehyde has a dual role in that it restricts fermentation by acting as a bacteriostat and also protects forage protein from degradation. This results in silages with high soluble carbohydrate and protein contents, low levels of fermentation acids and ammonia and higher intakes (Vanbelle *et al*, 1978).

Formaldehyde protects protein by forming methylene cross-bridges between certain amino acids resulting in intra- and inter-molecular linkages (Ferguson, 1975; Barry, 1976). Most of these linkages are thought to be hydrolysed under the acid-pepsin conditions in the abomasum (Barry, 1976) but some would appear to be stable, especially those involving tryptophan and histidine (Walker, 1964). Kreula and Rauramaa (1977) found that 84% of the added formaldehyde was recovered in grass/clover silage to which formalin had been added at 2.7 lt^{-1}. The remainder was lost by evaporation (12%) and in the effluent (6%). Of the formaldehyde recovered, 40-45% was found to be tightly bound.

Formaldehyde restricts rumen protein degradation, which results in an enhanced flow of amino acids into the small intestine (Beever *et al*,

1977; Vanbelle *et al*, 1978). The application rate is critical and is generally related to the protein content of the crop. If too much is added it is possible to starve the rumen micro-organisms of a supply of degradable nitrogen (Hinks and Henderson, 1977; Vanbelle *et al*, 1978; Siddons *et al*, 1979) and if free formaldehyde is present it could have an additional inhibiting effect (Wilkins *et al*, 1974). At high levels, irreversible binding of formaldehyde could also occur, which would reduce the protein digestibility in the small intestine (Siddons *et al*, 1979).

Vanbelle *et al* (1978) considered that for optimum protection of protein during ensiling it would be necessary to apply formaldehyde at a rate of 3-4gkg^{-1} or 60-100gkg^{-1} crude protein but this level of formaldehyde has been shown to have detrimental effects on silage utilization ie it reduces intake (Wilkins *et al*, 1974) and rumen fermentation (Faichney and Weston, 1971) as well as reducing the digestibility of dry matter (Brown and Valentine, 1972) and nitrogen (Waldo *et al*, 1973). Wilkinson *et al* (1976) proposed that a suitable level of formaldehyde application would be 30-50gkg^{-1} crude protein for grass, and 100-150gkg^{-1} for legumes. Unfortunately, at this level a clostridial fermentation may occur (Wilkins *et al*, 1974) as it seems that these organisms are more resistant to low levels of formaldehyde than lactic acid bacteria (Wilson and Wilkins, 1978). At low levels of formaldehyde application, it therefore becomes necessary to add acid to ensure conservation.

The two most common acids used are sulphuric and formic and a large number of experiments have been carried out to determine the optimum combination of formaldehyde and acid (Waldo *et al*, 1973; Barry, 1975; Wilson and Wilkins, 1974; Waldo, 1978; Vanbelle *et al*, 1978;

Hinks *et al*, 1978). Barry (1975) investigated the effects of formalin alone at 4.8 lt^{-1} (66 g formaldehyde per kg crude protein) and in combination with sulphuric or formic acid at 1 lt^{-1}. All the treated silages were well-preserved with high protein levels and a little fermentation had occurred. Treatment produced a large increase in intake and did not affect the digestibility of the dry matter, although that of the nitrogen was significantly reduced. Overall, the combination of formalin and formic acid produced the best silage. Vanbelle *et al* (1978) recommended that the level of formaldehyde should be limited to 1 kgt^{-1} (2.5 litres formalin per tonne), and applied along with formic acid at 3 lt^{-1}. Hinks *et al* (1978) applied formic acid at 2.9 lt^{-1} in combination with formalin at 1.4, 2.7 and 5.2 lt^{-1} (19, 37 and 72 g formaldehyde per kg crude protein) to wilted Italian ryegrass. In each case the addition of formalin produced silages with higher nutritive value than the untreated control. Increasing the level of formalin resulted in silages with lower acetate, lactate and volatile nitrogen contents, and higher levels of residual WSC and protein but there were no significant improvements on intake and animal performance. It was concluded that a low application rate of about 20 g formaldehyde per kg crude protein together with 3 lt^{-1} of formic acid ie 1:2 ratio was as effective in optimising intake and performance as higher levels of formalin in the ratio 1:1 or 2:1 with formic acid. Hinks *et al* (1978) also compared the effects of a low level of formaldehyde on its own with those of formic acid (2.9 lt^{-1}) alone or a combination of the two in the ratio 1:2. They found that the improvement in intake was largely brought about by formalin but stated that its use as a single additive could not be recommended since acid was required as an insurance against a clostridial fermentation.

3. OBJECTS OF STUDY

The object of this work was to study the effects of ensilage on grass with particular emphasis on the nitrogenous components. An attempt was made to differentiate between the effects of plant and microbial enzymes using gamma-radiation to inactivate the micro-organisms. The effect of pH on proteolysis was examined and the possibility of direct acid hydrolysis during prolonged storage at low pH investigated. Additives were studied using three different approaches:

(1) stimulating fermentation by inoculation and/or glucose addition;

(2) restricting fermentation by addition of formic acid and/or formaldehyde;

(3) raising the pH using either ammonia or buffer solutions.

4. GENERAL TECHNIQUES

4.1 EXPERIMENTAL GRASSES

The grasses used in the investigations were obtained from fields and experimental plots on the farms of the Edinburgh School of Agriculture. Further details are given in the appropriate sections on materials and methods.

The grass was harvested using a "Haldrup" reciprocal mower-type plot harvester. It was then hand chopped to approximately 20 mm lengths and well-mixed before taking representative samples for ensiling and analysis.

4.2 LABORATORY SILOS

Silage was made in Pyrex glass test-tubes, 255 mm in length and 44 mm in diameter, which held approximately 200 g of fresh grass when tightly packed. After filling, the tubes were sealed with a water-filled air-lock.

4.3 GAMMA-IRRADIATION

Fresh grass or grass extract was exposed to gamma-radiation from a cobalt-60 source at the Scottish Universities Research and Reactor Centre, East Kilbride. A total dose of approximately 3.25 Mrad was applied at a rate of 0.21 Mrad h^{-1}.

4.4 ANALYSES

Chemical analyses were carried out on the fresh material, or on material dried at 60°C in a forced-draught oven for 16h and hammer-milled to pass a 1 mm screen.

4.4.1 Dry matter

Fresh grasses and 24h silages were dried in an oven at 60°C.

The dry matter (DM) values of all other silages were determined using the toluene distillation method of Dewar and McDonald (1961). Corrections were made for acids and ethanol (East of Scotland College of Agriculture Annual Report 1978, p.75).

4.4.2 pH

The pH values of grasses and silages were determined on aqueous macerates of the fresh material using a Pye combined electrode and pH meter.

In experiment 2, the pH values of aqueous extracts of grass were determined using a Pye-Ingold micro-electrode.

4.4.3 Water-soluble carbohydrates

The water-soluble carbohydrates (WSC) of grasses and silages were measured in filtered, aqueous extracts by the method of McDonald and Henderson (1964), except for the final colorimetric determination where 2 ml of extract were pipetted into a boiling tube and 2 ml of Somogyi reagent (Somogyi, 1945) added. The tube was placed in a boiling-water bath for 20 minutes, removed and cooled under running water. Two ml of arsenomolybdate reagent (Nelson, 1944) were added to the tube with shaking and the contents transferred to a 50 ml volumetric flask and made up with distilled water. After 30 minutes the absorbance was read at 540 nm.

4.4.4 Ethanol

Ethanol was measured in filtered, aqueous extracts of fresh silages, by the method of Kent-Jones and Taylor (1954) using the Kozelka and Hine procedure.

4.4.5 Nitrogen

Brief outlines of the procedures for total nitrogen, non-protein nitrogen and amino acid nitrogen are given here. Further details may be obtained in Appendix 1.

4.4.5.1 *Total nitrogen (TN)* was determined in fresh grasses and silages by the Kjeldahl digestion method using a selenium catalyst, followed by steam-distillation of the digest with caustic soda.

In aqueous grass extracts (Experiment 2), TN was determined by Kjeldahl digestion using a mercury catalyst (Concon and Soltess, 1973), followed by colorimetric determination of the nitrogen in the digest using a weakly alkaline mixture of sodium salicylate and dichloroisocyanurate as the colour reagent (Crooke and Simpson, 1971).

4.4.5.2 *Non-protein nitrogen (NPN)* values for grasses and silages were determined by extracting samples of fresh material twice with boiling water and measuring the TN in the combined extracts by Kjeldahl digestion with the selenium catalyst.

In the grass extracts of Experiment 2, proteins were precipitated with 5% (w/v) trichloroacetic acid (TCA). The remaining NPN was measured by Kjeldahl digestion with the mercury catalyst, followed by colorimetric determination of the nitrogen in the digest (Crooke and Simpson, 1971).

4.4.5.3 *Volatile nitrogen (VN)* was measured in a sample of the aqueous extract prepared for the determination of NPN. Sodium borate (0.05 m) was used to adjust the pH to 9.2 and the VN determined by steam-distillation (Macpherson, 1952a).

4.4.5.4 *Amide nitrogen* was determined in a sample of the NPN extract using the method of Varner *et al* (1953) as modified by

Ohshima (1971). After precipitation of the protein with 70% ethanol, saturated borate buffer was added to adjust the pH to 10.0 and the ammonia removed by steam-distillation. Fifteen ml of 40% NaOH were added and the ammonia released from the amides measured by steam-distillation.

4.4.5.5 *Nitrate nitrogen* was determined on dried, milled samples of grasses and silages by the method of ap Griffiths and Johnston (1961).

4.4.5.6 *Individual amino acid nitrogen (AAN)* values for Experiment 1 were determined on an L.K.B. Model 4400 amino acid analyser equipped with an L.K.B. Model 2220 recording integrator. The determinations were carried out using protein hydrolysate methodology with a single 20 cm column of "Ultropac" resin and stepwise elution with sodium citrate buffers followed by detection as described in Protein Chemistry Notes No.10 in "Accelerated amino acid analysis using the L.K.B. 4400 Analyser with sodium buffer systems" (L.K.B. Biochrom Ltd). Some preliminary analyses (data not included here) were performed on a Locarte Model IV amino acid analyser according to the methodology of Gardner (1981).

Amino acids for Experiments 3 and 4 were determined on a modified Technicon Auto Analyser with a 65 cm column of Technicon C-2 resin, maintained at 60°C. A gradient system of sodium citrate buffers was used for eluting and 2,4,6-trinitrobenzene sulphonic acid (TNBS) for detection (D'Mello, 1972).

4.4.6 Lactic Acid

Fifty grams of finely chopped silage were tamped down firmly in a screw-capped bottle. Enough 0.3 m sulphuric acid was added to

cover, and a crystal of thymol, then the bottle was stored under refrigeration for at least 7 days. The contents were thoroughly mixed, squeezed through cloth and centrifuged. Aliquots of this acid extract were then used to determine lactic acid by the method of Barker and Sommerson (1941).

4.4.7 Volatile Fatty Acids

Individual volatile fatty acids (VFA) were measured in a sample of the acid extract of fresh silage prepared for the determination of lactic acid.

A Pye Unicam SP 30 gas chromatograph was used for separation and detection. It was equipped with a two metre glass column packed with Pye Unicam resin (Code No.: 1248/1WPT/105P1200-2915B). High purity argon was the mobile phase. The injection, column and detector temperatures were 145, 120 and 150°C respectively and a run lasted 35 minutes, detecting acetate through to hexane.

4.4.8 Gross Energy

Gross energy (GE) was determined on dried grass or fresh silage samples using an Adiabatic Bomb Calorimeter with polythene as primer (McDonald *et al*, 1973).

4.4.9 Microbiological

Microbiological analyses were carried out on samples of fresh grass or silage. Aseptic techniques were employed throughout.

A general viable count was made using plate count agar. Lactobacilli were counted on tween acetate agar, streptococci on sodium azide, coliforms on violet red bile agar, yeasts and moulds

on malt extract agar, lactate fermenters on a lactate medium and proteolytic species on a protein medium.

5. EXPERIMENT 1

THE ENSILAGE OF GAMMA-IRRADIATED AND INOCULATED GRASS

5.1 INTRODUCTION

The main aim of this experiment was to delineate the effects of plant and microbial enzymes on the nitrogenous changes during ensilage, especially with respect to amino acids.

The changes brought about during ensilage by plant and microbial enzymes are inter-related and thus difficult to distinguish. Some success has been achieved using microbe-free grass grown under asceptic conditions (Mabbitt, 1951; Stirling, 1953; Kemble, 1956; Playne *et al*, 1967; Woolford, 1972). Anti-microbial agents such as toluene, chloroform and various anti-biotics have also been used (Macpherson, 1952a; Brady, 1960; Gouet and Fatianoff, 1964; Woolford, 1972) but they are not entirely satisfactory, and the treated forage cannot be used to study the effects of inoculants. In the present experiment, an attempt was made to separate the two effects by using gamma-radiation to produce sterile grass. This technique has been used successfully with herbage by several workers (Gouet *et al*, 1970; Bousset *et al*, 1972; Gouet *et al*, 1972; Clark, 1974).

5.2 EXPERIMENTAL

An early perennial ryegrass (c.v. Cropper) sward was treated with a compound fertilizer (20% N, 6% P, 12% K) applied at the rate of 100kgNha^{-1} in mid April, and on the 28 May and 9 July 1980. The grass used in the experiment was the third cut from

the sward. It was harvested with an Allen scythe on the 20 August 1980 and was hand-chopped to a length of about 20 mm prior to ensiling.

Large Pyrex test-tubes (200 g capacity) which had previously been autoclaved (121°C for 15 mins) were used as silos. They were sealed with sterile plastic fermentation traps filled with water.

Silos 1-4 were filled with chopped, fresh grass and sealed (treatment A). Silos 5-8 were filled with herbage from the same source, sealed and gamma-irradiated (3.25 Mrad) as described in Section 4 (treatment B). Grass intended for silos 9-12 was placed in a polythene bag and, after irradiation (3.25 Mrad) was inoculated with a mixed culture of lactic acid bacteria (*L. plantarum* and *S. faecalis*) at the rate of 10^6 organisms g^{-1}. After thorough mixing, samples of inoculated material were ensiled in test-tubes, which were sealed immediately (treatment C). Representative samples of fresh grass were taken for analysis and the weight used in each silo recorded. All silos were incubated at 30°C for 153 days. At the end of this time the total weight of silage in each silo was recorded and representative samples taken for analysis using the techniques described in sections 4 and 13.

5.3 RESULTS AND DISCUSSION

Sterilization commenced about 3 hours after harvesting, and a dose of 3.25 Mrad was given over a period of 15.5 hours (0.21 Mrad h^{-1}). Very little gamma-radiation is required to inactivate most micro-organisms but some bacterial spores, such as those of

clostridia, are much more resistant (Bellamy, 1959; Goldblith, 1971; Davies, 1976). Gouet *et al* (1970) stated that 1.2 Mrad was sufficient to achieve sterility but later used 2.0 Mrad to ensure complete sterilization (Gouet *et al*, 1972). They considered that the process did not adversely affect plant enzyme activity nor the biochemical composition of the herbage. Clark (1974) used a dose of 2.5 Mrad given over an 11-hour period. In the present experiment a dose of 3.25 Mrad was given as circumstances dictated that samples had to be irradiated overnight at a predetermined rate.

It was considered that the three hour delay before irradiation would not result in significant growth of those organisms important during the ensiling process. On fresh herbage such organisms are scarce, especially the lactic acid bacteria (Kroulik *et al*, 1955a; Gibson *et al*, 1958; Stirling and Whittenbury, 1963) and various investigations have demonstrated a lag period before their multiplication. Ohyama *et al* (1973a) found a 24-hour delay before an increase in the numbers of *L. plantarum* and concomitant acid production, in Italian ryegrass. Greenhill (1964) reported a 10-hour delay before the start of acid production and delays of as much as two (Barnett, 1954) or even three days (Gouet *et al*, 1970) have been found.

A sample of irradiated grass was analysed immediately after irradiation, by the Microbiology Department of the Edinburgh School of Agriculture, and was pronounced sterile.

After irradiation of the grass in the polythene bag and application of the inoculum ($20 ml kg^{-1}$), the grass appeared very wet and

TABLE 5.1

Experiment 1. Weights of grass ensiled and dry matter losses

Treatment	Silo	Grass ensiled (g)	Dry matter loss (%)
A	1	212.4	0.3(+)
	2	211.5	2.1
	3	213.5	0.0
	4	204.8	0.0
B	5	222.0	2.3
	6	216.5	2.0
	7	219.6	2.9
	8	225.5	1.7
C	9	242.2	2.3
	10	243.0	4.7
	11	242.8	4.9
	12	242.7	3.9

limp and was much more easily compacted. This change in the texture of the grass probably resulted from plasmolysis, with a consequent loss of structure and release of cell contents. An attempt was made to ensile approximately the same amount of grass in each silo by applying less pressure when packing but, despite this, these silos contained about 10% more material than the others (Table 5.1).

In a laboratory silo, dry matter (DM) losses result from water and/or gas production (McDonald *et al*, 1962). Water can be produced from respiration or from various fermentations (Greenhill, 1964; McDonald *et al*, 1966a; McDonald and Whittenbury, 1967; Bousset *et al*, 1972). Although of considerable importance in farm silos, respiration is likely to be of little consequence in laboratory silos (McDonald, 1981). Greenhill (1964) has estimated that all oxygen trapped in laboratory silos is used up within two to five hours of ensiling. Bousset *et al* (1972) reported a close correlation between gaseous and DM losses in laboratory silos, and attributed them to bacterial fermentations rather than to respiration. Gross (1975) considered that in laboratory silos, DM losses were mainly the result of gaseous losses, chiefly carbon dioxide.

In this experiment the DM losses incurred were small (Table 5.1) and within the range expected for a lactate fermentation (McDonald and Whittenbury, 1973; McDonald, 1981). The inoculated silages had the highest losses. This was unexpected since only homofermentative organisms, which are generally regarded as being non-gas producing, were applied. It is, however, in keeping with the vigorous frothing observed in these silos during the first 24 hours of ensilage.

TABLE 5.2

Experiment 1. Composition of grass and silages

	GRASS*	SILAGES*			SED
		A	B	C	
DM(gkg^{-1})	159	158	157	154	-
pH	5.65^{a}	3.79^{b}	5.17^{c}	4.12^{d}	0.103
(gkg^{-1}DM)					
WSC	124^{a}	3.5^{b}	204^{c}	56^{d}	23.52
Total N	23.5	23.9	23.8	24.6	-
Lactic Acid	-	118.5^{a}	11.0^{b}	67.0^{c}	10.97
Acetic Acid	-	17.5^{a}	7.3^{b}	17.8^{a}	3.14
Propionic Acid	-	0.0^{a}	0.9^{b}	0.1^{a}	0.22
Butyric Acid	-	0.0^{a}	14.3^{b}	4.7^{a}	3.84
Ethanol	-	14.5^{a}	3.6^{b}	6.7^{c}	0.32
Gross Energy ($MJkg^{-1}$DM)	-	18.3^{a}	18.6^{a}	18.4^{a}	NS
(gkg^{-1}TN)					
Protein N	858^{a}	293^{b}	359^{c}	467^{d}	18.1
Volatile N	10.6^{a}	136^{b}	82^{c}	77^{c}	10.30
Amide N	9.8^{a}	15.7^{b}	10.2^{a}	12.2^{a}	1.21
Nitrate N	4.4	-	-	-	-

* Values on the same line with different superscripts differ significantly ($P<0.05$)

NS Not significant

The compositions of the grass and silages are given in Table 5.2. The results were statistically analysed using a one-way analysis of variance and the means and overall standard errors of difference (SED) are reported.

Silage A (control) appears to be a typical lactate silage (McDonald, 1981) with a low pH, and low water-soluble carbohydrate (WSC) and high lactic acid contents. Despite the low dry matter (DM) content ($159gkg^{-1}$) of the grass there was sufficient WSC present ($124gkg^{-1}$DM) to enable the lactic acid bacteria to produce enough acid to reduce the pH to 3.79. There was insufficient WSC present in the original crop to account for all the fermentation acids produced. This phenomenon has often been encountered during ensilage (Dewar *et al*, 1963; McDonald *et al*, 1964; Ohyama and Masaki, 1977) and suggests that substances other than soluble carbohydrates are being converted into acids.

Extensive proteolysis had occurred resulting in a low protein nitrogen (PN) content ($293gkg^{-1}$TN). There is also evidence of some amino acid breakdown, the volatile nitrogen (VN) content having risen substantially. It is possible that, owing to the high moisture content of the grass, the critical pH for the control of clostridia was not achieved rapidly enough to prevent their activity entirely.

The volatile fatty acid (VFA) profile and ethanol content are typical of a lactate silage (McDonald, 1981).

The gross energy (GE) content of the original grass was not measured but typically is about $18.4MJkg^{-1}$DM for such material

(McDonald *et al*, 1973; McDonald, 1981) and it appears that this had not changed significantly during ensilage. No energy loss was expected in such a closed system where lactic acid bacteria dominated the fermentation (McDonald and Edwards, 1976; McDonald, 1981). Indeed, increases in GE concentration of 10% or more are often experienced as a result of losses of DM without concomitant losses of energy, during ensilage (Alderman *et al*, 1971; McDonald *et al*, 1973; McDonald and Edwards, 1976).

Silage B, made from irradiated grass, was very different in composition from the control silage, having a high pH (5.17), a low lactic acid content (11.0gkg^{-1}DM) and a WSC content which was 65% higher (204gkg^{-1}DM) than that of the original grass. An increase in WSC after ensilage of gamma-irradiated grass has been demonstrated by Clark (1974), who obtained a 12% increase after seven days, and Bousset *et al* (1972) who found increases of 19 and 22% for meadow fescue and lucerne respectively, after 100 days. Increases of up to 73% have been reported by others, when fermentation has been restricted using sodium metabisulphite (Macpherson *et al*, 1957), antibiotics (Gouet and Fatianoff, 1964) or acids (McDonald and Henderson, 1974; Ohyama and McDonald, 1975; Ohyama and Masaki, 1977, 1979).

These increases in WSC are generally considered to result from the hydrolysis of plant structural carbohydrates such as cellulose, hemicelluloses, pectins and β-glucans. Macpherson *et al* (1957) found that the increase in WSC observed during ensilage of grass, which had been treated with sodium metabisulphite, was mainly due to an increase in the glucose fraction. They suggested that the

source of this had probably been cellulose since some hydrolysis of cellulose had occurred. The remainder was presumed to originate from the hydrolysis of hemicelluloses, a conclusion which was supported by the presence of pentoses in the silages. Ohyama and Masaki, (1977, 1979) observed similar increases in WSC but found that they were not due to glucose. They assumed that an increase in the pentose fraction was responsible although the results of chromatographic studies did not confirm this.

Some breakdown of cellulose does occur during ensilage but it is usually small compared to that of hemicelluloses (McDonald *et al*, 1960, 1962; Morrison, 1979). The hemicellulose content of grasses increases with maturity and varies from about 100 to 300 gkg^{-1}DM (Waite and Gorrod, 1959; McIlroy, 1967; Jones, 1970). Significant hydrolysis has been found during ensilage (McDonald *et al*, 1960; 1962; Bousset, 1967; Morrison, 1979). McDonald *et al* (1960) found that as much as half of the original hemicellulose content could be degraded. Morrison (1979) reported the effects of various additives on the hydrolysis of hemicellulose. He found that the addition of formaldehyde reduced the breakdown from 15% (control) to 10%. If, however, acids were added, hydrolysis increased to a maximum of 20% which occurred in the presence of sulphuric and formic acids. In all cases hydrolysis continued throughout the period of ensilage but the rate decreased gradually.

There are three possible sources of hemicellulose breakdown:
(1) hemicellulases present in the original herbage;
(2) bacterial hemicellulases and, (3) hydrolysis by organic acid produced during fermentation.

Dewar *et al* (1963) studied several species of lactobacilli and leuconostocs found in silage but could detect no hemicellulase activity. This confirmed the findings of a previous investigation by Wylam (cited by McDonald *et al*, 1960). They did, however, demonstrate the action of plant hemicellulases *in vitro*. The optimum pH was found to be 6 but the temperature optimum varied from 30 to 43°C depending on the source of the enzyme. Walker and Hopgood (1961) also found a pH optimum of 6 for plant hemicellulases. Wylam (cited by McDonald *et al*, 1960) found maximum activity at pH 5-6, but considerable decomposition also occurred at lower pH values.

Bousset *et al* (1972) failed to demonstrate hemicellulase activity *in vivo* but later concluded (Bousset, 1973) that plant hemicellulases do exist, but are relatively inactive owing to compartmentalization. They would be released only after plasmolysis when their activity would be short-lived because of the rapid fall in pH during fermentation. Thus, under normal circumstances, hemicellulase activity would not be expected to contribute significantly to an increase in WSC. In this experiment, however, the pH remained high (5.17) in the irradiated-grass silage and it is possible that hemicellulase activity continued for much longer.

It has been suggested (Dewar *et al*, 1963; Morrison, 1979) that during the prolonged storage period, direct acid hydrolysis of cell wall polysaccharides may contribute to the increase in WSC. Dewar *et al* (1973) demonstrated considerable breakdown of hemicelluloses at pH4 by incubating hemicellulose preparations at various pH levels (4-6) for 90 days. Morrison (1979) noted

increased hemicellulose breakdown when silages were made with certain acid additives, notably sulphuric and formic acids, but it is not known whether natural acid production in silages causes any hydrolysis.

In the irradiated silage the pH remained high owing to the absence of lactic acid bacteria. Neither lactic nor acetic acid are thought to be present in significant amounts in fresh grass (Playne and McDonald, 1966; Playne *et al*, 1967; McDonald *et al*, 1968; Gouet *et al*, 1970; Bousset *et al*, 1972) but small amounts were detected in the sterile silage. Other workers have also reported the production of these acids in sterile grass silage. (Playne *et al*, 1967; Bousset *et al*, 1972). A small amount of lactic acid is known to be produced during plant glycolysis (Davies, 1980) and both of these acids are produced from the breakdown of organic acids (McDonald, 1981).

The high level of butyric acid ($14.3 gkg^{-1}$ DM) found in this silage is very puzzling as it suggests the presence of saccharolytic clostridia. This is unlikely in view of the dose of gamma-radiation given, and the fact that the grass was sterilized after packing into the silos which were not opened again until the end of the experiment. If clostridia had been present, extensive breakdown of the WSC fraction would also have been expected. These samples were re-analysed using a different gas chromatograph but similar results were obtained. The source of this butyric acid is not known.

Almost as much proteolysis occurred as in the control silage. This was to be expected as plant enzymes have been shown to be responsible for most of the proteolysis during ensilage (Tracey, 1948; Mabbitt,

1951; Macpherson, 1952a; Kemble, 1956; Brady, 1961; Ohyama, 1970; Bousset *et al*, 1972; Clark, 1974). The total amount of protein hydrolyzed in the sterile silage was less than in the control despite the high pH. This suggests that some mechanism exists to limit the extent of proteolysis. McKersie and Buchanan-Smith (1982) have shown that the cessation of proteolysis after lucerne had been ensiled for one to two days was not the result of loss of enzyme activity and that the pH was not inhibiting (4.9-5.4). They suggested that substrate availability or end-product inhibition may have been responsible. Ohshima (1971) demonstrated that plant proteins are not equally degraded during ensilage. He found that the protein associated with the fibrous part of the plant was not degraded because of its firm fixation in the structural material. Pirie (1959) has stated that most of this protein is associated with trapped chloroplasts. Horigome and Kandatsu (1960) found that the cytoplasmic proteins of white clover were more easily degraded than the chloroplastic proteins. These and other possible causes of limited proteolysis have been discussed more fully in Section 2.2.2.8.

