THE EFFECT OF IN-SILO ADDITION OF CELLULASES AND HEMICELLULASES ON THE CHEMICAL COMPOSITION AND NUTRITIONAL VALUE OF SILAGE.

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

by

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A C K N O W L E D G E M E N T S

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SUMMARY

1. Aspects of digestion, control of food intake, plant cell wall structure and silage conservation are reviewed in relation to the breakdown of fibre constituents by the addition of polysaccharide-degrading enzymes.

2. A series of experiments with laboratory-scale silos was set up to examine the ability of the two cellulase/hemicellulase mixtures (ES 1 and ES 2; supplied by an industrial company) to degrade intact forage cell walls under conditions pertaining in the silo. Comparisons were made with activity-related additions of cellulase and hemicellulase preparations available commercially (Sigma Chemical Company). Three experiments examined the dose responses of the enzymes and the time course of their action. All silages were well preserved and there was no evidence of any adverse effects of enzyme addition on silage fermentation. Preparation ES 1 showed the greatest capacity to degrade cell walls as judged by reductions in neutral detergent fibre (NDF) and acid detergent fibre (ADF) concentrations; at 500 mlt^{-1} NDF and ADF concentrations in grass silage were reduced by 122 and 79 g $kgDM^{-1}$ respectively compared with the formic acid alone treatment. As the levels of enzyme addition were increased (up to a maximum of 2000 mlt^{-1}) it became increasingly difficult to account for the products of NDF and ADF breakdown in terms of water soluble carbohydrate (WSC) or fermentation products. The shortest period over which enzyme action was studied was 14 days and by this time there were measurable reductions in the concentrations of NDF and ADF.

3. Eight 1-tonne silos were made, four from perennial ryegrass (Lolium perenne) and four from lucerne (Medicago sativa). The grass silages were treated with formic acid (3 lt^{-1}) and increasing additions of ES 1 and ES 2 (100, 300 and 500 mlt^{-1}) used in a 50:50 combination. With the lucerne silages only the control had been treated with formic acid and the others with 125 mlt^{-1} ES 1, 250 mlt^{-1} ES 1/ES 2 and 10 mlt^{-1} ES 3 respectively, ES 3 being a third enzyme preparation supplied by the industrial company. All the silages were well preserved. The silages were used for studies on the disappearance of constituents from Dacron bags incubated in the rumen and in intake/digestibility trials with sheep.

4. Samples of the grass and lucerne silages were incubated in Dacron bags in the rumen in two cows fitted with rumen cannulas. The rate of loss of dry matter and fibre consituents were determined after 2, 7, 16, 24 and 48 h incubations. For the grass silages the rates of disappearance of DM and NDF were significantly greater ($P<0.01$) for the formic acid control treatment than for the enzyme treatments. For the lucerne silages the control treatment of formic acid alone had the lowest rates of disappearance of DM, NDF and ADF. Treatment with 125 mlt^{-1} ES 1 gave the greatest losses in DM, NDF and ADF at all incubation times ($P<0.01$).

5. The intake and digestibility of the silages was determined in vivo in sheep in two 4 x 4 Latin Square experiments; each period lasted 21 days with a faecal collection over the final 7 days of each period. The silages were offered ad-libitum and intakes were measured throughout the experiments. The digestibilities of the enzyme-treated grass silages were similar to those of the formic acid control and the DM and OM intakes

of the enzyme-treated silages were significantly lower ($P<0.01$) than those of the silage treated with formic acid only; the intake was lowest for the highest addition of enzyme (500 $ml\,t^{-1}$ ES 1/ES 2). A much greater effect of enzyme treatment was observed with the lucerne silages. There were reductions in the concentrations of NDF and ADF with all three enzyme treatments and there were increases in the digestibility of DM, OM and ADF for the silages treated with ES 1/ES 2 (150 $ml\,t^{-1}$) and ES 3 (10 $ml\,t^{-1}$) but not for the ES 1 treatment (125 $ml\,t^{-1}$). The intakes of DM and OM were highest for the lucerne treated with ES 1/ES 2 and lowest for ES 1 treatment; the intakes of digestible OM were 19% higher for the ES 1/ES 2 treatment than for the formic acid control.

6. A final experiment investigated the feeding value of silage made in farm-scale bunker silos (50 t) filled with late cut ryegrass ensiled without additive , with formic acid (2.6 $l\,t^{-1}$) or with the addition of ES 1/ES 2 (400 $ml\,t^{-1}$). Prior to the start of the experiment the digestibility of the silages was determined in sheep. The DOMD value of the silage was not increased by the enzyme additive. The feeding value of the silages was estimated using 12 lactating cows in an experiment of a cyclical design with 2 blocks of 6 animals and four 4-week periods per block. There were six dietary treatments consisting of each of the silages offered ad-libitum with concentrate supplements given at flat rates of either 6 $kg\,d^{-1}$ or 9 $kg\,d^{-1}$. Milk yield and food intake were recorded daily. The silages were well preserved and there was a reduction in the concentration of NDF and ADF with the enzyme-treated silage. At the 6 $kg\,d^{-1}$ level of concentrates the intakes of all three silages were similar. However, at the 9 $kg\,d^{-1}$ level the intake of the enzyme-treated silage was higher than that of the other two silages; the values ($kgDM\,d^{-1}$) were; 8.49, 8.74 and 9.29 for the

untreated, formic acid treated and enzyme-treated silage respectively. There were no differences ($P > 0.05$) between silages in milk yield and composition.

7. It is concluded that the addition of the cellulase/hemicellulase mixtures at ensilage has clear potential for the improvement of the nutritional value of the resulting silage. Whilst there was no clear-cut evidence of an increase in DOMD in response to enzyme addition in these studies, improvements in the voluntary intake of silage were measured in two experiments. Further work is required to examine factors such as dose-rate of enzyme in relation to crop characteristics in terms of species, stage of maturity and pre-ensiling treatments such as wilting. The need for further investigation of the mode of action of the enzymes and a more detailed examination of the end-products of fibre breakdown is also evident.

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L I S T O F F I G U R E S

SECTION I INTRODUCTION

RUMINANT DIGESTION

The breakdown and modification of their diet, comprised of mainly plant material, by ruminants occurs by both physical and chemical means. The contents of the rumen which include newly consumed and partially-digested feed, a mixture of salivary secretions and rumen fluid,are continually mixed by rhythmical contractions of the rumen walls. Food entering the rumen is subjected to intense microbial activity and from 70-80% of the DM is digested by a complex operation involving fermentation, regurgitation, eructation and absorption.

The chemical breakdown of food in the rumen is brought about enzymatically with the enzymes being secreted by the bacteria and protozoa existing symbiotically in the rumen. There is considerable information about the organisms found in the rumen, the substrates they attack and the products formed (Table 1).

Digestion of Carbohydrates

Carbohydrates in ruminant feeds can be divided into two groups: the available or intracellular carbohydrates and the unavailable or partially-available carbohydrates. The intracellular carbohydrates include: the simple sugars such as glucose, sucrose and fructose, found in the cell contents of most plants; the polymers such as fructosans and pentosans; and the storage polysaccharides of plants of which starch is the most widespread.

The unavailable or partially-available carbohydrates are the structural polysaccharides of the plant cell wall and are

TABLE 1: Substrates and fermentation products of some bacteria of the rumen (Hungate, 1966).

Organism	Substrates	Products
Bacteroides succinogenes	Cellulose, cellobiose glucose, CO_2	Succinate, acetate formate
Ruminococcus	Cellulose, cellobiose xylan, CO_2	Succinate, lactate acetate, formate ethanol, H_2
Butyrivibrio	10-20C carbohydrates, varying among strains	Butyrate, lactate, ethanol, formate, CO_2, and sometimes acetate and propionate
Bacteroides ruminicola	Many sugars, CO_2	Succinate, acetate formate
Bacteroides amylophilus	Starch, maltose, CO_2	Succinate, acetate lactate, ethanol
Succinimonas amylolytica	Starch, maltose, CO_2	Succinate, acetate propionate
Succinivibrio dextrinosolvens	Dextrin, maltose, xylose, pectin, CO_2	Acetate, succinate lactate
Lachnospira multiparus	Pectin, esculin, salicin, cellobiose, glucose, fructose	Formate, acetate, lactate, ethanol, CO_2, H_2
Peptostreptococcus elsdenii	Lactate, glucose, fructose, maltose, mannitol, sorbitol	2-6C fatty acids, H_2, CO_2
Selenomonas ruminantium	7-13C carbohydrates, esculin, sometimes salicin, glycerol, mannitol	Acetate, propionat CO_2 and often formate, butyrate, lactate, succinate
Streptococcus bovis	10-14C sugars, starch esculin salicin	Lactate
Eubacterium	Glucose, cellobiose and other 4-6C sugars	Formate, lactate, acetate, butyrate, CO_2, H_2
Methanobacterium	CO_2, H_2, formate	CH_4 and H_2O

commonly referred to as "fibre," which can be degraded and utilised to a large extent by ruminants. The plant cell wall is a complex unit containing a number of polysaccharides, namely cellulose, hemicelluloses and pectins and also variable amounts of the non-carbohydrate polymer, lignin. Active microbial hydrolysis in the rumen degrades cellulose, hemicellulose and pectin to eventually yield their component monomers, whilst lignin does not appear to be digested by rumen microbes.

Regardless of the carbohydrate ingested the main end products of rumen fermentation are the volatile fatty acids, acetic, propionic and butyric and the waste product, methane. The biochemical pathways for the formation of these end products are shown in Figure 1. The molar proportions of the individual acids produced are altered by factors such as changes in the composition and physical form of the diet. In animals fed roughages, acetic acid makes up 60-70%, propionic acid 15-20% and butyric acid 10-15% of the total acids produced (Thomas and Rook, 1977). Increasing the available carbohydrates in the diet, which occurs with starchy-concentrate feeding, reduces the proportion of acetic acid and raises the level of propionic acid, sometimes with increases in butyric acid also, (Storry and Rook, 1966; Thomas, Kelly, Chamberlain and Chalmers, 1980), whilst increasing the roughage content has the opposite effect (Baldwin and Allison, 1983). This acetate-propionate ratio is also affected by the physical form of the feed with the grinding of hay, for example, causing the proportion of acetic acid to fall (Balch, Broster, Rook and Tuck, 1965).

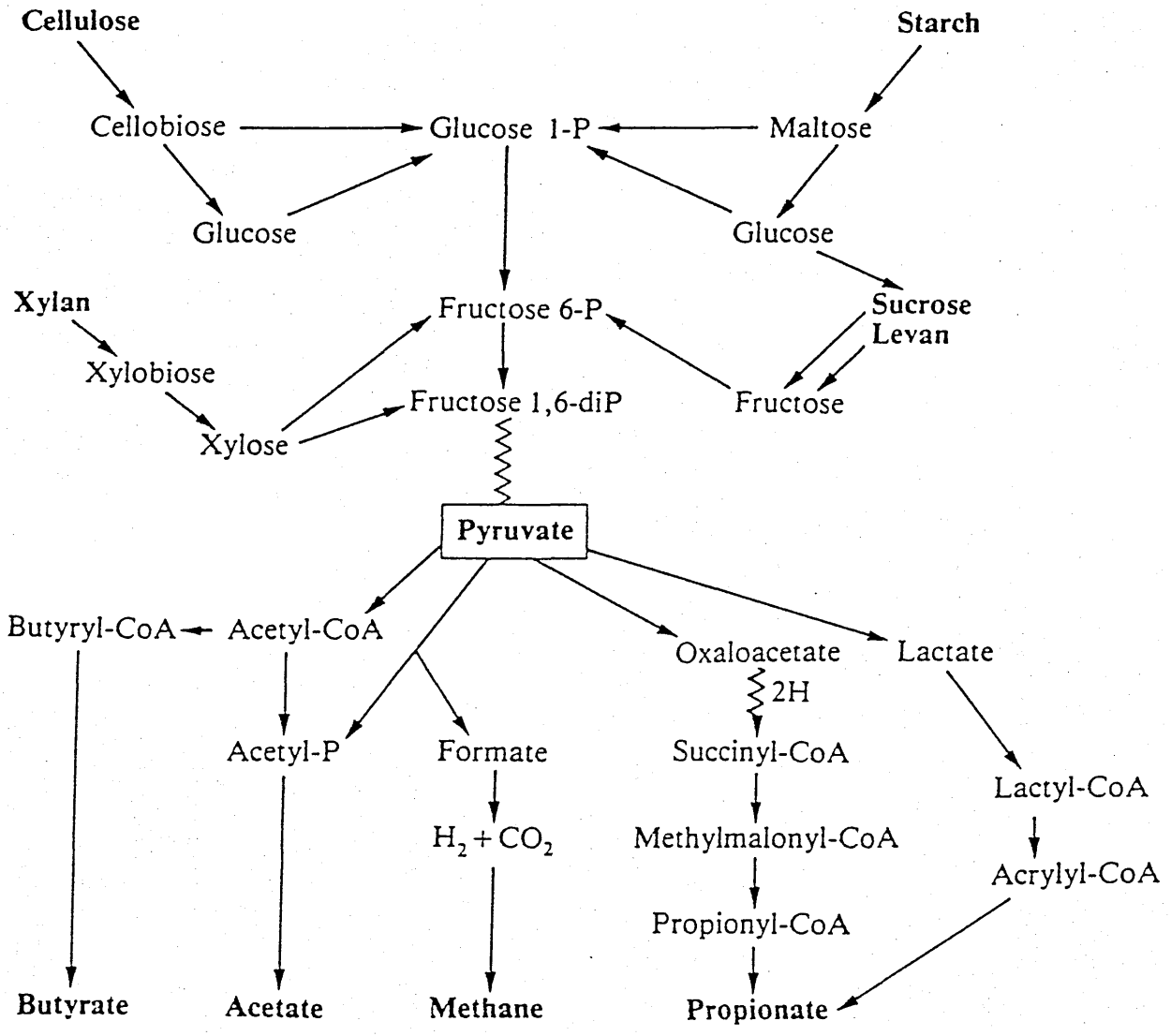


FIGURE 1: Metabolic pathways for the production of VFA (Lewis and Hill, 1983)

Most of the volatile fatty acids produced in the rumen are absorbed into the bloodstream and metabolised to provide energy for the animal; VFAs account for 55-60% of the digestible energy, (Thomas and Clapperton, 1972).

Carbohydrate leaving the rumen and passing to the small intestine is composed of three fractions: the portions of the plant structural carbohydrates, cellulose and hemicelluloses, and the plant storage polysaccharide, starch, which have escaped fermentation in the rumen; and the protozoal and bacterial carbohydrates synthesised in the rumen and passing out in the digesta.

The extent of ruminal digestion of structural carbohydrate is extensive but variable. As the plant material matures, the proportion of cellulose and hemicelluloses increase and their digestibilities decline (Waite, Johnstone and Armstrong, 1964). Processing of the forage such as drying, grinding and pelleting also depresses the overall digestibility of the structural carbohydrates, particularly cellulose and reduces the disappearance before the small intestine (Balch et al, 1965). In mixed rations cereal grain concentrates affect the site of digestion of the structural carbohydrates with increasing proportions of these carbohydrates passing undegraded from the rumen (MacRae and Armstrong, 1969).

The digestibility of starch in the rumen is generally complete. In mixed rations, however, variable proportions of grain starch may escape fermentation, the amounts of which may be influenced by dietary factors such as quantity, composition, degree and severity of processing and stage of maturity (Waldo, 1973). Barley grain starch is virtually all fermented unless grain is fed whole which

depresses its digestibility. Maize starch is less digestible but the amount digested is influenced by certain types of grain processing, eg. raw maize starch as opposed to flaked maize (steamed).

Appreciable amounts of microbial polysaccharides are synthesised in the rumen. The main component of bacterial polysaccharide is an α-linked glucose polymer, α-glucan. The carbohydrate content of rumen bacteria is influenced by the diet of the host animal and the time of sampling relative to the time of feeding. An increase in the ratio of concentrate : roughage in a mixed ration increased the α-glucan content of rumen bacteria (McAllan and Smith, 1974). The carbohydrate content of rumen protozoa, being mainly composed of glucose polymers, is also influenced by the amount and nature of the feed carbohydrate. However, only a small proportion of protozoal dry matter present in the rumen liquor enters the small intestine, contributing little to the carbohydrate content (Weller and Pilgrim, 1974).

Mammalian tissues do not synthesise enzymes with cellulase activity and therefore very little cellulose digestion occurs in the small intestine, the amount that does is probably due to microbial activity in the terminal ileum. The disappearance of hemicellulose is also very slight. Appreciable amounts of the starch entering the small intestine are removed before reaching the terminal ileum, although the capacity to digest starch is limited particularly when large amounts of it enter the duodenum. The enzymes amylase and maltase, involved in the hydrolysis of α-linked glucose polymers to glucose are found in both the pancreatic juice and intestinal mucosa of ruminants but in limiting amounts and activities, particularly

maltase (Mayes and Orskov, 1974).

Some microbial fermentation of carbohydrate occurs in the distal part of the ileum with further fermentation in the caecum, the principal substrates being the residual amounts of structural polysaccharides and α-glucan, the main component of microbial carbohydrate. The pathways of degradation are similar to those occuring in the rumen giving rise to volatile fatty acids, carbon dioxide and methane. There is a simultaneous synthesis of microbial cells and products occuring in the large intestine but owing to there being no further digestion this microbial mass is excreted in the faeces and wasted.