It is generally considered that the deamination of amino acids during ensilage arises mainly as a result of microbial action (McDonald, 1981), but in this experiment substantial quantities of volatile nitrogen were produced in the sterile silage, indicating the involvement of plant enzymes in deamination. Other workers have also demonstrated deamination by plant enzymes (Mabbitt, 1951; Macpherson, 1952a; Brady, 1960; Ohshima, 1971).

TABLE 5.3

Experiment 1. Composition of individual inoculated silages (treatment C

	SILAGES			
	9	10	11	12
DM(gkg^{-1})	157	149	155	154
pH	4.04	3.95	4.43	4.07
(gkg^{-1}DM)				
WSC	2	105	103	14
Total N	24.6	24.3	24.5	24.8
Lactic Acid	72	91	30	75
Acetic Acid	18.3	8.8	20.2	24.2
Propionic Acid	0	0	0.3	0.2
Butyric Acid	1.1	0	17.0	0.5
Ethanol	6.7	6.4	6.7	6.8
Gross Energy ($MJkg^{-1}$DM)	17.9	18.4	18.6	18.7
(gkg^{-1}TN)				
Protein N	458	471	477	462
Volatile N	80	58	65	104
Amide N	14.2	12.8	9.0	12.9

The inoculated silage (C) had a high residual WSC content ($56gkg^{-1}DM$) and a low lactic acid content ($67.0gkg^{-1}DM$) despite the fairly low pH attained (4.12). Acetic and propionic acid levels were normal for a lactate silage but the butyric acid content was high ($4.7gkg^{-1}DM$). There had been less proteolysis than in either silages A or B but the volatile nitrogen content was similar to that of the sterile silage. Some of these anomalies can be explained by the variability between individual replicates (Table 5.3).

The four replicates appear to have fermented in different ways, suggesting inadequate mixing of the inoculum with the irradiated grass. With hindsight, it would have been better to have divided the grass into four separate quantities before applying equal amounts of the inoculum to each. This would have ensured that a similar quantity of inoculum was applied to each replicate.

Silages 9 and 12 were similar to normal lactate silages, as one might expect from the application of a mixed homofermentative inoculum. The WSC had been utilized to produce lactic acid with a consequent reduction in the pH. The acetic acid content is relatively high but both *L. plantarum* and *S. faecalis* are capable of producing substantial quantities of acetic acid during fermentation (Huhtanen and Pensack, 1963; Bousset *et al*, 1972). This was demonstrated by Playne *et al* (1967) using microbe-free grass inoculated with these organisms. Both organisms are capable of limited amino acid deamination (Ohshima and McDonald, 1978) but there was no evidence of this in silo 9, the volatile nitrogen content being almost identical to that of the sterile silage. There

appeared to have been some microbial deamination in silage 12.

Silage 11 had a relatively high pH and low lactic and high butyric acid contents. It would appear that there had been clostridial activity. Although precautions were taken, it is possible that contamination occurred during ensiling as sterilization took place before the grass was inoculated and packed into the silos. It is surprising, however, that clostridia should have had a chance to become active because the addition of a homofermentative inoculum should have brought about a rapid acidification. The residual WSC of this silage was high and the amount of acids produced could not be accounted for by the apparent decrease in WSC. This would indicate that hydrolysis of hemicelluloses probably occurred. Activity by either clostridia or hemicellulases would suggest that the pH remained high for a period at the beginning of ensilage. The low level of proteolysis found in this silo does not support this conclusion, however, as this would indicate that rapid acidification had occurred (Carpintero *et al*, 1979; McDonald, 1981).

Despite substantial lactic acid production, silage 10 also had a high residual WSC indicating hemicelluloses had probably been hydrolysed in this silage too. No reason can be found to explain why the residual WSC of silages 9-12 should vary so much.

Proteolysis was inhibited to the same extent in all four replicates and indicated that inoculation had brought about rapid acidification, resulting in earlier inhibition of the proteases (Carpintero *et al*, 1979; McDonald, 1981).

TABLE 5.4

Experiment 1. Nitrogen balance sheet ($gkg^{-1}TN$)

	GRASS	SILAGES		
		A	B	C
Protein	858	293	359	467
Free amino acid*	42.6	285.8	302.3	232.8
Volatile	10.6	136	82	77
Amide	9.8	15.7	10.2	12.2
Nitrate	4.4	-	-	-
Unaccounted ($gkg^{-1}TN$)	74.6	269.5	246.5	211.0
Unaccounted ($gkg^{-1}NPN$)	525.4	381.2	384.6	395.9

*for those amino acids determined only.

A comprehensive analysis of the amino acids in the grass and silages was also carried out, the results of which are given in Tables 5.4-5.9.

The nitrogen distribution in the grass and silages is shown in Table 5.4 from which it can be seen that a fraction of the nitrogen remained unaccounted for. In the grass this represented only about 75gkg^{-1}TN but increased considerably in the silages. Nitrogenous compounds which were not measured but which would be included in this fraction are, small peptides, non-volatile amines, nucleic acids and those amino acids which were not determined in this study eg cysteine, methionine, histidine and tryptophan.

The values reported by other workers for unaccounted nitrogen vary considerably. Ohyama (1969a) examined a large number of silages for α-amino, peptide, amide and volatile nitrogen. He found that the fraction unaccounted for varied from 110-250gkg^{-1}TN. Similar figures have been reported elsewhere for silages (Kemble and Macpherson, 1954a; Ohyama, 1969b; Hughes, 1970; Bergen *et al*, 1974; Fujita, 1976). In some cases this fraction was found to decrease during ensilage (Bergen *et al*, 1974; Fujita, 1976) whereas in others, as with the current experiment, an increase was observed (Kemble and Macpherson, 1954a; Brady, 1960; Hughes, 1970). An increase may in part be accounted for by the production of γ-aminobutyric acid from glutamic acid (Ohyama, 1969a; Ohshima, 1971). This non-protein amino acid has been reported in silages at levels as high as 180gkg^{-1}TN (Kirchmeier and Kiermeier, 1962) although smaller amounts are more common (Macpherson and Slater, 1959; Brady, 1965; Ohshima *et al*, 1979.

TABLE 5.5

Experiment 1. Recovery of amino acid nitrogen

	GRASS*	SILAGES*		
		A	B	C
Protein N	858	293	359	467
Free amino acid N+	42.6	285.8	302.3	232.8
Amide N ($\frac{1}{2}$)	4.9	7.9	5.1	6.1
Sum of above	905.5	586.7	666.4	705.9
Total amino acid N+	657.0	492.4	598.4	583.6
Recovery (%)	71.8	83.9	89.8	82.7

* $gkg^{-1}TN$

+ for those amino acids determined on the amino acid analyser only.

If the total amino acid nitrogen measured on the amino acid analyser is compared with the total expected from the sum of the protein, free amino acid and amide nitrogen contents (Table 5.5), it would appear that 72% (grass) to 90% (irradiated silage) has been accounted for by amino acid analysis. Peptide nitrogen was not included in the calculated nitrogen, therefore, if significant peptide was present the recoveries would be slightly lower. These recoveries are within the range reported by others for analysis of total hydrolysates of herbage (Chibnall *et al*, 1963; Wilson and Tilley, 1965; De Vuyst *et al*, 1968b, 1971; Ohshima *et al*, 1979; Fishman *et al*, 1982; Moreau *et al*, 1982).

Part of the apparent loss results from the fact that certain amino acids were not determined in this study eg histidine, cysteine, methionine and tryptophan. Histidine elution was found to coincide with the second buffer change. Small losses of most amino acids occur during acid hydrolysis but in certain cases eg tryptophan, cysteine and methionine, these may be substantial, especially in the presence of carbohydrates and other physiological substances (Blackburn, 1978; Ambler, 1981; Gardner, 1981; Williams, 1981).

Analysis of grass and silage hydrolysates is subject to similar difficulties and sources of error to those known to be encountered in food analysis. These difficulties, summarized by Blackburn (1978), are greater than those found with pure or semi-pure proteins and mainly relate to side reactions between carbohydrates and amino acids.

TABLE 5.6

Experiment 1. The ratio of amino acid nitrogen in various grass fractions

	GRASS		
	Free Amino Acids	Protein Amino Acids	Total Amino Acids
Aspartic Acid	16.5	9.7+	10.2+
Threonine)	27.7	8.6	4.6
Serine)			5.2
Glutamic Acid	14.8	8.6+	9.0+
Proline	3.9	4.7	4.7
Glycine	1.1	10.6	10.0
Alanine	15.4	10.3	10.6
Valine	4.1	6.8	6.6
Isoleucine	2.0	3.6	3.5
Leucine	2.5	7.2	6.9
Tyrosine	0.8	2.3	2.2
Phenylalanine	1.6	3.7	3.6
Lysine	4.2	9.8	9.4
Arginine	5.4	14.1	13.5
Total	100.0	100.0	100.0

+ - includes amide nitrogen

TABLE 5.7

Experiment 1. The ratio of amino acid nitrogen in the fresh grass compared with results from other studies.

	Perennial Ryegrass	Italian[a] Ryegrass	Lucerne[a]	Lucerne[b]	Grass[b*] Protein
Asp[+]	10.2	9.3	16.4	12.2	8.6
Thr	4.6	4.6	4.0	4.7	4.9
Ser	5.2	5.1	5.0	5.3	5.1
Glu[+]	9.0	8.7	8.0	8.3	8.1
Pro	4.7	4.3	4.2	5.6	4.9
Gly	10.0	8.0	7.3	8.5	8.4
Ala	10.6	9.4	7.6	8.7	8.5
Val	6.6	6.4	5.8	6.2	6.8
Ile	3.5	3.9	3.8	4.3	4.5
Leu	6.9	8.7	7.3	7.2	7.6
Tyr	2.2	2.3	2.3	2.6	3.0
Phe	3.6	4.0	3.5	3.5	4.3
Lys	9.4	10.5	10.7	8.3	10.0
Arg	13.5	14.8	14.1	14.6	15.3
Total	100.0	100.0	100.0	100.0	100.0

a from Ohshima *et al* (1979)

b from Wilson and Tilley (1965)

* mean of five different grasses

+ includes amide nitrogen

TABLE 5.8

Experiment 1. Free amino acid nitrogen ($gkg^{-1}TN$) in grass and silages

	GRASS*	SILAGES*			SED
		A	B	C	
Asp	7.03^{a}	20.68^{b}	28.70^{c}	18.29^{bd}	2.693
Thr+ Ser+	11.79^{a}	10.89^{b}	11.72^{b}	13.48^{b}	4.225
Glu	6.29^{a}	6.31^{a}	12.26^{b}	10.39^{ab}	1.897
Pro	1.65^{a}	18.38^{b}	18.24^{b}	13.42^{c}	0.633
Gly	0.47^{a}	37.25^{b}	26.98^{c}	19.07^{d}	1.304
Ala	6.55^{a}	65.34^{b}	41.25^{c}	43.64^{c}	3.536
Val	1.75^{a}	28.93^{b}	24.41^{c}	17.93^{d}	0.837
Ile	0.87^{a}	19.09^{b}	15.97^{c}	10.71^{d}	0.387
Leu	1.08^{a}	32.09^{b}	25.11^{c}	19.97^{d}	1.072
Tyr	0.35^{a}	6.11^{b}	8.84^{c}	2.12^{d}	0.671
Phe	0.68^{a}	13.36^{b}	11.72^{c}	8.57^{d}	0.447
Lys	1.80^{a}	13.80^{b}	27.90^{c}	27.65^{c}	0.775
Arg	2.32^{a}	2.63^{a}	38.44^{b}	14.03^{a}	5.908
Total	42.63^{a}	285.75^{b}	302.26^{b}	232.75^{c}	10.505

* Values on the same line with different superscripts differ significantly ($P<0.05$)

\+ threonine and serine are likely to be over-estimates as asparagine and glutamine co-elute with them

TABLE 5.9

Experiment 1. Total amino acid nitrogen ($gkg^{-1}TN$) in grass and silages

	GRASS*	SILAGES*			SED
		A	B	C	
Asp+	66.88[a]	40.83[b] (61.1)	60.55[c] (90.5)	43.15[c] (64.5)	1.581
Thr	30.47[ac]	19.54[b] (64.1)	29.42[a] (96.6)	32.54[c] (106.8)	1.049
Ser	34.21[a]	20.81[b] (60.8)	32.00[a] (93.5)	28.52[c] (83.4)	1.432
Glu+	59.30[a]	31.67[b] (53.4)	58.72[a] (99.0)	48.98[c] (82.6)	2.388
Pro	30.58[a]	33.14[a] (108.4)	31.88[a] (104.3)	31.49[a] (103.0)	NS
Gly	65.59[a]	66.90[a] (102.0)	58.15[b] (88.7)	60.09[b] (91.6)	2.258
Ala	69.62[ac]	84.26[b] (121.3)	64.22[a] (92.2)	72.85[c] (104.6)	3.317
Val	43.49[a]	38.61[b] (88.8)	28.13[c] (64.7)	39.67[b] (91.2)	1.095
Ile	22.84[ab]	23.25[a] (101.8)	21.47[b] (94.0)	24.03[a] (105.2)	0.806
Leu	45.57[a]	46.51[a] (102.1)	42.56[a] (93.4)	45.02[a] (98.8)	NS
Tyr	14.50[a]	10.32[b] (71.2)	14.94[a] (103.0)	11.38[b] (78.5)	0.806
Phe	23.43[a]	22.57[a] (96.3)	26.12[b] (111.5)	22.32[a] (95.3)	0.894
Lys	61.89[a]	30.51[b] (49.2)	53.03[c] (85.7)	56.89[d] (91.9)	1.285
Arg	88.66[a]	23.46[b] (26.5)	77.21[ac] (87.1)	66.68[c] (75.2)	5.390
Total	657.03[a]	492.38[b]	598.40[c]	583.61[c]	11.185

* Values on the same line with different superscripts differ significantly ($P<0.05$). The data in parentheses show the % recovery after ensilage.

\+ includes amide nitrogen.

In order to compensate for general hydrolysis losses some users incorporate an internal standard eg norleucine but this practice is not universally adopted. A recent study (Gardner, 1984) has shown that more accurate and precise values are obtained by the use of internal, rather than external, standardization.

Studies have shown that the amino acid composition of plant proteins varies little, regardless of the species or stage of growth (Chibnall *et al*, 1963; Wilson and Tilley, 1965; Lyttleton, 1973). Since most of the nitrogen in fresh grass is in protein form (Ohshima and McDonald, 1978) the total amino acid composition would be expected to be similar to that of the protein. The figures given in Table 5.6 show that this was true for the perennial ryegrass in this experiment. Therefore it should be possible to compare its total amino acid composition with the compositions of leaf protein hydrolysates as well as with other whole leaf hydrolysates. When this is done (Table 5.7), it is found to compare well. The higher value for aspartic acid in lucerne is an indication of the importance of asparagine as a nitrogen transport compound in this species (Ohshima *et al*, 1979).

From the data presented in Tables 5.8 and 5.9 it can be calculated that in fresh grass only 6.5% of the amino acid nitrogen was found in free form, but during ensilage extensive proteolysis occurred causing a large increase in this fraction. The decrease in the total amino acid nitrogen content of the control silage (Table 5.9) suggests that further amino acid breakdown occurred and this conclusion is supported by the significant increase in volatile nitrogen (Table 5.4).

Proteolysis is mainly caused by plant enzyme activity but amino acid catabolism is generally thought to be chiefly the result of microbial activity (Ohshima and McDonald, 1978). The relatively small change in the total amino acid content of the sterile silage would appear to support this. Further confirmation is obtained from the inoculated silage which also showed little change, indicating that these lactic acid bacteria were not involved to any great extent in the process of amino acid breakdown.

In the fresh herbage (Table 5.9) more than 60% of the amino acid nitrogen was accounted for, in order of magnitude, by arginine, alanine, aspartic acid, glycine, lysine and glutamic acid. The pattern of free amino acids was different, however, as aspartic acid, glutamic acid, threonine, serine and alanine, all of which were present in similar amounts, accounted for more than 74% of the free amino acid nitrogen (Table 5.8). During ensilage selective amino acid degradation occurred (Table 5.9).

In general the amino acid changes observed in the control silage were typical of a lactate silage and were very similar to those reported by Ohshima *et al* (1979) for an Italian ryegrass lactate silage. Alanine was the only amino acid to increase. It can be formed by the decarboxylation of aspartic acid by both plant and microbial enzymes (Ohshima, 1971). The branched chain amino acids, valine, isoleucine and leucine showed little, if any, change. Proline, glycine and phenylalanine did not alter significantly either but there were significant decreases in aspartic acid, threonine, serine, glutamic acid, tyrosine, lysine and arginine. In particular, the decrease in arginine is interesting as this amino acid accounted for the largest fraction of amino acid nitrogen in

the original grass and after ensiling represented the smallest.

Very little amino acid catabolism had occurred in the sterile silage which supports the previous claim that it is the microbial enzymes which cause most of the breakdown. In the control silage there had been reductions in aspartic acid, threonine, serine, glutamic acid, valine, tyrosine lysine and arginine but in the sterile material there were no decreases in threonine, serine, glutamic acid, tyrosine and arginine, and only small decreases in aspartic acid and lysine. This indicates that microbial enzymes had been responsible for the changes in these amino acids in the control silage.

There was little change, if any, in proline, isoleucine, leucine and phenylalanine in either the control or sterile silages but there was a substantial decrease in valine in the sterile material, even more so than in the control, suggesting some microbial synthesis of valine normally occurs.

Alanine had been the only amino acid to increase significantly in the control silage and insignificant catabolism took place in the sterile material, although some deamination by plant enzymes is known to occur (Ohshima, 1971). This amino acid is formed from aspartic acid by decarboxylation, a reaction which during ensilage is considered to be mainly the result of microbial activity (Ohshima, 1971), a conclusion which is supported by these results.

On the whole these findings agree with those of Ohshima (1971) who carried out a detailed study of the individual changes in amino acids during incubation of clover juice with ^{14}C-labelled amino

acids. There are two main differences, however. Valine was found to decrease to a lower level in the sterile silage than in either of the other two treatments. This amino acid is usually well-preserved during ensilage (Hughes, 1970; De Vuyst *et al*, 1968b, 1971; Ohshima, 1971; Ohshima *et al*, 1979) and Ohshima *et al*(1979) suggested it may be synthesized to a certain extent by microbial enzymes. It is possible that in normal silages, catabolism by plant enzymes is balanced by microbial synthesis, although Kemble (1956) did not find any decrease in value in microbe-free grass silage.

The most surprising result was the lack of any change in glutamic acid in the sterile silage as this amino acid is considered to be decarboxylated to γ-aminobutyric acid (GABA) by plant enzymes (Macpherson and Slater, 1959; Hughes, 1970; Ohshima, 1971; Ohshima *et al*, 1979). Macpherson and Slater (1959) demonstrated the rapid formation of GABA and concomitant reduction in glutamic acid during ensilage of microbe-free grass. Ohshima (1971) showed that most of the glutamic acid in incubated clover juice was decarboxylated to GABA within four hours of incubation whereas if ^{14}C-labelled glutamic acid was added after the third day, its decarboxylation was very slow. Both of these studies indicate that the decrease in glutamic acid found during ensiling is a result of plant activity rather than microbial. The results of the current experiment disagree markedly with this conclusion.

Lactic acid bacteria are considered to have a very limited ability to catabolize (Beck, 1978) or synthesize (Stanier *et al*, 1976) amino acids. The results of the present experiment would appear to agree with this as there was no significant difference between the sterile

and inoculated silages with respect to the total amino acid nitrogen (Table 5.9). *L. plantarum* can deaminate serine while *S. faecalis* can deaminate arginine and decarboxylate tyrosine (Brady, 1966; Buchanan and Gibbons, 1974). Thus the main changes in the inoculated silages are in these amino acids as well as in aspartic and glutamic acids.

5.4 CONCLUSIONS

The results of this experiment support the general opinion that proteolysis is brought about by plant protease activity whereas further amino acid metabolism is mainly the result of microbial activity.

It was shown that substantial quantities of additional water-soluble carbohydrates could be made available during ensilage, presumably from polysaccharide breakdown.

The inoculation results were somewhat inconclusive owing to the variability between replicate silos but did indicate that inoculation may reduce proteolysis, presumably owing to rapid acidification. It was also shown that *L. plantarum* and *S. faecalis* were not proteolytic species and had a limited ability to catabolise amino acids.

6. EXPERIMENT 2

THE EFFECT OF pH ON PROTEOLYSIS

6.1 INTRODUCTION

This study was designed to investigate the effects of pH on plant protease activity during ensilage. The large number of treatments necessitated the ensilage of water-soluble extracts of grass, rather than fresh herbage. This method enables small test-tubes to be filled with the same material and ensures that other factors, such as compaction, dry matter content and temperature are eliminated or are identical and easily controlled. Macpherson (1952a) showed that the main effect of using grass extract instead of herbage was a higher initial rate of reaction owing to the substrates being more readily available. The final outcome after 14 days, however, was very similar in both cases.

Three separate experiments were carried out, covering different pH ranges and time scales.

6.2 GENERAL EXPERIMENTAL

In each of the three experiments an aqueous extract was made from ryegrass by macerating herbage with water and filtering through muslin. 5 ml aliquots were pipetted into test-tubes containing buffer solution, acid or water. The approximate quantities of buffer or acid necessary to achieve certain pH values had been determined previously on a similar ryegrass extract. After sealing, the tubes were gamma-irradiated to inactivate the micro-organisms, and incubated for varying lengths of time. Proteolytic

activity was expressed as the percentage of the original protein which had been hydrolysed. More specific details are given in the individual experimental sections.

6.3 EXPERIMENT 2.1

This preliminary experiment covered the pH range 6.6 to 3.4, with incubation times of 30h, 50h and 12 days.

6.3.1 Experimental

An aqueous extract was made from Italian ryegrass (DM 179gkg^{-1}; pH 6.0; WSC 204gkg^{-1}DM; PN 830gkg^{-1}TN) by macerating 100g samples of fresh grass twice with a total of 250 ml water and filtering the macerate through muslin. The filtrates from four extracts were combined and 5 ml aliquots pipetted into small test-tubes containing buffer solution or water. Phosphate buffer (40 mM) was used to maintain pH values above 6.0 and citrate buffer (40 mM) pH values between 6.0 and 3.0. The test-tubes were sealed, gamma-irradiated (2.25 Mrad) and incubated at 37°C for 30h, 50h and 12 days.

After each incubation period, eight replicate tubes were taken for each pH treatment. Total nitrogen was determined on four tubes. Non-protein nitrogen was determined on the other four tubes after first precipitating the protein with 5% w/v (final concentration) trichloroacetic acid. Protein nitrogen was estimated by difference.

6.3.2 Results and Discussion

Figure 6.1 shows the effects of pH and time on the extent of protein hydrolysis.

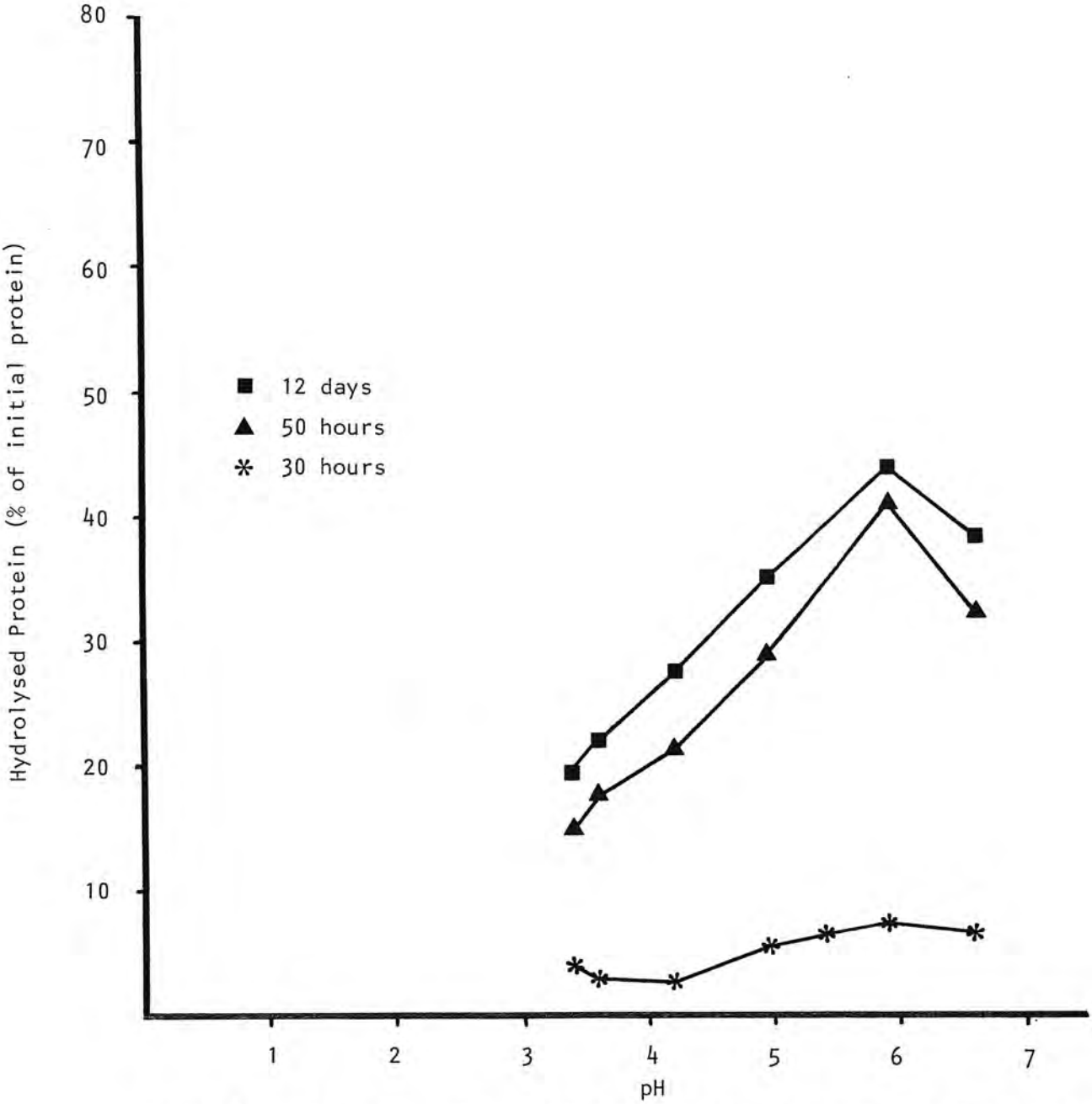


Figure 6.1. Experiment 2.1. Effect of pH on proteolysis in irradiated grass extract incubated at 37°C.

Most of the hydrolysis appeared to take place between 30h and 50h. It is surprising that so little occurred in the first 30h as the substrates should have been readily accessible to the proteases and the high incubation temperature would have been expected to encourage proteolysis (Macpherson, 1952a; Ohyama *et al*, 1973). Even in the normal silage situation, proteolysis usually begins almost immediately after harvesting (Kemble, 1956; Henderson *et al*, 1972; Bergen *et al*, 1974; Clark, 1974).

The pH optimum for proteolysis for Italian ryegrass is about 6.0, which is in agreement with the reports from several other studies on plant proteases using different species (Tracey, 1948; Brady, 1961; Singh, 1962). At this pH about 44% of the original protein had been hydrolysed after 12 days, but even at pH 3.4, more than 19% had been broken down.

Although most of the hydrolysis occurred within the first 50h, an appreciable amount took place during the following 10-day period, even at pH levels below 4.0.

These results do not agree with Virtanan's claim that plants do not contain proteases active below pH 4.0 (Virtanen, 1933) and Macpherson's report that a pH of less than 4.3 is sufficient to prevent significant proteolysis (Macpherson, 1952a).

6.4 EXPERIMENT 2.2

Owing to the unexpectedly high proteolytic activity at pH 3.4 in the previous experiment, another study was undertaken to investigate activity at lower pH levels, and also over a longer period of time. The aim was to determine whether prolonged storage at low pH levels would lead to non-enzymic hydrolysis of proteins.

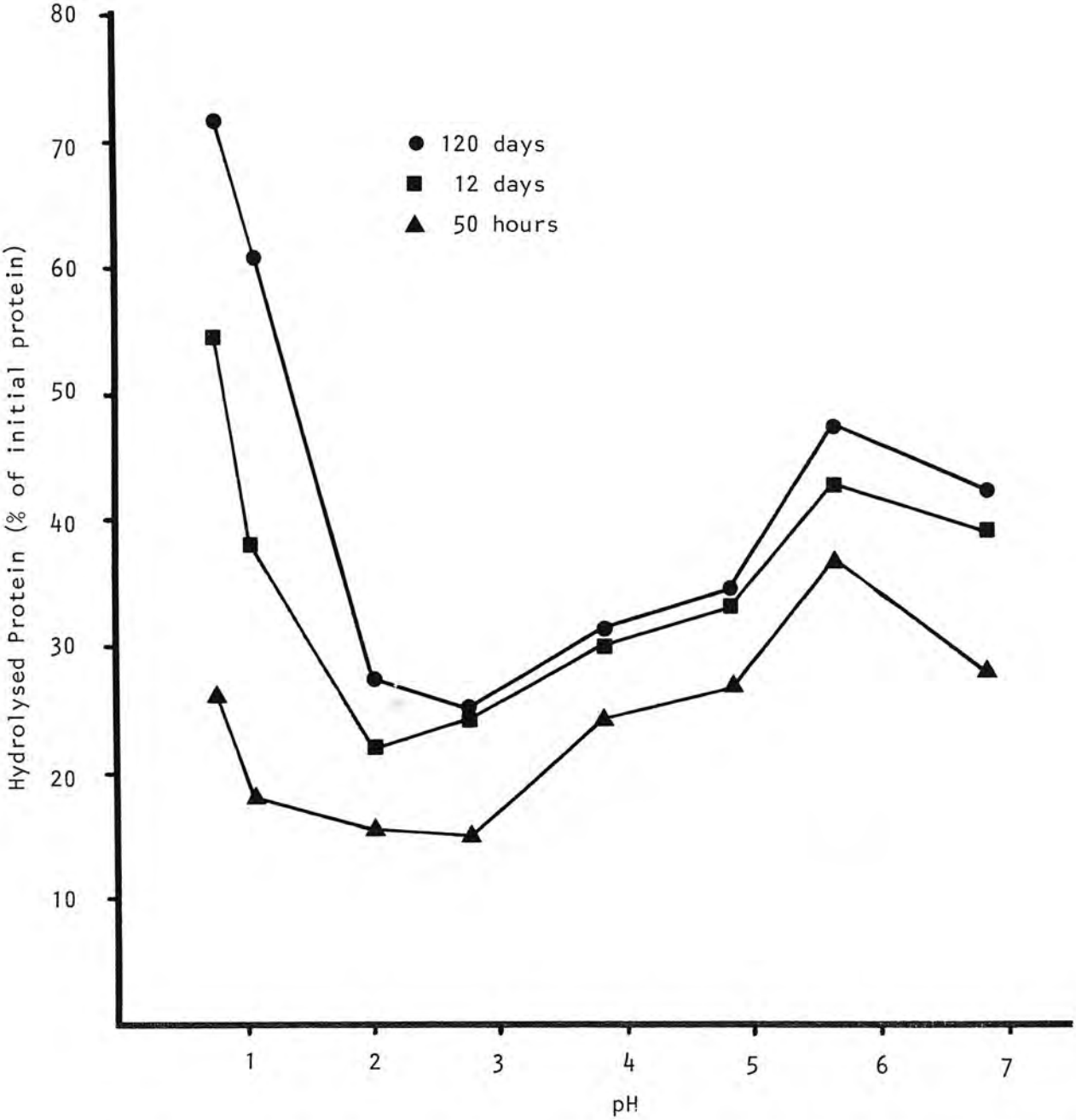


Figure 6.2. Experiment 2.2. Effect of pH on proteolysis in irradiated grass extract incubated at 37°C.

The pH range 6.80 to 0.75 was covered and tubes were opened after 50h, 12 days and 120 days.

6.4.1 Experimental

Using the same methods as in Experiment 2.1, an aqueous extract was made from perennial ryegrass (DM 156gkg^{-1}; pH 5.78; WSC 171gkg^{-1}DM; PN 900gkg^{-1}TN). Each 100g of grass was extracted with a total of 350 ml water. 5 ml aliquots were added to test-tubes containing 1 ml 1.0M phosphate (pH >6.0) or citrate buffer (pH 6.0 to 2.75), or sulphuric acid at predetermined concentrations (pH <2.75). The tubes were sealed, gamma-irradiated (3.25 Mrad) and incubated at 37°C for 50h, 12 days or 120 days. Four estimates of protein nitrogen were made.

6.4.2 Results and Discussion

As in the previous experiment, most of the protein hydrolysis occurred during the initial 50h period (Figure 6.2), although further breakdown did take place.

There was a peak of activity in the region of pH 5.0-6.0. Activity decreased with decreasing pH until about pH 2.0, when there was a dramatic increase. This is assumed to be the result of acid hydrolysis of proteins. Visual extrapolation of the data suggests that some acid hydrolysis could occur between pH 3.0 and 4.0 as well.

There had been considerable protein breakdown at normal silage pH (c. 4.0). The rate of decrease of proteolysis with pH was less than for the previous experiment and after 12 days at pH 3.4, hydrolysis was 65% of the maximum at pH 5.6.

After 12 days some further hydrolysis did occur at pH levels near the optimum, but proteolysis had virtually ceased at pH levels between 4.8 and 2.75.

These results suggest that, although reducing the pH to 4.0 will not prevent the initial burst of proteolysis during ensilage, it will inhibit it and will also prevent further significant proteolysis occurring during prolonged storage. Results of other studies have shown that reducing the pH to below 4.0 does not necessarily prevent proteolysis (Kemble, 1956; Macpherson *et al*, 1957). Carpintero *et al* (1979) acidified grass with formic acid to an initial pH of 3.5 which rose to a maximum of 3.8 during the ensiling period. After four days the initial protein content (819gkg^{-1}TN) had fallen to 338 in the control but only to 550 in the acid-treated materials, indicating that although the low pH had not prevented proteolysis, it had inhibited it to a considerable extent. During the next 46 days these values decreased to 265 and 462 gkg^{-1}TN respectively showing that proteolysis continued, despite the low pH.

6.5 EXPERIMENT 2.3

In the two previous experiments it was noted that there was a very high level of proteolysis at pH levels below 4.0, which apparently arose from plant activity. It seemed probably that the incubation temperature of 37°C was a contributing factor in this effect and another experiment was designed to investigate this. A larger number of pH treatments were also included in order to plot curves from which the pH values for zero acid and protease activity could be obtained by extrapolation. Heat was used to inactivate the plant enzymes in one set of tubes allowing acid hydrolysis alone to be measured.

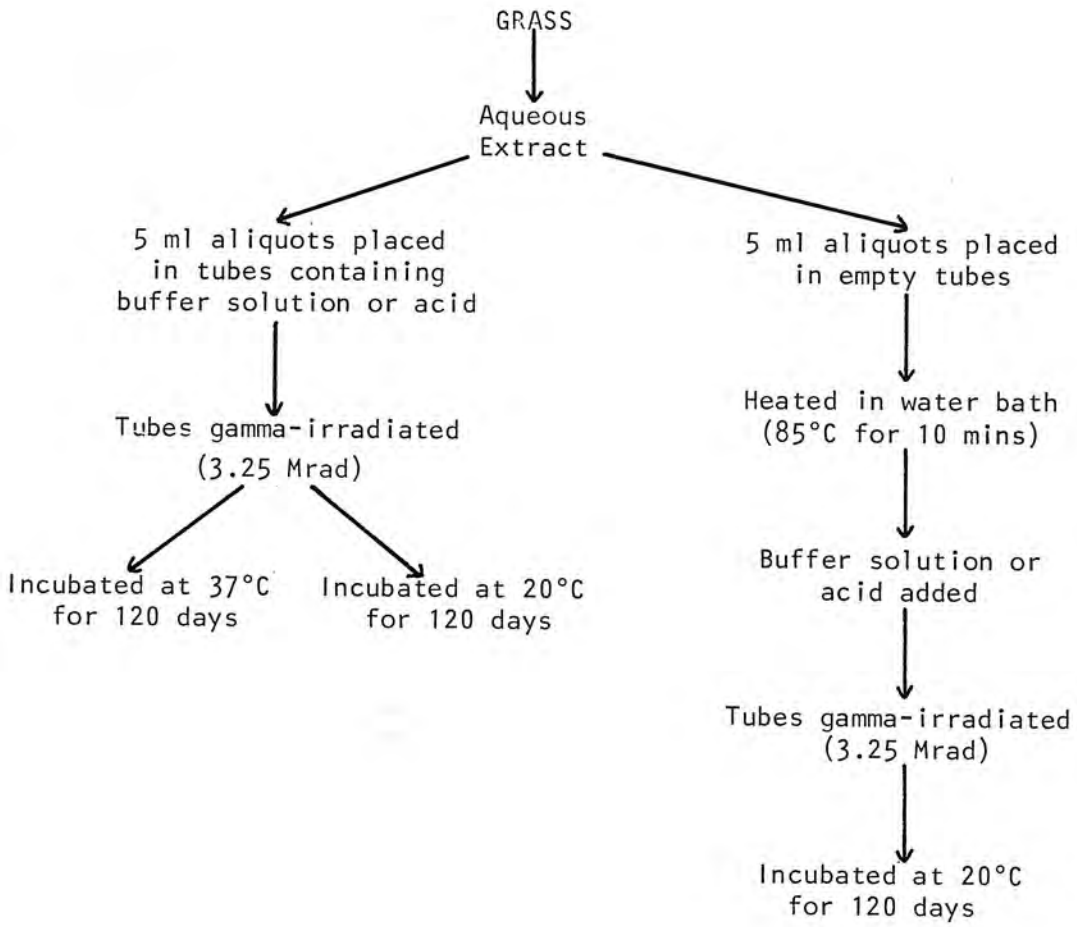


Figure 6.3. Experiment 2.3. Experimental design.

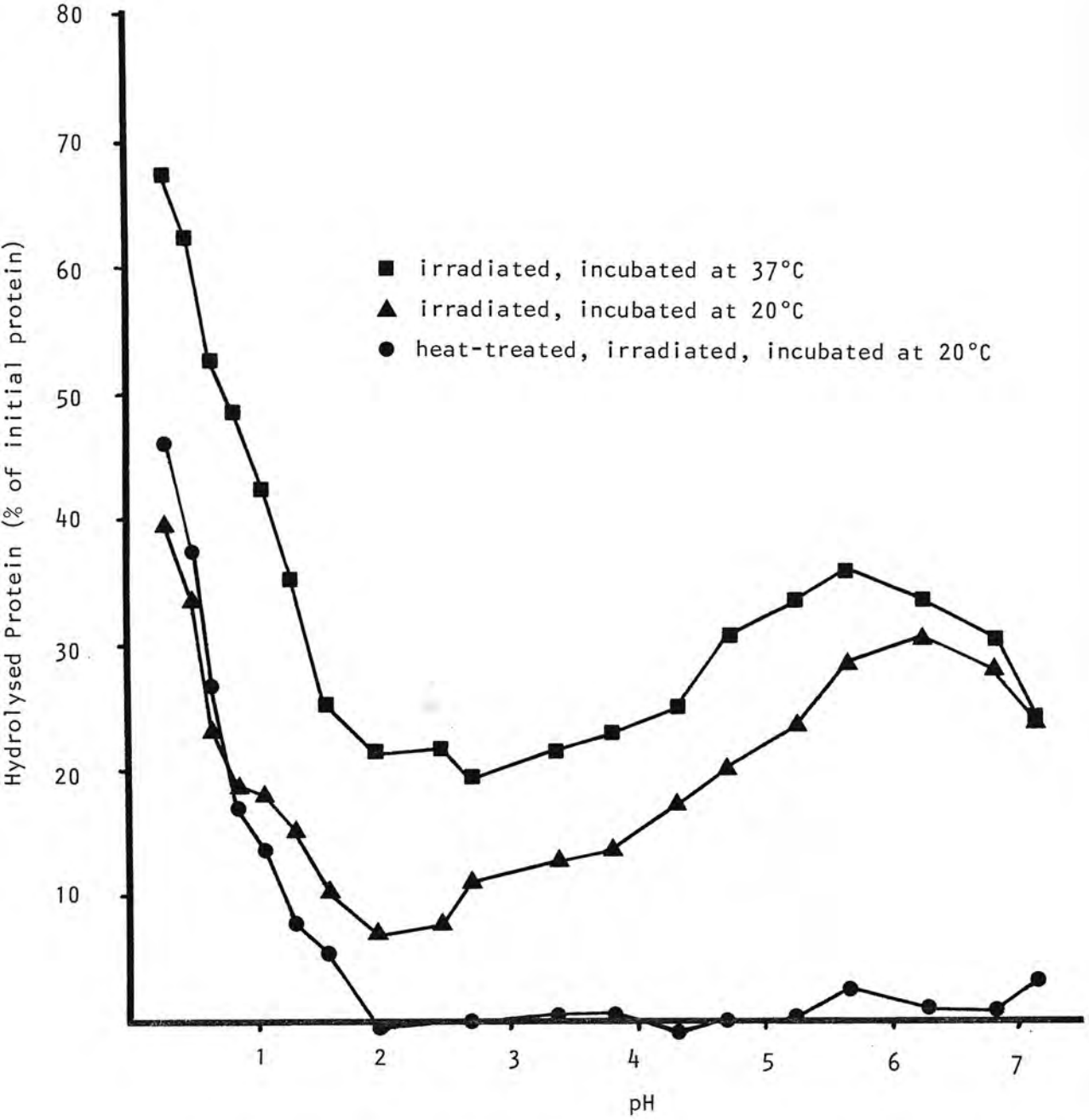


Figure 6.4. Experiment 2.3. Effect of pH on proteolysis in irradiated grass sap incubated for 120 days.

6.5.1 Experimental

An aqueous extract was made from perennial ryegrass (DM 195gkg^{-1}; pH 6.15; WSC 245gkg^{-1}DM; PN 910 gkg^{-1}TN) using 500 ml of water to extract each 100 g of grass. The experimental design is shown in Fig. 6.3. Except for the heating treatment, the techniques used were the same as for Experiment 2.2. Three estimates of protein nitrogen were made and the pH range covered was 7.15 to 0.30.

6.5.2 Results and Discussion

The results shown in Fig. 6.4 indicate that the temperature in the two previous experiments would have encouraged proteolysis, particularly at the lower pH levels, at which most of the hydrolysis would have been non-enzymic. Although the degree of proteolysis is affected, the two curves (■, ▲) have a similar shape and the pH optimum is about 6.0 in both cases.

The increase in the activity of the plant proteases at 37°C can be explained by the fact that most appear to have high temperature optima (Brady, 1961; Singh, 1962; Drivdahl and Thimann, 1977; Feller *et al*, 1977; Frith *et al*, 1978; Peoples and Dalling, 1978; Finley *et al*, 1980; McKersie, 1981).

Under the conditions of this experiment, an increase in temperature did bring about an increase in the extent of proteolysis but the situation in the silo is complicated by the effects of temperature on many other reactions. Macpherson (1952a) found that at higher temperatures the initial rate of proteolysis was greater but, because the pH fell faster, the proteases were inhibited more rapidly and the final outcome was much the same. He concluded that during ensilage the temperature

influenced the rate but not the course of proteolysis. Ohyama *et al* (1973) came to a similar conclusion, stating that the extent of proteolysis was influenced by temperature in the early stages but by pH in the later stages.

At both temperatures there was a large increase in protein hydrolysis below pH 2.0, which is probably due to direct acid hydrolysis. Visual extrapolation suggests that, at 37°C, there may be some acid hydrolysis between pH 3.0 and 4.0 ie the pH of most silages. This does not seem to be so at the lower temperature.

In order to investigate the extent to which acid hydrolysis could contribute to proteolysis during ensilage, some tubes were incubated after the grass extract had been subjected to both heat and gamma-radiation. These treatments would denature any existing proteases and inactivate the micro-organisms, and any breakdown could be validly attributed to non-enzymic activity. Heating to 85°C for 10 mins was used to inactivate plant proteases as other workers had demonstrated that this would be sufficient to inactivate them completely (Tracey, 1948; Brady, 1961; Preston and Kruger, 1976). The resulting curve, shown in Fig. 6.4 (●), demonstrates that at 20°C there was no acid activity above a pH of about 2.0. A comparison of these results with those found in the presence of plant enzyme activity (▲) suggests that the plant proteases were active below pH 3.0.

In order to separate the effects of acid hydrolysis and plant protease activity, and to determine the pH levels at which zero acid and protease activity occurred, the results shown in Fig. 6.4 were submitted to statistical analysis and smoothed

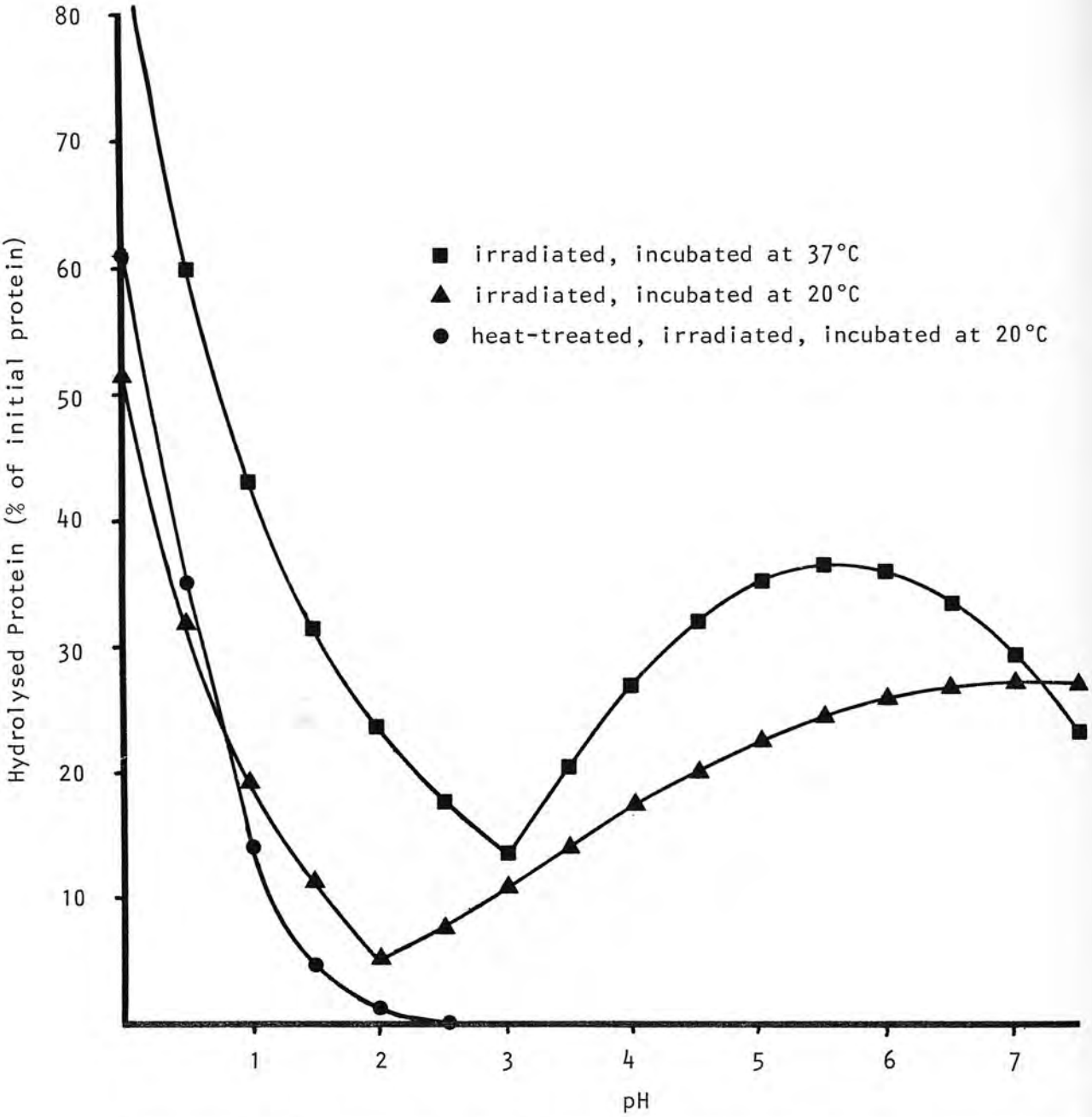


Figure 6.5. Experiment 2.3. Effect of pH on proteolysis in grass extract incubated for 120 days. Plot of estimated total response.

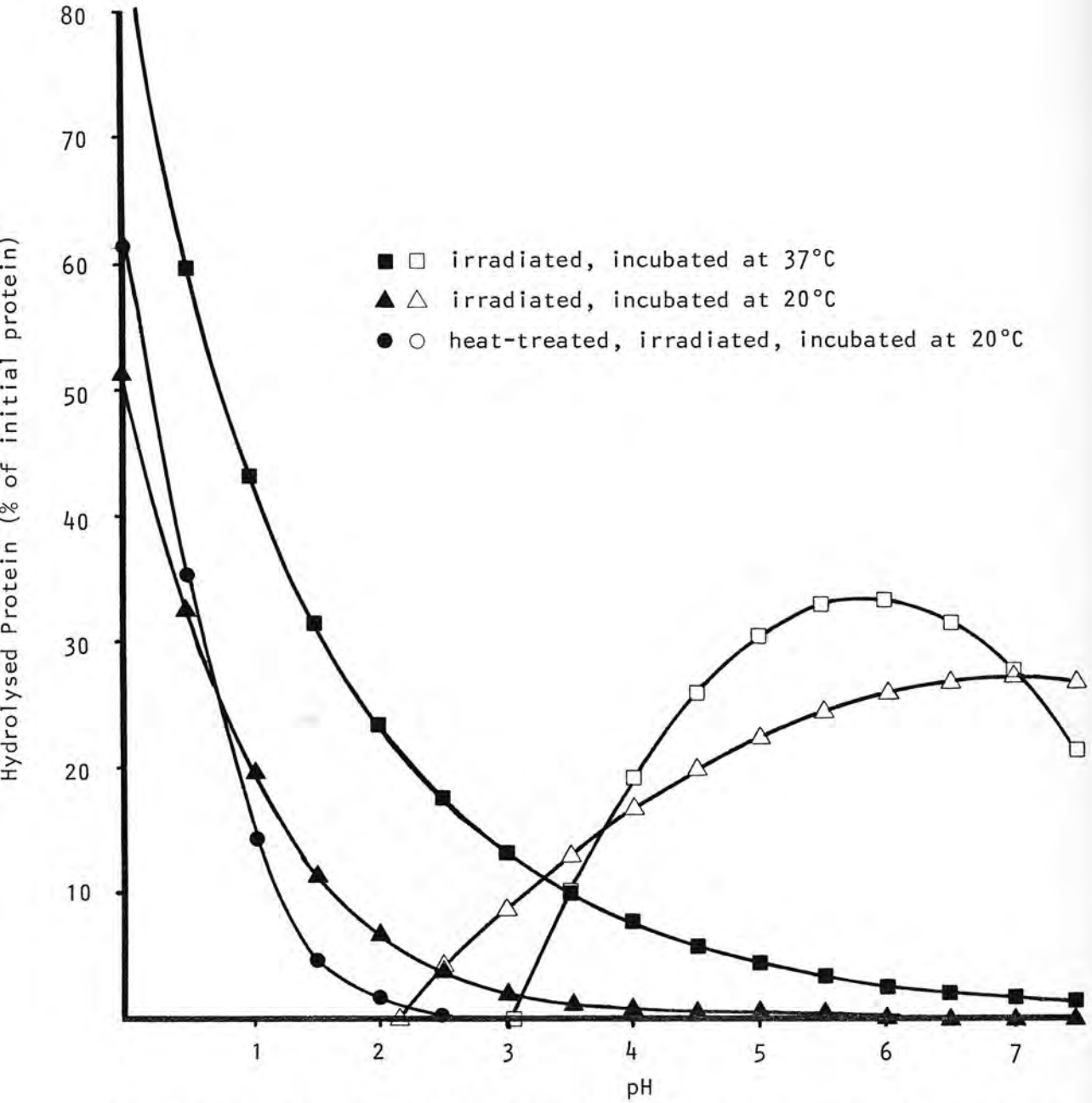


Figure 6.6. Experiment 2.3. Effect of pH on proteolysis in grass extract incubated for 120 days. Plot of estimated acid hydrolysis (■ ▲ ●) and plant protease activity (□ △ ○). The estimated values for ○ were all zero and are not shown.

TABLE 6.1

Estimated values of the parameters in the response curve equations and their standard errors.

	30°C for 120 days		20°C for 120 days		Heated then 20°C for 120 days	
Parameter	Est.	s.e.	Est.	s.e.	Est.	s.e.
A	87.62	6.39	51.36	6.18	61.28	4.85
B	0.71	0.08	0.98	0.15	1.47	0.10
C	0.89	0.10	1.10	0.18	1.41	0.15
L	111.49	12.26	28.80	5.77	-	-
M	49.69	4.69	15.92	2.46	-	-
N	4.26	0.44	1.13	0.25	-	-
Residual Mean Square (51 d.f.)	9.00		8.21		4.37	

curves produced. The best fit was found using the ARCUS-EUDOS program MINISQUARE, the optimising criterion being the minimum sum of squares of residuals. The estimated total responses are given in Fig. 6.5 and the separate estimates of acid hydrolysis and plant protease activity, in Fig. 6.6. The following equations apply:-

1) acid hydrolysis - fitted by the three parameter exponential $y_1 = A \exp^{(-Bx^C)}$
2) plant protease activity - fitted by the quadratic $y_2 = -L + Mx - Nx^2$ for positive values of y only
3) total response $y_1 + y_2$

The estimated values for the six parameters in the equations are given in Table 6.1.

The estimated curves fitted the observed results well as shown by the low values for the sum of squares of residuals (Table 6.1). From Fig. 6.6, zero protease activities at 20°C and 37°C were found to occur at about pH 2.2 and 3.0 respectively. Similarly, at lower temperatures there would be no acid hydrolysis at normal silage pH levels, but some hydrolysis may occur at higher temperatures.