Digestion of Proteins

The digestion of protein entering the rumen is brought about by microbial enzymes. A complex form of the nitrogen cycle exists in the ruminant as shown schematically in Figure 2. Enzymes from mixed populations of rumen organisms rapidly hydrolyse the dietary proteins to yield peptides, amino acids and ammonia, the rate and extent depending on the degradability of the dietary protein. These intermediates, amino acids and petides, may be further catabolised to ammonia, carbon dioxide and volatile fatty acids or other acids, or also used in the simultaneously occuring synthesis of microbial cells. Ammonia, however is the main nitrogen source of most rumen bacteria for their protein synthesis (Bryant and Robinson, 1962) and even when the diet contains adequate amino acids and peptides for microbial protein synthesis much catabolism to ammonia, carbon dioxide and acids still occurs. Studies of the dynamics of $^{15}NH_4^+$ metabolism in the rumen indicated that 50-70% of the microbial N was derived from ammonia (Pilgrim, Gray, Weller and Belling, 1970; Nolan, Norton and Leng, 1973), with peptides and

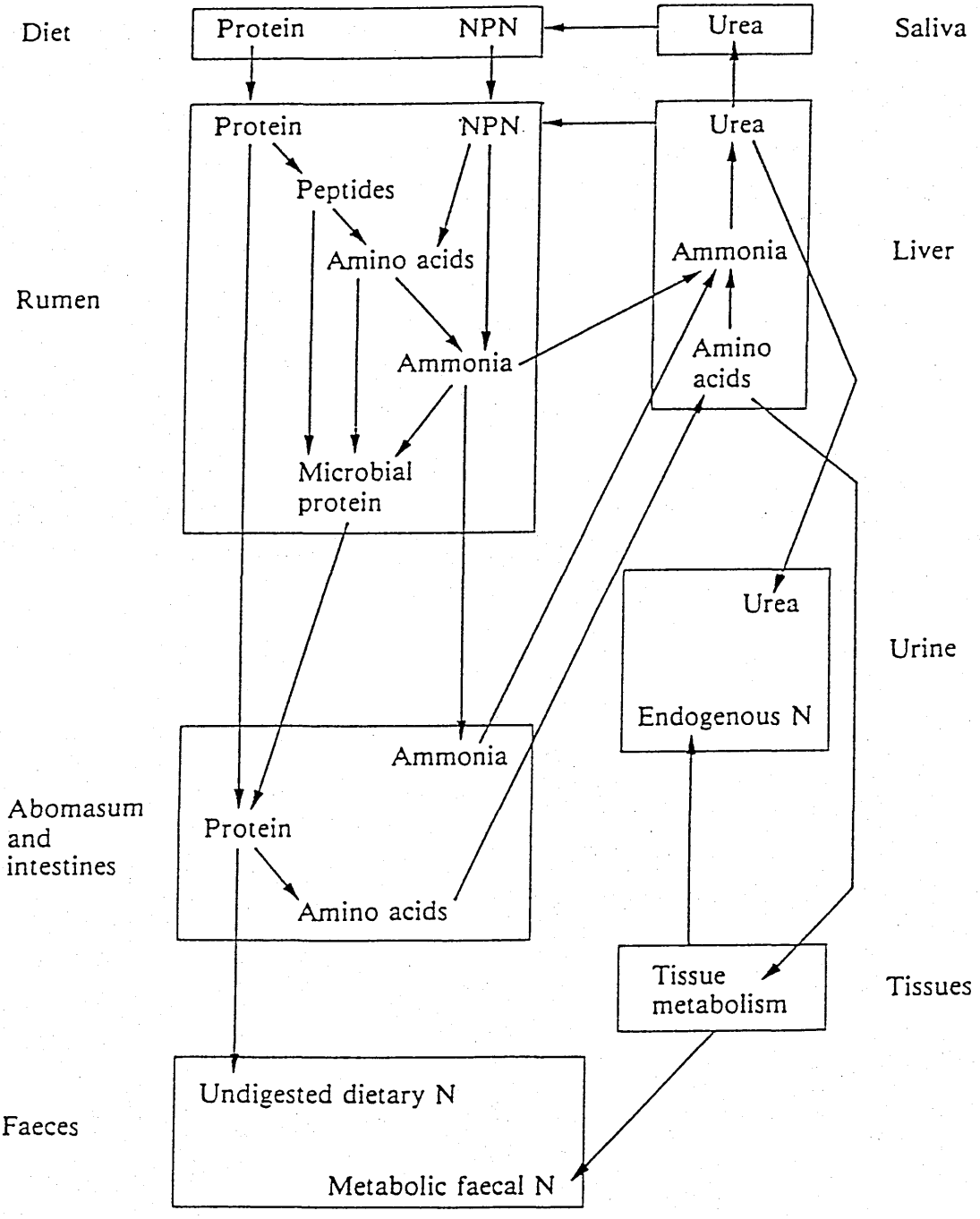


FIGURE 2: A schematic representation of nitrogen metabolism in the ruminant (Lewis and Hill, 1983)

amino acids from the feed probably providing the balance of the N required for microbial growth. The catabolism also ensures the supply of specific volatile fatty acids necessary for the growth of certain bacterial species and other functions and these are obtained from the deamination of specific amino acids.

The nutritional value to the animal of the protein given in the diet is controlled by the relative rapidity of the breakdown and synthetic reactions. This balance is regulated by several major factors such as the degradability of the ingested protein which is generally thought to be related to its solubility, although not invariably so (McDonald, 1952; Mangan, 1972) and the amount and type of carbohydrate present. The NH_3 produced in the rumen that is not used for growth in the micro organisms is absorbed into the bloodstream and converted to urea in the liver. Some of this urea is returned to the rumen in saliva (Somers, 1961), but the greater part is excreted in the urine. Diets rich in soluble protein but low in soluble carbohydrates result in high ammonia production and low utilisation and thus increased wastage. Heavy nitrogen fertilisation raises the protein content of fresh forage and lowers its content of soluble carbohydrates, whereas denaturing of the cellular protein of the feed eg. by heat processing, causes it to become highly insoluble and much less susceptible to rumen degradation. Nitrogenous compounds reaching the duodenum consist of undegraded dietary protein and microbial protein together with endogenous secretions from the abomasum. The amount of microbial protein present depends on the extent of microbial synthesis in the rumen, which is governed primarily by the availability of energy and nitrogen (Thomas, 1973; Okorie, Buttery and Lewis, 1977). The amount of undegraded dietary protein reaching the small intestine

depends on the level of proteolytic activity in the rumen, the residence time of the protein in the rumen and the susceptibility of the protein to enzymic attack, (ARC, 1984).

Protein digestion in the small intestine is brought about by a well-defined enzyme system to yield amino acids and small peptides, which are absorbed. Some of the breakdown products which would normally be wasted may be taken up and used by the animal eg. for nucleic acid sythesis. Protein passing to the large intestine undergoes microbial fermentation with the products released, particularly ammonia, being absorbed and partly reformed as urea or used for microbial growth and the synthesis of microbial protein, which is ultimately lost in faeces.

Digestion of Lipid

The lipid present in ruminant diets is mainly in the form of triglycerides and galactolipids with small amounts of phospholipid. Forages can have comparatively high proportions of mono- and digalactosyldiglycerides whilst cereal lipids are largely triglyceride. In the rumen there is a rapid hydrolysis of lipids by microbial lipase enzymes resulting in the release of free fatty acids and glycerol (Garton, Lough and Vioque, 1961). The glycerol released yields mainly propionic acid and the galactose released can be fermented to volatile fatty acids which are then absorbed (Hobson and Mann, 1961). The free fatty acids undergo biohydrogenation which, in the case of forages, results in much stearic acid as the unsaturated fatty acids predominantly found in forage lipid are C18:1, 18:2 and 18:3 (Bickerstaffe, Annison and Linzell, 1974).

Ruminal micro organisms also synthesise long chain fatty acids for the lipids of their own cells. These lipids are characterised by comparatively high proportions of acids with branched-chains and odd-numbers that are esterified to phospholipids (Emmanuel, 1974 and 1978). The stages of absorption and resynthesis involved in lipid metabolism do not occur in the rumen (Garton, 1969). The microbial cells and the hydrolysed and hydrogenated dietary lipids, therefore, pass from the rumen through the omasum and abomasum, where the bacteria and protozoa largely disintegrate under the acid conditions and release their lipids. All these products of the free fatty acid fraction bound with the food particles to form an insoluble complex plus small amounts of unhydrolysed dietary lipid and microbial lipid pass to the small intestine.

Digestion of lipid in the small intestine involves a transfer of fatty acid from the insoluble, particulate phase to the soluble micellar phase. This transfer is brought about by the action of pancreatic juice, which contains enzymes involved in the solubilisation of digesta lipids and creates conditions which are conducive to lipid absorption. Pancreatic juice also contains bile, the salts of which are essential for micellar formation and fat absorption (Heath and Morris, 1963). Considerable quantities of the triglyceride that have escaped rumen degradation are hydrolysed by a powerful lipase present in the pancreatic juice. Any lipid passing to the large intestine is subjected to microbial fermentation but the fatty acids released, along with any microbial lipid synthesised in the large intestine, passes out in the faeces (Harrison and Leat, 1975).

CONTROL OF FOOD INTAKE

Short Term Regulation

The absorption of nutrients from the digestive tract and their presence in the circulating blood constitute a set of primary signals which in turn may influence the satiety centre. Several blood constituents have been suggested as possible signals including glucose, free fatty acids, amino acids, vitamins and minerals. The glucostatic theory (Mayer, 1955) has received most attention. Reduced concentrations of blood glucose cause the animal to feel hungry while raised blood glucose levels such as found after eating, remove the desire to eat. Blood glucose may exert its action through the hypothalamus which may contain "glucoreceptors" sensitive to blood glucose. However, since under usual dietary circumstances, little glucose is taken up from the ruminant gut, it seems unlikely that a glucostatic mechanism is important in ruminants.

In ruminants, the three major fermentation acids, acetic, propionic and butyric may be involved in a chemostatic mechanism of control. Intraruminal injections of acetate and propionate have been shown to depress intake of concentrate diets (Baile and Mayer, 1969) and also of roughage-based diets (Montgomery, Schultz and Baumgardt, 1963; Egan, 1966). Receptors for acetate and propionate regulation are suggested to occur on the luminal side of the reticulorumen. Butyric acid is less effective in reducing intake (Baile and Mayer, 1969) which may be a consequence of its normal metabolism to acetoacetate and β-hydroxybutyrate by the rumen epitheluim.

The thermostatic theory (Brobeck, 1948) proposed that animals eat to keep warm and stop eating to prevent hyperthermia. One of the signals thus used in the short-term regulation of food intake is that provided by the heat increment produced during digestion and metabolism of food. Thermoreceptors have been established to be present in the anterior hypothalamus and also peripherally in the skin. From observations with a number of species it has been found that food intake increases in cold and decreases in hot environments.

A further theory of short-term regulation is based on a single gut hormone, cholecystokinin, which has been invoked as a satiety factor (Smith, Gibbs and Young, 1974). Infusions of cholecystokinin at low rates into the cerebroventricles of sheep have been sufficient to cause significantly large depressions in food intake.

Long Term Regulation

A long-term regulation of food intake is implied from the preservation of a relatively constant body weight in most adult animals combined with an animal's desire to return to that body weight if it is altered by starvation or forced feeding. The lipostatic theory suggests that fat deposition is the agent which is associated with this energy storage and thus acts as a signal for long-term regulation. It has also been proposed that the hypothalamus may receive the lipostatic signal by way of a natural steroid (Hervey, 1969). In cattle, fatness has been found to reduce intake suggesting that in terms of energy balance, the thin animal has a requirement for nutrients for fat synthesis which is reduced or absent from the fat animal. Alternatively, in the very fat animal, the space in the

abdominal cavity into which the rumen can expand during feeding may be reduced by fat deposition (McDonald, Edwards and Greenhalgh, 1966).

The effects of the sensory appraisal of food on voluntary intake in animals are difficult to measure. Palatability describes the degree of readiness with which a particular food is selected and eaten, involving the senses of smell, touch and taste. It can be influenced by the inclusion in the feed of aromatic additives which may appear to give short-term increases in food intake but there is no direct evidence to indicate any long-lasting effects of overall increased food intake. In ruminants too, although the senses are important in grazing and eating behaviour, they do not appear to influence the overall intake. Palatability may limit the intake of poor-quality forage like cereal straws (Greenhalgh and Reid, 1967), but not of better quality material.

Nutritional deficiencies of indispensable amino acids, vitamins and minerals are likely to affect the intake of food by directly causing a reduction in intake. They may also have an indirect involvement such as in the efficient functioning of the metabolic pathways that are involved in the utilisation of the absorbed products of digestion by the tissues. In the ruminant, the effects of nutritional deficiencies may operate via effects on the nutritional status of the micro organisms in the rumen or on the nutritional status of the host, or both. Egan and Moir (1965) clearly demonstrated the difference between nitrogen deficiency in the rumen and protein deficiency in the animal. In sheep given a low protein straw diet, intraruminal infusion of urea resulted in an increased voluntary intake accompanied by an increased rate of digestion and passage

from the rumen apparently relieving a physical limitation in intake (see later). However, duodenal infusion of casein resulted in an increased voluntary intake that was not accompanied by increased digestion and rate of passage from the rumen. The suggestion is that casein, infused at a site where its amino acids could be absorbed without degradation, relieved a metabolic limitation of intake.

Physical Limits

The relation between the voluntary intake of food by sheep and the digestibility of the food is shown in Figure 3 (Blaxter, Wainman and Wilson, 1961). The relationship implies two mechanisms for the control of intake: a physical control operating up to around 70% digestibility and a "physiological" control mechanism operating with diets of higher quality (digestibility).

Under most practical circumstances, the nature of the diet fed to ruminant animals means that the emphasis on intake control is on physical limitations. The capacity of the reticulo-rumen and the rate of disappearance of digesta from it limit the voluntary intake of diets containing a high proportion of roughages. Reduction in the physical capacity of the reticulo-rumen by additions of inert material or digesta or distension by water-filled balloons decreases the voluntary intake of roughage. It would, therefore seem that there is a certain threshold of rumen distension which the animal will not exceed even though its energy requirements have not been satisfied. Stretch and tension receptors present in the reticulo-rumen probably function as a limiting factor when bulky diets are consumed.

The digestibility of the food and its rate of disappearance

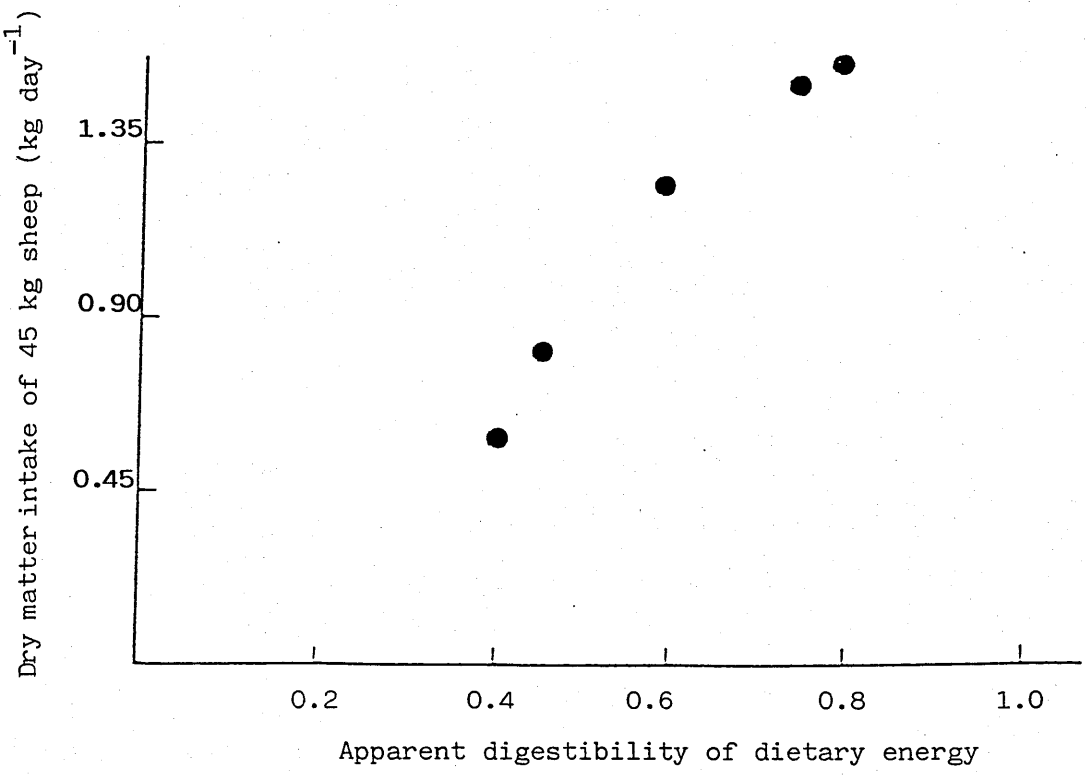


FIGURE 3: Food consumption and digestibility in sheep fed on roughages (After Blaxter et al, 1961).

from the reticulo-rumen are important in determining food intake. The rate of disappearance of digesta depends primarily on its rate of digestion which in turn depends upon the chemical and physical composition of the food eaten. Fibrous foods of low digestibility are broken down slowly as they are less susceptible to physical comminution and microbial enzymic breakdown. This delayed digestion results in a more lengthy retention of food in the rumen, for only particles of small size are permitted to pass down the tract. The larger quantities of cell wall constituents, particularly cellulose, in fibrous foods are responsible for this slow rumen digestion and rate of passage through the gut.

There is, therefore, a relation between digestibility and the rate of digestion which in turn leads to a relation between digestibility and food consumption. The more digestible and higher quality the food is and the faster it is removed from the rumen (Blaxter et at, 1961), the greater will be the ruminal space cleared between meals and the more the animal will be able to eat.

This relationship between digestibility and food consumption in ruminants is a general one and it may be modified by the influence on intake of properties of foods other than digestibility. Reduction in the particle size of forage material by grinding, for example, will give a decrease in the digestibility but a subsequent increase in intake. This is due to an increased rate of passage of material through the rumen. Supplementing a roughage-based diet with a concentrate will give an increase in total food intake which is often greater than can be accounted for by the higher digestibility of the supplemented diet. For highly digestible roughages, differences in digestibility

may have a relatively smaller effect on intake than for less digestible materials. At such high levels of digestibility food intake in ruminants may no longer be limited by the rate at which digesta can be removed from the tract.

However, on certain roughage diets, ruminants do not eat to a constant rumen fill and the general relationship between intake and digestibility is not valid. This is particularly true for certain silage diets where dry matter intakes are lower than those obtained with animals consuming herbage of similar digestibility and preserved as hay or dried grass. Such silages do not, therefore, follow the general pattern of improvements in digestibility up to around 0.7 resulting in markedly increased voluntary intakes. It would therefore appear that with high-quality silage diets there is a limiting physical component in the mechanism for the control of voluntary food intake possibly at the level of the rate of digestion of the fibre constituents. Attempts to improve the intakes of these silages such as reducing the particle size, for example, by mincing before feeding, may alleviate the apparent restrictive physical limitations on intake although associated depressions in digestibility have been recorded for minced silages over unminced controls (Thomas, Kelly and Wait, 1976). The control of intake of silage is further complicated by the effects of the end-products of fermentation such as high organic acid contents or high ammonia levels. Further attempts at improving the intake of such forage-based diets may need to be centred on an improvement in the susceptibility of the structural carbohydrates of the forage to microbial breakdown in the rumen.

Physiological State

The physiological state of the ruminant influences food intake according to the demand for energy. In growing animals, the abdominal volume increases with growth and food intakes are maintained accordingly over a weight range. Lighter animals of the same age, breed and sex have lower maintenance requirements than heavier animals and thus gain weight faster as a greater proportion of energy is available for liveweight gain.

Pregnancy influences food intake by two opposing effects. The demand for nutrients for foetal development causes intake to rise but as pregnancy progresses the abdominal capacity is reduced as the foetus increases in size and similarly rumen expansion is restricted thus depressing intake. The onset of lactation increases food intake due to both physiological and physical effects as the reduction in fat deposits increases the abdominal capacity.

STRUCTURE OF PLANT CELL WALLS

From the foregoing discussions, it is clear that the polysaccharide components of plant cell walls are an important energy source to ruminant animals. The degradation and utilisation of these structural carbohydrates within the rumen is dependent on a number of factors, the most important being the species of plant material and its stage of maturity.

Cellulose makes up the greatest proportion of plant cell wall polysaccharides. It is a linear homopolymer based on 1$\rightarrow$4 β-D

glucopyranosyl residues with the chain of a cellulose molecule being up to 10,000 units in length. Each chain is in the form of a ribbon. Hydrogen bonding exists between chains in such a way that a number of cellulose molecules will pack very tightly together in parallel to give a very stable multi-chain structure. Further regions of bonding exist between sets of cellulose chains.

The two most abundant hemicelluloses in plant cell walls are the xylans and glucomannans. They both possess, like cellulose, the 1→4 β structure based on D-xylopyranosyl residues although their chain lengths are much smaller than cellulose and they can contain quite a number of side chains. The xylans have side chains of 1→3 α-L arabinofuranosyl and 1→2α-D-(4-O-methyl) glucuronosyl residues or even more complex side chains. The glucomannans have side chains which are predominantly of 1→6 α-D galactose residues.

There are other hemicelluloses such as xyloglucans which are found in primary cell walls from a variety of plants, and mixed β 1→3 and β 1→4 glucans which are found in young grasses but their concentration declines rapidly with maturity. The hemicellulose composition varies betwen plant species and within a species there is not just a single type of hemicellulose as grasses, for example, contain more that one type of xylan (Morrison, 1974 a).

The third group of polysaccharides in plant cell walls are the pectins. They are based on 1→4 α-D galacturonosyl residues although a wide variety of neutral sugars are also associated with pectins and some may be present in the main chain. The importance

of pectins varies between species being major components of legume cell walls but minor components in grasses.