The pH optima for plant proteases are known to be affected by temperature (Wittenbach, 1978) and in this experiment it appeared that the optimum pH at 20°C was slightly higher than at 37°C. In both cases, however, the optimum lay between pH 5.5 and 6.5 which agrees with the results of the previous two experiments. Similar pH optima have been found by other workers when measuring the activity of crude protease preparations from different species using endogenous substrate (Brady, 1961; Singh, 1962; Finley *et al*, 1980).

In most studies of plant proteases, artificial substrates have been used and these may not give a true representation of the pH optima of such enzymes when acting upon their natural substrates (Drivdahl and Thimann, 1977; Frith *et al*, 1978; Peoples and Dalling, 1978). This was clearly demonstrated by Wittenbach (1978) who incubated a crude extract from senescing wheat leaves, with several different substrates including ribulose diphosphate carboxylase, a natural substrate. The pH optimum, level of activity and pH range over which the proteases were active, were all found to differ according to the substrate.

It is also true to say that the activities of purified proteases *in vitro* may not give a true indication of their activities *in vivo* as the conditions of assay are unlikely to reflect the conditions in the leaf. In the leaf itself it is probable that many of the proteases are working at sub-optimal pH (Thomas, 1978).

In each of the three experiments reported here, protease activity took place over a wide pH range. This has also been found by other investigators assaying crude enzyme preparations (Brady, 1961; Singh, 1962; Finley *et al*, 1980) and it is probable that such pH profiles represent the combined activities of several proteases with different pH optima. Tracey (1948) suggested that the single peak, which he found with a partially purified tobacco leaf preparation, represented several proteases. In some studies in which only one peak was found, the presence of "shoulders" on the curve may indicate the existence of more than one protease (Peoples and Dalling, 1978). Frith *et al* (1978) found protease activity with an optimum at pH 4.2 using haemoglobin as substrate. When separated and purified the material was found to contain at least six separate proteases.

Proteolytic enzymes are always present in leaves (Thimann, 1980) and are active over a wide pH range (Frith and Dalling, 1980). Virtanen's claim (Virtanen, 1933) that plants do not contain proteases active below pH 4 has now been disproved as the recent surge of interest in leaf proteases has led to the discovery of many such enzymes (Martin and Thimann, 1972a; Preston and Kruger, 1976; Drivdahl and Thimann, 1977; Feller *et al*, 1977; Frith *et al*, 1978; Frith and Dalling, 1980). The results of this experiment show that perennial ryegrass contains proteases which are active below pH 2.5. Other studies have also demonstrated activity at these low pH levels (Feller *et al*, 1977; McKersie, 1981).

Most studies on leaf proteases have been related to their role in protein mobilization during seed germination and leaf senescence (McKersie, 1981). In both, rapid, extensive proteolysis occurs. Although some protease synthesis does take place, much of the activity results from the existing proteases. It follows therefore that these enzymes must be controlled in some way during most of the lifetime of the plant, only limited activity being necessary for protein turnover. The possible mechanisms for such control have already been discussed (section 2.2.2.7).

Various studies have shown that specific types of protease are more active at certain times. For instance, carboxypeptidases (acid pH optima) appear to be very important during seed germination (Mikola and Kohlemainen, 1972; Preston and Kruger, 1976). Acid proteases have been shown also to be particularly active in the early stages of leaf senescence in oats

(Martin and Thimann, 1972a) and Darnel grass (Thomas, 1978). In both cases the activity of the acid proteases fell sharply within a few days, and proteases with neutral and alkaline pH optima became dominant. Thomas (1978) suggested that this was because the acid proteases were less resistant to protein degradation.

A recent study by McKersie and co-workers (McKersie, 1981; McKersie and Buchanan-Smith, 1982) investigated the changes in the activities of specific proteases during the ensilage of lucerne. They identified several different proteases in the fresh herbage with pH optima at 4.5, 5.2, 5.5 and 7.0. Each showed activity over a broad pH range. During ensilage, proteolysis was virtually complete after two days, by which time the pH had fallen below 5.5. An examination of the individual protease activities showed that those with optima at 4.5 and 7.0 decreased very rapidly to less than 10% of their initial value. Despite the cessation of proteolysis, the other two enzymes remained potentially active. It was concluded that other factors such as substrate availability had been responsible for the lack of further proteolysis.

It has been shown that plants which are commonly ensiled do contain proteases which would be active at the pH levels encountered during ensilage. Although many of these enzymes are likely to be compartmentalized in the living cell, it is probably that, during ensilage, they will be released from the cells by mechanical disruption and plasmolysis. If they are stable under the conditions of ensilage, they may remain active for a considerable time unless other factors such as substrate accessibility become limiting.

6.6 CONCLUSIONS

Several conclusions can be drawn from the three experiments reported here:

1) Proteolysis is largely completed within the first few days of ensilage.
2) Ryegrass contains proteases active at the pH levels found in silage.
3) The overall optimum pH for ryegrass proteases is between 5.5 and 6.5.
4) Acid hydrolysis is unlikely to contribute significantly to proteolysis during ensilage, unless the temperature in the silo rises appreciably.
5) Lowering the pH by direct acidification before ensiling should inhibit proteolysis but may not prevent it.
6) The temperature in the silo will affect protease activity.

7. EXPERIMENT 3

THE EFFECTS OF INOCULATION, MINCING AND GLUCOSE ADDITION ON FERMENTATION DURING ENSILAGE

7.1 INTRODUCTION

The main objective in silage fermentation is the rapid achievement of a pH at which all biological activity virtually ceases (McDonald, 1984). In particular, it is important to bring about a rapid inhibition of coliforms and clostridia. If the decline in pH is delayed, allowing acetate production by these organisms, heterofermentative lactic acid bacteria may be encouraged. Beck (1978) has shown that these are more acetate-tolerant than the homofermentative species. The heterolactics are less desirable as they are less efficient at producing acid conditions (Gouet *et al*, 1979a) and, if they dominate and the sugar content is low, the pH may be lowered too slowly or to an insufficiently low level to prevent clostridial growth. (Woolford, 1972). In addition, if the water-soluble carbohydrate (WSC) content is low, the fructose/glucose ratio may become limiting, since heterolactic fermentation is particularly inefficient when fructose is the substrate (Woolford, 1972).

There are three main criteria for achieving natural preservation of crops as silage. These are (1) an adequate supply of fermentable substrate, (2) the presence of sufficient numbers of suitable lactic acid bacteria and (3) the rapid achievement and maintenance of anaerobic conditions within the silo (McDonald and Whittenbury, 1973; Burghardi *et al*, 1980).

The rate at which the pH is lowered is important as well as the

final value achieved (Whittenbury *et al*, 1967; McDonald, 1981; Woolford, 1984), since clostridia are capable of multiplying in silage almost as soon as the other bacteria (Gibson *et al*, 1958; Gouet *et al*, 1972).

It is frequently assumed that there are plenty of lactic acid bacteria on fresh crops but this may not be the case (Stirling, 1953; Kroulik *et al*, 1955a; Anderson, 1956; Gibson *et al*, 1958; Stirling and Whittenbury, 1963; Gouet *et al*, 1979b). In addition, those that are present may not be the most desirable from the point of view of an efficient fermentation (Whittenbury, 1968; McDonald and Whittenbury, 1973). Although inoculation will probably occur, to a certain extent, as a result of the presence of plant sap on harvesting machinery (Stirling and Whittenbury, 1963; Henderson *et al*, 1972), this is by no means certain. It would seem, therefore, that application of a suitable inoculum might improve the likelihood of a lactate fermentation. There has recently been a renewal of interest in the use of cultures of lactic acid bacteria, despite the disappointing results of earlier studies (Watson and Nash, 1960).

The longer an inoculum takes to establish itself and reach optimum numbers, the longer it will be before the development of undesirable anaerobes is arrested. It is, therefore, imperative that suitable species are chosen (McCullough, 1977). Whittenbury (1961) laid down a list of criteria to which such organisms should conform (see page 73). *Lactobacillus plantarum* proved to be the most suitable but, owing to the fact that its growth is rather slow above about pH 5 (Buchanan and Gibbons,

1974), another species, which grows better at the higher pH levels, is usually incorporated into the inoculum. Such a homofermentative inoculum should overwhelm the resident bacterial population and ensure a rapid acidification of the medium.

Frequently, crops do not ensile satisfactorily because they have a low WSC content. The problem is exacerbated if the dry matter is low (McDonald *et al*, 1962), as a lower pH is then necessary in order to inhibit the clostridia (Watson and Nash, 1960; Carpintero *et al*, 1969), or if the buffering capacity is high as in legumes (McDonald and Whittenbury, 1973). If this is the case, and particularly when the fermentation is dominated by heterolactics, the production of acid may be too slow or too low to prevent clostridial growth. Even in cases where the WSC content is relatively high, they may not be immediately available for fermentation, owing to the necessity for some plasmolysis to release the cellular contents (Greenhill, 1964, 1964a).

The problem of substrate availability may be overcome directly by adding a source of sugars, or indirectly by mechanically damaging the crop to release the cell contents (Wieringa, 1960; Weise, 1968; Crawshaw, 1977; Marsh, 1978). In addition to releasing the plant juices, mechanical treatment (chopping, bruising, lacerating, mincing etc) allows the crop to be consolidated more easily and hence encourages rapid anaerobiosis (Gibson *et al*, 1961; McDonald *et al*, 1964; Zimmer, 1977).

The experiment described here was devised to investigate the effects of inoculation, glucose addition and mincing on the efficiency of fermentation.

7.2 EXPERIMENTAL

Grass from a mixed Italian/perennial ryegrass sward was harvested using a "Haldrup" reciprocal mower-type plot harvester and, after hand-chopping to approximately 20mm lengths, was ensiled in 200g capacity laboratory silos directly (A) and after three levels of inoculation i.e. 10^4(B), 10^6(C) and 10^8(D) organisms g^{-1} fresh material. The inoculum applied was a new commercial product manufactured by EWOS AB, Södertälje, Sweden. It is currently (1984) being distributed under the name "Clampdown" but was previously known as either "Siloferm" or "S99". The inoculum contains approximately equal numbers of *Lactobacillus plantarum* and *Pediococcus acidilactici* and the manufacturers recommended application rate is 10^6 organisms g^{-1}. The product is supplied in a freeze-dried form and is reconstituted in water one hour before application, in order to allow the bacteria to reach the exponential phase of growth. The inoculum was made up to different strengths and applied at a rate of 20 ml kg^{-1} fresh grass.

The effects of mincing (M) and glucose addition (G) were also investigated. Mincing was achieved using a domestic meat mincer (screen diameter 8mm). Glucose was added as a 71.4% solution at the rate of 28 ml kg^{-1} (20 gkg^{-1}) of fresh material.

Equal volumes of liquid (48 ml kg^{-1}) were added for each treatment, water being added when necessary.

The silos described were sealed with plastic fermentation traps filled with water, and incubated at room temperature (c. 20°C).

TABLE 7.1

Experiment 3. Dry matter losses

Treatment*	Dry matter loss (%)	
	24h	76d
AO	2.81(+)	1.58(+)
AM	2.27(+)	4.26
AG	0.80	5.94
BO	1.16(+)	0.38(+)
BM	1.49(+)	7.69
BG	3.90	8.33
CO	2.95(+)	0.45(+)
CM	2.74(+)	3.00
CG	5.21	9.00
DO	1.40	3.53
DM	2.06(+)	5.13
DG	2.73	7.30

* A - no inoculum
B - 10^4 organisms g^{-1}
C - 10^6 organisms g^{-1}
D - 10^8 organisms g^{-1}

O - chopped (c. 20 mm)
M - minced (8 mm screen)
G - chopped (c. 20 mm) + glucose ($20gkg^{-1}$)

They were opened after 24h and 76 days when comprehensive analyses were made using the techniques described in sections 4 and 13. Each treatment was replicated three times.

7.3 RESULTS AND DISCUSSION

In laboratory silos, dry matter (DM) losses are considered to result mainly from the production of CO_2 during fermentation (see page 95). The losses incurred in this experiment are given in Table 7.1.

There were negligible losses in the untreated control (A0) and, on the whole, inoculation had little effect. This was to be expected since the inoculum was homofermentative.

There were no losses in the minced silages after 24h but significant losses did occur during the later stages of fermentation. It was noted that, in these silos, considerable quantities of effluent were lost through the fermentation traps and this would have accounted for some of the losses of dry matter. Mechanical pre-treatment might be expected to increase effluent production owing to tissue breakage (Woolford, 1984) and in most studies this does appear to be the case, although the effects seem to vary with the species and DM content of the herbage (McDonald, 1981). In the farm situation the increased losses would tend to be offset by a reduction in respiration losses owing to better consolidation, but in laboratory silos respiration losses are negligible. In this experiment the production of effluent was probably encouraged by the low dry matter content of the fresh material (153 gkg^{-1}).

The remainder of the dry matter losses in the minced silages may have been caused by yeast activity since ethanol production was quite high

in these silages (Table 7.4).

Glucose addition also brought about an increase in the dry matter losses and this was apparent even after 24h. Higher losses might have been expected in the non-inoculated silages (A) since it is likely that some of the added glucose would be fermented by heterolactic pathways. This would lead to higher CO_2 losses than if fructose was the substrate (McDonald and Whittenbury, 1973). This does not explain the high losses in the inoculated silages in which presumably the homolactic bacteria dominated. It was noted, however, that the ethanol contents of these silages were particularly high which suggests yeast activity. The numbers of yeasts were high in all silages (Table 7.5) but were not any higher in these particular silages. It would seem likely that the immediate availability of fermentable substrate in the minced and glucose supplemented silages, particularly the latter, made it possible for yeasts to produce ethanol despite the rapidly growing population of lactic acid bacteria.

The main biochemical and microbial constituents of the grass and silages are given in Tables 7.2 to 7.8. In the microbial analyses the pH of the tween acetate plates (for counting lactic acid bacteria) was found to be too high owing to a mistake in the buffer concentration. This meant that the counts were more representative of a total count. Since this was already determined on plate count agar, the tween acetate counts are not shown.

The pH of the untreated control silage (A0) declined very little during the first 24h (Table 7.2) but inoculation brought about a rapid decrease in pH, the effect increasing with the rate of

TABLE 7.2

Experiment 3. Dry matter, pH, water-soluble carbohydrates and lactic acid

Treatment	DM (gkg^{-1})		pH		WSC (gkg^{-1}DM)		Lactic Acid (gkg^{-1}DM)	
Grass	153		5.88		180		ND	
Silages*	24h	76d	24h	76d	24h	76d	24h	76d
AO	154	156	5.75	3.75	188	9	ND	150
AM	156	157	5.75	3.77	215	1		113
AG	170	166	5.70	3.76	267	45		135
BO	153	157	5.43	3.70	181	71		166
BM	157	149	5.71	3.68	207	76		160
BG	166	164	5.76	3.66	283	62		155
CO	156	154	4.54	3.57	155	46		166
CM	157	157	5.07	3.61	212	63		188
CG	166	165	4.79	3.59	227	39		165
DO	163	157	4.03	3.56	139	51		180
DM	164	156	4.19	3.53	180	85		166
DG	179	168	4.20	3.53	200	29		164
SED (24 d.f.)								
A, B, C, D	-	-	0.039	0.015	4.9	9.4		6.5
O, M, G	-	-	0.034	NS	4.2	NS		NS
Interaction	-	-	0.068	NS	8.5	16.2		NS

* see Table 7.1 for key

ND not determined
NS not significant

TABLE 7.3

Experiment 3. The initial WSC content of ensiled herbage and the amount of WSC apparently utilized during fermentation

Treatment*	Initial WSC ($gkg^{-1}DM$)	WSC utilized+			
		24 hour		76 day	
		gkg^{-1} fresh	% of initial	gkg^{-1} fresh	% of initial
AO	180	1.9(+)	7.0(+)	24.9	90.6
AM	180	6.0(+)	21.9(+)	27.4	99.3
AG	277	2.0	4.4	38.7	84.6
BO	180	0.4(+)	1.5(+)	15.9	57.7
BM	180	5.1(+)	18.4(+)	16.9	61.2
BG	277	3.4	7.4	36.4	79.6
CO	180	3.0	10.8	19.5	70.8
CM	180	5.8(+)	21.0(+)	18.3	66.3
CG	277	10.3	22.5	37.8	82.7
DO	173	4.9	17.7	18.8	68.2
DM	173	1.8(+)	6.4(+)	14.6	53.1
DG	267	12.4	27.1	41.0	89.7

* see Table 7.1 for key

\+ This figure does not take into account any extra WSC made available from polysaccharide breakdown

inoculation. A pH of 4.03 was reached at the highest rate ($10^8 g^{-1}$). The comparable figure for the untreated control was 5.75 which demonstrates clearly the potential of inoculation for bringing about rapid acidification.

Both mincing and glucose addition inhibited the rate of decline of pH significantly. This was surprising as both of these treatments had been expected to stimulate a lactate fermentation, and hence acidification, by providing a readily available supply of sugars.

After 76 days, the pH values of all the silages were low and similar, although they tended to be lower with increasing rates of inoculation.

The apparent quantities of water soluble carbohydrate utilized during ensilage, were calculated from the difference between the WSC ensiled and that present after opening the silos, taking into account the added glucose (Table 7.3). These figures, together with the WSC contents of the silages (Table 7.2), indicate that during the first 24h, little fermentation had occurred in the non-inoculated (A) and low inoculum (B) silages. In fact, an increase in WSC had taken place in treatments O and M, which suggests that hydrolysis of polysaccharide had occurred. The increase was particularly noticeable in the minced silages and was apparent at the higher inoculation rates too. The higher levels of WSC in the minced, compared with the chopped silages, suggests either that WSC fermentation was slower in the former or that mincing caused an acceleration of plant polysaccharide hydrolase activity. The fact that the decline in pH was slower

TABLE 7.4

Experiment 3. The acetic acid, ethanol and gross energy contents of the 76-day silages

Treatment*	Acetic+ Acid ($gkg^{-1}DM$)	Ethanol ($gkg^{-1}DM$)	Gross Energy ($MJkg^{-1}DM$)
AO	26.55	21	18.6
AM	53.10	25	19.3
AG	39.86	34	18.9
BO	11.92	16	18.6
BM	6.67	24	19.3
BG	11.61	49	19.3
CO	10.46	11	18.6
CM	5.66	18	18.9
CG	8.76	39	19.3
DO	7.92	13	17.2
DM	3.80	27	18.4
DG	6.54	52	18.5
SED (24 d.f.)			
A, B, C, D	1.770	NS	NS
O, M, G	NS	3.9	NS
Interaction	3.066	NS	NS

* see Table 7.1 for key

\+ no other acids were detected

NS not significant

in the minced silages supports the former conclusion but the latter is supported by the observation that immediately after mincing (ie before ensiling) a 14% increase in WSC took place. It is probable that the final outcome was the result of a combined effect.

The figures in Table 7.3 suggest that more fermentation had occurred in the silages to which glucose had been added but this did not result in a lower pH. It is possible that neutral products were formed and, in fact, the ethanol contents were higher (Table 7.4).

Inoculation led to a higher residual sugar content, presumably because of a more efficient fermentation. The effects of mincing and glucose were inconsistent.

After 76 days, all the silages had high lactic acid contents (Table 7.2), particularly the inoculated materials. Increasing the level of inoculation did not increase the concentration of lactic acid. Neither did mincing nor the addition of glucose, despite the fact that more sugar appeared to have been utilized in the latter. As already mentioned, however, the ethanol content of these silages was somewhat higher.

As expected, the homofermentative inoculum led to a reduction in the concentration of acetic acid (Table 7.4). When an inoculum was not applied, mincing brought about an increase in acetate concentration. This phenomenon has been found by others (Wieringa, 1959b; Seale *et al*, 1982) and suggests activity by coliforms and/or heterofermentative lactic acid bacteria. After

TABLE 7.5

Experiment 3. Microbial analyses (log_{10} organisms g^{-1} fresh herbage)

Treatment*	Total	Streptococci	Coliforms	Yeasts	Lactate fermenters	Proteolytic species
Grass						
Standing Crop	7.20	3.95	4.26	5.20	3.78	<3.00
Chopped	7.92	4.81	5.60	6.23	4.00	<3.00
24h Silages						
AO	7.73	7.58	6.79	7.40	6.49	<3.00
AM	7.67	7.11	6.65	7.45	5.89	<3.00
AG	8.23	7.52	7.00	7.77	5.67	<3.00
BO	8.00	7.48	6.76	7.60	3.66	<3.00
BM	7.71	7.23	6.59	7.18	6.56	<3.00
BG	8.08	7.67	6.83	7.56	6.15	<3.00
CO	8.34	8.18	6.40	7.18	6.28	<3.00
CM	8.00	7.90	6.65	7.08	6.48	<3.00
CG	8.00	8.04	6.52	7.28	6.11	<3.00
DO	8.62	7.92	4.00	5.36	5.80	<3.00
DM	8.38	7.79	4.08	4.75	6.28	<3.00
DG	8.51	8.49	4.43	4.57	6.26	<3.00
76d Silages						
AO	8.95	<2.00	<2.00	c.7.70	<2.00	<2.00
AM	7.68	<2.00	<2.00	c.7.70	<2.00	<2.00
AG	7.94	<2.00	<2.00	c.7.70	<2.00	<2.00
BO	6.32	<2.00	<2.00	4.60	<2.00	<2.00
BM	6.60	<2.00	<2.00	5.67	<2.00	<2.00
BG	7.08	<2.00	<2.00	5.20	<2.00	<2.00
CO	6.26	<2.00	<2.00	5.23	<2.00	<2.00
CM	6.30	<2.00	<2.00	5.34	<2.00	<2.00
CG	6.63	<2.00	<2.00	5.23	<2.00	<2.00
DO	6.36	<2.00	<2.00	4.52	<2.00	<2.00
DM	6.11	<2.00	<2.00	5.52	<2.00	<2.00
DG	5.78	<2.00	<2.00	5.11	<2.00	<2.00

* see Table 7.1 for key

24h the numbers of coliform bacteria in the minced non-inoculated silage were relatively high but were similar in those which were not minced or had had glucose added (Table 7.5).

Despite the high counts after 24h for lactate fermenters (Table 7.5), no butyric acid was detected. This is unusual but it may be that these organisms were present as inactive spores, rather than in vegetative form.

Ethanol concentration was unaffected by inoculation with homofermentative bacteria but both mincing and the addition of glucose caused an increase, especially the latter. This is likely to be the result of activity by yeasts or heterofermentative lactic acid bacteria. As has already been mentioned, it is unlikely that heterolactics were present in appreciable numbers in the inoculated silages therefore yeasts were probably responsible.

The results show that inoculation was very successful in producing a rapid lowering of the pH. Owing to a more efficient fermentation the lactic acid content was greater despite a higher concentration of residual sugars than in the non-inoculated silages. The mincing and glucose treatments were unsuccessful. There was no improvement in the rate of acidification and, in the case of the non-inoculated material, mincing appeared to encourage a heterofermentative fermentation as shown by the lower lactate and higher acetate contents of the silages. Addition of glucose had a similar though lesser effect. Glucose treatment appeared to favour ethanol production probably as a result of the activity of yeasts. The fact that the decline in pH was slow in the untreated control, that inoculation increased the rate of decline and that mincing and the addition of glucose did not, suggests that the limiting factor

TABLE 7.6

Experiment 3. The nitrogenous constituents of the grass and silages

Treatment	TN ($gkg^{-1}DM$)		PN ($gkg^{-1}TN$)		VN ($gkg^{-1}TN$)		Amide N ($gkg^{-1}TN$)	
Grass	26.2		797		18.7		22.4	
Silages*	24h	76d	24h	76d	24h	76d	24h	76d
AO	26.5	26.9	619	264	35	114	ND	9.6
AM	27.2	28.2	603	349	32	104		13.8
AG	24.7	24.6	644	255	30	108		15.9
BO	24.5	27.8	626	286	29	94		13.2
BM	27.0	26.9	609	353	28	82		18.9
BG	24.6	25.0	650	262	28	106		13.6
CO	28.3	27.2	644	358	23	60		28.2
CM	26.2	27.3	579	389	26	51		26.6
CG	25.2	25.6	677	355	26	53		25.9
DO	25.1	26.0	596	301	21	48		27.8
DM	25.3	26.8	577	376	23	41		35.3
DG	23.8	24.0	623	312	21	47		43.9
SED (24 d.f.)								
A,B, C, D	-	-	10.3	6.0	1.5	2.6		2.1
O, M, G	-	-	8.9	5.2	NS	2.3		1.8
Interaction	-	-	NS	10.3	NS	4.6		3.6

* see Table 7.1 for key

ND not determined
NS not significant

was the population of lactic acid bacteria, rather than substrate availability.

In another experiment using this inoculum on lucerne of low water-soluble carbohydrate content (Henderson, *et al*, 1984), the control silage was badly preserved but inoculation produced a lower pH and the effect was greatly enhanced in the presence of added sugar.

The effect of the treatments on the main nitrogenous constituents are shown in Tables 7.6.

Inoculation did not reduce greatly the amount of proteolysis which took place in the first 24h but, after 76 days, least protein breakdown had occurred at the inoculation rate of $10^6 g^{-1}$(C). No further improvement was evident when the rate was increased to $10^8 g^{-1}$. After 24h, the greatest breakdown was in the minced silages. The addition of glucose appeared to have a beneficial effect at this stage but, after 76 days, it was the minced material which had the highest residual protein content. There were no significant differences between treatments 0 and G. The most effective treatment, with respect to protein preservation, was inoculation of minced grass at a rate of $10^6 g^{-1}$ of fresh grass. The addition of glucose was not as effective as mincing despite a slightly more rapid decline in pH in the former case.

Inoculation helped to reduce deamination reactions throughout the period of ensilage. Mincing and the addition of glucose had no effect during the initial stages but mincing did reduce deamination significantly in the later stages.

TABLE 7.7

Experiment 3. The ratio of amino acid nitrogen in fresh grasses

Amino Acid	Mixed Ryegrass	Perennial[a] Ryegrass	Italian[b] Ryegrass
Aspartic Acid+	9.6	10.2	9.3
Threonine	5.1	4.6	4.6
Serine	4.8	5.2	5.1
Glutamic Acid+	10.6	9.0	8.7
Proline	-	4.7	4.3
Glycine	8.3	10.0	8.0
Alanine	10.6	10.6	9.4
Valine	5.9	6.6	6.4
Isoleucine	4.0	3.5	3.9
Leucine	7.1	6.9	8.7
Tyrosine	2.4	2.2	2.3
Phenylalanine	3.9	3.6	4.0
Histidine	5.0	-	-
Lysine	8.0	9.4	10.5
Arginine	14.8	13.5	14.8
Total	100.0	100.0	100.0

a - from experiment 1

b - from Ohshima *et al* (1979)

+ - includes amide nitrogen

TABLE 7.8

Experiment 3. Total amino acid nitrogen ($gkg^{-1}TN$) in grass and silages

	GRASS*	SILAGES*				SED
		AO	DO	DM	DG	
Asp+	58.66	56.82	60.84	61.19	61.75	NS
Thr	31.02	28.17	25.89	26.29	28.73	NS
Ser	29.14	27.93	27.20	27.81	29.86	NS
Glu+	64.90^{a}	37.23^{b} (57.4)	54.22^{a} (83.5)	60.77^{a} (93.6)	60.29^{a} (92.9)	5.124
Gly	50.50	56.08	62.38	52.16	55.23	NS
Ala	64.54^{ab}	71.12^{a} (110.2)	61.77^{b} (95.7)	57.36^{b} (88.9)	65.84^{ab} (102.0)	3.926
Val	36.29^{a}	40.97^{b} (112.9)	36.34^{a} (100.1)	35.02^{a} (96.5)	37.43^{ab} (103.1)	4.282
Ile	24.48	26.36	24.52	24.38	24.53	NS
Leu	43.28	46.03	43.48	50.50	43.00	NS
Tyr	14.64^{a}	8.98^{b} (61.3)	14.18^{a} (96.9)	14.96^{a} (102.2)	15.79^{a} (107.9)	3.330
Phe	23.55	23.77	22.89	22.55	23.14	NS
His	30.43	24.55	29.21	28.66	30.55	NS
Lys	49.02^{a}	40.94^{b} (83.5)	57.77^{c} (117.9)	54.50^{ac} (111.2)	56.82^{c} (115.9)	6.126
Arg	90.25^{a}	26.89^{b} (29.8)	68.13^{c} (75.5)	72.52^{c} (80.4)	72.54^{c} (80.4)	5.224
Total	610.67^{a}	515.84^{b}	588.82^{a}	588.67^{a}	605.50^{a}	62.646

* Values on the same line with different superscripts differ significantly ($P<0.05$). The data in parentheses show the % recovery after ensilage.

\+ includes amide nitrogen

NS not significant

The concentration of amides decreased in the non-inoculated silages and those inoculated at low levels, but increased at the higher inoculation rates. This suggests that heterofermentative lactic acid bacteria may be responsible for amide breakdown during ensilage, probably by deamination. Silages with low concentrations of amides tended to have high volatile nitrogen contents. Conversely, it would appear that homolactics are responsible for an increase in amides.

The fresh grass and a few of the silages were subjected to amino acid analysis to examine the changes taking place in the individual amino acids during ensilage.

It is of interest to compare the ratio of amino acids in the fresh material with those of several other similar grasses (Table 7.7). The results show clearly how consistently uniform these ratios are. This is in agreement with the theory that the amino acid ratio varies little between plants, regardless of species (Lyttleton, 1973). In view of this, it is not surprising to find that the same six amino acids (arginine, glutamic acid, alanine, aspartic acid, glycine and lysine) as in Experiment 1, account for the same percentage (c. 60%) of the total amino acid nitrogen and their order of concentration is the same (Table 7.8).

Very little amino acid degradation has occurred in any of the silages, including the untreated control (A0).

Extensive amino acid degradation is usually associated with the activities of proteolytic clostridia and the microbial analyses (Table 7.5) showed these to be absent in all of the silages throughout the period of ensilage. The only significant changes in the control were large decreases in the concentrations of arginine, glutamic acid

and tyrosine, a smaller decrease in the concentration of lysine and a small increase in the concentration of valine. It is interesting to note that the amino acids which were catabolized to the greatest extent were those present at the highest concentrations originally.

The recoveries of arginine and glutamic acid were similar to those found for the control silage in Experiment 1. Extensive breakdown of arginine by micro-organisms is known to occur during ensilage, even in the absence of clostridial activity (Macpherson and Violante, 1966, 1966a; De Vuyst *et al*, 1968c; Ohshima, 1971; Ohshima *et al*, 1979; Moreau *et al*, 1982; Mortensen, 1982). Arginine is first deaminated to citrulline then decarboxylated to ornithine (Ohshima *et al*, 1979). Glutamic acid is considered to be decarboxylated to γ-aminobutyric acid mainly by plant enzymes in the early stages of ensilage (Macpherson and Slater, 1959; Ohshima, 1971; Ohshima and McDonald, 1978).

The decreases in tyrosine and lysine were probably the result of decarboxylation to their respective amines (Macpherson, 1962; Macpherson and Violante, 1966, 1966a; Hughes, 1969, 1970; Ohshima *et al*, 1979). Ohshima *et al* (1979) found substantial decreases in tyrosine and lysine concentrations during ensilage of lucerne and Italian ryegrass, but most was accounted for by increases in the respective amines tyramine and cadaverine. Hughes (1970) reported similar findings.

Apart from an increase in lysine in silages DO and DG, the only significant change in the amino acid concentrations of the other three silages, was a small decrease in arginine. This reduction in amino acid catabolism is presumably because of rapid acidification

brought about by inoculation. Macpherson and Violante (1966a) showed that degradation of lysine and arginine could be reduced markedly by rapid acidification and concluded that the same would apply to other amino acids. Mortensen (1982) also stated that lowering of the pH helped to preserve the amino acids.

The results of the analysis of the nitrogenous constituents indicate that inoculation did increase protein preservation to a certain extent, but had a greater effect in preventing deamination. The rapid attainment of a low pH appeared to prevent amino acid degradation. Mincing reduced proteolysis to a limited extent but had little effect on deamination. Glucose additon proved to be entirely unsatisfactory with respect to reducing either proteolysis or deamination.

These results demonstrate that even a decline in pH to 4.0 within 24h is insufficient to prevent proteolysis, and provides further evidence to refute the claim that proteolysis is negligible at pH 4 (Virtanen, 1933; Macpherson, 1952a). This is in agreement with the results of Carpintero *et al* (1979) who showed that even direct acidification to a pH of less than 4.0 did not prevent proteolysis although it did inhibit it.

7.4 CONCLUSIONS

Inoculation with homofermentative lactic acid bacteria stimulated a more efficient fermentation, leading to a more rapid acidification and producing silages with higher lactate and lower acetate contents. There was some reduction in proteolysis and a considerable decrease in deamination. This, together with the higher residual WSC content of the inoculated silages, may increase the efficiency of nitrogen utilization by the ruminant. The rate of 10^6 organisms g^{-1} fresh material appeared

to be adequate.

Although mincing did not encourage acidification, it did reduce proteolysis, particularly in the non-inoculated herbage. It did not, however, reduce deamination or increase residual sugar levels in such material. When used in conjunction with inoculation at 10^6 g^{-1}, it brought about further small decreases in proteolysis and deamination but not enough to warrant the combined treatments.

In this experiment, the addition of glucose was ineffective but this may have been because the original WSC content was relatively high. Other studies have shown the addition of readily available carbohydrate to be beneficial particularly when the WSC content of the original herbage was low (Lanigan, 1961; Carpintero *et al*, 1969; Ohyama *et al*, 1973, 1973a; Gouet *et al*, 1979b).

8. EXPERIMENT 4

THE EFFECTS OF FORMIC ACID AND FORMALDEHYDE ON FERMENTATION DURING ENSILAGE

8.1 INTRODUCTION

Although ideal from the point of view of preservation, there is considerable evidence to indicate that the products of a lactate fermentation may have certain undesirable nutritional characteristics (McDonald, 1984). Ensiling generally has little effect on either digestibility or net energy value of the herbage but it may result in substantial reductions in voluntary dry matter intake and in the efficiency of nitrogen utilization (Wilkinson *et al*, 1976; Wilkins, 1980). The products of silage fermentation which inhibit intake have not yet been identified (McDonald, 1984) but high concentrations of fermentation acids and the products of clostridial fermentation have both been implicated (Wilkins, 1980). The depressed efficiency with which the nitrogenous constituents of silage may be utilized is associated with their highly soluble nature. This, coupled with a low content of water-soluble carbohydrates (WSC), may result in high ammonia concentrations in the rumen, substantial losses of nitrogen in the urine and low levels of nitrogen retention by the animal (McDonald and Edwards, 1976; Wilkins, 1980). In addition, a higher proportion of dietary protein will be broken down in the rumen and absorbed and this may result in a reduced supply of amino acids to the small intestine (Barry, 1976; Beever, 1980).

In the present economic climate there is pressure for conserved forages to satisfy an increasing proportion of the needs of the animal. This requires that their nitrogen be utilized with

greater efficiency, which involves protection of the original protein against breakdown, both during ensilage and in the rumen, and conservation of the water-soluble carbohydrates of the herbage.

Control of fermentation and improvement of the nutritive value of silage can be achieved by the application of formic acid to the crop. This brings about an immediate decrease in pH, and the amount of fermentation which occurs subsequently is reduced. The silage which results contains lower concentrations of acids and higher residual protein and WSC contents (McDonald, 1981). Formic acid treatment of silage improves dry matter intake and nitrogen utilization (Waldo *et al*, 1969; Castle and Watson, 1970b; Barry, 1975; Waldo, 1977; Wilkins, 1980).

Although formic acid used at the commercial application rate (c. 2.5 kgt^{-1}) inhibits proteolysis, it is still extensive (McDonald, 1984). Crawshaw (1977) considered that protection of protein during ensiling was of equal importance in silage research studies with efforts to restrict fermentation, and could be of greater importance on the commercial farm where silage was often in limited supply and maximum intake was not always desirable.

Formalin (40% w/v formaldehyde) inhibits fermentation and has a greater effect than formic acid in protecting the protein fraction from degradation during ensilage and subsequently in the rumen (Wilson and Wilkins, 1977; Siddons *et al*, 1979). Silages made with formalin have high residual protein and WSC contents and contain low concentrations of acids and volatile nitrogen (Wilkins *et al*, 1974; Thomas, 1978a; Vanbelle *et al*, 1978).

The effects of formaldehyde on intake and nitrogen utilization are dependent upon the level applied (Wilkins *et al*, 1974). Vanbelle *et al* (1978) found that, at a rate of 3-4 kgt^{-1} (60-100 g formaldehyde per kg crude protein), formaldehyde was an excellent preservative, but had adverse effects on rumen fermentation and nitrogen digestibility. These detrimental effects were reduced at 2 kt^{-1} but they recommended that no more than 1 kgt^{-1} should be applied. At such low levels, however, it is possible to stimulate a clostridial fermentation (Wilkins *et al*, 1974; McDonald *et al*, 1983) and it is necessary therefore to include an acid to ensure successful preservation. The acid most commonly used is formic but economic factors may lead to its replacement by the cheaper sulphuric acid which has been shown to be equally effective (Wilson and Wilkins, 1977).

There are reports in the literature of many different combinations of formic acid and formaldehyde, applied at various rates (Valentine and Brown, 1973; Waldo *et al*, 1973; Wilkins *et al*, 1974; Barry, 1975; Hinks and Henderson, 1977; Wilson and Wilkins, 1977; Arnould *et al*, 1978; Vanbelle *et al*, 1978; Van Bockstaele *et al*, 1978; Hinks *et al*, 1978; Moreau *et al*, 1982) but the trend appears to be towards the use of a low level of formaldehyde with a moderate level of acid (McDonald, 1984). The experiment described here investigates the effects on fermentation of a low level of formaldehyde applied alone, with various levels of formic acid, and the same levels of acid applied without formaldehyde.

TABLE 8.1

Experiment 4. Dry matter losses

Treatment*	Dry matter loss (%)	
	24h	125d
0A	3.59	2.51
0B	2.28(+)	4.10
0C	1.94(+)	1.83
0D	1.31(+)	0.36(+)
FA	2.75(+)	0.67(+)
FB	2.76(+)	0.98
FC	3.58(+)	0.25
FD	1.33(+)	0.56

* A - no Add-F
B - 2.3 lt^{-1} Add-F
C - 4.6 lt^{-1} Add-F
D - 6.9 lt^{-1} Add-F

0 - no formalin
F - 1.8 lt^{-1} formalin (17.6 g formaldehyde per kg crude protein)

8.2 EXPERIMENTAL

Italian ryegrass was harvested using a "Haldrup" reciprocal mower-type plot harvester and, after hand-chopping to approximately 20 mm lengths, was ensiled directly in 200 g capacity laboratory silos (treatment 0A), after addition of formalin* at the rate of 1.8 lt^{-1} (17.6 g formaldehyde per kg crude protein) (treatment FA) or after addition of "Add-F"* at 2.3, 4.6 and 6.9 lt^{-1} (treatments 0B, 0C and 0D respectively). The three acid treatments were repeated with the addition of formalin at the previous level (treatments FB, FC and FD respectively).

The silos were sealed with plastic fermentation traps filled with water, and incubated at room temperature (c. 20°C). Some were opened after 24h and others after 125 days when comprehensive analyses were made using the techniques described in Sections 4 and 13. Each treatment was replicated three times.

8.3 RESULTS AND DISCUSSION

The dry matter losses incurred in this experiment are shown in Table 8.1. In most cases there was an apparent increase in the amount of dry matter recovered after 24h. This is not possible, and presumably reflects inaccuracies in the dry matter determinations of these silages which were carried out by drying in an oven. The results in Table 8.2 suggest that the estimates were rather high which indicates that either the samples were not completely dried initially or they were allowed to pick up water before weighing.

*formalin (40% w/v formaldehyde)
Add-F (85% w/w formic acid) - manufactured by BP Chemicals International Ltd.

TABLE 8.2

Experiment 4. Dry matter, pH, water-soluble carbohydrates and lactic acid

Treatment*	DM (gkg^{-1})		pH			WSC ($gkg^{-1}DM$)		Lactic Acid ($gkg^{-1}DM$)	
Grass	243		6.11			144		ND	
Silages	24h	125d	0h	24h	125d	24h	125d	24h	125d
OA	238	238	6.11	5.71	3.77	160	22	ND	114
OB	250	236	4.49	4.72	3.83	182	158		58
OC	251	243	3.98	4.19	4.05	179	230		11
OD	252	249	3.75	3.83	3.90	173	211		8
FA	250	246	6.05	5.86	3.85	165	93		80
FB	252	242	4.44	4.71	4.59	165	229		13
FC	255	248	4.05	4.15	4.13	170	213		16
FD	251	249	3.72	3.91	3.85	172	192		16
SED (16 d.f.)									
A, B, C, D	-	-	-	0.018	0.018	3.6	9.9		5.6
O, F	-	-	-	0.013	0.013	2.5	7.0		4.0
Interaction	-	-	-	0.026	0.026	5.0	14.0		7.9

* see Table 8.1 for key

ND not determined

The dry matter losses after 125 days were all low. There was a tendency for them to be somewhat lower in the silages treated with formalin although such treatment does not usually result in improved recoveries (Thomas, 1978a; Woolford, 1984). Formic acid treatment generally results in improved recoveries of dry matter (Waldo, 1977) provided a yeast ethanolic fermentation has not been stimulated (Henderson and McDonald, 1971; Henderson *et al*, 1972) but, since all recoveries were high, no marked improvement was discernible.

The biochemical and microbial constituents of the grass and silages are given in Tables 8.2 to 8.7. There are no counts for lactic acid bacteria in Table 8.4 for reasons given previously (page 128).

The pH of the control silage began to fall within the first 24h resulting in a well-preserved lactate silage after 125 days. Formic acid was added at three levels. The lowest, which corresponded to the commercial rate (2.3 lt^{-1} of Add-F), reduced the pH immediately to 4.5. According to Woolford (1975), this should be sufficient to inhibit coliforms and clostridia. Pedersen *et al* (1973) showed this to be the case in practice. At this pH the lactic acid bacteria were still active and a restricted fermentation occurred, producing a silage with a high WSC content and a low concentration of fermentation acids.

Increasing the level of acid applied, to 4.6lt^{-1} (Add-F), restricted fermentation further, so that the content of fermentation acids was very low. After 125 days, the WSC content was 60% higher than that present initially, indicating that hydrolysis of polysaccharides had occurred. This phenomenon has been noted by others

TABLE 8.3

Experiment 4. The acetic acid, propionic acid, ethanol and gross energy contents of the 125-day silages.

Treatment*	Acetic Acid ($gkg^{-1}DM$)	Propionic Acid ($gkg^{-1}DM$)	Ethanol ($gkg^{-1}DM$)	Gross Energy ($MJkg^{-1}DM$)
OA	25.44	0	5.44	17.5
OB	11.33	0	1.62	18.1
OC	2.42	0.12	0.82	17.6
OD	2.16	0.09	1.37	17.0
FA	17.22	0	4.19	17.3
FB	2.34	0.13	1.57	17.3
FC	2.09	0.10	1.55	16.5
FD	2.24	0.12	1.63	17.4
SED (16 d.f.)				
A, B, C, D	0.837	0.000	0.183	NS
O, F	0.592	0.000	NS	0.183
Interaction	1.183	0.000	0.258	NS

* see Table 8.1 for key

NS not significant

TABLE 8.4

Experiment 4. Microbial analyses - grass and 125-day silages ($\log_{10}$ organisms g^{-1} fresh herbage).

Treatment*	Total	Streptococci	Coliforms	Yeasts	Lactate Fermenters	Proteolytic Species
Grass						
Standing Crop	-	-	4.20	7.07	-	-
Chopped	-	-	4.64	7.32	-	-
Silages						
OA	5.81	4.26	<3.00	<2.00	c.6	-
OB	6.00	5.83	<3.00	<3.00	c.6	c.4
OC	4.28	<3.00	<3.00	<3.00	c.3	c.3
OD	3.85	<3.00	<3.00	<3.00	<3.00	-
FA	7.23	5.76	<3.00	c.4	c.7	c.5
FB	4.38	<3.00	<3.00	<3.00	<3.00	c.3
FC	4.08	<3.00	<3.00	<3.00	<3.00	c.3
FD	3.95	<3.00	<3.00	<3.00	<3.00	c.3

* see Table 8.1 for key

for formic acid-treated silages (Carpintero *et al*, 1969, 1979; McDonald *et al*, 1983). Woolford (1975) stated that this level of formic acid should be sufficient to inhibit all silage micro-organisms and these results would appear to support that conclusion. It has been noted, however, that formic acid is less inhibitory to yeasts than to lactic acid bacteria, and sometimes their growth is stimulated in formic acid-treated silages, leading to high concentrations of ethanol and high dry matter losses (Henderson and McDonald, 1971; Henderson *et al*, 1972). Although the yeast count on the fresh grass was high (c. 10^7 organisms g^{-1}), there was no evidence of yeast activity in any of these silages as the concentrations of ethanol were low (Table 8.3) and the yeast count was reduced considerably (Table 8.4).

The highest rate of application of formic acid (6.9 lt^{-1} Add-F) produced a silage with very similar fermentation characteristics to that from treatment C.

The addition of 1.8 lt^{-1} (1.9 kgt^{-1}) of formalin (17.6 g formaldehyde per kg crude protein) did not restrict fermentation markedly. In fact, it had less effect than the lowest level of formic acid. Despite the low final pH, it did result in a higher residual content of water-soluble carbohydrates than the control and a lower level of fermentation acids. Wilson and Wilkins (1977) reported that at least 3 kgt^{-1} of formaldehyde was necessary in order to restrict fermentation effectively on its own and in the current experiment only 0.7 kgt^{-1} was applied. It is important not to apply too much formaldehyde because of the risk of over-protecting the protein fraction and Wilkinson *et al* (1976) suggested 30-50 g formaldehyde per kg crude protein would provide a safe, effective rate. The level used in this experiment was

TABLE 8.5

Experiment 4. The nitrogenous constituents of the grass and silages

Treatment*	TN ($gkg^{-1}DM$)		PN ($gkg^{-1}TN$)		VN ($gkg^{-1}TN$)		Amide N ($gkg^{-1}TN$)	
Grass	27.1		951		13		17.8	
Silages	24h	125d	24h	125d	24h	125d	24h	125d
OA	27.4	28.0	725	356	13	67	ND	20.6
OB	26.7	28.2	710	282	15	34		21.4
OC	26.2	27.0	765	446	15	19		22.1
OD	27.0	26.3	786	479	9	22		20.5
FA	26.1	27.1	767	379	9	55		17.2
FB	27.0	27.9	792	584	9	25		12.8
FC	26.0	27.6	801	658	10	14		15.1
FD	26.7	26.5	811	691	8	16		15.8
SED (16 d.f.)								
A, B, C, D	-	-	5.5	10.8	1.1	2.3		NS
O, F	-	-	3.9	7.6	0.8	1.6		0.52
Interaction	-	-	7.7	15.2	NS	NS		1.03

* see Table 8.1 for key

ND not determined

NS not significant

considerably lower than this (17.6 gkg^{-1} CP) and at this low level there is a risk of stimulating a clostridial fermentation as it is thought that clostridia may be more resistant to formaldehyde than lactic acid bacteria (Wilson and Wilkins, 1978). The low level used here had little effect on fermentation and a low pH, lactate silage was produced. Although the counts for both saccharolytic and proteolytic clostridia were high (Table 8.4), there was no biochemical evidence of clostridial activity. This suggests that most of these clostridia were present in spore form as the method used for detection does not distinguish between spore and vegetative forms.

The effects of adding a mixture of formaldehyde and formic acid were investigated using the same levels of formic acid as for treatments 0B, 0C and 0D.

The combination of formalin (1.8 lt^{-1}) with formic acid (2.3 lt^{-1} Add-F) restricted fermentation to a greater extent than either treatment on its own and resulted in a silage with a relatively high pH and residual WSC content, and very low levels of fermentation acids. Increasing the rate of formic acid lowered the final pH level, but the WSC and acid contents were little different.

After 125 days, substantial proteolysis had occurred in the control silage (0A) although the level of deamination was low (Table 8.5). The addition of the lowest level of formic acid (0B) resulted in an even lower protein content, despite the more rapid acidification. It did, however, decrease the level of deamination. At the higher levels of addition of formic acid there was a significant decrease in proteolysis and a further reduction in deamination. The

TABLE 8.6

Experiment 4. The ratio of amino acid nitrogen in fresh grasses

	Italian Ryegrass	Mixed[a] Ryegrass	Perennial[b] Ryegrass	Italian[c] Ryegrass
Aspartic Acid[+]	9.2	9.6	10.2	9.3
Threonine	4.5	5.1	4.6	4.6
Serine	4.9	4.8	5.2	5.1
Glutamic Acid[+]	10.6	10.6	9.0	8.7
Proline	-	-	4.7	4.3
Glycine	9.2	8.3	10.0	8.0
Alanine	9.2	10.6	10.6	9.4
Valine	6.3	5.9	6.6	6.4
Isoleucine	4.4	4.0	3.5	3.9
Leucine	7.9	7.1	6.9	8.7
Tyrosine	2.8	2.4	2.2	2.3
Phenylalanine	4.5	3.9	3.6	4.0
Histidine	4.8	5.0	-	-
Lysine	6.0	8.0	9.4	10.5
Arginine	15.8	14.8	13.5	14.8
Total	100.0	100.0	100.0	100.0

a - from experiment 1

b - from experiment 3

c - from Ohshima *et al* (1979)

+ - includes amide nitrogen

TABLE 8.7

Experiment 4. Total amino acid nitrogen ($gkg^{-1}TN$) for grass and silages

Amino Acid	GRASS*	SILAGES*			SED
		OA	OD	FD	
Asp+	55.17[a]	42.98[b] (77.9)	56.83[a] (103.0)	61.03[a] (110.6)	2.852
Thr	27.00	27.71	29.98	32.45	NS
Ser	29.08[ab]	27.03[a] (93.0)	31.95[bc] (109.9)	34.33[c] (118.1)	1.897
Glu+	63.63[a]	47.43[b] (74.5)	66.04[ac] (103.8)	69.04[c] (108.5)	1.461
Gly	54.88	53.07	53.82	55.40	NS
Ala	54.94[a]	69.86[b] (127.2)	58.67[ac] (106.8)	61.60[c] (112.1)	2.530
Val	37.77	38.11	36.53	38.51	NS
Ile	26.19	26.27	27.02	27.13	NS
Leu	47.21	45.77	47.08	49.03	NS
Tyr	16.47	15.97	16.21	16.09	NS
Phe	26.99	27.01	26.54	26.17	NS
His	28.86	30.11	30.02	27.78	NS
Lys	36.15[a]	57.75[b] (159.8)	62.96[c] (174.2)	52.63[d] (145.6)	1.897
Arg	94.40[a]	51.54[b] (54.6)	89.88[a] (95.2)	98.18[a] (104.0)	12.250
Total	598.40[ab]	560.59[a]	633.54[b]	649.36[b]	23.802

* Values on the same line with different superscripts differ significantly ($P<0.05$). The data in parentheses show the % recovery after ensilage.

\+ includes amide nitrogen

NS not significant

differences between the effects of the two highest levels were small. These results are consistent with those of others who have found that formic acid has a limited effect in reducing proteolysis despite the rapid acidification, but does tend to reduce deamination (Saue and Brierem, 1969; Henderson and McDonald, 1971; Henderson *et al*, 1972; Wilson and Wilkins, 1973; Hinks *et al*, 1976; Hinks and Henderson, 1977; Waldo, 1977; Wilson and Wilkins, 1977; Carpintero *et al*, 1979; McLellan and McGinn, 1981; Henderson *et al*, 1982).

On its own, formaldehyde did not have much effect on the degree of proteolysis which occurred but, in combination with formic acid, there was a marked reduction so that, with the highest level of acid, the protein nitrogen content was 691 gkg^{-1} TN. This synergistic effect has been noted elsewhere (Valentine and Brown, 1973; Henderson *et al*, 1972; Moreau *et al*, 1982; Siddons *et al*, 1982).

Amino acid analyses were carried out on the fresh grass, the control silage (OA), and on those silages treated with the highest level of formic acid (OD and FD).

As with experiments 1 and 3, the ratio of amino acid nitrogen in the fresh grass was compared with that of similar grasses (Table 8.6). Once again the uniformity is apparent.

The total amino acid nitrogen content of the control silage was not significantly different from that of the fresh material (Table 8.7), indicating that little amino acid catabolism had occurred, despite the extensive proteolysis which took place. This conclusion was supported by the relatively low volatile

nitrogen content (Table 8.5). The only significant changes were in aspartic acid, glutamic acid, alanine, lysine and arginine. Decreases in aspartic acid, glutamic acid and arginine, and increases in alanine are typical for a lactate silage (Macpherson and Violante, 1966; Ohshima *et al*, 1979). The large increase in lysine was unexpected as this amino acid is usually degraded during ensilage (Slater, 1959; Macpherson and Violante, 1966; Ohshima *et al*, 1979). From a comparison of the ratios of amino acid nitrogen reported in Table 8.6, it would appear that the lysine value for this grass was somewhat low. If this figure is erroneous, it would explain the increase in lysine which seems to have occurred during ensilage.

Several investigators have reported that amino acid catabolism is inhibited by the rapid attainment of a low pH (Macpherson and Violante, 1966a; Barry *et al*, 1978b; Ohshima *et al*, 1979; Moreau *et al*, 1982; Mortensen, 1982) and in the current experiment there were no significant changes in any of the amino acids in the formic acid-treated silage, except for lysine. Similar results were obtained for the silage treated with both acid and formaldehyde.

8.4 CONCLUSIONS

These results demonstrated that formic acid was a very effective inhibitor of fermentation but had a limited effect in preventing proteolysis when used on its own. Formaldehyde, at the very low level used, had very little effect on either fermentation or protein protection, unless acid was added too. The combination of the two, however, produced a synergistic effect with respect to

protein protection. This effect was most apparent at the commercial rate of application of formic acid (2.3 lt^{-1} Add-F). At this rate the mixed additive produced a relatively high pH silage with high protein and WSC contents and very low concentrations of fermentation acids and volatile nitrogen. Increasing the level of acid applied produced a lower pH and a somewhat higher protein content but did not increase the level of residual carbohydrates. It is doubtful whether the small increase in protein protection would warrant the application of a higher level of acid as this would make the treatment considerably more expensive.