There are several non-carbohydrate components present in the structure of plant cell walls of which the most important is lignin. Lignin is an inert polymer based on phenylpropane residues and acts as a cementing agent in the cell wall. It is found in the thicker secondary wall of the plant cell laid down after the finish of elongation. In cell wall preparations phenolic acid and acetic acid groups have been found. Ester linkages convalently bind the ferulic, p-coumaric and diferulic phenolic acids to both carbohydrate and lignin, and bind the acetyl groups exclusively to hemicellulose (Bacon, Gordon, Morris and Farmer, 1975; Hartley and Jones, 1977). Lignin itself has been found to be covalently bound, entirely or in part, to hemicellulose (Morrison, 1974b).

Changes occur in the structure of the cell wall with stage of growth which have profound effects on the digestibility of the cell wall carbohydrates. Chemically the structure of neither cellulose nor the hemicelluloses alters significantly during the growing period yet as the plant matures, cell wall carbohydrate digestibility declines with hemicellulose digestibility falling at a greater rate than that of cellulose. Therefore changes in the arrangements of components within the cell wall are vitally important. The cellulose microfibrils are not found to be linked to any other cell wall component but they are embedded in a ligno-hemicellulosic macromolecule which protects the cellulose from attack by rumen micro organisms by forming a "cage" effect. In young tissue lignification is very sparse and the "bars"

of the "cage" are too far apart to prevent access by cellulose degrading enzymes. However as the plant matures, the ligno-hemicellulosic complex develops enclosing the cellulose more tightly and restricting cellulolysis to just the ends of broken fibres. This theory is supported in grinding which leaves more broken ends and causes more extensive breakdown of cellulose. Cellulose is thus available to rumen microflora to a variable degree ranging from about 25 to 90% depending mainly on the amount of lignification.

The breakdown of hemicelluloses also declines as the plant matures. Initially the degree of lignification is low and the hemicelluloses are easily recognised by their respective hydrolytic enzymes. As lignification increases and the ligno-hemicellulosic complex is built up the hemicellulose polysaccharides undergo modification and are no longer recognised by and subjected to the action of hydrolases (Morrison, 1980; Brice and Morrison, 1982). The overall effect, therefore, of increasing lignification with maturity is a reduction in the digestion of cell wall carbohydrates.

The proportion of the major cell wall components vary between species of plant material as shown in Table 2. The content and composition of the fibre determines its nutritional value. However the degree of lignification of the plant material has an over-riding effect as it determines both the extent and rate of digestion of the plant cell wall. The increase in lignin content with maturity, see Figure 4, and the formation of the ligno-hemicellulosic complexes reduces the extent of cell wall digestion by exerting both physical and chemical influences on the degradation of the cellulose and hemicellulose components.

TABLE 2: Percentage of cell wall components of dried plant samples (Morrison, 1980)

Plant Sample	Cellulose	Hemicellulose *(+starch)	Lignin
Timothy stem cell wall	53.3	43.8	10.1
Timothy leaf cell wall	36.1	48.5	9.3
Sainfoin stem cell wall	40.7	39.7	6.2
Sainfoin leaf cell wall	22.4	71.4	4.3
Sainfoin flower cell wall	35.6	35.9	4.7
Lucerne stem cell wall	54.7	27.6	10.4
Lucerne leaf cell wall	32.4	32.7	5.7
White clover stem cell wall	41.8	24.3	6.1
White clover leaf cell wall	33.2	53.9	1.7
Redclover stem cell wall	45.8	34.9	7.4
Redclover leaf cell wall	31.1	48.6	3.1

*
Hemicellulose determined also contained variable small amounts of starch as the method of preparation did not remove all of the starch.

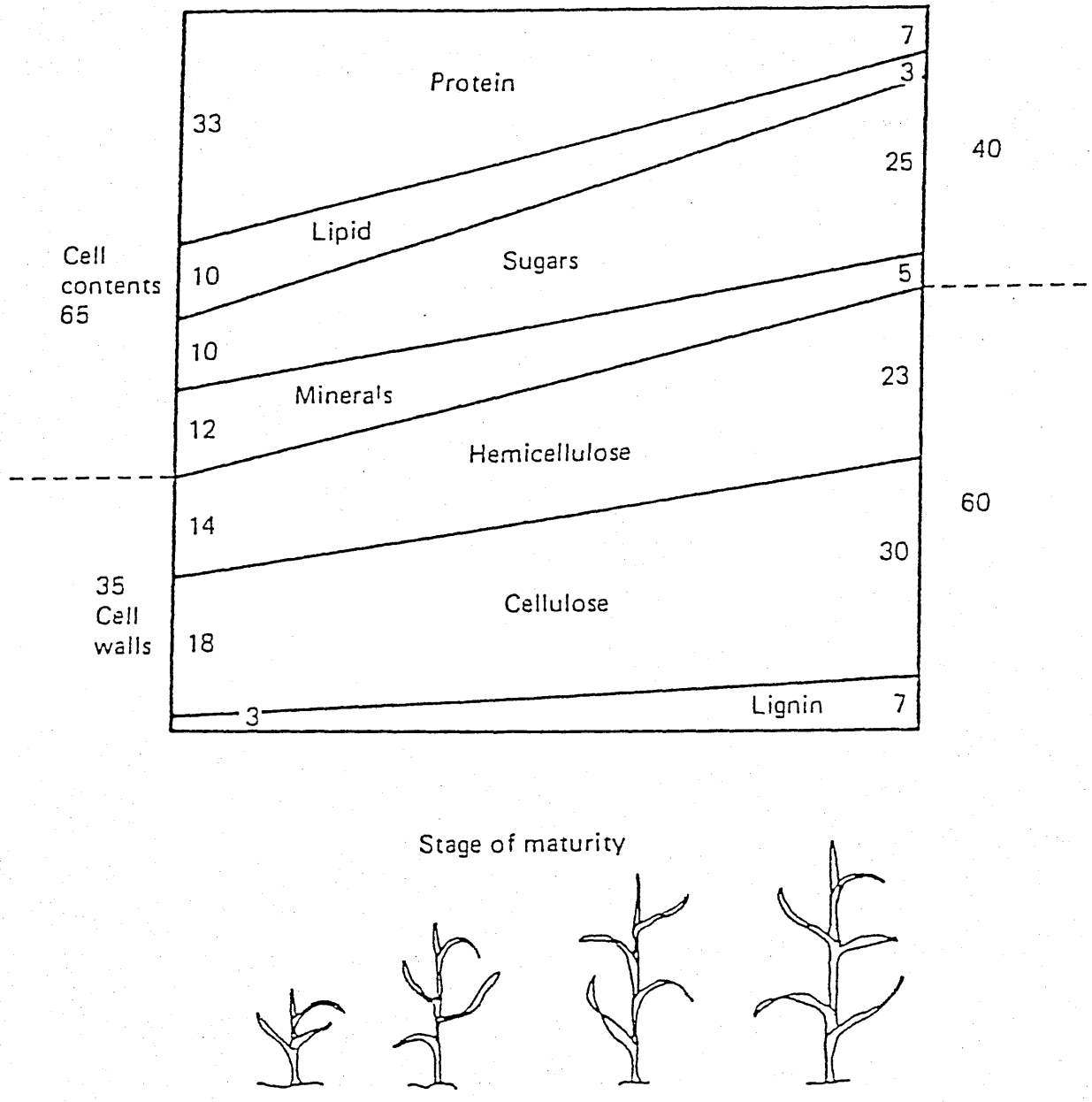


FIGURE 4: Schematic representation of the changes in chemical composition of grasses which accompany advancing maturity. All values are in percentages (Osbourn,1980).

With this increase in lignification the rate of degradation of the cell wall is also reduced. A much longer period of fermentation by rumen microorganisms is required to digest the structural carbohydrates. The supply of readily fermentable carbohydrate made available from the cell wall components is reduced restricting the growth of the micro organisms. The overall effect is a slowing down of the rate of passage of the plant digesta in the rumen which in turn reduces the feed intake of the animal. Such detrémental effects have an overall influence on the energy supply and hence production level of the animal.

Methods for Improving the Rate and Extent of Digestion of Fibre

This reduction in the rate of plant digestion raises the possibility of pretreatment of the fibre before feeding to improve its nutritional value. Chemical treatments such as with sodium hydroxide or ammonium hydroxide have been used to delignify mature, heavily lignified crops such as straw. The alkalis react with the crops in such a way as to cause physical swelling of the lignin crust bound to the hemicellulose component or encasing the cellulose component. This swelling results in a separation of lignin from the other components making them more susceptible to microbial and enzymic degradation. Improvements in the digestibility of the roughage itself have been recorded with overall improvements in the digestibility of the whole diet. This has resulted in an increase in dietary intake and a concurrent improvement in animal production (Greenhalgh, Pirie and Reid, 1976; Jackson, 1977). There also exists the possibility of treating forages with cell-wall degrading enzymes to breakdown, at least partially, the structural polysaccharides and increase their susceptibility to digestion in the rumen.

Enzymic Degradation of Plant Cell Walls

A large variety of polysaccharide-degrading enzymes have been isolated from higher plants, fungi and bacteria. These enzymes act in a complex manner such that each of the structural polysaccharides is hydrolysed, not by a single enzyme but by a number of enzymes acting on the same linkage in different ways. The mode of action of these enzymes is further complicated by the need of an, as yet unidentified, "wall-modifying enzyme" as a prerequisite to the degradation of many cell wall polymers. The isolation and proposed action of cellulases and hemicellulases have been investigated in many circumstances.

Cellulases isolated from species such as Trichoderma and Aspergillus have found to differ in their ability to degrade native cellulose derivatives (Reese, Sui and Levinson, 1950; Jones and Hayward, 1975). These microbial cellulase systems have also been shown to consist of several cellulolytic components (Reese et al, 1950; Gilligan and Reese, 1954). Similarly, active hemicellulases have been isolated, particularly from cell-free supernatants of rumen contents, which can hydrolyse the main chain of isolated purified xylans from grasses (Morrison, 1975). These enzymes, however are not capable of hydrolysing hemicelluloses present either in intact or delignified cell walls.

Karr and Albersheim (1970) have shown that this occurrence of polysaccharide-degrading enzymes, that cannot degrade naturally occuring cell wall polymers, is a general phenomenon. The organisation of the cell wall prevents degradation of the structural polysaccharides and only after the prior action of a "wall-modifying enzyme", a yet unidentified factor, can the polysaccharide-degrading enzymes attack plant cell walls.

The mode of action of the wall-modifying enzyme is such that it catalyses alteration of the cell wall which permits subsequent degradation of the wall polymers by enzymes. The wall-modifying enzyme appears to act by cleaving critical linkages, important in the structural integrity of the wall, particularly involved in the cross-linking of the cell wall polymers. The action of the wall-modifying enzyme is not dependent on the presence of further polysaccharide-degrading enzymes. However, the enzymes carrying out further degradation of the cell wall can only effectively do so when acting on walls which have had critical linkages broken. They are unable to attack unaltered, undegraded cell walls (Karr and Albersheim, 1970).

The degradation of cellulose is carried out by a complex enzyme system of multiple components. The most frequently studied cellulases are from fungal sources, such as Trichoderma and Myrothecium (Gilligan and Reese, 1954) and Sporotricum (Ayers, Ayers and Eriksson, 1978) and these have been shown to contain six or seven enzymes. The components of the cellulase complex are divided into two main systems, CI and CX, comprised of endo- and exo- (1→4) β glucanase and other β-glucosidase enzymes. The CI system is thought to act on the native cellulose molecule, being concerned, possibly, with a splitting of the cross-linkages, to produce linear polysaccharide anhydroglucose chains. The enzyme components of the CX system carry out further hydrolysis to produce glucose as an end product from the long-chain compounds. In some circumstances the complete hydrolysis of native cellulose may require the action of a cellobiohydrase enzyme, as the CX system is unable to hydrolyse any cellobiose that may be produced.

Cellulose-degrading systems differ in complexity according to the nature of the cellulose they are capable of degrading. Many of the fungal systems studied degrade highly lignified woods, whereas in the rumen ecosystem, cellulolysis of less highly lignified material is required. Also the splitting of native cellulose is restricted to cellulolytic organisms. There are however, many micro orgamisms, particularly in the rumen, with the ability to develop an enzyme capable of hydrolysing the (1→4) β glucosidic linkage.

After the action of a wall-modifying enzyme resulting in the release of hemicellulose into a hydrolysable form, hemicellulase enzymes bring about the degradation of these hemicelluloses. The action of hemicellulases has been considerably less investigated although a series of hemicellulases have been isolated from cell-free rumen fluid (Morrison, 1975). Such enzyme systems contain active xylanases capable of hydrolysing the main chain of xylans typically found in grasses. More linear xylans are hydrolysed to a greater extent than branched xylans.

Hydrolysis takes place at the bond between two unsubstituted xylose residues and thus substitution of xylose residues by arabinose residues, which occurs in the side chains of young grass, will reduce hydrolysis. The extent of hydrolysis does not result in xylose but in oligosaccharides from xylobiose upwards. The hemicellulose fragments may be degraded by glycolytic enzymes or transported as they are across the bacterial membrane for further metabolism. The increases in lignin content and the formation of ligno-hemicellulose complexes have a profound effect on hemicellulase activity. This is seen in the reduced rate of hydrolysis found in older tissue with a higher lignin content. The microbial population of ruminant animals also shows the ability

to adapt the enzymic complexes required for hydrolysing the hemicelluloses present in the diet.

The enzymic degradation of the cell wall polysaccharides is thus a multi-step process requiring modification factors and complex enzyme systems. However, it is an important process in ruminant digestion with the amount of time and energy incurred in the breakdown of fibrous dietary components being variable and sometimes extensive depending on the nature and presentation of the cell wall components of the plant. Therefore, the possibility of treatment, before feeding, of the fibrous plant constituents with cell-wall degrading enzymes to enhance their digestibility has enormous potential in the efficiency of the ruminant digestion.

SILAGE

The provision of feed for the winter period is a major concern to farmers in the United Kingdom. Forage production and conservation are of vital importance in the planning and management of livestock-based farms. In the U.K., forage production of hay and silage alone amounts to over 12.5×10^6 tonnes of dry matter each year, with over half being conserved as silage (Woolford, 1984). Silage-making is now, therefore, the preferred method of conservation on most dairy farms and also on the larger and more extensive beef and mixed-livestock units.

Principles of Ensilage

The object of storing green crops of high moisture content as silage is to preserve the material with a minimum loss of nutrients

via the controlled fermentation of the crop.

Biochemical Changes During Ensilage

Immediately after cutting and during the early stages of ensiling, the biochemical changes occuring in the herbage mainly arise from the activities of the plant enzymes being responsible particularly for respiration and proteolysis (Kemble, 1956; Brady, 1960). The hexose sugars are the main substrates for respiration which will continue as long as conditions in the silo are favourable with both oxygen and a supply of substrates being available. Plant respiration is thus restricted by efficient compaction and sealing and by the fall in pH brought about by the rapid growth of lactic acid bacteria. The attainment of a low pH (3.8-4.0) also prevents further proteolysis during ensilage as it restricts the activity of the plant protease enzymes which have an optimum pH of 5.0-6.0 (Tracey 1948). However, even in a well-preserved silage the amount of the protein broken down to free amino acids and small peptides may be 50-60% (Whittenbury, McDonald and Bryan-Jones, 1967).

Microbial Changes During Ensilage

The sequence of microbial changes in the fermentation of ensiled herbage is shown in Figure 5. Soluble carbohydrates surviving aerobic metabolism are fermented by a variety of micro organisms of which lactic acid becteria are the most important and dominate the fermentation in well-preserved silages. On growing crops there are normally very few lactic acid bacteria (Stirling and Whittenbury, 1963) but by the time the crop reaches the silo the numbers usually increase greatly (Henderson, McDonald and Woolford, 1972).

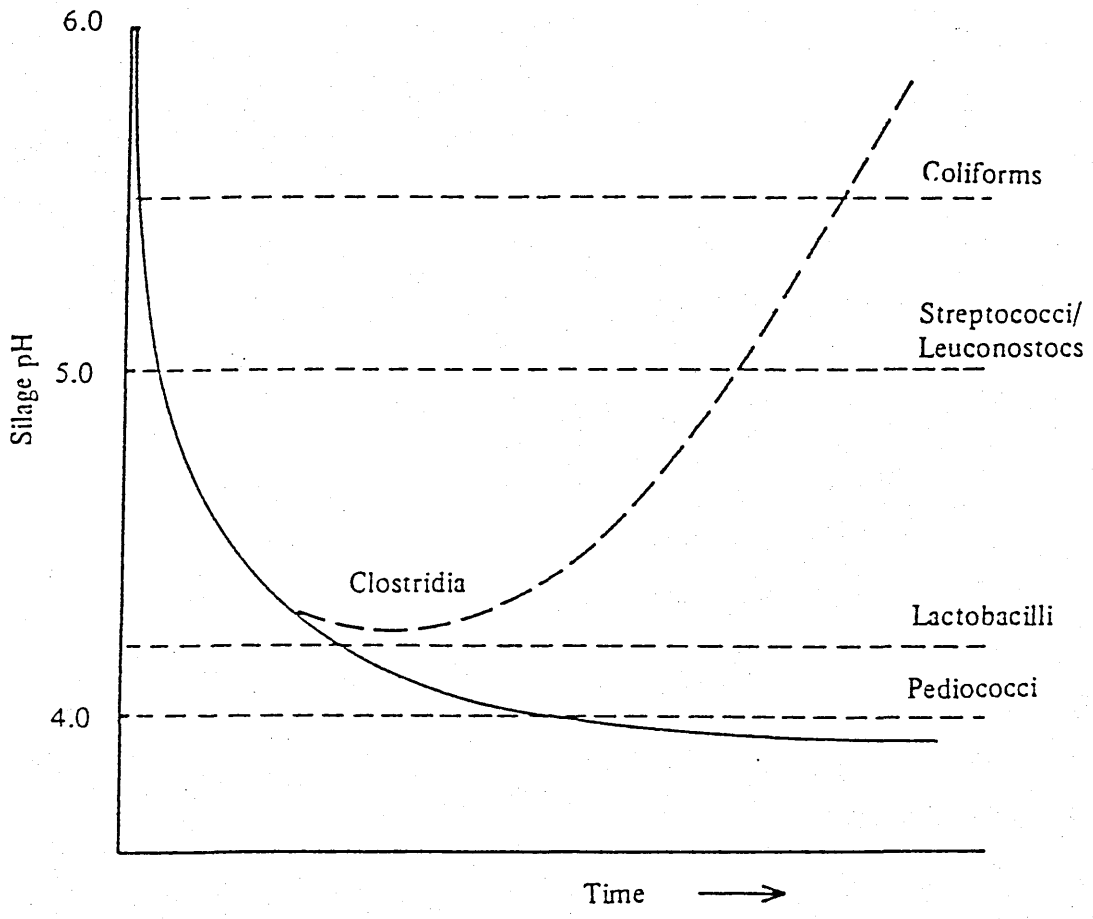
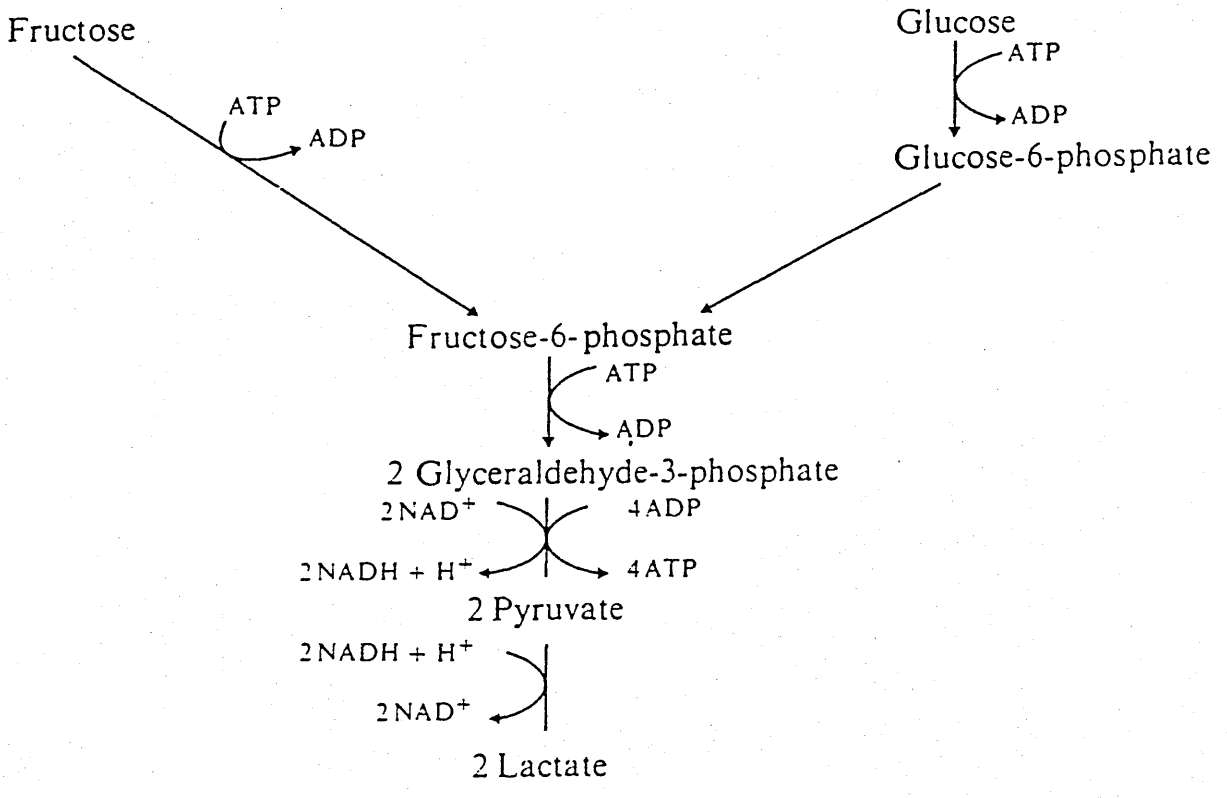


FIGURE 5: Qualitative changes which take place in the silage microflora during fermentation (Woolford, 1984).