The most satisfactory treatment, from the preservation point of view, therefore, appeared to be a combination of formic acid and formaldehyde in a 3 to 1 ratio applied at a rate of 3 to 4 lt^{-1}, which agrees with the recommendations of others (Vanbelle *et al*, 1978; McDonald, 1984).

9. EXPERIMENT 5

THE EFFECTS OF ALKALI TREATMENT ON FERMENTATION DURING ENSILAGE

9.1 INTRODUCTION

Minimising losses of nutrients is a major aim during conservation of forages. Recently attention has turned to the possibility of achieving a conserved product of higher nutritive value than the original herbage (Wilkins, 1980; Wilkinson, 1982). This has involved treating the crop with additives which bring about some "pre-digestion" of the material before it is offered to the animal.

It is well established that the digestibility of graminaceous forages is increased by treatment with an alkali. This results from an increase in the digestibility of the cell walls, thought to be related to the release of phenolic compounds (Hartley and Keene, 1982). Such techniques have been used commercially for many years to improve the nutritive value of low quality roughages, notably straw (Jackson, 1977; Thomsen, 1980; Wilkins, 1980).

Dulphy *et al* (1982) found that the beneficial effects of sodium hydroxide treatment of straw were greater with straws of lower nutritive value. Similar results have been found with grasses (Wilkinson, 1982). This technique thus has particular potential for use with forages cut at a mature stage of growth.

Owing to the hazardous nature of sodium hydroxide and problems which may occur in the animals owing to the increased mineral content of the treated material, its use on the farm has been

limited (Wilkins, 1980; Wilkinson, 1982). Similar results, however, have been obtained using ammonia (Witt and Mølle, 1981; Mason *et al*, 1982). The addition of ammonia raises the nitrogen content of the forage and is particularly beneficial with mature crops, which tend to have low nitrogen contents (Waite and Gorrod, 1959). In addition, whole-crop cereal silage is becoming more popular in many countries (Witt and Mølle, 1981) and such crops are also low in nitrogen (McDonald *et al*, 1981).

The present study was designed to investigate the effects of alkali treatment on fermentation during ensilage, particularly on the nitrogenous constituents since plant protease activity is inhibited by alkaline pH (section 2.2.2.6). Two methods of alkali treatment (ammonia and borax buffer) were investigated and compared with two methods of direct acidification (sulphuric acid and citric acid. Citric acid was included because it is a relatively safe material to handle and is an important intermediate in energy metabolism, and yet very little work has been undertaken to assess its potential as a silage additive.

9.2 EXPERIMENTAL

Grass from a mixed Italian/perennial ryegrass sward was harvested on 30 September 1981 using a "Haldrup" reciprocal mower-type plot harvester and, after hand-chopping to approximately 20 mm lengths, was ensiled in 200 g capacity laboratory silos. The following treatments were applied to the fresh grass.

1. Control (C) - 25 ml water.
2. Sulphuric acid (SA) - 25 ml 15.9% w/v (4.0 gkg^{-1}).
3. Citric acid (CA) - 25 ml 64.0 w/v (16.0 gkg^{-1}).
4. Borax buffer (B1) - 20 g borax + 25 ml water.
5. Borax buffer (B2) - 20 g borax + 25 ml 19.2% w/v NaOH (4.8 gkg^{-1}).
6. Borax buffer (B3) - 20 g borax + 25 ml 32.0% w/v NaOH (8.0 gkg^{-1}).
7. Ammonia (A1) - 25 ml 5.15% w/w NH_3 (1.1 gkg^{-1}).
8. Ammonia (A2) - 25 ml 12.4% w/w NH_3 (2.7 gkg^{-1}).
9. Ammonia (A3) - 25 ml 30.9% w/w NH_3 (6.8 gkg^{-1}).

The silos were sealed with plastic fermentation traps filled with water, and incubated at room temperature (c. 20°C). Silos were opened after 24h and 140 days, when comprehensive analyses were carried out, using the techniques described in sections 4 and 13. Each treatment was replicated three times.

9.3 RESULTS AND DISCUSSION

Owing to the highly volatile nature of ammonia, substantially less ammonia was applied than had been intended, particularly in treatment A3. The levels of nitrogen applied were calculated from the increase in the total nitrogen contents in the 24-hour silages, and found to be 54, 68 and 47% of that intended for treatments A1, A2 and A3 respectively. It is difficult to explain why the recovery for A1 should be so low. In most investigations involving ammonia treatments, it has been applied in gaseous form to material

TABLE 9.1

Experiment 5. Dry matter losses

Treatment*	Dry matter loss (%)	
	24h	140d
C	3.32	2.13
SA	0.73(+)	2.80
CA	0.96	7.61
B1	3.59	8.26
B2	2.75	7.10
B3	3.79	9.45
A1	3.20	3.05
A2	2.29	3.48
A3	0.50	1.80

* C - control
SA - sulphuric acid (4.0 gkg^{-1})
CA - citric acid (16.0 gkg^{-1})
B1 - borax (20 gkg^{-1})
B2 - borax (20 gkg^{-1}) + NaOH (4.8 gkg^{-1})
B3 - borax (20 gkg^{-1}) + NaOH (8.0 gkg^{-1})
A1 - ammonia (1.1 gkg^{-1})
A2 - ammonia (2.7 gkg^{-1})
A3 - ammonia (6.8 gkg^{-1})

TABLE 9.2

Experiment 5. Dry matter, pH, water-soluble carbohydrates and lactic acid

Treatment*	DM (gkg^{-1})			pH		WSC (gkg^{-1})		Lactic Acid (gkg^{-1})	
Grass	174			5.87		14.1		ND	
Silages	0h	24h	140d	24h	140d	24h	140d	24h	140d
C	170	164	168	5.28	3.99	11.3	1.6	ND	23.9
SA	174	175	170	4.13	3.77	15.3	1.8		18.2
CA	185	183	172	3.85	3.64	17.5	3.0		20.0
B1	186	179	171	8.03	6.52	14.2	19.7		15.2
B2	190	185	177	8.85	6.53	14.9	4.4		12.5
B3	193	186	175	9.27	7.40	11.8	8.2		6.5
A1	171	166	168	5.65	4.14	10.7	0.8		21.7
A2	172	168	168	7.13	4.55	14.9	0.6		18.9
A3	174	173	173	8.69	4.75	17.0	1.0		15.7
SED+	-	-	-	0.100	0.116	0.93	1.10		1.79

* see Table 9.1 for key

\+ WSC (20 d.f.); Lactic Acid (18 d.f.)

ND not determined

TABLE 9.3

Experiment 5. The volatile fatty acid, ethanol and gross energy of the 140-day silages

Treatment*	Acetic Acid (gkg^{-1})	Propionic Acid (gkg^{-1})	Butyric Acid (gkg^{-1})	Ethanol (gkg^{-1})	Gross Energy ($MJkg^{-1}$)
C	6.6	0.8	0.0	0.8	3.3
SA	4.8	0.7	0.0	0.6	3.0
CA	4.8	0.6	0.0	0.4	3.2
B1	1.6	0.6	0.0	0.6	3.0
B2	2.5	0.5	0.0	0.7	2.8
B3	3.3	0.5	0.0	0.7	3.1
A1	7.5	0.6	0.0	1.4	3.1
A2	9.1	0.7	0.0	1.4	2.9
A3	9.6	0.8	0.3	1.1	3.2
SED (18 d.f.)	0.26	0.04	-	0.11	0.09

* see Table 9.1 for key

in sealed containers, and the recoveries have been much higher than in the present study (Huber *et al*, 1979; Thonney *et al*, 1980; Witt and Mølle, 1981; Halverson and Emerick, 1982). The low recoveries in the present experiment suggest that the application of an ammonia solution to exposed herbage is not a satisfactory method.

The dry matter losses incurred as a result of ensiling are shown in Table 9.1. After 24h, the dry matter losses were small in all cases. After 140 days they had increased substantially particularly in the citric acid and borax buffer-treated silages.

The results of the biochemical and microbial analyses are shown in Tables 9.2 to 9.7. Most of the results are presented in terms of the fresh material rather than the dry matter or total nitrogen, owing to the complicating effect of the addition of dry matter and nitrogen in some of the additives.

After 24h, the sulphuric and citric acids had reduced the pH to 4.13 and 3.85 respectively (Table 9.2). This rapid acidification slowed down the initial fermentation, as evidenced by the increase in the WSC content of these silages, compared with the control, during the initial 24h (Table 9.2). Despite this, extensive fermentations did take place. Thus the residual WSC contents after 140 days were low, particularly in the sulphuric acid-treated herbages, and the levels of fermentation acids (Tables 9.2 and 9.3) were almost as high as for the control silage, which had undergone a normal lactate fermentation.

TABLE 9.4

Experiment 5. The nitrogenous constituents of the grass and silages (gkg^{-1})

Treatment*	TN		PN		VN		Amide-N	
Grass	6.9		6.2		0.07		0.16	
Silages	24h	140d	24h	140d	24h	140d	24h	140d
C	6.9	6.7	4.8	1.3	0.17	0.68	ND	0.21
SA	6.9	6.6	5.3	2.1	0.06	0.48		0.24
CA	6.8	6.8	5.1	2.0	0.05	0.39		0.22
B1	6.5	6.8	5.1	2.2	0.10	0.48		0.09
B2	6.7	6.6	5.5	3.2	0.10	0.46		0.07
B3	6.6	6.7	5.3	3.2	0.07	0.17		0.07
A1	7.4	7.5	5.3	2.7	0.66	1.20		0.17
A2	8.4	8.3	5.9	3.4	1.19	2.06		0.15
A3	9.5	9.9	6.1	4.0	2.13	2.93		0.13
SED (20 d.f.)	0.14	0.26	0.16	0.26	0.082	0.100		0.118

* see Table 9.1 for key

ND not determined

TABLE 9.5

Experiment 5. The utilization of WSC during ensilage

Treatment*	WSC utilized (% WSC ensiled)	
	24h	140d
C	17.6	88.7
SA	11.6(+)	87.3
CA	27.1(+)	79.5
B1	5.5(+)	45.7(+)
B2	11.0(+)	67.4
B3	12.3	38.6
A1	22.2	94.0
A2	8.1(+)	97.7
A3	23.3(+)	92.9

* see Table 9.1 for key

Both acid treatments reduced proteolysis (Table 9.4) but, despite the rapid acidification, to below pH 4.0 brought about by the citric acid treatment, considerable protein breakdown had occurred. The treatments did, however, have a significant effect in reducing deamination reactions.

The results presented here for sulphuric acid are similar to those found by Carpintero *et al* (1979) who used the same rate of application on lucerne. They, however, obtained a more significant reduction in proteolysis. Carpintero (unpublished work) also applied citric acid (30 gkg^{-1}) to lucerne, but found that there was little effect on proteolysis despite the rapid acidification. The final protein nitrogen contents were 419 and 504 gkg^{-1}TN for the control and citric acid treatments respectively.

The addition of borax buffers to the grass resulted in pH values of 8.0 to 9.3 after 24h (Table 9.2). Despite the presence of the buffer, and the low initial WSC content of the grass, sufficient fermentation occurred to lower the pH to between 6.5 and 7.4 after 140 days. The addition of the buffer at the two higher levels (B2 and B3) did restrict fermentation, resulting in a higher residual WSC concentration and lower content of fermentation acids compared to the control. Treatment B1 resulted in a similar production of acids to B2 but instead of a reduction in WSC there was an apparent 45% increase (Table 9.5). Although this result seems highly improbable, it is correct.

Several workers have stressed the potential problem with clostridial fermentations occurring in alkali-treated materials, particularly when the dry matter is low (Bolsen *et al*, 1980; Witt and Mølle,

TABLE 9.6

Experiment 5. Microbial analyses (log_{10} organisms g^{-1} fresh herbage)

Treatment*	Total	Streptococci	Coliforms	Yeasts	Lactate Fermenters	Proteolytic Species
Grass						
Standing Crop	8.08	<3.00	4.18	6.67	<3.00	c.4
Chopped	8.08	4.99	4.48	7.04	4.80	c.5
24h-Silages						
C	8.18	6.08	5.78	7.36	6.23	c.5
SA	7.18	6.85	4.76	5.49	6.36	-
CA	5.96	5.38	4.34	4.76	5.81	c.4
B1	7.68	5.62	4.52	6.90	5.32	c.5
B2	7.58	5.26	4.48	5.48	5.18	c.5
B3	6.72	4.18	4.23	4.52	5.26	c.5
A1	8.28	6.87	7.15	7.80	6.70	c.5
A2	8.08	7.88	6.83	7.75	6.70	c.5
A3	7.38	6.43	3.81	4.60	5.53	c.3
76d-Silages						
C	7.88	5.32	3.53	4.51	6.72	c.5
SA	7.93	6.58	4.15	5.58	>7.00	c.5
CA	7.94	3.45	4.74	6.52	6.32	c.4
B1	7.83	6.00	<2.00	6.85	5.79	c.4
B2	7.86	7.40	c.2	6.40	5.88	c.4
B3	6.94	6.38	c.2	6.26	>6.00	c.4
A1	7.78	6.15	<2.00	6.57	7.57	c.4
A2	7.71	6.63	2.97	6.65	>6.00	c.4
A3	7.59	6.32	<2.00	6.91	>6.00	c.4

* see Table 9.1 for key

TABLE 9.7

Experiment 5. Proteolysis during ensilage

Treatment*	Protein hydrolysed (% PN ensiled)	
	24h	140d
C	20.0	78.7
SA	11.8	65.0
CA	15.5	65.6
B1	12.6	63.3
B2	6.4	45.6
B3	10.5	45.0
A1	12.8	55.0
A2	2.0	44.5
A3	1.7	34.2

* see Table 9.1 for key

1981; Kung *et al*, 1984) but, despite the high pH levels and low dry matter contents of these silages, no butyric acid was detected (Table 9.3). The counts of lactate fermenters in silages B1, B2 and B3 were high (Table 9.6) but no higher than in the other silages. This, together with the lack of biochemical evidence to support clostridial activity, suggests that the clostridia were present in spore form only.

The addition of borax buffers at the two higher levels (B2 and B3) had a very significant effect on the degree of proteolysis (Table 9.7) reducing the amount of protein hydrolysed to about 45% of the initial protein ensiled (control - 79%). There was a reduction in the amount of deamination which occurred, especially with treatment B3. Unlike the control and acid treatments, which cause an increase in the amide nitrogen content, the alkaline buffer treatment brought about a significant decrease in amide nitrogen.

The low ammonia treatment (A1) did not markedly alter the pH (5.65) compared with the control (5.28) after 24h but, at the higher levels, ammonia significantly increased the pH to 7.1 and 8.7 (A2 and A3 respectively). After 140 days the pH values had decreased considerably but were still higher than in the control.

Many studies have found that ammonia treatment prolongs fermentation, resulting in low residual WSC and high lactic acid contents (Huber *et al*, 1973; Mowat *et al*, 1976; Phipps and Fulford, 1977; Halverson and Emerick, 1982; Kung *et al*, 1984). In the present study the lactic acid content was not higher than that of the control. This is presumably the result of the low initial WSC content which would limit the extent of fermentation.

The higher acetic acid and ethanol contents (Table 9.3) suggest that ammonia treatment encouraged heterolactic acid bacteria.

All the ammonia treatments brought about significant decreases in the extent of proteolysis (Table 9.4). This effect has been noted by others (Mowat *et al*, 1976; Huber *et al*, 1979; Halverson and Emerick, 1982; Johnson *et al*, 1982; Kung *et al*, 1984). The higher true protein content could be the result of either microbial protein synthesis from the added ammonia, or reduced proteolysis. Huber *et al* (1979) have used ^{15}N-labelled ammonia to show that some of the added ammonia becomes incorporated into the insoluble protein fraction.

Although it is generally considered that a rapid decrease in pH is desirable if proteolysis is to be inhibited, it would appear that a high initial pH will also bring this about. It must be stressed, however, that the pH fell to a relatively low level during the ensiling period. In doing so, it must have passed through the pH optimum of the proteases. Since this did not seem to result in increased proteolysis, it must be assumed that either the pH fell through the optimum very rapidly, or that other factors were involved. Bergen *et al* (1974) have suggested that some form of end-product inhibition may be acting.

9.4 CONCLUSIONS

Both sulphuric and citric acids reduced the initial pH to a low level but despite this considerable proteolysis took place. The acid treatments did, however, have a significant effect in reducing deamination reactions.

The borax buffers raised the initial pH level to between 8.0 and 9.3 but fermentation reduced the final pH levels to 6.5 to 7.4. The two higher levels of buffer reduced proteolysis to a greater extent than the acid treatments and also reduced deamination.

Although the two higher levels of ammonia treatment resulted initially in alkaline pH values (7.1 and 8.7 for A2 and A3 respectively), these pH levels fell to values slightly above those of the control after 140 days. All of the ammonia treatments resulted in higher residual protein levels than in the control silage, the effect increasing as the level of application increased. Of all the treatments, the ammonia resulted in the highest degree of protein protection.

10. CONCLUDING DISCUSSION

Silage-making is becoming an increasingly popular method of forage conservation. It has the advantages, over hay-making, of being relatively independent of the weather, and being more suitable for large-scale mechanisation. In the early 1960s only 10% of all grass conserved in the United Kingdom was made into silage but in the late 1970s this figure had risen to about 45% (Wilkins, 1980).

The major objectives of the conservation of a green crop as silage are, to preserve the material with a minimum loss of nutrients and to provide a palatable material for ruminant animals. The outcome of a natural silage fermentation depends on many factors (McCullough, 1977) and is difficult to predict with any certainty. The use of additives has helped to increase the probability of a desirable fermentation.

It is well known that intakes of the dry matter of even well-preserved lactate silages, are frequently lower than those of fresh or dried crops of comparable digestibilities (McDonald, 1976; Wilkins, 1980). Various studies have attributed this to the presence of large quantities of fermentation acids, or highly soluble nitrogenous substances, produced during fermentation (Wilkins, 1974; Wilkinson *et al*, 1976). In addition, silage nitrogen is used with a lower efficiency than that of the original material owing to its high solubility and to a shortage of dietary energy (McDonald and Edwards, 1976).

These problems have stimulated research into the silage fermentation process and its manipulation, using various additives.

Means are being investigated, not only to improve preservation but also to increase the protein and soluble carbohydrate contents of silages (McDonald, 1984).

In order to decide how best to manipulate the fermentation process, it is necessary to understand the nature of the reactions taking place. The first part of the present study investigated the main nitrogenous changes occurring during ensilage, the causes of such changes and the effects of pH and temperature.

In order to separate the effects of plant and microbial enzyme activity, it was necessary to find a method which would inactivate the micro-organisms without affecting the activity of the plant enzymes. Various methods involving the use of toluene, chloroform, antibiotics or asceptically-grown grass have been used (Mabbit, 1951; Macpherson, 1952a; Kemble, 1956; Brady, 1960, 1961; Gouet and Fatianoff, 1964; Woolford, 1972) but they are unreliable and, in most cases, the treated plant material cannot be used for inoculation studies. By far the most successful method has been sterilization using gamma-irradiation (Gouet *et al*, 1970, 1972). The use of this technique in experiment 1 allowed a comparison to be made of the separate contributions of the plant and microbial enzymes in bringing about changes in the nitrogenous fractions.

Plant proteases were found to be responsible for most of the proteolysis which agrees with the results of other studies (Mabbitt, 1951; Macpherson, 1952a; Kemble, 1956; Brady, 1960, 1961; Ohyama, 1970; Bousset *et al*, 1972; Clark, 1974).

The changes in amino acids which occurred in the control silage, were typical of a lactate silage (Hughes, 1970; De Vuyst *et al*, 1971; Ohshima *et al*, 1979). It is generally considered that micro-organisms are responsible for these changes, particularly the deamination reactions. Nevertheless, several workers have reported ammonia production resulting from plant enzyme activity (Mabbitt, 1951; Macpherson, 1952a; Brady, 1960, 1961; Ohshima, 1971), and in the present study substantial quantities of ammonia were produced in the sterile silage.

In a lactate silage most of the ammonia is believed to come from microbial deamination of serine and arginine (McDonald and Whittenbury, 1973) and in this investigation both of these amino acids underwent extensive degradation. Since no significant decreases in the amounts of these amino acids were observed in the sterile silage, it would appear that plant enzymes were not involved and that the deamination of these amino acids did not contribute to the production of ammonia in the sterile silage.

The only amino acids whose concentration did decrease appreciably in the sterile silage were valine, lysine and aspartic acid. Although lysine is degraded extensively during ensiling, it is usually by decarboxylation to cadaverine (Macpherson and Violante, 1966; Hughes, 1970; Ohshima *et al*, 1979). Aspartic acid has been shown to undergo some deamination by plant enzymes (Ohshima, 1971) but it is more commonly broken down by decarboxylation to alanine, a reaction which is carried out chiefly by micro-organisms (Ohshima, 1971). The large decrease in valine was surprising as this amino acid is normally well-preserved during ensiling (Hughes, 1970; De Vuyst *et al*, 1968b, 1971;

Ohshima, 1971; Ohshima *et al*, 1979). This result is in agreement with the suggestion of Ohshima *et al* (1979) that some synthesis of valine may occur during ensiling, presumably by microbial enzymes.

Lactic acid bacteria are considered to have a very limited ability to catabolize (Beck, 1978) or synthesize (Stanier *et al*, 1976) amino acids. The results of the inoculation study using *L. plantarum* and *S. faecalis* were in accordance with this as there was no significant difference between the sterile and inoculated silages with respect to the total amino acid contents. *L. plantarum* is known to deaminate serine while *S. faecalis* can deaminate arginine and decarboxylate tyrosine (Brady, 1966; Buchanan and Gibbons, 1974), and all three amino acids were found to be degraded to a limited extent. In addition, *L. plantarum* and *S. faecalis* would appear to be involved in the breakdown of aspartic and glutamic acids, especially the former.

Other studies have shown that glutamic acid is extensively decarboxylated to γ-aminobutyric acid by plant enzymes during the initial stages of ensilage (Macpherson and Slater, 1959; Ohshima, 1971). The results of the current experiment suggest that this amino acid is broken down not by plant enzymes but by microbial enzymes, during ensiling.

In order to reduce the amount of proteolysis occurring during ensilage it is necessary to inhibit the activity of plant enzymes. Virtanen (1933) stated that plants did not contain proteases active at pH values below 4.0 and Macpherson (1952a) claimed that if the pH was reduced to less than 4.3, proteolysis would be negligible. However, a recent study by Carpintero *et al* (1979) has shown that

even an initial pH of 3.5 is not sufficient to prevent proteolysis during subsequent ensilage, although it will inhibit it to some extent. Other studies on plant proteases (section 2.2.2.6) have shown that many forage plants contain proteases which are not only active at pH 4.0, but may have their pH optimum at or below this level. Most of this work has been carried out on cereal and legume crops, rather than grasses, and it was decided therefore to investigate the activity of ryegrass proteases (experiment 2).

The results of three separate studies showed that the optimum pH for ryegrass proteases lies between 5.5 and 6.5 which is similar to the range reported for other plant species (Brady, 1961; Singh, 1962; Finley *et al*, 1980). This emphasises the necessity of encouraging a rapid decrease in pH during ensiling.

In the present experiment, as well as those of others (Brady, 1961; Singh, 1962; Finley *et al*, 1980), activity has been found over a broad pH range. It is probable that these pH profiles represent the combined activities of many proteases with different pH optima. Frith *et al* (1978) found that protease activity with a single pH optimum of pH 4.2, in fact represented the activities of six separate proteases. The apparent optima in these curves may represent the combined activities of many proteases, each with a low level of activity or a few, each with a high level of activity.

As the pH decreases during ensiling, the relative activities of the proteases would be modified. Proteases active at very low pH have been found by others (Feller *et al*, 1977; McKersie, 1981) and this experiment demonstrated proteolytic activity at pH 2.5 (20°C). Considerable activity was present at pH 4.0, the pH of a well-preserved

lactate silage, and proteolysis might, therefore, be expected to continue indefinitely during ensilage, if pH was the only controlling factor. It is found, however, that the level of protein nitrogen in silage rarely falls below about 250 gkg^{-1}TN, which suggests that other factors are involved. McKersie and Buchanan-Smith (1982) examined several proteases with different pH optima, extracted from lucerne silages at various stages during ensiling. They found that their activities during ensiling declined independently of each other and that the cessation of proteolysis was not the result of the loss of enzyme activity, since at least two of the proteases were still activite and the pH was not inhibiting. This suggests that other factors, such as substrate availability or end-product inhibition, may have been responsible. This subject was discussed in sections 2.2.2.6 and 2.2.2.7 of the literature review.

The effect of temperature on proteolysis was also studied and protease activity over the pH range 4.0 to 7.0 was found to be substantially greater at 37°C than at 20°C. At lower pH levels the higher temperature resulted in a rapid loss of activity. Plant proteases have generally been found to have relatively high (c.45-55°C) temperature optima (Brady, 1961; Drivdahl and Thimann, 1977; Feller *et al*, 1977; Peoples and Dalling, 1978), and an increase in temperature resulting from respiration during the initial stages of ensiling, could accelerate proteolysis greatly. Ohyama *et al* (1973) stated that proteolysis was affected by temperature during the early stages of ensiling and by pH during the later stages.

An interesting feature of this study, which has not been reported previously, is the chemical hydrolysis of proteins at low pH. This was found to be negligible above pH 2.0 at 20°C, but at 37°C there was

appreciable hydrolysis, even at pH 4.0. This introduces the possibility of acid hydrolysis of proteins occurring during prolonged storage of silages in which a rise in temperature has taken place.

In the latter part of this study three different approaches to manipulating silage fermentation were investigated.

One of the main objectives of fermentation during ensilage is the rapid achievement of a stable, low pH, in order to inhibit undesirable organisms and reduce the extent of proteolysis.

Inoculants have been used for some time in an attempt to encourage a rapid lactate fermentation but, on the whole, the results have been disappointing (Watson and Nash, 1960; Thomas, 1978a). Recently there has been renewed interest and several inoculant-based commercial additives have been introduced.

Most crops have a very low indigenous population of lactic acid bacteria (Gibson *et al*, 1958; Stirling and Whittenbury, 1963). Inoculation with a rapidly-growing culture of suitable species should overwhelm the resident population of bacteria and ensure a rapid, efficient fermentation. Provided an adequate supply of water-soluble carbohydrates is available, a stable pH should then be reached.

The effect of an inoculant containing *L. plantarum* and *P. acidilactici*, which is now being sold commercially as "Clampdown", were investigated in experiment 3. The effects of mincing and the addition of glucose, were also studied.

Inoculation was found to have a very significant effect on the rate of acidification which increased as the level of application

increased from 10^4 to 10^8 organisms g^{-1}.

Although the water-soluble carbohydrate content of the original grass was high (180 gkg^{-1}DM), most of the sugars would be expected to remain relatively unavailable to the micro-organisms until some plasmolysis of the plant cells had occurred. It was, therefore, anticipated that mincing, to release the substrate immediately, or the addition of an alternative readily-available substrate, would encourage a rapid fermentation. In fact, neither treatment succeeded in encouraging fermentation. This suggests that, in this particular case, lactic acid bacteria had been very scarce on the fresh plant, or had been slow to develop. Unfortunately, counts of lactic acid bacteria, which might have allowed identification of the factors responsible, were not available.