There are two catagories of lactic acid bacteria, the homofermentative lactic acid bacteria (homolactics) and the heterofermentative lactic acid bacteria (heterolactics). Subdividing the catagories into rod and coccus forms the homofermentative lactic acid bacteria commonly associated with ensilage contain certain species of Lactobacilli and the Streptococci and Pediococci, whereas the heterofermentative lactic acid bacteria are made up of the Leuconostocs and other species of Lactobacilli.

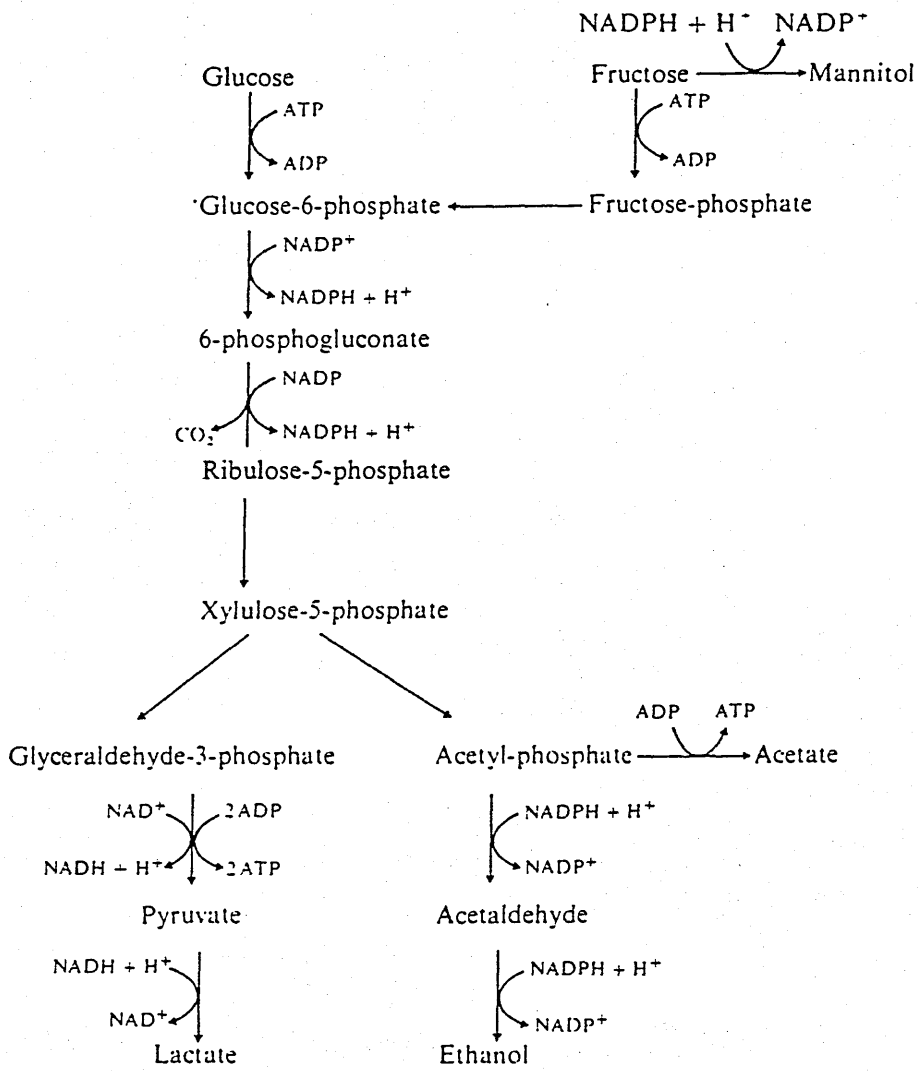
Homolactic organisms ferment both glucose and fructose via the glycolytic pathway as shown in Figure 6 to yield two moles of lactic acid per mole of sugar fermented. Heterolactic organisms produce a number of products depending upon the type of hexose substrate fermented. Both glucose and fructose are degraded along the pentose phosphate pathway followed by reduction to lactate in the homolactic pathway. In the fermentation of glucose the products are lactate, ethanol and CO_2 as shown in Figure 7. If fructose is fermented mannitol is a chief product with the reduction of two moles of fructose to mannitol being coupled with the oxidation of one mole of fructose to produce lactate, acetate and CO_2 (Fig. 7). Xylose and arabinose may become available as substrates from the hydrolysis of plant hemicelluloses. These pentoses are fermented by both homofermentative and heterofermentative lactic acid bacteria to yield one mole of lactate and one mole of acetate per mole of pentose fermented (Figure 8). The homofermentative organisms are, however, the more efficient as acid producers and are thus more desirable for preservation.

Of the other species of micro organisms found in the silo during the first few days of ensilage, clostridia are the most important



Sum: $C_6H_{12}O_6 + 2\,ADP \rightarrow 2\,CH_3CHOHCOOH + 2\,ATP$

FIGURE 6: Fermentation of glucose and fructose by homofermentative lactic acid bacteria (McDonald, 1981).

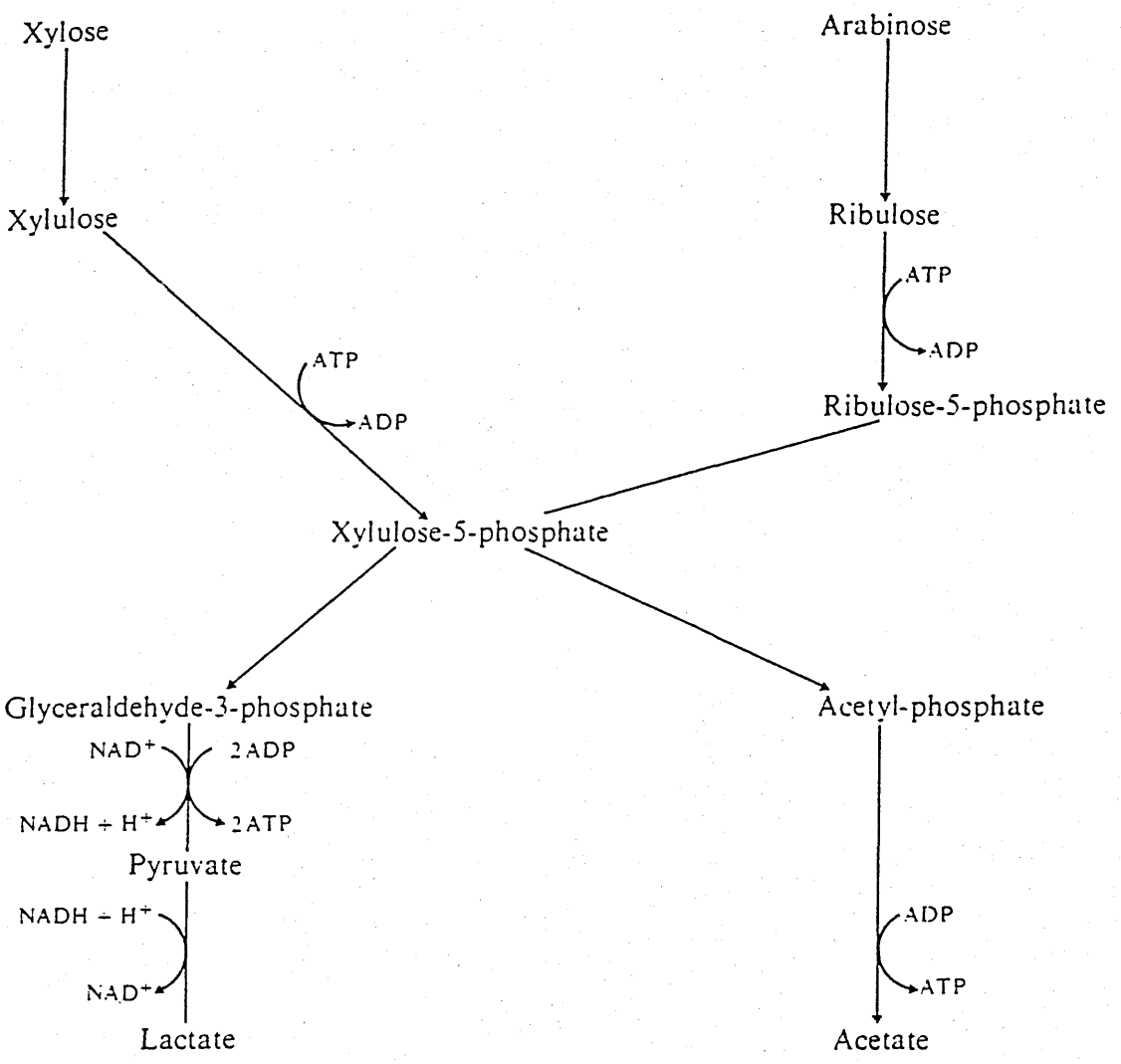


Sum: (1) $C_6H_{12}O_6$ (glucose) + ADP → $CH_3CHOHCOOH + C_2H_5OH + CO_2$ + ATP

(2) $3\,C_6H_{12}O_6$ (fructose) + H_2O + 2 ADP → $CH_3CHOHCOOH + 2\,C_6H_{14}O_6$ + CH_3COOH^- + CO_2 + 2 ATP

(3) $C_6H_{12}O_6$ (glucose) + $2\,C_6H_{12}O_6$ (fructose) + H_2O + 2 ADP → $CH_3CHOHCOOH$ + CH_3COOH + $2\,C_6H_{14}O_6$ + CO_2 + 2 ATP

FIGURE 7: Fermentation of glucose and fructose by heterofermentative lactic acid bacteria (McDonald, 1981).



Sum: $C_5H_{10}O_5 + 2\,ADP \rightarrow CH_3CHOHCOOH + CH_3COOH + 2\,ATP$

FIGURE 8: Fermentation of pentoses by homofermentative and heterofermentative lactic acid bacteria (McDonald, 1981).

as they can bring about anaerobic deterioration of the silage. Clostridia are present on harvested forage, usually in the form of spores. Gibson (1965) identified seven species of clostridia which play a major role in silage fermentation and designated them to two main groups, lactate fermenters and amino acid fermenters. In the anaerobic conditions of the silo they can begin to multiply utilising the sugars and lactic acid to produce butyric acid and degrading the amino acids to products of poor nutitional value with the overall production of an unacceptable, putrid silage.

Two important factors operate in supressing the growth of Clostridia. Their optimum pH is 7.0-7.4 and they cannot tolerate acidic conditions ($<$ pH 4.2). A sufficient production of lactic acid, by domination of the fermentation by the lactic acid bacteria, will lower the pH to a critical level and therefore inhibit their growth. Clostridia also prefer very wet conditions (Gibson, 1965) and the wilting of crops, preferably to dry matter levels above 300 gkg^{-1} and the adequate sealing of the silo to prevent the penetration of rain will restrict their activity.

Silage Additives

Improvements in the intake and feeding value of silage may be achieved by treatments applied in conjuction with or separate to physical measures such as chopping. The addition of substances to the forage at the time of ensilage is aimed at ensuring the success of the process either by stimulating the natural fermentation or by restricting fermentation and achieving preservation without lactic acid production, the mechanism of action depending on the type and level of additives used.

Silage additives can be classified, broadly, into two main catagories: inhibitors and stimulants (Table 3). Inhibitors are those containing

TABLE 3: Classification of silage additives (McDonald, 1981).

Silage Additives

Fermentation stimulants		Fermentation inhibitors		Aerobic deterioration inhibitors	Nutrients*
Bacterial cultures	Carbohydrate sources*	Acids	Others		
Lactic acid bacteria	Glucose	Mineral acids	Formaldehyde	Propionic acid	Urea
	Sucrose	Formic acid	Paraformaldehyde	Caproic acid	Ammonia
	Molasses	Acetic acid	Sodium nitrite	Sorbic acid	Biuret
	Cereals	Lactic acid	Sulphur dioxide	Pimaricin	Minerals
	Whey	Benzoic acid	Sodium metabisulphite	Ammonia	
	Beet pulp	Acrylic acid	Ammonium bisulphate		
	Citrus pulp	Glycollic acid	Sodium chloride		
	Potatoes	Sulphamic acid	Antibiotics		
	Cellulases	Citric acid	Carbon dioxide		
		Sorbic acid	Carbon bisulphide		
			Hexamethylenetetramine		
			Bronopol		
			Sodium hydroxide		

* Most substances listed under carbohydrate sources can also be listed under nutrients

chemical agents which act at the fermentation stage of the silage process or else aid the preservation of the silage from aerobic deterioration. Stimulants are those additives which contain fermentable substrates, such as sugars or other carbohydrate sources, and/or biological agents.

Chemical agents concerned with fermentation control act by inhibiting partially, or completely, microbial growth. Although a variety of chemical additives is currently in use, see Table 3, the majority are based on sulphuric acid, formic acid and formaldehyde, alone or in combination. Formic acid is the most extensively studied silage additive in terms of its effects on both the microbial population (Henderson et al, 1972; McDonald and Henderson, 1974; Woolford, 1975) and chemical composition (Wilson and Wilkins, 1973; Barry, Cook and Wilkins, 1978) of the silage and also in animal production studies (Thomas and Thomas, 1985). Little difference has been reported between formic acid and sulphuric acid when both are applied at similar rates (Flynn and O'Kiely, 1984).

Formaldehyde has known bacteriostatic properties and also the property of protecting plant protein from microbial breakdown. The level of addition of formaldehyde is closely related to the protein content of the ensiled material (Wilkinson, Wilson and Barry, 1976) as at high levels of addition intake and digestibility are depressed. Also silages made with formaldehyde only tend to be susceptible to secondary clostridial fermentation and aerobic deterioration and thus formaldehyde is now always used in combination with an acid.

Owing to the corrosive nature of acids and their general unpleasantness in handling, many silage makers dislike using chemical additives and non-chemical additives or biological additives are gaining support.

There is still only limited research information on the effectiveness of such fermentation stimulants as bacterial inoculants although there are occasions when they should prove beneficial (McDonald, Stirling, Henderson and Whittenbury, 1964) as should the addition of carbohydrate-rich material (McCullough and Neville, 1960; Jones, 1970).

A shift of emphasis in silage-making is required from just an achievement of a satisfactory fermentation, aided by the diverse range of additives available, to a greater consideration of nutritive value and ways of improving the nutritional characteristics of the silage.

Use of Cell Wall Degrading Enzymes in Ensilage

The optimum temperature for the activity of a commercial cellulase preparation has been claimed to be 40°C (Toyama, 1968) with the activity of the enzyme being greatly reduced when the temperature exceeds this level. This is supported by the work of Henderson, McDonald and Anderson (1982) who obtained maximum cellulose hydrolysis in ensiled forage by a commercial cellulolytic enzyme preparation in silage held at 35°C. The optimum pH value for cellulolytic enzymes is around 4.5-5.0 (Bemiller, Tegtmeier and Pappelis, 1968) again supported by the work of Henderson *et al* (1982). Investigating hemicellulase preparations extracted from forages Dewar, McDonald and Whittenbury (1963) found that the enzymes had an optimum pH of 6.0 and an optimum temperature around 37°C.

The addition of cell-wall degrading enzyme preparations to crops at the time of ensilage introduces them to generally disadvantageous conditions as regards their optimum pH and especially temperature. There are compensations, however, in that the enzymes are afforded a long period

in the silo in which to perform their hydrolysis and they are placed in direct contact with their substrates.

Significantly lower cellulose contents were obtained by Leatherwood, Mochrie and Thomas (1961) in first cut alfalfa and whole barley on addition of a cellulase preparation before ensilage; a 20% greater hydrolysis of cellulose was found in the alfalfa over the control after 56 days ensilage. In further work with alfalfa treated with an enzyme preparation from Aspergillus oryzae there was a tendency for increased voluntary intakes (not statistically significant) by cows of alfalfa silage compared to untreated silage. The daily gains of calves fed on similarly treated haylage were significantly higher comparing treated and untreated material (Olson and Voelker, 1961).

The effect of cellulase preparations on sorghum forage was proposed by McCullough (1964) to be influenced by fermentation factors such as the type of fermentation, silage acidity, initial pH and the need for a buffer, particularly in conventional silos. He found that silage made with a cellulase preparation and a $CaCO_3$ buffer had 20% less cellulose than the control silage. This resulted in an increase of 4 percentage units in digestibility when the silages were fed to dairy heifers. Owen (1962) however, found no apparent degradation of the complex polysaccharides of sorghum silage treated with cellulase, hemicellulase and pectinase enzyme preparations.

Corn silage, treated with cellulase prepared from Trichoderma viride, after one year in the silo was found to have a lower cellulose content, approximately 13% reduction, than the untreated material (Autrey, McCaskey and Little, 1974). These workers also found that the pH of

the ensiled forage varied inversely with the cellulase concentrations used of 0, 0.5, 1.0 and 2.0 g cellulase $kgDM^{-1}$, indicating a possible increase in fermentation of carbohydrate at higher enzyme concentrations. This could have resulted from some breakdown of cellulose in the silo although cellulose digestibility did not differ significantly between cellulase-treated and control forage. They treated other forages and cellulosic materials such as hay and newsprint by soaking them for seven days in a cellulase preparation. The mean coefficients of cellulose digestion showed an increase between 5% and 10% for the enzyme-treated material as compared to control material.

Autrey et al (1974) also investigated the effect on cellulose digestibilty of presoaking hay with 2% NaOH prior to a cellulase treatment. These findings showed that soaking with NaOH alone followed by washing was sufficient to give an increase in cellulose digestibilty. Treatment with only the cellulase preparation did in fact give a reduction in cellulose digestibility. The combined effect however, of NaOH, washing and cellulase addition was to give a much greater increase in cellulose digestibility which was further increased by increasing enzyme concentration.

These experiments illustrate the need to consider the nature of the material treated and the interaction of dietary components as well as the source of the enzyme preparation used. Jones and Hayward (1975) tested different cellulase preparations and found that the enzyme derived from T. viride was more active than the enzyme from A. niger on substrates of dried, milled herbage and cellulose paper. These findings showing that too high a level of enzyme may actually reduce cellulose digestibility accentuates the importance of enzyme concentration suggesting that from a nutritional standpoint there is likely to be a critical level of addition.

Henderson and McDonald (1977) sought to increase the digestibility of grass silage for pigs by treating the silage with a range of cellulase preparations. The findings of their experiments showed that in all cases the addition of cellulase to grass resulted in hydrolysis of cellulose during the period of ensilage. In some cases the water soluble carbohydrate levels in the cellulase-treated silages were much lower than in the original herbage, but the lower pH values of these silages compared with untreated silages indicated an increased fermentation of free sugar by lactic acid bacteria.

The losses of cellulose reported for some enzyme preparations were as high as 300-400 g/kg fresh herbage. Enzymes prepared from T. viride were again reported to be more active than other fungal preparations. The feeding of silages, made with an acid additive with and without a cellulase treatment, to pigs showed that there were no differences in digestibility of the gross energy as a result of silage treatment or cellulase addition (Whittemore and Henderson, 1977).

In further work Henderson et al (1982) studied the effect of formic acid (4.5 lt^{-1}) and a commercial cellulase preparation (4 gkg^{-1}) on ensiled grass, lucerne and clover. Results showed that treatment with a cellulase preparation caused a significant breakdown of cellulose during ensilage. The losses of cellulose were greatest when the silages were minced and stored at 35°C. Also there was a significantly greater effect of cellulase treatment on the hydrolysis of cellulose in grass silages than in legume silages; maximum values for the hydrolysis of cellulose for grass, lucerne and clover were 450 gkg^{-1}, 318 gkg^{-1} and 249 gkg^{-1} cellulose ensiled respectively.

The hydrolysis of cellulose should increase the water soluble carbohydrate content in silages with its recovery depending on the extent to which it is utilised by the bacteria or yeasts. An excess of water soluble carbohydrate will have little effect on the fermentation pattern of well-preserved silages but in situations where water soluble carbohydrate is limiting for a satisfactory lactic acid fermentation, the addition of a cellulase preparation may be beneficial.

From most of the experimental work carried out the addition of cellulolytic enzymes to crops at the time of ensiling has been found to cause an increased hydrolysis of the cellulose content and improved digestibilty of the ensiled crop. Commercial cellulases used in most experiments are known to contain hemicellulases and may have resulted in the hydrolysis of some hemicellulose in the crops to which they were added.

Most of the work carried out to investigate the enzymic hydrolysis of hemicellulose has been on purified hemicellulose substrates isolated by extraction processes and not performed on the intact crops. The hemicellulase preparations used in such studies have been derived from microbial sources such as bacteria (Whistler and Masak, 1955), fungi (Gasgoigne and Gasgoigne, 1960) or rumen micro organisms (Walker and Hopgood, 1961). Hydrolysis of hemicelluloses such as xylan and other pentosans by these enzyme preparations resulted in a considerable release of monosaccharides such as xylose and glucose, disaccharides and higher oligosaccharides. It has been observed (Laidlaw and Reid, 1952) that, in silage studies, the production of total volatile acids and lactic acid may be greater than

the loss in soluble carbohydrates. One of the sources for acid production may be the hemicellulose fraction (Dewar et al, 1963).

AIMS AND OBJECTIVES

The aim of the work described in this thesis was to further investigate the use of enzymes, especially those which degrade cellulose and hemicellulose, as components in silage additives. The application of these enzymes at a high enough rate to achieve a significant breakdown of the plant cell wall in the silo has great potential. It could lead to beneficial effects on the rate or extent of cell-wall digestion in the cow's rumen with consequences in improved silage intake, improved microbial protein synthesis and improved overall digestibility of dietary organic matter. Such upgrading of harvested forage and improvements in feeding value would make it feasible to harvest crops at a later stage of growth when the DM yield hectare^{-1} was high, without suffering the fall in digestibility which normally accompanies such a change in cutting strategy.

SECTION II MATERIALS AND METHODS

LABORATORY SILOS

Test-tubes and plastic measuring cylinders of 300 ml capacity were used in a series of laboratory ensilage experiments. The silos were fitted with water-seal fermentation traps and kept at room temperature throughout.

PILOT SCALE SILOS

Fibre glass tank silos of one tonne capacity and lined with a large plastic bag were used in a series of intake and digestion studies. On filling and compressing the silos were sealed and kept air-tight until required, being stored in the outside environment.

ANALYTICAL METHODS

Dean and Starke Dry Matter

The dry matter was determined by distillation of a minced silage sample with toluene following the procedure of Dewer and McDonald (1961).

Reagents

(1) Toluene, distilled (110-120°C) to remove any water.

(2) Neutral ethanol - 25 ml ethanol was titrated against 0.1 M NaOH and then a stock solution prepared according to the values obtained.

Procedure

25 g of minced silage was placed in a round-bottomed 1 litre flask and immediately covered with 300 ml of redistilled toluene. The Dean and Starke apparatus was set up to give a good flow of water and the degree of heating was arranged so that the toluene vapour condensed about 1-2" up the condenser.

The refluxing was continued until the level of water in the receiver did not change over a period of 15 minutes. The water receiver was then disconnected and left for an hour. After an hour the volume of water was read and the toluene removed by means of a Pasteur pipette.

10 ml of the water was pipetted into a 25 ml volumetric flask and diluted to volume. The acidity of the water was measured using 10 ml of the diluted liquid. 40 ml of neutral ethanol was added and titrated with 0.1N NaOH using phenolphthalein as the indicator.

Calculation

W = weight of fresh silage (g)

V = observed vol of water (ml)

T = titre of 0.1M NaOH (ml)

Vol correction = 2.5 x V/10 x T x 0.0055 ml

= 0.001375VT ml

At 20°C the wt of water = 0.998V (1 - 0.001375T)

wt of silage DM = W - 0.988 (1 - 0.001375T)

$$\% \text{ Dry Matter} = \frac{100 \times W - 0.998V\ (1 - 0.001375T)}{W}$$

Determination of Silage pH

On opening each silo the pH of the silage was measured using a PT1-6, universal digital pH meter.

Procedure

A representative sample of 20 g of wet silage was taken and mixed in 20 ml of distilled water. The resulting pH was read on the aforementioned pH meter.

Total Nitrogen

The analysis was carried out using a Kjeltec system apparatus, which includes a digestion unit, a distilling unit and a titration unit (Tecator Ltd, Thornbury, Bristol).

Procedure

Samples containing 1-2 mg of nitrogen were first digested with nitrogen-free concentrated sulphuric acid, using tablets containing 2 g potassium sulphate and 0.02 g selenium as a catalyst. The digested samples were distilled and the ammonia collected in a 250 ml conical flask containing 25 ml boric acid solution (40 gl^{-1}). The total nitrogen was determined by titration with 0.01M hydrochloric acid.

Calculation

$$\% N = \frac{\text{Molarity of acid} \times 14 \times (\text{titre-blank})}{\text{sample DM(g)} \times 10} = \text{T.Ng/100g DM}$$

Estimation of True Protein

The method used was a modification of the tannic acid method of C. Van Roth.

Reagents

The reagent was prepared by dissolving 4.4g of tannic acid in water and adding 0.1 ml of concentrated sulphuric acid and making up to 100 ml in a volumetric flask. The reagent was allowed to stand for 24 hours and before use, was filtered using a No.42 GF/A Whatman filter.

Procedure

Wet silage (1g) was weighed into a centrifuge tube and 20 ml of boiling tannic acid solution was added to it and thoroughly mixed. The tube and its contents were then heated in a boiling water bath for 15 minutes. The tube was then allowed to cool for a period of about 15 minutes. The tube and contents were centrifuged for 10 minutes at 1500 g.

The supernatant was sucked out through muslin to avoid any of the particles escaping. Cold water was added to the tube

to make a volume of 25 ml and the residue stirred and centrifuged as before and the washing repeated. The centrifugation was repeated twice more. The silage residue was finally washed into a Kjeldahl digestion tube ready for nitrogen determination using the procedure already described.

Calculation

$$\frac{\text{Titre-blank} \times 14}{\text{wt sample DM} \times \frac{\text{DM\%}}{100}} = \text{gTP-N/100 g DM}$$

Silage Extract Preparation

The extract was prepared by taking 20 g of wet silage and adding 200 ml of distilled water. The extract was then heated at 40°C in a water bath for 30 minutes and then filtered through muslin. The filtrate was centrifuged at 1700 g for 20 minutes and the solution decanted, bottled, labelled and stored in a freezer until used.

Ammonia Nitrogen in Silage

This method also uses the steam distillation apparatus as described in the analysis of true proteins.

Procedure

Sodium hydroxide (10 M, 10 ml) was added to 10 ml of silage extract in a Kjeldahl tube. The tube was placed into the distilling unit and the ammonia distilled over was collected in 25 ml of boric acid solution (40 gl^{-1}). The ammonia was determined by titration with 0.01 M hydrochloric acid.

Calculation

$$A = 20 \times \frac{DM}{100} \qquad B = 200 + 20 \times \frac{(100-DM)}{100}$$

$$\frac{\text{Titre-blank} \times 14 \times \text{molarity of acid}}{10/B \times A} = \text{g } NH_3\text{-N}/100 \text{ g DM}$$

Total Reducing Sugars

Total reducing sugars were determined by a method similar to that of Somogyi (1945).

Reagents

(1) Somogyi reagent

Prepared by dissolving 28 g of anhydrous di-sodium hydrogen-orthophosphate + 40 g potassium sodium tartrate in 700 ml H_2O. Added to the solution:

100 ml 1N sodium hydroxide

80 ml of 10% hydrated copper sulphate solution and

180 g anhydrous sodium sulphate. Then made up to 1 litre.

(2) Arsenomolybdate reagent.

Prepared by dissolving 25 g of ammonium molybdate in 450 ml distilled water then adding 21 ml analar concentrated sulphuric acid. A solution of 3 g di-sodium hydrogen arsenate in 25 ml distilled water was then added in a water bath at 55°C for 25 minutes with continuous stirring. To prevent deterioration the reagent was kept in a brown bottle.

(3) 0.3N sodium hydroxide

(4) 5% (w/v) zinc sulphate

(5) 0.5M sulphuric acid

(6) 1M sodium hydroxide

(7) A and B reagents

i) Reagent A

Prepared by dissolving 25 g of sodium carbonate + 25 g of Rochelle salt (potassium sodium tartrate) + 20 g of sodium hydrogen-carbonate + 200 g of sodium sulphate (anhydrous), in 800 ml of distilled water and diluting to 1 litre. Filtered if necessary.

ii) Reagent B

15% copper sulphate solution containing 1 or 2 drops of concentrated sulphuric acid per 100 ml.
A and B reagent was made up as 1 part B to 25 parts A.

Procedure

5 ml of silage extract was taken and 0.1 ml of 0.5M sulphuric acid was added. The mixture was then hydrolised by boiling for 30 minutes in a Quickfit test-tube. The tubes were cooled in a water bath and then 0.1 ml of 1M sodium hydroxide was added. 2 ml of each extract was transferred into a 15 ml centrifuge tube and deprotenised by adding 4 ml of 5% zinc sulphate solution and 4 ml of 0.3M sodium hydroxide. The tubes were centrifuged for 10 minutes at 1500 g. The samples and standards were carried out

in duplicate. The standards went through the same procedure but instead of the silage extract varying concentrations of D-glucose were used.

2 ml of sample or standard was added to 2 ml of A and B reagent and the tubes put in a boiling water bath for 10 minutes. The tubes were then cooled and 2 ml of arsenomolybdate reagent added. The solution was transferred to a 50 ml volumetric flask (making sure all the solution was washed out using distilled water), and made up to the mark. The absorbances of the resulting solutions were measured by a spectrophotometer at 500 nm against a distilled water blank which had been prepared in the same way as the standards. The concentration of reducing sugars was calculated graphically by reference to the absorbance of the standard glucose solutions.

Calculation

where R = concentration from graph.

$$\text{Soluble sugar} = \frac{R \times 5.2 \times 11}{100} - \frac{\%\ DM}{100} \quad (\text{mg gDM}^{-1})$$

Lactic Acid

Lactic acid was determined by the Barker and Summerson (1941) method outlined by Pryce (1969).

Reagents

(1) Protein precipitating reagent.

Prepared by dissolving 10 g of sodium tungstate in 800 ml of distilled water, adding 22ml of 90% (w/v) orthosphosphoric

acid + 5 g hydrated copper sulphate and made up to 1 litre.

(2) Concentrated sulphuric acid.

(3) Colour reagent

Prepared by dissolving 1.5 g of parahydroxybiphenyl in 100 ml of dimethyl formamide.

(4) Lactic acid stock solution

1.065 g pure lithium lactate + 1 ml concentrated sulphuric acid made up to 1 litre.

Procedure

0.1 ml of silage extract was added to 3.9 ml of protein precipitating reagent, the tubes were shaken and centrifuged at 1500 g for 5 minutes. 1 ml of the supernatant was transferred to a boiling tube and 5 ml of the concentrated sulphuric acid was quickly added and the tubes left to sit for 2 minutes and then cooled in a water bath.

0.1 ml of p-hydroxybiphenyl (colour reagent) was added to the tubes and they were shaken and left standing for 10 minutes in order to allow the colour to develop.

The tubes were placed in a boiling water bath for 90 seconds and then cooled. The absorbance of the solution was read at 565 nm against a distilled water blank. The concentration of lactic acid in the silage was obtained from a standard calibration graph.

Determination of Volatile Fatty Acids

Reagents

Preservative: 30 ml metaphosphoric acid (25% w/v)

10 ml formic acid (90% (w/v) Analar)

10 ml distilled water

Internal Marker: Hexanoic acid (2 gl^{-1})

Procedure

2 ml of silage extract was taken and to it 2 ml of hexanoic acid and 1 ml of preservative was added. At the same time 2 ml of standard mixture was taken and hexanoic acid and preservative were added to it in the same amounts. The mixture was placed in a centrifuge tube and shaken and allowed to stand for 20 minutes. The supernatant (1-3 μl) was injected in the column of the gas liquid chromatograph. The separation of the acids was carried out using a Shimadzu G.C. 8A with a flame ionization detector. The column packing was 5% Carbowax 20M TPA. The oven temperature was 100°C stepped up at a rate of 4°/minute to 150°C. The N_2 flow rate was 60 ml/min.

The molar concentration of each acid was calculated from the peak area of each acid on the chromatogram relative to the area of hexanoic acid after allowances had been made for differences in the detector response for each acid determined from analysis of standard mixtures containing known concentrations of each acid.

Dry Matter and Ash

A known weight of sample was oven dried at 100°C for 3 hours and then re-weighed. The dry matter was expressed as a percentage of the fresh weight. Ash was determined by ignition of a known weight of dry sample in a muffle furnace at 550°C overnight.

Neutral Detergent Fibre (NDF)

(1) Neutral Detergent solution

The following chemicals were dissolved and made up to 1 litre.

30 g	Sodium lauryl sulphate
18.61 g	Ethylenediaminetetra-acetic acid (di-sodium salt-EDTA).
6.81 g	Di-sodium tetraborate
4.56 g	Di-sodium hydrogen orthophosphate
10 ml	2-Ethoxyethanol (analar)

(2) Decahydronaphtalene - Dekalin

(3) Acetone

(4) Sodium sulphite (anhydrous)

Procedure

Approximately 1 g of sample was added to a conical flask to which 100 ml of neutral detergent solution, 2 ml of Dekalin and 0.5 g of sodium sulphite was also added. The flask was then refluxed for 60 minutes after the onset of boiling and the contents then transferred to a pre-weighed sintered glass crucible. The contents were then filtered using a Buckner funnel and the flask and the sides of the crucible washed twice with hot water and twice with acetone. The crucible was put in

the oven at 100°C for 8 hours and re-weighed. The residue (NDF) was expressed as a percentage of the original weight of sample DM taken. Ash-free NDF values were obtained from the loss in weight after 3 hours in a muffle furnace at 550°C.

Acid Detergent Fibre(ADF)

The acid detergent fibre in silage was determined by the method of Goering and Van Soest (1970).

Reagents

(1) Acid Detergent solution

This solution consists of 20 g of CTAB (cetyl-trimethyl ammonium bromide) per litre of 0.5M sulphuric acid.

(2) Decahydronaphthalene - Dekalin

(3) Acetone

Procedure

Approximately 1 g of sample was weighed and transferred to a conical flask. Then 100 ml of acid detergent solution and 2 ml of Dekalin were added. The flask was refluxed for 60 minutes from the onset of boiling. The contents were transferred from the flask to a pre-weighed glass crucible and washed with hot water, (90°C-100°C). The flask and sides of the crucible were washed twice with hot water and then the wash was repeated with acetone.

The crucibles were then dried at 100°C for 8 hours in an oven and then re-weighed after cooling in a dessicator. The residue (ADF) was expressed as a percentage of the original weight of sample DM taken. Ash-free ADF values were obtained from the loss in weight after 3 hours in a muffle furnace at 550°C.

Acetyl Bromide Method for the Determination of Lignin

The original method was from Johnston (1961) but has been modified and was used in the Morrison (1972) form.

Reagents

(1) Acetone

(2) Diethyl ether

(3) Absolute ethanol 99.7-100%

(4) Acetic acid

(5) Acetyl bromide - acetic acid reagent

Prepared by mixing 25 ml of acetyl bromide, minimum assay 99%, with acetic acid in a 100 ml volumetric flask and diluted to volume with acetic acid.

(6) Hydroxylammonium chloride solution

Prepared by dissolving 3.5 g hydroxylammonium chloride in distilled water and made up to 100 ml.

(7) Sodium hydroxide 2M

Procedure

Approximately 0.05 g of dried sample was taken and to it 20 ml of distilled water was added. The tubes were then placed in a 70°C water bath for 30 minutes, the contents being shaken once every minute. The contents were filtered through a 2.5 cm GF/A filter paper in the nylon filter unit and washed with four 5 ml portions of distilled water followed by four 5 ml portions of absolute ethanol, then four 5 ml of acetone and finally four 5 ml portions of diethyl ether.

The filter and contents were transferred back to the Quick-fit tube and placed in a 100°C oven for 5 minutes. A blank determination was included and all determinations were carried out in triplicate.

5 ml of acetyl bromide-acetic acid reagent was added to the residue in the tube, mixed and placed in a 70°C water bath for 30 minutes. The tubes were transferred to a 20°C water bath for 30 minutes, swirled and stirred as above every 10 minutes. Using a Zippette, 20 ml of glacial acetic acid was added to the tubes. 5 ml was taken from the tubes and transferred to a 50 ml graduated volumetric flask containing 1 ml of 2M sodium hydroxide solution and 7.5 ml of glacial acetic acid.

Absolute ethanol was added until the volume was approximately 45 ml, then 1.5 ml of hydroxylammonium chloride solution was added and the flask was then made up to the volume with absolute ethanol. The contents of the flask were mixed well and the flask allowed to stand for 1 hour in order that the proteinaceous sediment should settle out.

The supernatant liquid was decanted through a 9 cm GF/A filter paper and the optical density read in a 10 mm silica cell at 280 nm.

Calculation

a = optical density of sample - optical density of blank

b = wt. DM x 4

A = a/b

Lignin % DM = 3.37A - 1.05 (Morrison, 1972)

Determination of Enzyme Activity

Preparations of cell-wall degrading enzymes, ES 1 and ES 2, in solution, prepared from Trichoderma sp., were supplied by an industrial company for use in ensilage experiments. Enzyme preparations of purified cellulase (C) and crude hemicellulase (H) were obtained from Sigma Chemical Company. The cellulase enzyme was prepared from T. viride and the hemicellulase from A. niger. From recommended activity levels of the Sigma preparations a 2% (2 g per 100 ml) solution of a cellulase preparation and a 7% solution of the hemicellulase preparation were made up for use in the experiments. The activities of all enzyme preparations were determined and standardised by several enzyme assays.