An interesting observation made during this study was that, after 24 hours, the water-soluble carbohydrate content of most silages had increased. This suggests that structural carbohydrates are broken down during ensilage. This has been observed elsewhere (Macpherson *et al*, 1957; Dewar *et al*, 1963; McDonald *et al*, 1964; Ohyama and Masaki, 1977, 1979; Morrison, 1979) and was also found to occur in sterile silage (experiment 1). Most of the initial increase is probably the result of hemicellulases, which have an optimum pH of about 6.0 (Dewar *et al*, 1963). Further breakdown of hemicelluloses may occur at a later stage owing to direct acid hydrolysis (Dewar *et al*, 1963; Morrison, 1979). It must be noted that although these results suggest that a considerable increase in the water-soluble carbohydrate content of the material may occur, most of these sugars may not be available to the bacteria during the initial stage of ensilage.

Although inoculation did produce a rapid decrease in pH, to 4.0 after 24h at the highest rate of inoculation, this had very little effect on the amount of proteolysis which occurred either within the initial 24-hour period or during the subsequent ensilage period. It did, however, have a significant effect in reducing deamination.

Very few amino acids had been broken down in the control silage indicating that the activities of proteolytic clostridia had been minimal. The only amino acids to decrease significantly were glutamic acid, tyrosine and arginine. Most of the increase in volatile nitrogen in the control silage probably resulted from deamination of arginine, a reaction which occurred to a much lesser extent in the inoculated silage.

It is interesting to note that the amide concentration appears to be affected by inoculation since these compounds decreased in the control treatment but increased at the higher levels of inoculation.

Glucose addition was found to have practically no effect on either proteolysis on deamination, regardless of whether it was applied on its own or in addition to the inoculant which, presumably reflected its lack of effect on acidification. Mincing, on the other hand, did reduce proteolysis singificantly despite its similar lack of effect on the rate of acidification. This suggests that mincing may have released a compound which is preventing some of the protein from being broken down. This may be a protease inhibitor, or a substance such as a tannin (section 2.2.2.8) which physically prevents enzyme access to the substrate. The fact that the effect is not apparent after 24h would seem to favour the latter suggestion.

In conclusion, inoculation with "Clampdown" stimulated a more efficient fermentation, leading to a more rapid acidification and a higher residual water-soluble carbohydrate content. Its effect on proteolysis was small but it did reduce deamination significantly. The effectson amino acid metabolism were less easy to determine as very little amino acid catabolism had occurred in the control silage. However, rapid acidification should reduce amino acid catabolism (Macpherson and Violante, 1966; Mortensen, 1982). It is thought that inoculation may prove to be more beneficial for crops which have low water-soluble carbohydrate contents when it is important that the available substrates are used efficiently. The relationship between inoculation and the forage water-soluble carbohydrates content is controversial, however, as some studies have reported that inoculation is only successful when the water-soluble carbohydrates content is above a certain level (Wieringa, 1960) whereas others have found an improvement only when the level was low (McDonald *et al*, 1964). Wieringa (1960) also stated that, if the herbage was extensively lacerated, the effects of inoculation were considerably reduced but that was not found to be the case in the present experiment.

The fact that rapid acidification to pH4 did not prevent proteolysis provided further evidence to show that proteolysis during ensiling is limited to a certain extent by a low pH but is not prevented.

Most inoculants in use at present concentrate on rapid acidification but the development of genetic engineering techniques provides many possibilities for manipulating lactic acid bacteria, for example

giving them the ability to hydrolyse cellulose. It is possible that such organisms will provide the basis for more successful inoculants in the future.

In the second experiment with additives (experiment 4), the effects of adding formic acid and formaldehyde were examined.

It was found that formic acid, used on its own, was a very effective inhibitor of fermentation, an effect which increased with the rate of application. Formaldehyde, on the other hand, had little effect on fermentation at the low rate used (1.8 lt^{-1}) unless acid was added also.

Formic acid had a limited effect on proteolysis when used on its own, as did formaldehyde, but using them together resulted in a synergistic effect in reducing proteolysis. Formic acid reduced deamination when used on its own or in the presence of formaldehyde.

Despite the extensive proteolysis which took place in the control silage, little amino acid catabolism occurred, except for a large decrease in arginine and smaller decreases in aspartic acid, glutamic acid and serine.

It would seem that a combination of formic acid and formaldehyde encourages the production of a well-preserved silage with a high residual content of water-soluble carbohydrates, and in which little proteolysis or amino acid breakdown occurs. Unfortunately, both of these chemicals are hazardous and require great caution in their use. In addition, the application rate of formaldehyde is critical and depends upon the crude protein content of the fresh herbage. If too much is added it is possible to starve the rumen micro-organisms of a

supply of degradable nitrogen (Hinks and Henderson, 1977; Vanbelle *et al*, 1978; Siddons *et al*, 1979) and if free formaldehyde is present, it could have an additional inhibitory effect on the rumen micro-organisms (Wilkins *et al*, 1974). It is also possible to cause irreversible binding of the protein, which reduces the digestibility of the protein in the small intestine (Siddons *et al*, 1979).

The problems associated with the use of formic acid and formaldehyde as additives in silage-making have stimulated the search for safer, more reliable additives.

The final study was inspired by the fact that proteases are inhibited by alkaline pH, and far fewer plant proteases have alkaline pH optima (Frith and Dalling, 1980). In addition, attention has recently turned towards the possibility of using alkalis to "pre-digest" fibrous material before it is offered to the animal (Wilkins, 1980; Wilkinson, 1982).

In experiment 5 borax buffer and an aqueous solution of ammonia were used to raise the pH before ensiling. The alkali treatments were compared with more conventional acid treatments (sulphuric and citric acids).

The addition of both sulphuric and citric acids to grass produced a low initial pH. Despite this, extensive fermentation and proteolysis took place, although deamination was reduced.

The addition of borax buffers raised the initial pH to between 8.0 and 9.3, depending on the level applied. These decreased to 6.5-7.4 during the period of ensiling, as a result of fermentation. Never-

theless, the borax-treated silages had higher residual contents of water-soluble carbohydrates than the control silage and the levels of proteolysis and deamination were considerably reduced.

The method chosen for the addition of ammonia proved to be unsatisfactory owing to its highly volatile nature. This resulted in the amount of ammonia added being much less than intended. The pH values after 24h ranged from 5.7 to 8.7 but extensive fermentation then occurred, resulting in final pH values varying from 4.1 to 4.8. Despite the fact that the pH must have passed through the optimum for protease activity during its decline, all the ammonia treatments resulted in higher protein levels than the control. In fact, treatment with the highest level of ammonia resulted in the highest residual protein content of all the treatments. It must be assumed, therefore, that either the decrease in pH was very rapid, which would seem unlikely, or that other factors, such as end-product inhibition, were involved, as has been suggested by Bergen *et al* (1974).

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13. APPENDIX 1

METHODS OF ANALYSIS

13.1 TOTAL NITROGEN (grass and silage)

About 10g of fresh material is accurately weighed into a pre-weighed polythene bag and placed into a 500 ml kjeldahl flask. Five grams of K_2SO_4/Se (435:15) catalyst is added then 40 ml conc. H_2SO_4. The flask is placed on the digestion stand and boiling continued for two hours after clearing.

When cool, the flask is half-filled with distilled water, shaken well and a few anti-bumping granules added before being set aside temporarily.

To a 600 ml conical flask are added 8 ml 0.5M H_2SO_4, three drops of methyl red indicator and water up to about 200 ml. The flask is placed under the condenser tip of the distillation unit.

Slowly, 130 ml 10M NaOH is added to the kjeldahl flask and the flask quickly attached to the distillation apparatus. The flask is swirled gently to mix the contents thoroughly before heat is applied. Distillation is continued for 30 minutes then the distillate is back-titrated using 0.1M NaOH.

13.2 TOTAL NITROGEN (grass extract)

The contents of a test-tube are quantitatively transferred to a 500 ml kjeldahl flask. Six ml conc. H_2SO_4 and 2 ml 30% (w/v) H_2O_2 are added then four kjeltabs containing a total of 6g K_2SO_4 and 0.3g HgO. The flask is placed on the digestion stand and

boiled for a further 20 minutes after clearing. Should frothing occur, a few drops of hot 50% lauric acid in ethanol are added.

After cooling the digest is made up to 75 ml and a sample analysed colorimetrically (section 13.5) for nitrogen.

13.3 NON-PROTEIN NITROGEN (grass and silage)

Twenty-five grams of fresh material are extracted twice with boiling water. The extracts are filtered through muslin, added together and the total volume noted. Total nitrogen is determined in 15 ml aliquots of the extract by Kjeldahl digestion (section 13.1) using 4g catalyst and 25 ml conc. H_2SO_4 for digestion, 90 ml 10M NaOH for distillation and 10 ml 0.05M H_2SO_4 in the collection flask.

13.4 NON-PROTEIN NITROGEN (grass extract)

One ml of 30% trichloroacetic acid (TCA) is added to the extract in the test tube to give a final TCA concentration of 5% (w/v). The tube is restoppered, shaken and left for 30 minutes after which the contents are transferred to a centrifuge tube, using a minimal amount of water. The extract is centrifuged for 10 minutes at 4000 rpm and the supernatant filtered through Whatman No.1. filter paper into a 500 ml kjeldahl flask. The precipitate is washed twice with distilled water and the washings transferred to the flask. Six ml conc. H_2SO_4 and 2 ml 30% (w/v) H_2O_2 are added and digestion and calorimetric nitrogen determination carried out as in sections 13.2 and 13.5 respectively.

13.5 COLORIMETRIC DETERMINATION OF NITROGEN IN KJELDAHL DIGESTS

A sample (250 µl) of the acid digest is diluted with water to 5 ml in a 6 ml plastic cup.

A Hook and Tucker T40 sample processor is set up with a diluter and two dispensers. The diluter is set to sample 100 µl of the prediluted sample and dispense 1.5 ml, dilution being with 1.12% (w/v) NaOH. The dispensers are set to dispense 1.5 ml 9.33% (w/v) sodium salicylate and 0.4 ml 0.4% (w/v) sodium dichloroisocyanurate - 0.04% sodium nitroprusside.

After reagent addition the samples are left for at least 40 minutes before reading the absorbances at 660 nm.

13.6 AMINO ACID NITROGEN (experiment 1)

Free amino acids are determined in a sample of the NPN extract after deproteinization with solid sulphosalicylic acid ($30 mg ml^{-1}$).

Total amino acids are determined in an oven dried (60°C) sample of grass or silage which has been kept in a vacuum dessicator. A correction is made for the loss of dry matter in the case of silages. An accurately known weight of about 0.3 g is taken and 150 ml 6M HCl added. After hydrolysis over nitrogen for 22 hours at 110-115°C the hydrolysate is made up to 500 ml with deionised, distilled water and filtered through a scintered funnel. Five ml are taken and dried down three times with water in a

ANALYSIS DATA SHEET

LKB BIOCHROM

SAMPLE NORMAL OPERATING CONDITIONS

VOLUME LOADED ..... AMOUNT LOADED .....

DATE ANALYSED .....

RUN NUMBER .....

OPERATOR .....

	Fluor	440nm	570nm
Base Line Pot Setting	—	550	640
Absorbance/Gain	—	0–1	0–1

Buffer p.H.

A = pH 3·2 Na Citrate

B = pH 4·25 Na Citrate

C = —

D = —

E = pH 10 Na Borate

F = 0·4 M NaOH

MINS.			BUFFER						PUMP	T2	T3		NIN O/R	FAN	LOAD	SOR	REC				X 10	HOLD	
			A	B	C	D	E	F															
		•1	1	●	○	○	○	○	○	●	○	○	○	○	○	○	●	●	○	○	○	●	○
	4·2		2	●	○	○	○	○	○	●	○	○	○	○	○	○	○	●	○	○	○	○	○
	7·7		3	●	○	○	○	○	○	●	●	○	○	○	○	○	○	●	○	○	○	○	○
2	1·0		4	○	●	○	○	○	○	●	●	○	○	○	○	○	○	●	○	○	○	○	○
	2·1		5	○	●	○	○	○	○	●	○	●	○	○	○	○	○	●	○	○	○	○	○
1	4·0		6	○	○	○	○	●	○	●	○	●	○	○	○	○	○	●	○	○	○	○	○
	2·8		7	○	○	○	○	○	●	●	○	●	○	○	○	○	○	●	○	○	○	○	○
	7·0		8	●	○	○	○	○	○	●	○	●	○	○	○	○	○	●	○	○	○	○	○
	4·2		9	●	○	○	○	○	○	●	○	○	○	●	●	○	○	○	○	○	○	○	○
1	3·3		10	●	○	○	○	○	○	●	○	○	○	●	○	○	○	○	○	○	○	○	○
		•5	11	●	○	○	○	○	○	●	○	○	○	●	○	○	○	○	○	○	○	○	○
		1·0	12	●	○	○	○	○	○	●	○	○	○	●	○	●	○	○	○	○	○	○	○
			13	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
			14	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
			15	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○

TOTAL RUN-TO-RUN TIME: 77·9 min.

Temperature setting

T1 = 55·0 °C

T2 = 58·0 °C

T3 = 82·0 °C

Operating Pressures at T1

Buffer = 25–35 Bar

Nin/~~OPA~~ = ca. 40 Bar

Coil = 7–8 Bar

Flow Rates

Buffer = 28nom ml/h

Nin/~~OPA~~ = 20nom ml/h

Chart Speed = 300mm/h

Pump Settings

Buffer Pump 24·0

Nin/~~OPA~~ Pump 16·5

Bubble time combined flow: 42sec.

Column No: L330 (Gold)

Analytical Column

Column Length 202 mm

Column i.d. 4·6 mm

Resin Type Ultropac 8 (Batch 10-62)

Figure 13.1. Operating conditions for the L.K.B. 4400 Amino Acid Analyser (Gardner, 1984).

rotary evaporator (35-40°C) then dried down completely and reconstituted in 2 ml pH 2.2, 0.2M sodium citrate buffer, ready for loading on to the analyser.

A mixed standard containing 10 n moles each of 17 amino acids is loaded before each run of 12 samples. An internal norleucine standard (10 n moles) is also loaded with each sample.

The determinations are carried out on an L.K.B. Model 4400 amino acid analyser (L.K.B. Biochrom Ltd, Cambridge Science Park, Cambridge, England) with a single 20 cm column of L.K.B. "Ultropac" resin. The analyser is equipped with an L.K.B. Model 2220 recording integrator. The manufacturer's standard methodology for protein hydrolysates is used (see Protein Chemistry Notes No.10 "Accelerated amino acid analysis using the L.K.B. 4400 Analyser with sodium buffer systems", L.K.B. Biochrom Ltd). The elution programme is given in Fig. 13.1.

13.7 AMINO ACID NITROGEN (Experiments 3 and 4)

Total amino acids are determined in hydrolysed samples of grass and silage which are prepared as above with two minor modifications: (1) Before drying down 5 ml of hydrolysate, 2 ml of norleucine standard (0.25 μMml^{-1}) is added; (2) the hydrolysate is reconstituted in 2 ml 10% sucrose solution in 0.1M HCl.

The determinations are carried out on a modified Technicon Auto Analyser (Technicon Instruments Co. Ltd., Hamilton Close, Houndmills, Basingstoke, Hants) with a single 65 cm column of

Technicon C-2 resin. The column is maintained at 60°C and the analyses carried out using protein hydrolysate methodology with a gradient elution system (pH 2.75-6.1 sodium citrate). Regeneration is with 0.2M NaOH for 30 minutes and re-equilibriation with pH 3.1 sodium citrate buffer for 90 minutes. Detection is with 0.1% (w/v) trinitrobenzene sulphonic acid (TNBS). The flow rates are 0.8 ml min^{-1} and 0.23 ml min^{-1} for buffers and TNBS respectively and a run is completed in 5.0-5.5 hours excluding regeneration and re-equilibriation.

13.8 MICROBIOLOGICAL ANALYSES

Plate count agar (CM 325), malt extract agar (CM 59), violet red bile agar (CM 107) and azide blood agar base (CM 259) are obtained from Oxoid Ltd, Wade Road, Basingstoke, Hants and are made up according to the manufacturers instructions.

Tween acetate agar, protein medium, lactate agar and anaerobic seal are made up as follows:

Tween acetate agar: peptone, 15 g; beef extract (Lab-lemco), 15 g; yeast extract, 15 g; Tween '80', 1.5 g; glucose, 15 g; agar, 45 g; water, 100 ml. The medium is bottled and sterilized at 121°C for 15 minutes. Just before pouring the plates, 10 ml pH 5.4, 2M sodium acetate buffer are added. After the inoculated medium has solidified an anaerobic seal is added.

Protein medium: peptone, 15 g; yeast extract, 2 g; gelatin, 120 g; L-cysteine hydrochloride, 0.5 g; water, 1000 ml; pH 7.0. Ten ml aliquots are poured into test tubes and sterilized at 121°C for

15 minutes. The medium is liquified before inoculating and after cooling an anaerobic seal is applied.

Lactate agar: agar, 2 g; resazurin, 5 mg; yeast extract, 6 g; L-cysteine hydrochloride, 0.5 g; thioglycollic acid, 0.5 ml; sodium lactate, 14.3 ml of 70% syrup; sodium acetate, 8 g; ammonium sulphate, 1 g; biotin, 0.1 µg; p-aminobenzoic acid, 100 µg; water, 1000 ml; pH 6.0. Ten ml aliquots are poured into test tubes and sterilized at 121°C for 15 minutes. The medium is liquified before inoculating and after cooling an anaerobic seal is applied.

Anaerobic seal: agar, 10 g; L-cysteine hydrochloride, 0.5 g; thioglycollic acid, 0.5 ml; resazurin, 5 mg; water, 1000 ml; pH 7.0. The medium is bottled and sterilized at 121°C for 15 mins.

All the media are incubated at 27°C for 5 days except for violet red bile agar which is incubated at 37°C overnight.

14. APPENDIX 2

RESULTS

14.1 EXPERIMENT 1

TABLE 14.1.1

Dry matter, pH, water-soluble carbohydrates and lactic acid

	Replicate No.	Grass	SILAGES A	SILAGES B	SILAGES C
Dry matter (gkg^{-1})	1	159	158	156	157
	2	158	158	157	149
	3	159	157	156	155
	4	-	159	158	154
pH	1	5.60	3.77	5.20	4.04
	2	5.70	3.79	5.07	3.95
	3	5.65	3.78	5.27	4.43
	4	-	3.81	5.13	4.07
Water-soluble carbohydrates (gkg^{-1}DM)	1	124	1	213	2
	2	124	10	202	105
	3	123	2	222	103
	4	-	1	178	14
Lactic acid	1	ND	119	16	72
	2		116	6	91
	3		115	7	30
	4		124	15	75

ND - not determined

TABLE 14.1.2

Volatile fatty acids, ethanol and gross energy

	Replicate No.	SILAGES		
		A	B	C
Acetic Acid ($gkg^{-1}DM$)	1	12.28	6.04	18.31
	2	17.35	9.59	8.76
	3	19.99	6.04	20.15
	4	20.20	7.47	24.19
Propionic Acid ($gkg^{-1}DM$)	1	0	0.62	0
	2	0	1.18	0
	3	0	0.52	0.29
	4	0	1.45	0.22
Butyric Acid ($gkg^{-1}DM$)	1	0	11.22	1.11
	2	0	16.77	0
	3	0	9.79	17.01
	4	0	19.37	0.52
Ethanol ($gkg^{-1}DM$)	1	13.9	3.0	6.7
	2	14.3	3.7	6.4
	3	14.5	3.8	6.7
	4	15.3	3.8	6.8
Gross Energy ($MJkg^{-1}DM$)	1	18.5	18.8	17.9
	2	18.0	18.6	18.4
	3	18.5	18.5	18.6
	4	18.2	18.3	18.7

TABLE 14.1.3

Total N, protein N, volatile N and amide N

	Replicate No.	Grass	SILAGES		
			A	B	C
Total N (gkg^{-1}DM)	1	23.1	23.3	23.9	24.6
	2	23.8	25.0	23.9	24.3
	3	23.6	23.8	23.2	24.5
	4	-	23.5	24.3	24.8
Protein N (gkg^{-1}TN)	1	861	289	365	458
	2	855	325	373	471
	3	859	281	303	477
	4	-	278	393	462
Volatile N (gkg^{-1}TN)	1	10.6	133	75	80
	2	10.6	158	88	58
	3	10.6	126	82	65
	4	-	127	82	104
Amide N (gkg^{-1}TN)	1	9.7	15.5	8.8	14.2
	2	9.8	15.6	10.5	12.8
	3	9.8	15.1	8.6	9.0
	4	-	16.6	12.8	12.9

TABLE 14.1.4

Free amino acid nitrogen ($gkg^{-1}TN$)

Amino Acid	Replicate No.	Grass	SILAGES		
			A	B	C
Aspartic Acid	1	6.96	21.38	29.59	13.20
	2	7.19	22.23	27.56	26.51
	3	7.07	21.36	29.67	11.23
	4	6.89	17.76	27.96	22.20
Threonine*	1	11.50	10.37	14.95	18.55
	2	12.30	12.52	8.72	14.83
	3	11.83	12.42	14.60	14.73
	4	11.51	11.09	10.16	17.08
Serine*	1	-	10.78	16.75	11.18
	2	-	11.45	4.22	17.73
	3	-	10.99	14.85	13.73
	4	-	7.51	5.51	0
Glutamic Acid	1	6.13	10.78	12.08	10.90
	2	6.42	10.15	11.12	10.18
	3	6.35	1.07	14.71	11.84
	4	6.27	3.22	11.11	8.65
Proline	1	1.60	19.06	19.37	13.41
	2	1.90	19.24	18.12	13.04
	3	1.52	17.64	19.27	14.15
	4	1.59	17.59	16.21	13.08
Glycine	1	0.46	38.46	24.61	21.82
	2	0.48	34.51	29.41	16.86
	3	0.46	38.05	26.51	17.05
	4	0.46	37.97	27.39	20.53
Alanine	1	6.59	67.30	42.96	51.59
	2	6.41	59.39	45.22	37.34
	3	6.66	64.89	38.34	49.93
	4	6.53	69.78	38.49	35.69
Valine	1	1.74	29.64	24.40	19.31
	2	1.74	27.97	24.41	17.85
	3	1.78	29.24	21.86	17.40
	4	1.74	28.85	26.95	17.16
Isoleucine	1	0.85	19.81	15.94	11.91
	2	0.83	17.89	16.05	10.39
	3	0.91	19.42	16.07	10.12
	4	0.87	19.22	15.81	10.40
Leucine	1	1.10	33.21	27.35	20.96
	2	1.04	30.77	26.50	20.83
	3	1.12	32.52	24.23	20.73
	4	1.07	31.86	22.37	17.34
Tyrosine/					

TABLE 14.1.4 (cont'd)

Amino Acid	Replicate No.	Grass	SILAGES		
			A	B	C
Tyrosine	1	0.26	6.11	10.05	3.36
	2	0.36	6.24	9.59	2.94
	3	0.39	6.57	8.12	2.19
	4	0.38	5.53	7.61	0
Phenylalanine	1	0.62	13.86	12.38	9.06
	2	0.66	12.89	11.95	8.77
	3	0.73	13.52	10.29	8.62
	4	0.69	13.17	12.27	7.83
Lysine	1	1.81	14.74	26.60	28.28
	2	1.78	14.01	28.88	28.10
	3	1.82	15.18	27.93	27.82
	4	1.78	11.28	28.18	26.39
Arginine	1	2.22	0	38.69	0
	2	2.31	1.30	42.59	27.68
	3	2.49	5.16	37.95	28.44
	4	2.24	4.07	34.54	0

* the results for serine are included in the threonine figure.