Polysaccharide Assay

1 mg ml^{-1} solutions of polysaccharide substrates in 0.01 M MES [2(N Morpholino) ethane sulphonic acid] buffer (pH 6.5) were prepared. The polysaccharides used were Xylan (from Oat Smelts)

obtained from Sigma and Avicell, a microcrystalline form of cellulose obtained from Honeywell and Stein Ltd, Wallington, Surrey. Xylan went into solution after 20-30 minutes sonication at higher power (18000 microns) but Avicell was insoluble.

4 ml of the enzyme solutions were added to 30 ml of the appropriate pre-warmed substrate with ES 1 and Sigma cellulase solutions being added to Avicell substrate and ES 2 and Sigma hemicellulase solutions being added to xylan substrate. 8 ml of the mixture was then taken and incubated at 39°C for 2 hours for the xylan tubes and overnight for the Avicell tubes. All tubes were boiled for 5 minutes to inactivate the enzymes and rapidly cooled on ice. Once cooled, the tubes were centrifuged at 3000 g on a bench centrifuge for 5 minutes to remove all particulate material which would interfere with the reducing sugar assay. 5 ml of the supernatant was taken and assayed for total reducing sugars using glucose as a standard for the Avicell assays and xylose for the xylan assays.

Alternatively, the cellulose-degrading ability of the ES 1 and Sigma cellulase preparations was determined using filter paper. 2 ml of the enzyme solutions was added to 8 ml of 0.05M citrate buffer (pH 4.8) and 50 mg filter paper. After incubation for 1 hour at 50°C, the tubes were boiled for 5 minutes and the assay procedure followed as outlined above.

A further method of assay to determine cellulase and hemicellulase activity was based on the measurement of the released reducing sugars using the DNS reagent.

Cellulase Activity

Reagents

1) 1M sodium acetate buffer adjusted to pH 5.0 with glacial acetic acid.
2) 1% carboxymethyl cellulose (CMC) solution (sodium salt obtained from Sigma) adjusted to pH 5.0 by adding 10 ml sodium acetate buffer, pH 5.0 per 100 ml solutions.
3) DNS solution

   Prepared by adding 1 g of 3.5 dinitro salicyclic acid and 30 g potassium sodium tartrate tetrahydrate (Rochelle salt) to 16 ml 10% sodium hydroxide. 50 ml distilled water was then added and the mixture warmed to dissolve and then cooled and added to a 100 ml volumetric flask and made up to the mark with distilled water.

Procedure

Enzyme dilutions of the ES 1 and C preparations were made up in distilled water. 1 ml of the CMC substrate was placed in a series of test-tubes prepared in duplicate and placed in a water bath at 30°C. After 10 minutes, 1 ml of enzyme solution was added to each tube and left for exactly 10 minutes. The reaction was then stopped by adding 2 ml of DNS reagent. The tubes were then placed in a boiling water bath for 10 minutes. They were rapidly cooled in iced water and 10 ml distilled water added to each tube. The absorbance at 540 nm of the resulting solutions was measured against a distilled water blank prepared in the same way. The concentration

of reducing sugars was calculated graphically by reference to the absorbance of standard glucose solutions of varying concentrations.

Xylanase Activity

Reagents

1) 0.5% xylan solution adjusted to pH 4.5 by adding 5 ml 1M sodium acetate buffer pH 4.5 per 100 ml solution.
2) DNS solution.

Procedure

The assay procedure was similar to that followed for the cellulase assay except that it was carried out using enzyme dilutions of the ES 2 and H preparations with xylan as the substrate. The assay temperature was 40°C and the tubes were boiled for 5 minutes. The standard graph was constructed using varying concentrations of xylose.

From the results of these assays the ability of the enzyme preparations to produce reducing sugars from polysaccharides was determined. Also the activity of the industrial preparations, ES 1 and ES 2, in relation to the Sigma preparations was determined and therefore activity-equated additions of the enzyme solutions for laboratory ensilage experiments could be calculated.

Enzyme Activity

From the methods described the activities for the enzyme preparations were as follows:

for ES 1 and Sigma cellulase (C) using Avicell/filter paper (polysaccharide assay) the activities were found to be

ES 1 = 16.4 U (where U (units) = μM glucose hr^{-1} ml^{-1})

C = 11.8 U

and using DNS reagent (cellulase activity) the activities were found to be

ES 1 = 19.5 U (where U (units) = mM glucose hr^{-1} ml^{-1})

C = 14.0 U

for ES 2 and Sigma hemicellulase (H) using Xylan (polysaccharide assay) the activities were found to be

ES 2 = 2.8 U (where U (units) = μM xylose hr^{-1} ml^{-1})

H = 0.2 U

and using DNS reagent (xylanase activity) the activities were found to be

ES 2 = 2.1 U (where U (units) = mM xylose hr^{-1} ml^{-1})

H = 0.2 U

SECTION III EXPERIMENTAL SECTION

EXPERIMENTAL SECTION 1: Experiments with Laboratory Silos

Grass from a pure sward of perennial ryegrass (Lolium perenne) was cut, minimum wilted for 2 hours, minced and ensiled in a series of laboratory experiments. The laboratory silos were kept sealed for 100 days except in Experiment 1 where a time response was studied. The silos were set up in triplicate in all experiments and formic acid solution (850 gkg^{-1}) was applied at a level of 3 lt^{-1}.

Experiment 1. Time Response

Silos were prepared at one level of addition (125 mlt^{-1}) of cell-wall degrading enzyme preparations, ES 1 and ES 2. Control silos were prepared using formic acid only. The silos were opened in series after periods of 2, 8 and 14 weeks in the silo. All treatments were prepared in triplicate with the total number of silos prepared being 27.

Results and Discussion

The chemical compositions of the final fourteen week silages are given in Table 4. All the silages were well preserved with low pHs, low NH_3-N concentrations and butyric acid was not detected. The two enzyme-treated silages contained significantly ($P<0.001$) higher concentrations of lactic acid but acetic acid concentrations were similar for all silages. The content of water soluble carbohydrates (WSC) was higher for the silages treated with ES 1 but the difference did not reach statistical significance ($P>0.05$). The concentration of NDF in the silage treated with ES 1 was significantly ($P<0.01$) lower than either of the other two treatments; ADF concentrations

TABLE 4: Chemical composition (g $kgDM^{-1}$) of the silages after 14 weeks in the silo.

[Each value is a mean for 3 replicates.]

	CONTROL	ES1	ES2	SED
DM (g kg^{-1})	239	213	225	6.4
pH	4.2	4.1	4.1	0.4*
Total N	34	34	33	2.4
NH_3-N (g kg TN^{-1})	45	51	46	0.2
WSC	105	110	85	5.2
Lactic Acid	51	78	66	4.8***
Acetic Acid	19	20	19	0.6
Butyric Acid	0	0	0	
NDF	474	428	523	0.8**
ADF	263	230	263	0.5
NDF/lignin	11.4	9.7	9.7	0.7
ADF/lignin	6.3	5.2	4.9	0.4

*** ($P < 0.001$)

were also lower in the ES 1 silage but these differences were not statistically significant ($P>0.05$).

The changes with time in the concentration of various constituents in the silages are shown in Figs 9-11. Although the pattern of change was similar for all treatments, there was a significantly ($P<0.01$) greater rate of production of lactic acid with the two enzyme treatments compared with the control: for treatments ES 1 and ES 2 the lactic acid concentrations after eight weeks in the silo were 49 and 35 $gkgDM^{-1}$ respectively whereas the corresponding value for the control was only 19 $gkgDM^{-1}$. However, despite these higher concentrations of lactic acid with the enzyme treatments, the WSC contents after eight weeks were very similar for all three treatments. This suggests either a more efficient production of lactic acid from WSC in the enzyme-treated silages, or alternatively, which seems more likely, more WSC available for fermentation with the enzyme treatments. That the enzymes may indeed have increased the supply of WSC by way of a breakdown of plant fibre is also suggested by the changes in NDF/lignin and ADF/lignin ratios (Fig 11); although the differences did not reach statistical significance ($P>0.05$) there was a clear tendancy for reduced ratios with enzyme additions.

The experiment showed the addition of the enzyme mixtures to have no deleterious effects on the fermentation. However, it should be remembered that in this experiment formic acid (850 gkg^{-1}) was added to all silages as a safeguard against poor fermentation. With regard to the effectiveness of the enzyme in degrading plant cell walls,

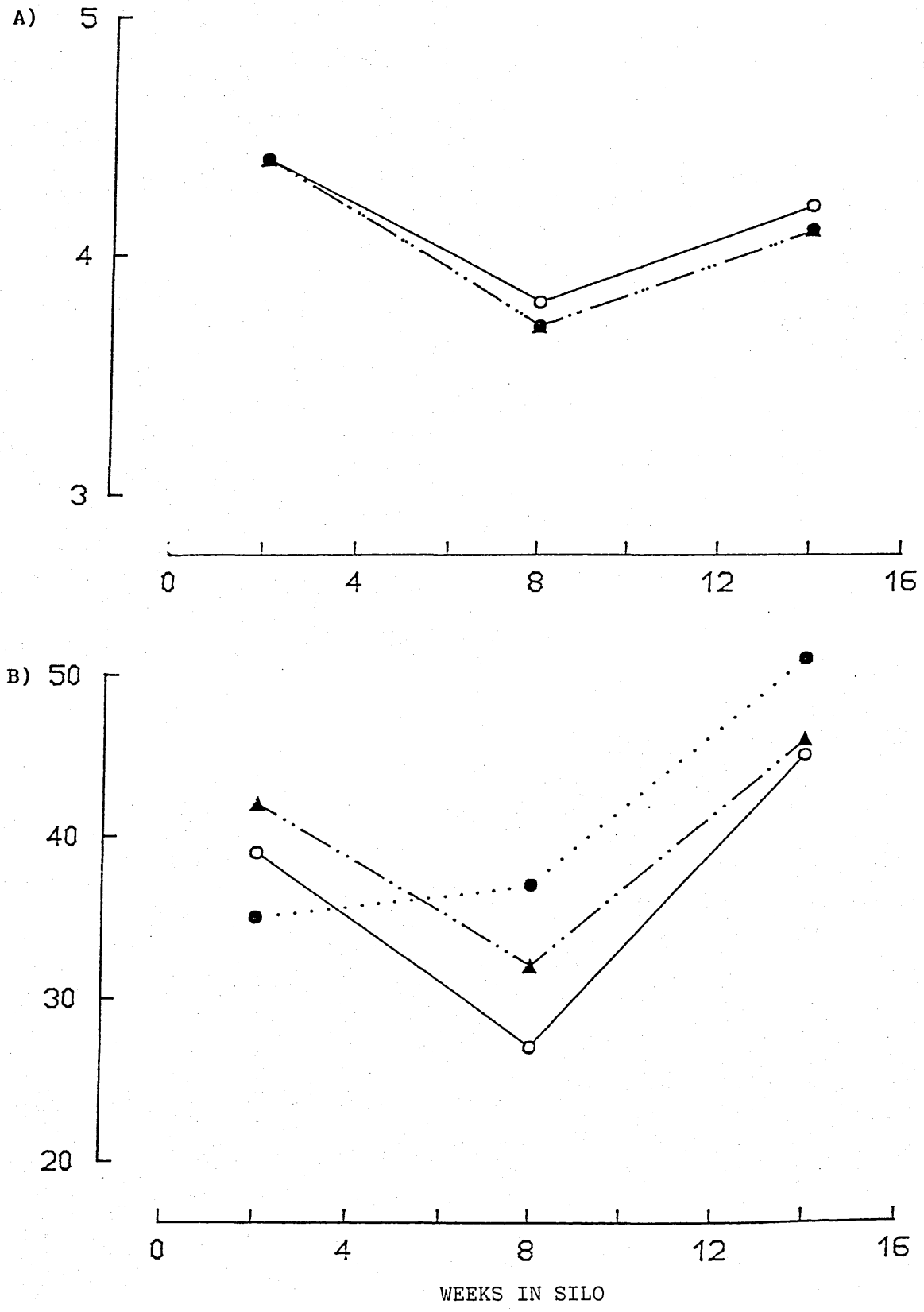


FIGURE 9: The changes with time in A) pH and B) concentration (g kg TN^{-1}) of NH_3N in the silages prepared in Experiment 1.

○ Formic Acid ($3lt^{-1}$); ● Formic Acid + 125 mlt^{-1}ES1; ▲ Formic Acid + 125 mlt^{-1}ES2

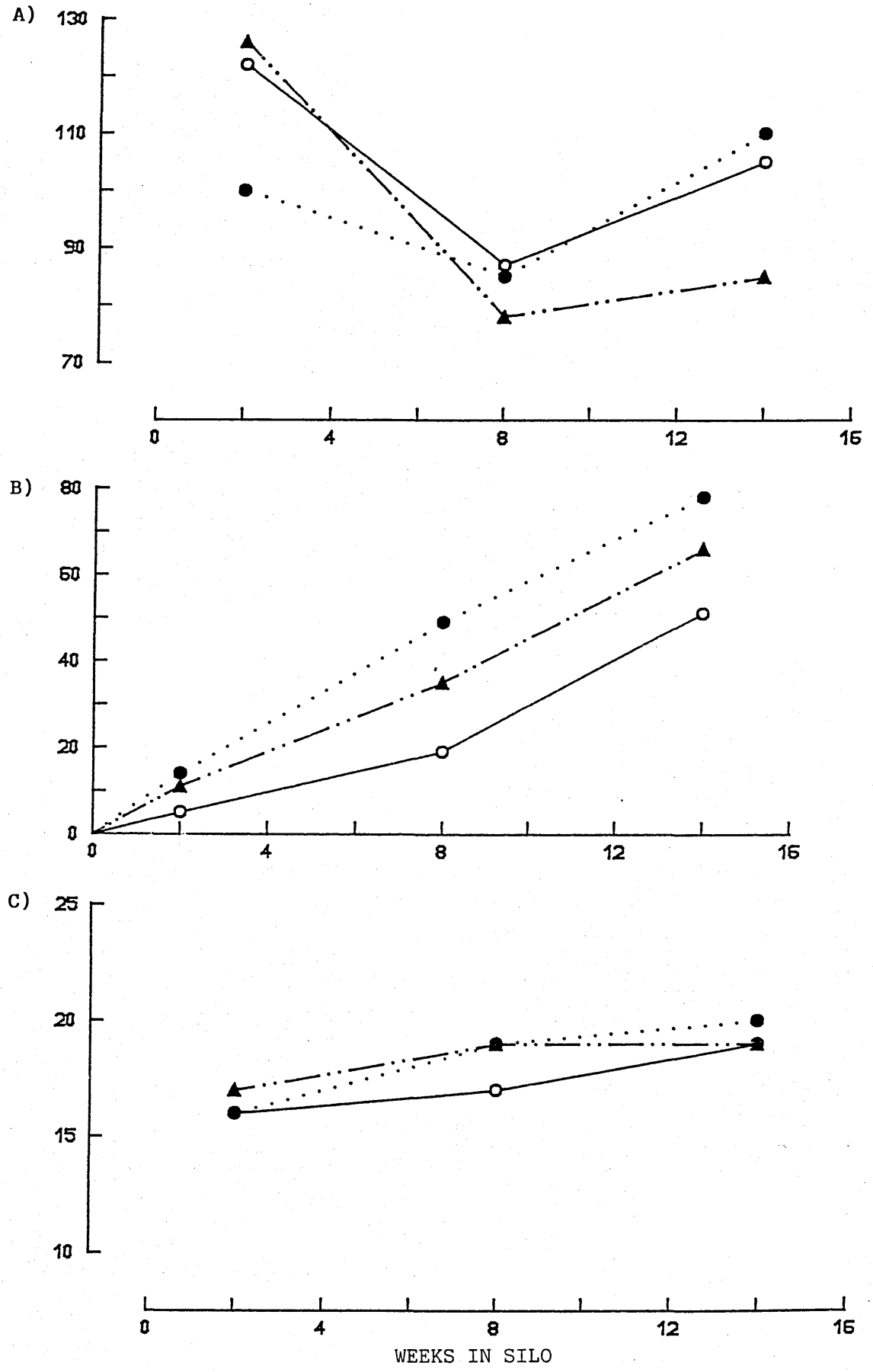


FIGURE 10: The changes with time in the concentration (g kg DM^{-1}) of: A) WSC; B)Lactic Acid; and C) Acetic Acid in the silages prepared in Experiment 1. ○ Formic Acid (3 l t^{-1}) ● Formic Acid + 125 ml t^{-1} ES1; ▲ Formic Acid +125 ml t^{-1} ES2

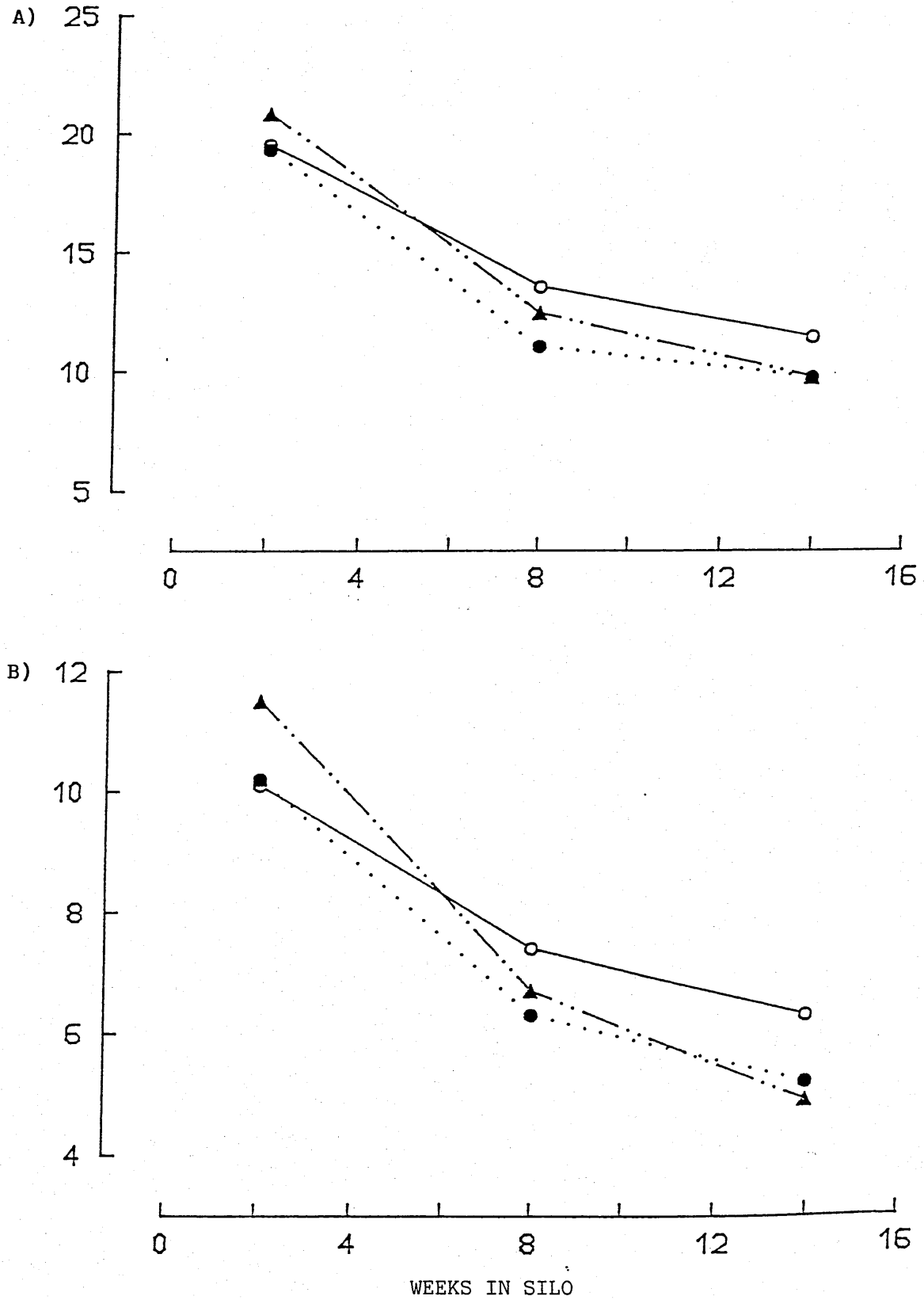


FIGURE 11: The changes with time in the concentration of A) NDF/lignin and B) ADF/lignin in the silages prepared in Experiment 1. ○ Formic Acid (3 lt^{-1}); ● Formic Acid + 125 mlt^{-1} ES1; ▲ Formic Acid + 125 mlt^{-1} ES2.

there are clear suggestions from the results for the concentrations of fibre components and lactic acid of an increased degradation of fibre and a release of fermentable substrates in response to the addition of the enzymes.

Experiment 2. Dose Response

Silos were prepared at increasing levels of addition of enzyme preparation. The levels studied for the preparations ES 1 and ES 2 were equivalent to enzyme additions of 100, 200, 300, 400 and 500mlt^{-1}. Activity equated additions of 133, 266, 400, 553 and 667 mlt^{-1} and 1390, 2780, 4170, 5560 and 6950 mlt^{-1} were used when adding purified cellulase (C) and crude hemicellulase (H) enzyme preparations respectively. Control silos of formic acid only were also set up. Each treatment was replicated three times with the total number of silos prepared being 63.

Results and Discussion

The chemical compositions of the silages treated with increasing levels of addition of the enzyme preparations or with 3 lt^{-1} formic acid (850 gkg^{-1}) alone are given in Tables 5 and 6. Low pHs and low NH_3-N concentrations and an absence of a detectable level of butyric acid indicate that all the silages were well preserved. The WSC concentration of the enzyme-treated silages differed significantly ($P<0.001$) from each other with the silage treated with ES 1 having the highest concentration of WSC whilst that treated with ES 2 had

TABLE 5: Chemical composition (g $kgDM^{-1}$) of the silages after 100 days in the silo.

[Each value is a mean of 3 replicates.]

		pH	NH_3-N (g $kgTN^{-1}$)	WSC	LACTIC ACID	ACETIC ACID
CONTROL		4.2	47	107	25	18
ES1	(mlt^{-1})					
	100	4.4	56	135	10	17
	200	4.2	56	132	37	19
	300	4.2	55	106	51	24
	400	4.4	55	123	45	18
	500	4.3	69	101	42	26
ES2	100	4.0	55	58	63	17
	200	4.0	62	27	77	18
	300	4.0	64	31	64	18
	400	4.0	60	57	75	20
	500	4.1	58	45	87	21
C	133	4.4	47	110	10	18
	266	4.1	49	111	56	20
	400	4.2	48	90	36	17
	533	4.1	50	75	59	18
	667	4.2	51	81	68	19
H	1390	4.1	61	44	74	19
	2780	4.1	57	58	64	19
	4170	4.1	60	62	65	19
	5560	4.1	56	57	86	19
	6950	4.1	50	70	72	21
SED		0.04***	0.3***	10.7***	10.6***	1.5*

*** ($P < 0.001$)

TABLE 6: Chemical composition (g $kgDM^{-1}$) of the silages after 100 days in the silo.

[Each value is a mean for 3 replicates.]

		NDF	ADF	NDF/ Lignin	ADF/ Lignin
CONTROL		461	251	9.2	5.0
ES1	(mlt^{-1})				
	100	476	272	9.7	5.5
	200	434	229	10.4	5.5
	300	405	219	9.0	4.9
	400	392	216	9.6	5.3
	500	354	193	8.2	4.5
ES2					
	100	475	263	11.8	6.5
	200	476	268	10.9	6.1
	300	471	267	11.7	6.6
	40	471	251	10.4	5.5
	500	462	254	9.5	5.2
C					
	133	465	261	11.5	6.4
	266	469	261	10.8	6.0
	400	480	262	10.7	5.9
	533	480	262	9.4	5.2
	667	464	254	9.9	5.4
H					
	1390	481	263	9.8	5.4
	2780	478	261	9.1	5.0
	4170	477	269	8.9	5.1
	5560	466	264	9.4	5.3
	6950	477	257	9.8	5.3
SED		0.8***	0.5***	0.5***	0.3***

*** ($P < 0.001$)

the lowest. Also when compared with the control ES 1 and purified cellulase (C) treatments still gave significantly ($P < 0.001$) higher levels of WSC than the ES 2 and crude hemicellulase (H) treatments. Conversely, the opposite effect was seen with the lactic acid levels with ES 2 and H treatments giving significantly ($P < 0.001$) higher production of lactic acid than the ES 1, C and formic acid alone treatments. However, these differences were generally less apparent at the higher levels of addition of the enzymes. The concentrations of acetic acid were generally similar for all the silages although the treatment with ES 1 gave slightly higher ($P < 0.05$) levels than the other treatments.

The concentration of NDF in the silage treated with ES 1 was significantly ($P < 0.001$) lower than in any of the other treatments with slightly higher concentrations of NDF being observed in the other enzyme treatments than in the control. Similarly ADF concentrations were also significantly ($P < 0.001$) lower in the ES 1 treated silage than in the other treatments and again the concentrations for the other enzyme treatments were higher than in the formic acid only treated silage. The differences in NDF/lignin and ADF/lignin ratios were highly significant ($P < 0.001$) with those of the control, ES 1 and H treatments being lower than those of the ES 2 and C treatments. Only at the higher level of addition (500 mlt^{-1}) of ES 1 were there any apparent effects of additional treatment of the silage with an enzyme as compared to treatment with formic acid alone.

Overall, little appeared to be accomplished by the addition of purified cellulase or hemicellulase enzymes. Significant ($P < 0.001$) increases in WSC may have been obtained with purified cellulase treatment

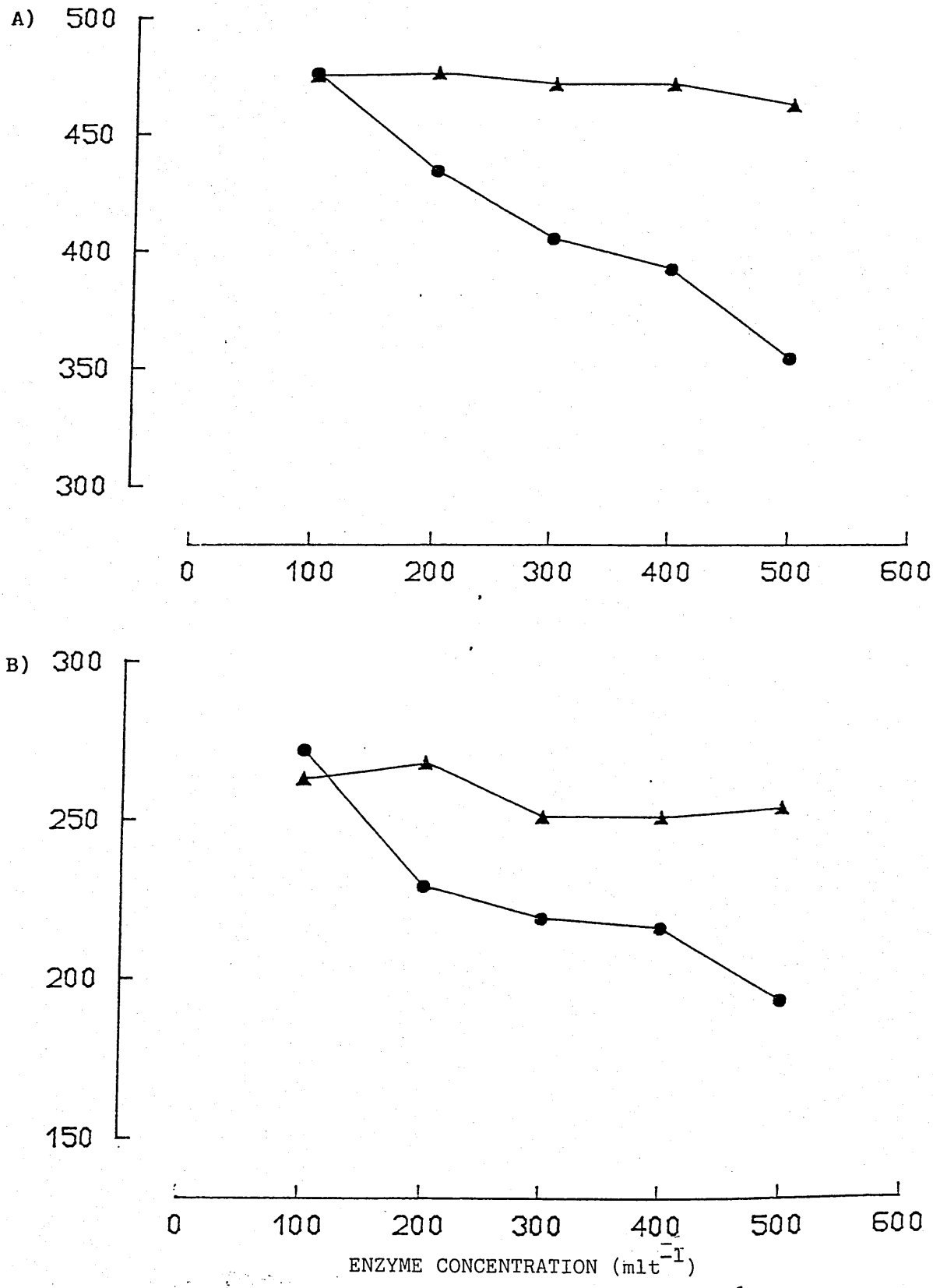


FIGURE 12: The changes in concentration (g kg DM^{-1}) in A) NDF and B) ADF with increasing levels of addition (mlt^{-1}) of ● ES1 and ▲ ES2 in the silages prepared in Experiment 2.

and significant increases ($P < 0.001$) in lactic acid production with hemicellulase addition but there was no apparent evidence of a reduction in the fibre content of the silages treated with these enzymes. Similarly, the ES 2 preparation had little significant effect on the chemical composition over that of formic acid treatment alone although a significant ($P < 0.001$) increase in lactic acid production may afford it some benefit in enabling a more efficient conversion of WSC to lactic acid to achieve a desired fermentation.

A comparison of the changes in the concentrations of NDF and and ADF contents of the silages as a result of increasing concentrations of ES 1 and ES 2 preparations is shown in Fig 12. Increase in the concentration of ES 1 gave accompanying reductions in NDF and ADF with an increase from 100 to 500 mlt^{-1} ES 1 resulting in a reduction of 122 $gkgDM^{-1}$ and 79 $gkgDM^{-1}$ in NDF and ADF respectively. The corresponding changes with ES 2 were very slight. From the effects of addition of ES 1 it would appear that the enzymes contained in the preparation are capable of degrading plant fibre with a release of fermentable substrates although an increased conversion of these substrates to lactic acid was not apparent.

Experiment 3. ES 1 Dose Response

Silos were prepared to study the effect of higher levels of addition of the ES 1 preparation. The levels used were 500, 1000, 1500 and 2000 mlt^{-1}. Control silos of formic acid only were also prepared. Each treatment was replicated three times with the total number of silos prepared being 15.

Results and Discussion

The chemical compositions of the silages treated with increasing levels of addition of ES 1 preparation or with formic acid (850 gkg^{-1}) alone are given in Table 7. The fermentation quality obtained in all the silages was very good with the silages having low pHs, low NH_3-N concentrations and an absence of detectable butyric acid. The highest contents in WSC were obtained with an addition of 500 mlt^{-1} ES 1 and with formic acid alone. The WSC contents obtained with the other levels of enzyme addition were lower and significantly ($P<0.05$) so at 2000 mlt^{-1}. The concentrations of lactic acid obtained in the silages were generally low and variable. However, silage treated with 1000 mlt^{-1} ES 1 contained a significantly ($P<0.001$) higher concentration of lactic acid than the other treatments. The acetic acid concentrations were similar for all silages.

The concentration of NDF in the silage treated with ES 1 was significantly ($P<0.001$) lower at all levels of enzyme addition than treatment with formic acid alone. An addition of 500 mlt^{-1} ES 1 gave a reduction in NDF of 90 $gkgDM^{-1}$ and this reduction had increased to 139 $gkgDM^{-1}$ with an addition of 2000 mlt^{-1} ES 1. Similarly, significant ($P<0.001$) reductions in the concentration of ADF of the silages treated with ES 1 at all levels of addition over treatment with formic acid alone were obtained. The extent of this reduction was a loss of ADF of 48 $gkgDM^{-1}$ at 500 mlt^{-1} ES 1 increasing to 71 $gkgDM^{-1}$ at 2000 mlt^{-1}. That treatment with the enzyme preparation may indeed have caused a breakdown of plant fibre is also suggested by the changes in NDF/lignin and ADF/lignin ratios. Significant ($P<0.001$) reductions in both

TABLE 7: Chemical composition (g $kgDM^{-1}$) of the silages after 100 days in the silo.

[Each value is a mean for 3 replicates.]

	CONTROL	ES1 (mlt^{-1})				SED
		500	1000	1500	2000	
DM (g kg^{-1})	188	192	184	182	188	
pH	4.5	4.4	4.2	4.5	4.4	0.8*
Total N	29	32	29	30	29	1.8
NH_3-N (g $kgTN^{-1}$)	51	55	57	60	58	0.5
WSC	172	174	161	150	140	10.3*
Lactic Acid	5	16	62	8	16	8.1***
Acetic Acid	20	20	25	23	26	2.5
Butyric Acid	0	0	0	0	0	
NDF	461	371	350	330	322	1.3***
ADF	251	203	190	173	180	0.8***
NDF/lignin	11.2	9.1	8.6	9.3	8.9	0.4***
ADF/lignin	6.1	5.0	4.7	4.9	4.9	0.3***

*** ($P < 0.001$)

ratios were obtained with enzyme addition than those obtained with treatment with formic acid alone.

A dose-related loss in NDF and ADF was observed for ES 1 (Fig 13). From additions of the preparation ES 1 to silage it would seem apparent that enzymes contained in the preparation are effective in degrading plant cell walls due to the significant ($P<0.001$) reductions obtained in the fibre components. However, any subsequent benefit from this increased degradation of fibre was not reflected in an increased concentration of fermentable substrates made available for fermentation or preserved in the silage.

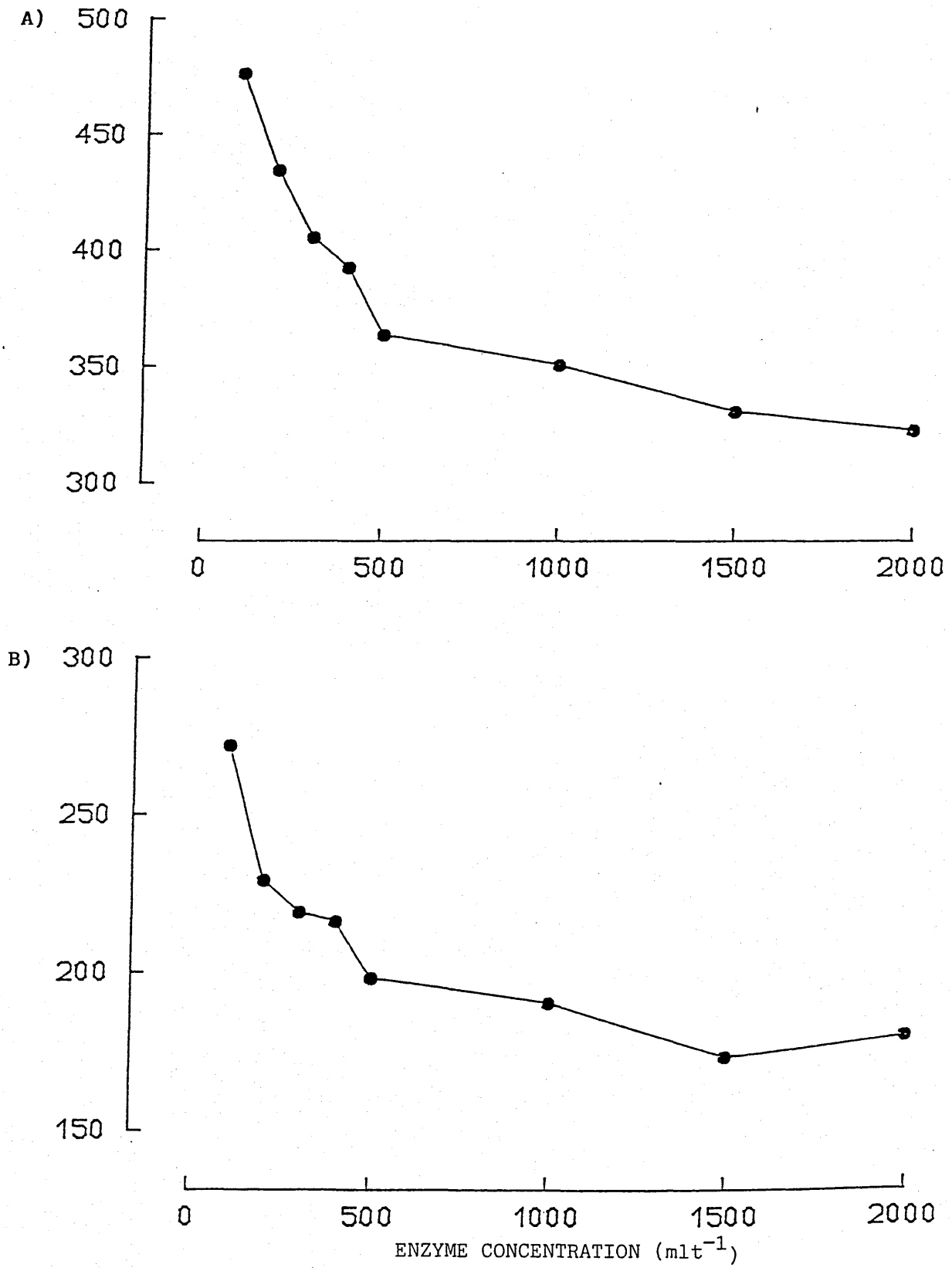


FIGURE 13: The losses in concentration (g kg DM^{-1}) in A) NDF and B) ADF with increasing levels of addition (mlt^{-1}) of ES1 in the silages prepared in Experiment 3.

EXPERIMENTAL SECTION 2 : Intake and Digestion Studies with One-tonne Silos

Eight 1-tonne silos were set up. Four of these silos were used to study the effect of increasing additions of ES 1 and ES 2 enzyme preparations used in a 50:50 combination. The silos were filled with precision chopped perennial ryegrass treated with formic acid at 3 lt^{-1} and the appropriate enzyme addition. For ease of application the volumes of the enzyme solutions were increased to 2 lt^{-1} by dilution with water. The four treatments were as follows:

1. 3 lt^{-1} Formic Acid (control)
2. 3 lt^{-1} Formic Acid + 50 mlt^{-1} ES 1 + 50 mlt^{-1} ES 2
3. 3 lt^{-1} Formic Acid + 150 mlt^{-1} ES 1 + 150 mlt^{-1} ES 2
4. 3 lt^{-1} Formic Acid + 250 mlt^{-1} ES 1 + 250 mlt^{-1} ES 2

The remaining four silos were filled with lucerne (Medicago sativa). Formic acid was used only on the control silage and the others were treated with various levels or combinations of the enzyme preparations ES 1, ES 2 and ES 3. ES 3 was a third enzyme preparation supplied by the industrial company. It was known to contain glucose oxidase but it was also thought likely to contain enzymes with cell-wall degrading abilities. The four treatments were as follows:

1. 3 lt^{-1} Formic Acid (control)
2. 125 mlt^{-1} ES 1
3. 125 mlt^{-1} ES 1 + 125 mlt^{-1} ES 2
4. 10 mlt^{-1} ES 3

Compositional Data: Results and Discussion

On opening the corresponding 1-tonne silos used in the intake/digestion studies the physical characteristics of the grass silages treated with cell-wall degrading enzymes, in particular, were visibly different from the control silage treated with formic acid alone. Although it was not possible to quantify the volume of effluent produced there was clearly an increasing rate in effluent production with the grass silages which was related to the increasing enzyme dose rate. With the lucerne silages there was no evidence of increases in effluent volume with the addition of cell-wall degrading enzymes.