TABLE 14.1.5

Total amino acid nitrogen ($gkg^{-1}TN$)

Amino Acid	Replicate No.	Grass	SILAGES		
			A	B	C
Aspartic Acid	1	68.66	38.64	60.37	39.94
	2	65.28	40.52	59.75	46.10
	3	63.21	41.86	61.11	43.64
	4	70.38	42.30	60.98	42.93
Threonine	1	31.62	18.13	29.57	32.23
	2	32.08	19.16	30.17	31.00
	3	30.17	20.37	28.86	35.38
	4	28.01	20.51	29.06	31.54
Serine	1	39.15	20.59	31.80	29.19
	2	31.98	22.31	31.61	29.42
	3	34.56	19.04	33.02	27.39
	4	31.15	21.28	31.55	28.09
Glutamic Acid	1	60.33	35.31	57.50	45.56
	2	61.06	36.07	58.09	46.50
	3	57.88	26.28	57.71	54.52
	4	57.91	29.00	61.58	49.32
Proline	1	31.48	36.86	31.39	30.44
	2	29.09	29.23	30.52	30.83
	3	30.53	32.82	32.75	33.11
	4	31.21	33.64	32.84	31.58
Glycine	1	66.36	63.40	56.16	54.93
	2	66.14	64.15	57.14	58.87
	3	65.79	72.63	58.10	64.57
	4	64.06	67.42	61.20	61.97
Alanine	1	72.18	82.12	61.23	73.89
	2	72.83	78.44	65.00	68.59
	3	66.82	81.73	63.38	70.44
	4	66.63	94.74	67.25	78.47
Valine	1	40.72	38.07	27.20	38.26
	2	44.34	38.86	29.01	42.24
	3	45.43	37.29	27.57	39.10
	4	43.47	40.21	28.75	39.09
Isoleucine	1	22.86	23.93	21.29	22.16
	2	23.35	21.51	20.64	24.06
	3	22.23	23.01	21.37	23.91
	4	22.93	24.53	22.59	26.00
Leucine	1	45.82	50.67	42.57	42.67
	2	45.86	43.94	41.00	44.20
	3	47.68	42.47	42.35	47.85
	4	42.92	48.97	44.31	45.35
Tyrosine/					

TABLE 14.1.5 (cont'd)

Amino Acid	Replicate No.	Grass	SILAGES		
			A	B	C
Tyrosine	1	14.18	10.13	14.37	11.99
	2	14.71	11.12	14.97	12.19
	3	13.73	10.43	14.93	12.88
	4	15.37	9.59	15.47	8.45
Phenylalanine	1	22.87	23.05	25.97	20.51
	2	23.94	22.84	26.56	21.12
	3	23.46	21.56	24.99	25.51
	4	23.45	22.82	26.94	22.13
Lysine	1	61.94	31.35	54.35	56.36
	2	60.53	29.36	49.30	54.42
	3	61.44	31.75	53.90	58.06
	4	63.66	29.57	54.57	58.72
Arginine	1	87.13	23.68	83.00	55.98
	2	83.97	26.19	71.69	76.13
	3	89.08	23.61	72.17	79.67
	4	94.44	20.36	81.97	54.93

14.2 EXPERIMENT 2

TABLE 14.2.1

Experiment 2.1 The percentage of the initial protein hydrolysed during incubation of grass extract at 37°C for varying lengths of time.

pH	Replicate No.	INCUBATION TIME		
		30h	50h	12d
6.60	1	4.73	32.87	39.97
	2	7.23	32.36	36.15
	3	6.44	32.27	38.61
	4	7.59	32.58	39.44
5.90	1	7.19	38.83	46.22
	2	7.47	40.03	40.83
	3	8.64	42.83	46.15
	4	6.13	42.97	42.79
4.95	1	4.17	29.69	33.29
	2	4.86	28.70	38.43
	3	7.98	27.54	34.58
	4	4.74	29.02	34.52
4.20	1	2.67	19.11	27.60
	2	2.57	22.36	28.28
	3	3.46	21.57	28.08
	4	1.25	20.81	26.27
3.60	1	2.43	18.43	21.70
	2	2.67	15.15	19.78
	3	3.93	18.93	22.19
	4	2.76	17.81	23.83
3.40	1	3.36	13.36	18.69
	2	3.80	15.34	19.65
	3	3.57	14.35	19.36
	4	4.97	16.32	20.26

TABLE 14.2.2

Experiment 2.2 The percentage of the initial protein hydrolysed during incubation of grass extract at 37°C for varying lengths of time

pH	Replicate No.	INCUBATION TIME		
		50h	12d	120d
6.80	1	25.82	37.79	42.80
	2	28.40	39.22	42.38
	3	29.04	40.11	42.38
	4	27.49	39.16	42.91
5.60	1	31.48	41.66	43.20
	2	32.86	44.70	45.77
	3	39.95	42.67	51.21
	4	43.76	42.67	49.61
4.80	1	26.36	34.69	35.43
	2	28.18	32.57	34.57
	3	23.41	33.12	33.51
	4	29.04	32.28	34.21
3.80	1	21.78	26.96	34.57
	2	23.91	32.38	30.93
	3	26.48	31.30	29.72
	4	25.26	30.69	30.26
2.75	1	14.55	26.58	26.72
	2	15.83	25.55	26.81
	3	15.40	23.96	24.00
	4	13.95	21.70	22.95
2.00	1	13.29	19.18	28.85
	2	17.71	23.49	25.11
	3	16.37	21.66	29.62
	4	14.29	23.16	26.23
1.05	1	14.99	38.68	62.64
	2	20.81	35.08	58.41
	3	17.94	39.77	64.34
	4	17.14	38.68	57.56
0.75	1	26.16	58.17	67.97
	2	30.38	53.40	72.39
	3	24.98	54.05	71.96
	4	22.60	52.59	72.59

TABLE 14.2.3

Experiment 2.3 The percentage of the initial protein hydrolysed during incubation of grass extract for 120 days

pH	Replicate No.	37°C	20°C	20°C (Heated)
7.15	1	23.74	23.06	1.28
	2	23.67	22.94	3.81
	3	24.05	24.32	4.81
6.80	1	30.80	26.02	0.12
	2	29.47	26.91	0.42
	3	30.80	29.77	2.12
6.25	1	32.34	30.74	1.47
	2	33.38	29.67	0.88
	3	33.91	30.61	0.83
5.65	1	37.40	27.83	2.33
	2	35.84	29.38	2.31
	3	34.13	27.43	3.31
5.25	1	31.97	24.62	1.31
	2	35.56	22.65	-1.26
	3	32.16	22.66	0.88
4.70	1	30.72	20.08	0.85
	2	30.79	19.49	0.07
	3	29.80	20.38	-1.15
4.30	1	24.66	17.67	-0.88
	2	27.02	14.96	-0.72
	3	22.65	18.11	-1.21
3.80	1	22.63	11.84	1.29
	2	23.57	12.08	-0.22
	3	21.91	16.32	0.99
3.35	1	21.23	13.52	0.17
	2	22.39	12.35	0.69
	3	20.94	12.20	0.22
2.70	1	18.63	10.60	-2.31
	2	20.86	11.82	1.21
	3	18.09	10.56	1.03
2.45	1	22.44	7.56	-
	2	21.56	7.47	-
	3	21.40	6.85	-
1.95	1	21.28	7.09	-0.22
	2	22.37	7.09	-0.84
	3	20.44	6.35	-0.35
1.55	1	26.58	13.88	5.20
	2	25.41	7.59	6.13
	3	24.00	8.90	5.52

1.30/

TABLE 14.2.3 (contd.)

pH	Replicate No.	37°C	20°C	20°C (Heated)
1.30	1	35.47	14.57	9.72
	2	34.91	15.97	6.47
	3	34.91	15.03	6.87
1.05	1	43.35	17.41	13.92
	2	41.89	16.89	12.59
	3	41.99	19.29	14.41
0.85	1	48.89	17.01	14.30
	2	49.56	19.68	18.91
	3	47.28	19.14	17.39
0.65	1	51.74	22.38	22.74
	2	53.00	22.81	31.12
	3	53.04	23.48	25.47
0.50	1	59.60	33.56	39.17
	2	62.73	33.33	37.51
	3	65.25	32.93	35.55
0.30	1	66.61	40.60	42.94
	2	69.60	38.73	48.33
	3	66.26	38.63	46.87

14.3 EXPERIMENT 3

TABLE 14.3.1

Dry matter, pH, water-soluble carbohydrates and lactic acid

Treatment	Replicate No.	DM (gkg^{-1})		pH		WSC ($gkg^{-1}DM$)		Lactic Acid ($gkg^{-1}DM$)	
Grass	1	153		5.89		179		ND	
	2	153		5.87		179			
	3	152		5.89		183			
Silages		24h	76d	24h	76d	24h	76d	24h	76d
AO	1	152	148	5.61	3.77	181	25	ND	155
	2	153	153	5.89	3.71	189	1		145
	3	157	166	5.75	3.77	194	2		151
AM	1	162	160	5.74	3.71	203	1		141
	2	147	157	5.77	3.82	231	2		99
	3	159	154	5.75	3.79	211	1		99
AG	1	171	166	5.72	3.78	263	64		135
	2	170	170	5.71	3.71	271	61		136
	3	168	163	5.67	3.80	267	9		133
BO	1	149	154	5.40	3.70	180	102		178
	2	150	157	5.42	3.71	184	77		170
	3	159	159	5.47	3.69	178	35		150
BM	1	158	151	5.69	3.70	205	79		179
	2	158	150	5.73	3.68	205	75		156
	3	156	145	5.71	3.67	211	73		146
BG	1	167	163	5.77	3.66	270	103		167
	2	166	161	5.75	3.63	287	34		156
	3	165	167	5.77	3.68	293	50		141
CO	1	155	149	4.73	3.57	152	38		186
	2	160	160	4.45	3.57	160	49		149
	3	152	153	4.45	3.58	153	51		163
CM	1	154	151	5.19	3.57	216	79		195
	2	159	154	4.92	3.56	207	65		196
	3	159	165	5.10	3.56	213	45		174
CG	1	175	159	4.70	3.59	208	27		172
	2	167	168	4.77	3.60	226	50		167
	3	155	167	4.90	3.59	248	111		155
DO	1	165	161	4.06	3.57	137	36		187
	2	165	155	4.02	3.57	142	49		156
	3	160	154	4.00	3.53	138	69		196
DM	1	163	154	4.21	3.57	189	84		169
	2	162	157	4.18	3.50	173	70		166
	3	168	156	4.18	3.52	179	100		163
DG	1	180	163	4.18	3.55	185	23		167
	2	182	176	4.21	3.55	194	44		163
	3	174	164	4.21	3.53	221	21		162

ND - not determined

TABLE 14.3.2

Volatile fatty acids, ethanol and gross energy

Treatment	Replicate No.	Acetic Acid ($gkg^{-1}DM$)	Ethanol ($gkg^{-1}DM$)	Gross Energy ($MJkg^{-1}DM$)
Silages				
AO	1	21.78	30	19.0
	2	25.96	14	19.1
	3	31.91	18	17.8
AM	1	54.90	19	18.2
	2	47.84	32	19.9
	3	44.17	24	19.9
AG	1	27.53	42	18.9
	2	47.88	9	18.3
	3	44.17	51	19.4
BO	1	12.11	9	19.3
	2	12.07	21	18.7
	3	11.58	17	19.8
BM	1	6.44	20	20.3
	2	6.96	26	18.5
	3	6.62	27	19.0
BG	1	13.68	38	18.1
	2	10.88	63	18.9
	3	10.26	46	18.7
CO	1	10.86	12	18.1
	2	10.10	11	18.9
	3	10.43	11	18.7
CM	1	5.88	11	18.7
	2	5.67	22	19.3
	3	5.42	20	18.7
CG	1	9.03	44	19.4
	2	8.12	48	19.5
	3	9.14	26	18.9
DO	1	7.91	19	16.3
	2	7.78	10	17.7
	3	8.08	11	17.6
DM	1	3.73	22	18.8
	2	3.98	35	17.6
	3	3.68	23	18.8
DG	1	6.08	57	18.9
	2	6.42	48	16.5
	3	7.13	50	20.2

TABLE 14.3.3

Total N, protein N, volatile N and amide N

Treatment	Replicate No.	TN (gkg^{-1}DM)		PN (gkg^{-1}TN)		VN (gkg^{-1}TN)		Amide N (gkg^{-1}TN)	
Grass	1	26.4		800		22.4		29.4	
	2	26.8		793		15.6		24.0	
	3	25.4		798		-		13.7	
Silages		24h	76d	24h	76d	24h	76d	24h	76d
AO	1	26.3	26.3	586	245	38	118	ND	10.3
	2	27.1	26.9	633	278	34	112		9.3
	3	26.5	27.4	639	268	34	113		9.1
AM	1	24.7	27.9	585	340	30	104		12.9
	2	30.2	28.6	619	363	34	101		13.3
	3	26.8	28.2	606	345	33	106		15.3
AG	1	25.6	24.3	655	225	27	115		14.0
	2	24.5	24.4	639	273	24	98		18.9
	3	23.9	25.0	639	267	38	112		14.8
BO	1	27.2	27.7	629	289	29	87		13.0
	2	25.8	28.6	626	289	31	98		13.6
	3	24.5	27.2	622	280	28	96		12.9
BM	1	27.1	27.2	611	364	26	81		17.3
	2	27.0	27.1	614	350	28	81		18.8
	3	26.9	26.4	603	345	29	83		20.5
BG	1	24.1	25.0	634	263	30	120		12.0
	2	25.3	25.3	653	274	24	103		14.6
	3	24.3	24.6	663	248	30	94		14.2
CO	1	26.8	26.3	620	354	23	59		32.7
	2	25.9	27.5	626	362	26	61		24.7
	3	28.3	27.7	686	357	20	60		27.1
CM	1	27.4	27.6	601	402	26	53		27.5
	2	25.7	27.6	570	386	29	45		26.1
	3	25.5	26.6	566	379	23	55		26.3
CG	1	23.7	26.3	670	367	24	59		25.9
	2	25.2	25.9	686	354	29	48		25.1
	3	26.6	24.7	674	345	24	51		26.7
DO	1	26.5	25.4	616	305	18	47		25.2
	2	26.1	26.8	627	295	19	49		23.9
	3	25.1	25.7	545	302	25	47		34.2
DM	1	23.7	26.6	566	380	24	41		35.3
	2	25.2	27.3	563	386	24	40		34.1
	3	26.6	26.5	601	361	22	42		36.6
DG	1	24.6	23.5	624	307	19	47		37.5
	2	23.3	24.0	618	311	22	50		35.4
	3	23.4	24.5	627	318	23	45		58.8

ND - not determined

TABLE 14.3.4

Total amino acid nitrogen ($gkg^{-1}TN$)

Amino Acid	Replicate No.	GRASS	SILAGES			
			AO	DO	DM	DG
Aspartic Acid	1	54.64	56.07	60.30	61.72	59.91
	2	62.67	53.61	59.00	60.34	56.21
	3	-	60.78	63.23	61.52	69.12
Threonine	1	23.37	27.86	26.51	26.83	28.71
	2	38.67	25.59	24.43	25.25	25.85
	3	-	31.07	26.74	26.78	31.62
Serine	1	27.15	26.33	28.46	28.09	28.41
	2	31.12	26.67	24.79	26.13	27.67
	3	-	30.80	28.35	29.21	33.50
Glutamic Acid	1	60.54	38.05	56.04	60.90	57.99
	2	69.26	43.85	48.39	59.32	55.11
	3	-	29.78	58.23	62.10	67.77
Glycine	1	50.25	58.00	52.50	51.88	55.33
	2	50.75	53.87	47.94	51.08	51.05
	3	-	56.36	86.70	53.52	59.31
Alanine	1	62.74	68.81	61.89	57.75	63.10
	2	66.34	68.48	57.19	56.25	61.33
	3	-	76.08	66.23	58.09	73.10
Valine	1	34.30	42.44	35.57	36.02	36.17
	2	38.27	37.05	35.90	34.28	37.48
	3	-	43.41	37.56	34.76	38.65
Methionine	1	7.81	7.18	4.94	7.54	6.57
	2	0	7.82	4.91	6.42	6.48
	3	-	7.79	5.27	6.60	6.46
Isoleucine	1	24.56	26.81	25.06	24.91	25.02
	2	24.40	24.49	25.06	23.57	23.15
	3	-	27.78	23.43	24.66	25.43
Leucine	1	42.72	45.05	44.19	67.73	43.37
	2	43.83	42.88	43.30	41.52	39.25
	3	-	50.17	42.94	42.25	46.39
Tyrosine	1	13.89	12.44	15.30	14.46	16.41
	2	15.39	6.40	13.60	15.32	15.07
	3	-	8.11	13.64	15.10	15.89
Phenylalanine	1	23.45	23.33	24.95	22.94	24.01
	2	23.65	22.97	21.92	22.52	21.58
	3	-	25.01	21.80	22.20	23.82
Histidine	1	28.83	28.07	30.26	27.35	31.05
	2	32.03	29.21	28.00	29.23	28.00
	3	-	16.38	29.36	29.40	32.59
Lysine	1	50.15	41.89	63.03	56.10	58.53
	2	47.88	39.25	55.05	55.36	53.01
	3	-	41.68	55.24	52.03	58.93
Arginine	1	89.45	26.17	66.60	71.83	76.13
	2	91.04	24.02	69.79	72.95	68.55
	3	-	30.47	68.00	72.77	72.95

14.4 EXPERIMENT 4

TABLE 14.4.1

Dry matter, pH, water-soluble carbohydrates and lactic acid

Treatment	Replicate No.	DM (gkg^{-1})		pH		WSC ($gkg^{-1}DM$)		Lactic Acid ($gkg^{-1}DM$)	
Grass	1	242		6.11		148		ND	
	2	244		6.09		144			
	3	243		6.12		140			
Silages		24h	125d	24h	125d	24h	125d	24h	125d
OA	1	235	236	5.67	3.75	164	19	ND	113
	2	238	234	5.71	3.78	162	27		112
	3	242	244	5.76	3.78	154	21		118
OB	1	253	234	4.72	3.80	175	144		67
	2	246	235	4.70	3.88	186	178		52
	3	252	238	4.74	3.82	186	151		54
OC	1	249	243	4.19	4.08	186	235		12
	2	251	240	4.19	4.05	173	229		20
	3	253	245	4.19	4.02	178	226		2
OD	1	251	253	3.85	3.90	180	204		10
	2	251	249	3.81	3.90	172	219		7
	3	253	245	3.82	3.90	167	209		7
FA	1	249	248	5.85	3.80	163	76		97
	2	250	239	5.86	3.95	164	135		66
	3	251	250	5.87	3.80	168	68		76
FB	1	252	243	4.71	4.59	176	234		10
	2	251	240	4.71	4.59	164	249		21
	3	252	244	4.72	4.60	156	204		7
FC	1	256	251	4.12	4.13	174	210		2
	2	255	249	4.12	4.13	174	212		19
	3	255	244	4.22	4.12	163	217		28
FD	1	250	250	3.90	3.88	172	182		5
	2	248	248	3.91	3.83	172	199		26
	3	256	248	3.92	3.83	172	194		17

ND - not determined

TABLE 14.4.2

Volatile fatty acids, ethanol and gross energy

Treatment	Replicate No.	Acetic Acid ($gkg^{-1}DM$)	Propionic Acid ($gkg^{-1}DM$)	Ethanol ($gkg^{-1}DM$)	Gross Energy ($MJkg^{-1}DM$)
Silages					
OA	1	26.99	0	5.20	18.1
	2	24.69	0	5.60	17.6
	3	24.63	0	5.53	16.9
OB	1	12.73	0	1.61	18.3
	2	9.76	0	1.63	18.0
	3	11.51	0	1.61	17.9
OC	1	3.18	0.17	0.95	17.8
	2	2.01	0.10	0.66	17.3
	3	2.06	0.10	0.84	17.7
OD	1	1.88	0.10	1.87	17.2
	2	2.28	0.08	1.61	16.4
	3	2.31	0.10	0.64	17.5
FA	1	19.31	0	4.33	16.9
	2	13.20	0	4.01	17.4
	3	19.15	0	4.24	17.6
FB	1	2.42	0.13	1.75	16.7
	2	2.28	0.16	1.37	18.1
	3	2.32	0.10	1.59	17.1
FC	1	2.28	0.08	1.45	16.6
	2	2.00	0.13	1.66	16.8
	3	2.00	0.10	1.54	16.2
FD	1	2.14	0.10	1.31	17.3
	2	2.26	0.13	1.91	17.7
	3	2.33	0.13	1.66	17.3

TABLE 14.4.3

Total N, protein N, volatile N and amide N

Treatment	Replicate No.	TN ($gkg^{-1}DM$)		PN ($gkg^{-1}TN$)		VN ($gkg^{-1}TN$)		Amide-N ($gkg^{-1}TN$)	
Grass	1	26.8		975		13		17.4	
	2	27.4		940		13		18.0	
	3	27.0		937		12		17.9	
Silages		24h	125d	24h	125d	24h	125d	24h	125d
OA	1	27.7	27.9	727	362	16	73	ND	20.4
	2	28.2	28.4	733	363	12	65		20.1
	3	26.4	27.6	714	344	12	64		21.4
OB	1	27.0	28.3	707	364	13	36		21.6
	2	26.9	28.2	722	400	14	30		20.6
	3	26.2	28.0	702	382	19	35		22.1
OC	1	26.3	26.8	767	429	14	16		23.5
	2	26.5	27.1	773	439	14	20		21.8
	3	25.9	27.1	755	469	18	20		21.0
OD	1	27.4	25.7	798	483	9	26		20.2
	2	26.5	26.8	777	448	9	16		23.5
	3	26.3	26.5	783	506	10	23		17.7
FA	1	25.7	27.0	784	363	11	59		17.8
	2	26.8	27.3	750	374	7	51		15.8
	3	25.9	27.0	768	400	9	54		18.1
FB	1	26.6	28.1	794	582	9	19		12.8
	2	27.4	28.0	793	571	10	31		12.9
	3	27.0	27.6	790	598	9	24		12.7
FC	1	26.1	27.0	801	644	9	13		15.2
	2	25.8	27.7	804	644	11	14		14.8
	3	26.0	28.0	798	665	9	14		15.4
FD	1	26.9	26.0	808	609	6	14		16.1
	2	26.8	26.7	812	704	8	17		15.3
	3	26.3	26.7	814	700	10	17		15.9

TABLE 14.4.4

Total amino acid nitrogen ($gkg^{-1}TN$)

Amino Acid	Replicate No.	GRASS	SILAGES		
			OA	OD	FD
Aspartic Acid	1	53.48	38.72	52.47	63.56
	2	56.86	47.07	57.50	58.70
	3	-	43.16	60.53	60.83
Threonine	1	29.14	27.38	27.94	35.98
	2	24.85	26.24	30.26	30.02
	3	-	29.51	31.75	31.34
Serine	1	28.71	25.21	30.01	38.38
	2	29.45	27.25	32.16	32.18
	3	-	28.62	33.68	32.43
Glutamic Acid	1	63.73	46.79	64.94	70.63
	2	63.52	45.80	64.97	66.96
	3	-	49.71	68.21	69.53
Glycine	1	52.64	51.23	51.88	56.81
	2	57.12	51.09	55.53	54.62
	3	-	56.89	54.05	54.76
Alanine	1	52.30	69.84	54.73	62.56
	2	57.58	66.18	60.98	60.33
	3	-	73.56	60.31	61.92
Valine	1	36.38	35.98	36.55	39.76
	2	39.16	39.30	36.21	37.37
	3	-	39.06	36.84	38.40
Isoleucine	1	26.47	25.67	25.83	27.79
	2	25.91	26.53	28.28	26.22
	3	-	26.61	26.94	27.38
Leucine	1	45.73	44.91	45.27	48.28
	2	48.68	46.19	46.36	48.65
	3	-	46.21	49.62	50.17
Tyrosine	1	15.82	15.54	16.24	16.61
	2	17.11	15.56	15.71	15.25
	3	-	16.80	16.68	16.41
Phenylalanine	1	27.27	26.31	28.62	28.03
	2	26.71	27.10	24.95	24.35
	3	-	27.61	26.06	26.12
Histidine	1	27.66	29.79	30.44	30.61
	2	30.06	29.64	30.40	26.26
	3	-	30.89	29.21	26.46
Lysine	1	34.70	55.33	62.01	54.51
	2	37.60	56.31	62.46	50.72
	3	-	61.62	64.40	52.65
Arginine	1	93.56	46.90	58.67	103.12
	2	95.24	52.97	106.04	100.29
	3	-	54.74	104.93	91.14

1.45 EXPERIMENT 5

TABLE 14.5.1

Dry matter, pH, water-soluble carbohydrates and lactic acid

Treatment	Replicate No.	DM (gkg^{-1})		pH		WSC (gkg^{-1})		Lactic Acid (gkg^{-1})	
Grass	1	173		5.83		12.35		ND	
	2	175		5.88		14.27			
	3	175		5.89		15.66			
Silages		24h	140d	24h	140d	24h	140d	24h	140d
C	1	163	170	5.40	3.99	11.25	0.68	ND	24.12
	2	169	168	5.20	4.00	10.31	2.18		24.85
	3	161	166	5.23	3.98	12.40	1.83		22.76
SA	1	177	171	4.21	3.80	16.46	1.88		15.70
	2	176	169	4.10	3.80	14.78	1.69		21.65
	3	173	171	4.09	3.72	14.71	1.71		17.10
CA	1	183	173	3.85	3.65	17.39	3.46		20.92
	2	182	170	3.89	3.62	17.65	2.89		19.58
	3	185	174	3.82	3.66	17.39	2.78		19.56
B1	1	179	170	8.00	6.75	13.96	21.76		18.19
	2	181	168	8.07	6.50	11.95	19.32		16.62
	3	178	174	8.02	6.30	16.73	17.92		10.84
B2	1	183	177	8.84	6.39	15.01	5.66		13.06
	2	188	179	8.90	6.71	15.04	5.73		11.12
	3	183	174	8.90	6.48	14.64	1.74		13.28
B3	1	186	174	9.20	7.42	11.72	8.53		6.11
	2	183	174	9.30	7.20	11.71	5.74		8.39
	3	188	176	9.31	7.59	11.84	10.38		4.89
A1	1	169	167	5.70	4.12	10.65	0.17		23.81
	2	168	168	5.50	4.21	10.92	1.18		18.98
	3	161	168	5.75	4.10	10.63	1.18		22.26
A2	1	170	167	6.81	4.51	13.77	0.67		20.44
	2	169	167	7.49	4.52	14.70	0.17		17.28
	3	166	170	7.10	4.62	16.27	1.02		18.97
A3	1	174	179	8.79	4.70	16.18	1.61		17.06
	2	172	169	8.60	4.99	17.72	1.18		13.60
	3	173	171	8.68	4.56	17.13	0.17		16.57

TABLE 14.5.2

Volatile fatty acids, ethanol and gross energy

Treatment*	Replicate No.	Acetic Acid (gkg^{-1})	Propionic Acid (gkg^{-1})	Ethanol (gkg^{-1})	Gross Energy ($MJkg^{-1}$)
Silages					
C	1	6.62	0.78	0.77	3.23
	2	6.64	0.78	0.79	3.21
	3	6.67	0.75	0.76	3.42
SA	1	5.21	0.76	0.64	3.01
	2	4.82	0.69	0.72	2.97
	3	4.37	0.66	0.52	3.10
CA	1	4.79	0.62	0.35	3.24
	2	4.83	0.59	0.30	3.21
	3	4.90	0.61	0.52	3.24
B1	1	1.37	0.67	0.36	3.04
	2	2.04	0.61	0.36	3.06
	3	1.28	0.50	0.48	3.01
B2	1	2.45	0.52	0.46	2.71
	2	2.60	0.52	0.76	2.61
	3	2.31	0.52	0.51	2.92
B3	1	3.20	0.50	0.74	3.10
	2	3.49	0.48	0.62	2.94
	3	3.19	0.50	0.86	3.12
A1	1	7.58	0.60	0.68	3.17
	2	7.68	0.58	0.79	3.06
	3	7.37	0.56	0.75	2.99
A2	1	9.13	0.73	1.41	2.92
	2	9.29	0.79	1.21	2.84
	3	8.88	0.67	1.47	3.07
A3	1	8.97	0.70	0.98	3.28
	2	10.05	0.75	1.37	3.14
	3	9.62	0.87	0.98	3.25

TABLE 14.5.3

Total N, protein N, volatile N and amide-N

Treatment*	Replicate No.	TN (gkg^{-1})		PN (gkg^{-1})		VN (gkg^{-1})		Amide-N (gkg^{-1})	
Grass	1	7.13		6.41		0.08		0.16	
	2	6.96		6.19		0.07		0.16	
	3	6.62		5.91		0.06		0.16	
Silages		24h	140d	24h	140d	24h	140d	24h	140d
C	1	6.99	6.76	4.95	1.11	0.18	0.68	ND	0.14
	2	7.00	6.85	4.84	1.21	0.18	0.67		0.24
	3	6.66	6.34	4.66	1.57	0.17	0.68		0.25
SA	1	7.01	6.28	5.33	1.86	0.06	0.48		0.24
	2	6.98	7.16	5.32	2.62	0.07	0.48		0.25
	3	6.80	6.36	5.17	1.91	0.06	0.47		0.22
CA	1	6.69	6.58	5.11	1.87	0.06	0.37		0.20
	2	6.98	6.64	5.23	2.09	0.04	0.41		0.21
	3	6.61	6.77	4.88	2.06	0.04	0.40		0.23
B1	1	6.46	6.81	4.99	2.08	0.11	0.44		0.08
	2	6.70	6.81	5.30	2.10	0.11	0.48		0.10
	3	6.45	6.84	5.11	2.29	0.10	0.52		0.09
B2	1	6.74	6.71	5.55	3.24	0.07	0.47		0.07
	2	6.75	6.67	5.60	3.19	0.12	0.46		0.06
	3	6.58	6.54	5.32	3.11	0.10	0.46		0.07
B3	1	6.49	6.53	5.15	3.07	0.08	0.20		0.07
	2	6.70	6.80	5.32	3.41	0.08	0.18		0.06
	3	6.72	6.81	5.32	3.19	0.06	0.12		0.07
A1	1	7.35	7.36	5.33	2.51	0.06	1.18		0.16
	2	7.63	7.48	5.63	2.90	0.06	1.24		0.17
	3	7.21	7.68	4.86	2.82	0.07	1.18		0.18
A2	1	8.44	8.11	5.98	3.15	0.87	1.87		0.16
	2	8.44	8.33	5.84	3.46	1.31	2.06		0.13
	3	8.40	8.45	5.99	3.58	1.39	2.24		0.16
A3	1	9.29	9.83	5.81	4.03	2.21	2.84		0.11
	2	9.68	10.09	6.28	3.82	2.21	3.27		0.15
	3	9.64	9.62	6.05	4.25	1.97	2.69		0.14