The chemical compositions of the grass and lucerne silages are given in Tables 8 and 9 respectively. The fermentation quality of the grass silages was good (Table 8) with low pHs, low NH_3-N concentrations and low or negligible butyric acid levels. The content of water soluble carbohydrates was highest in the control silage of formic acid alone and substantially lower for the enzyme-treated silages, particularly at the 300 and 500 mlt^{-1} ES 1/ES 2 levels of addition. The concentration of lactic acid was higher in the enzyme-treated silages, particularly with the addition of 500 mlt^{-1} ES 1/ES 2. The acetic acid concentration was similar in all four silages.

There was a reduction in the NDF contents of the silages treated at the 100 and 300 mlt^{-1} rates of enzyme application over the control of 26 and 41 gNDF $kgDM^{-1}$ respectively. However, at the highest level of enzyme treatment the NDF content was higher than that of the control. Similarly, the ADF content of the enzyme-treated silages at all levels of application was higher than that of the formic acid control. It

TABLE 8 : Chemical composition (g kg DM^{-1}) of the grass silages.

	FORMIC ACID $3lt^{-1}$	ES1/ES2 (mlt^{-1})		
		100	300	500
DM g kg^{-1}	269	256	247	231
pH	3.9	3.8	3.8	3.8
Total N	16	17	16	18
NH_3-N (g kg TN^{-1})	80	80	100	120
NPN (g Kg TN^{-1})	630	620	580	700
WSC	81	60	26	39
Lactic Acid	70	82	73	85
Acetic Acid	37	37	37	39
Butyric Acid	2	0	0	3
Ethanol	23	23	21	7
NDF	498	472	457	562
ADF	269	268	271	316
(NDF-ADF)	229	204	186	246

therefore appears that there was a substantial loss of soluble materials from the silage in the increased volumes of effluent production.

All four lucerne silages were well preserved (Table 9). The pHs, although higher than those of the grass silages, were typical of well-preserved legume silages. The NH_3-N concentrations were also low and butyric acid was not detected. The content of water soluble carbohydrates was very low in all four silages, but again this was not unexpected for ensiled legume crops. The lactic acid concentrations of the silages treated with ES 1 (125 mlt^{-1}) and a 50:50 mixture of ES 1 and ES 2 (125 mlt^{-1}) were considerably higher than in the treatment with formic acid alone. Treatment with ES 3 (10 mlt^{-1}) resulted in a slightly lower lactic acid concentration than that of the control. The acetic acid concentration was slightly higher in the enzyme-treated silages than with the formic acid treatment.

The pattern of change with enzyme treatment in the NDF and ADF contents of the lucerne silages was different from that of the grass silages. There was a reduction in the NDF content of the lucerne silages at all levels of enzyme treatment over the formic acid control treatment. The addition of 10 mlt^{-1} ES 3 gave the greatest reduction in NDF of 67 $gkgDM^{-1}$ over the control whilst the losses with the addition of 125 mlt^{-1} ES 1 and 250 mlt^{-1} ES 1/ES 2 were 30 gNDF $kgDM^{-1}$ and 55 gNDF $kgDM^{-1}$ respectively. Similarly, the reduction in ADF was greatest with the addition of ES 3 resulting in a loss of 42 gADF $kgDM^{-1}$ over the control with the addition of ES 1 and ES 1/ES 2 giving losses in ADF of 14 $gkgDM^{-1}$ and 34 $gkgDM^{-1}$ respectively.

It should be remembered that with the lucerne silages formic acid (850 gkg^{-1}) was only added to the control silage and therefore a good

TABLE 9 : Chemical composition (g kg DM^{-1}) of the lucerne silages.

	FORMIC ACID $3lt^{-1}$	ENZYME SYSTEM		
		ES1 $125mlt^{-1}$	ES1 $125mlt^{-1}$ + ES2 $125mlt^{-1}$	ES3 $10mlt^{-1}$
DM g kg	253	234	239	271
pH	4.8	4.6	4.5	4.9
Total N	24	25	25	28
NH_3-N (g kg TN^{-1})	120	160	140	180
NPN (g kg TN^{-1})	560	650	630	660
WSC	4	2	3	2
Lactic acid	22	39	37	16
Acetic Acid	41	52	51	50
Butyric Acid	0	0	0	0
NDF	529	499	474	462
ADF	444	430	410	402
(NDF-ADF)	85	69	64	60

fermentation was obtained with addition of the enzyme preparations by themselves. Also from the results for the concentrations of fibre components and, to some extent, lactic acid there was a much clearer indication of an increased degradation of plant cell walls and release of fermentable substrates in response to the addition of the enzymes to the lucerne silages in comparison with the grass silages.

Experiment 1. The rate of ruminal digestion of grass and lucerne ensiled with the addition of cell-wall degrading enzymes as determined by the disappearance of dry matter and forage fibre from Dacron bags incubated in the rumen.

Two cows fitted with rumen cannulas were given two equal meals daily of a diet consisting of 3 kg dried grass, 3 kg hay and 2 kg barley. Samples of grass and lucerne silage (8g bag^{-1}) were incubated in Dacron bags (Mehrez and Orskov, 1977) in the rumen. Measurements of the rate of loss of fibre constituents and dry matter were made over 48 h periods, with bags being withdrawn after 2, 7, 16, 24 and 48 h. Each series of incubations was repeated in each animal. The residues from the dacron bags were analysed for dry matter, organic matter, NDF and ADF.

Results and Discussion

The rates of disappearance of DM, NDF and ADF with time from Dacron bags of the selected grass and lucerne silages are shown in Figs 14-19. The data for these plots and the best fit curves are contained in Appendix Tables 1-6 and Appendix Figs 1-6 respectively. For the grass silages the greatest rates of DM disappearance were obtained for the silage treated with formic acid ($3lt^{-1}$) alone with statistically significant differences in DM digestion rates being obtained after most incubation periods, particularly after 7 h ($P<0.001$), 16 h ($P<0.01$) and even after 48 h ($P<0.01$). DM disappearances for the grass silages treated with formic acid and increasing levels of addition of ES 1 and ES 2 enzyme preparations, in combination, were least at the lowest level of enzyme addition and increased as the concentration of the enzymes was stepped up. However, the differences in DM digestion between formic acid applied alone and with enzyme combinations were apparently less as the period of incubation was increased, although only at the 24 h period was the difference not statistically significant ($P>0.05$).

Similarly with the disappearances of NDF the greatest rates of digestion were obtained for the silage treated with formic acid ($3lt^{-1}$) only. The differences between the treatment of formic acid alone and those of the enzyme additions were significant (at least $P<0.01$) at all incubation periods up to 24 h ($P>0.05$) and again at 48 h ($P<0.01$). These differences in NDF disappearance were particularly apparent between formic acid alone and the lower levels ($100\ mlt^{-1}$ and $300\ mlt^{-1}$) of enzyme addition.

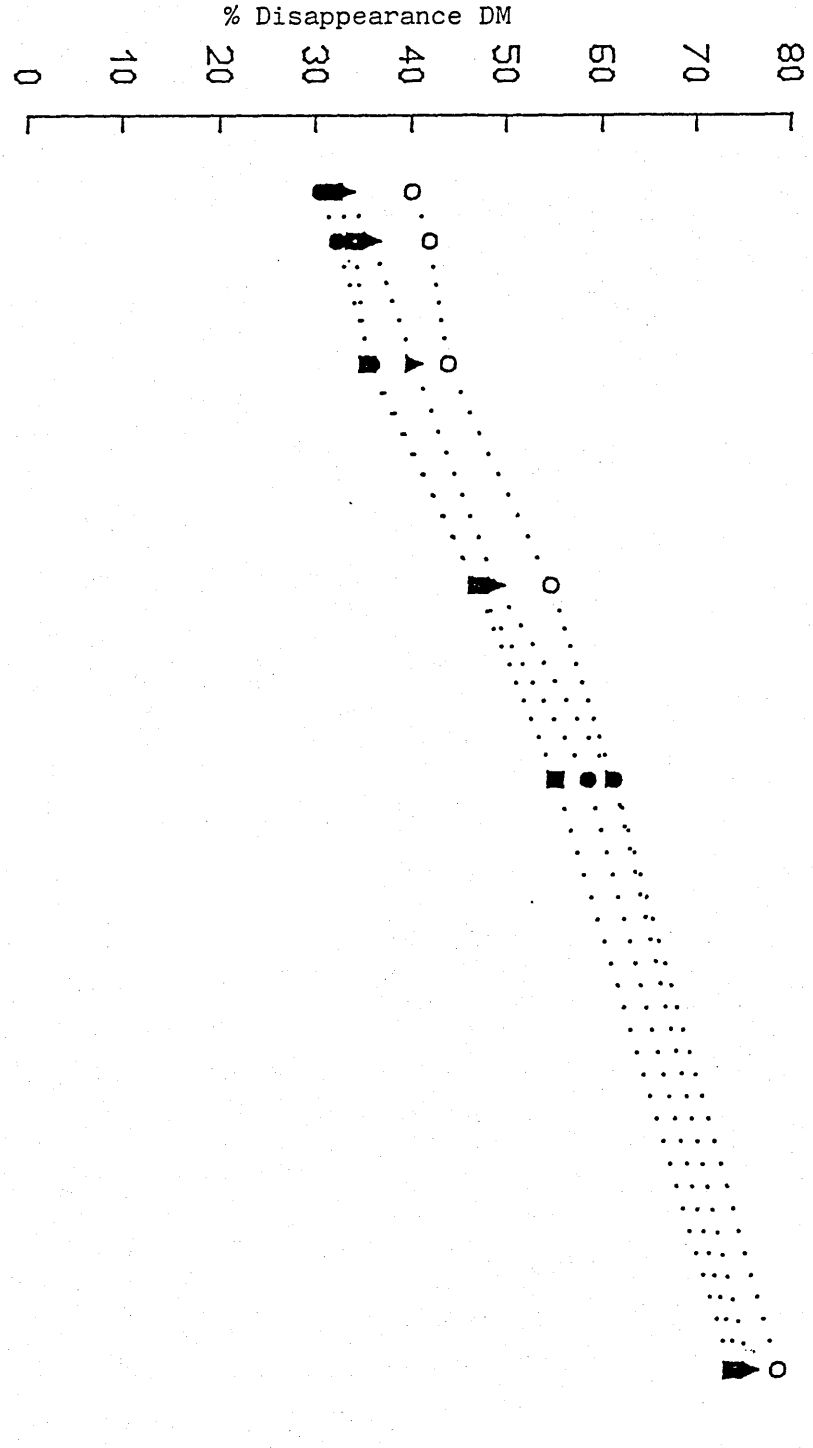


FIGURE 14: The disappearance (%) of dry matter with time for grass silages incubated in dacron bags. Silages were prepared as follows:
o Formic Acid ($3lt^{-1}$); ● Formic Acid + 100 mlt^{-1} ES1/ES2;
■ Formic Acid + 300 mlt^{-1} ES1/ES2; ▲ Formic Acid + 500 mlt^{-1} ES1/ES2.

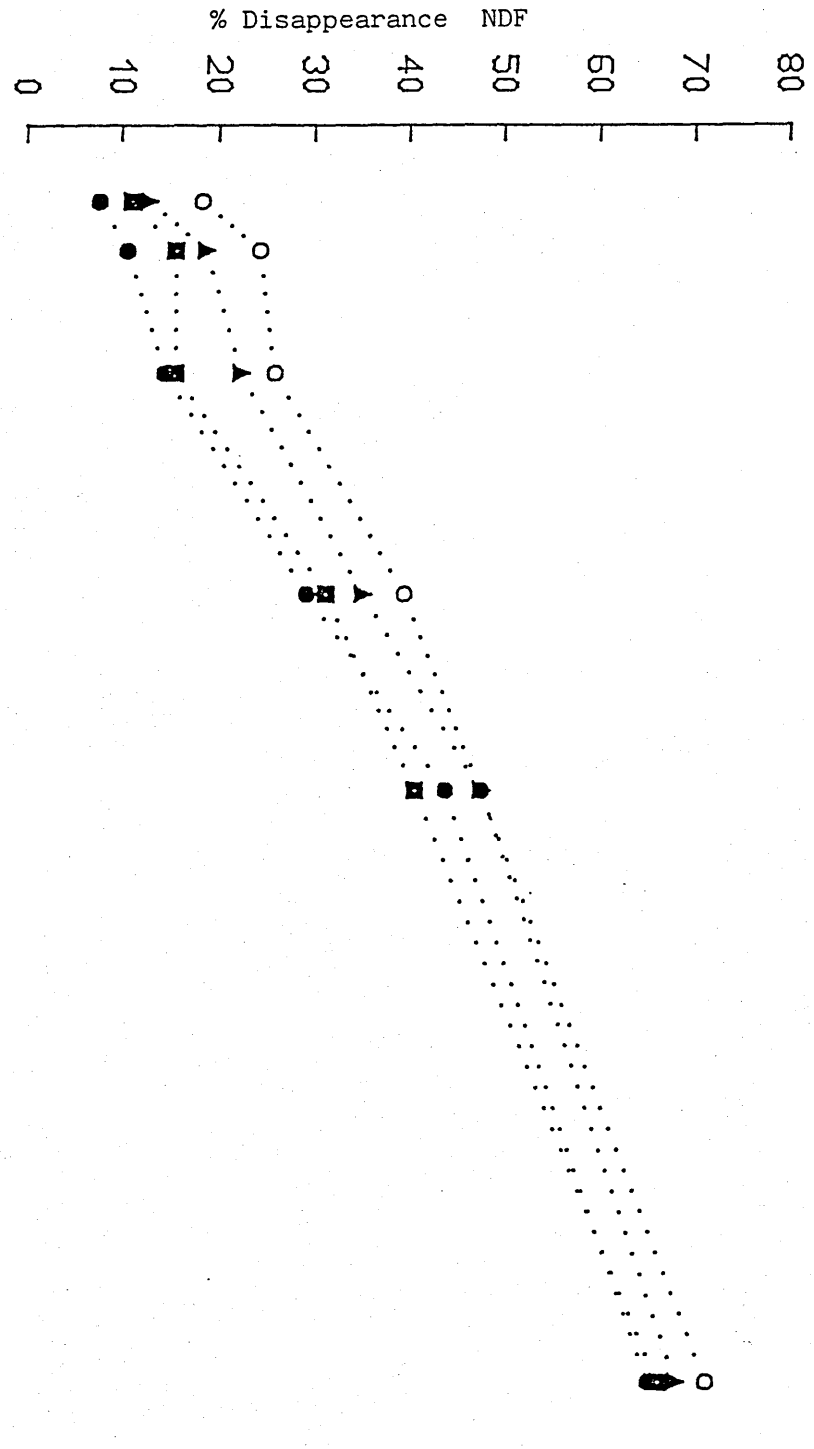


FIGURE 15: The disappearance (%) of NDF with time for grass silages incubated in dacron bags. Silages were prepared as follows: ○ Formic Acid ($3l\,t^{-1}$) ● Formic Acid + 100 $ml\,t^{-1}$ ES1/ES2; ■ Formic Acid + 300 $ml\,t^{-1}$ ES1/ES2; ▲ Formic Acid + 500 $ml\,t^{-1}$ ES1/ES2.

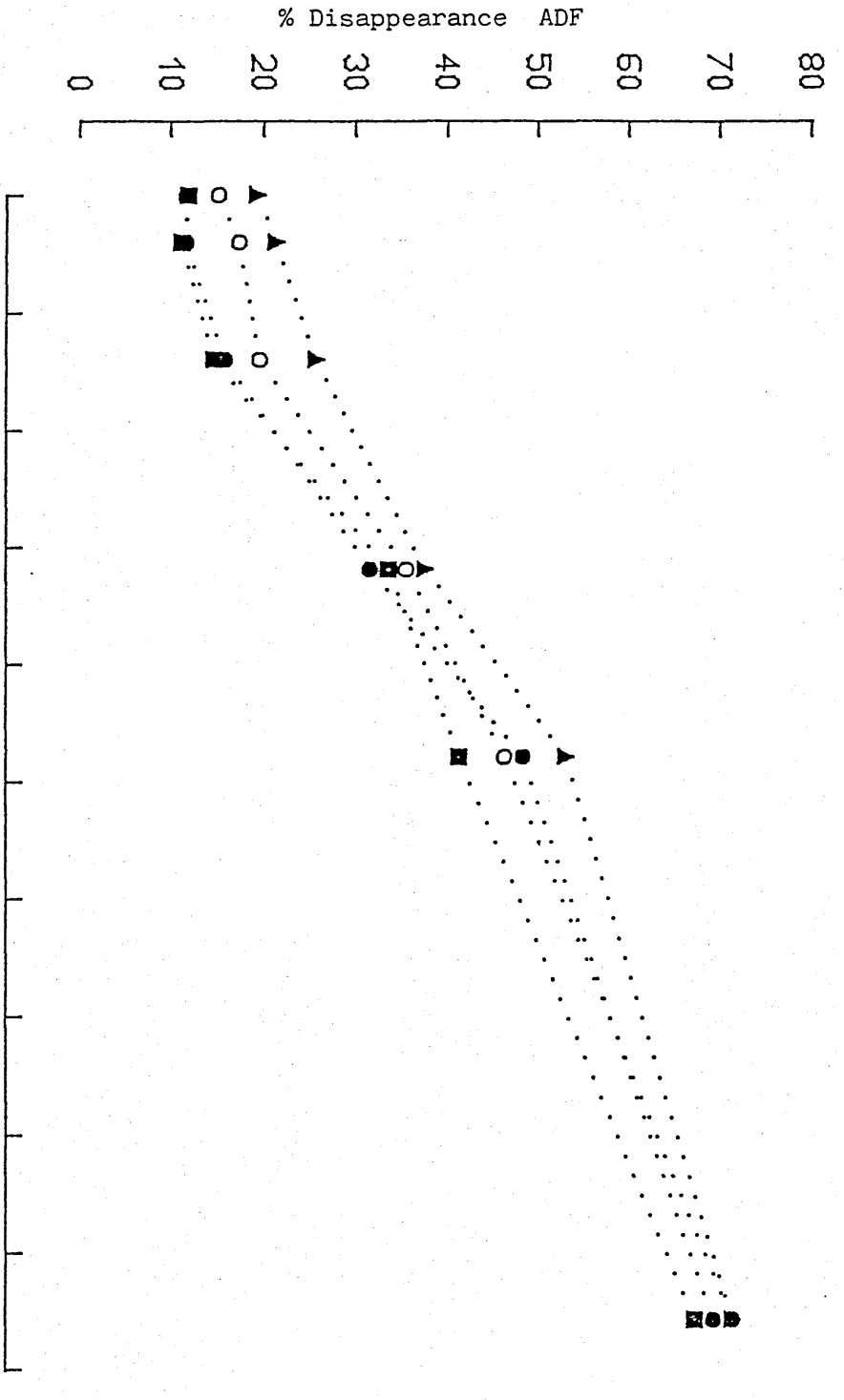


FIGURE 16: The disappearance (%) of ADF with time for grass silages incubated in dacron bags. Silages were prepared as follows: ○ Formic Acid ($3lt^{-1}$); ● Formic Acid $100\ ml\ l^{-1}$ ES1/ES2; ■ Formic Acid + $300\ ml\ t^{-1}$ ES1/ES2; ▲ Formic Acid + $500\ ml\ t^{-1}$ ES1/ES2.

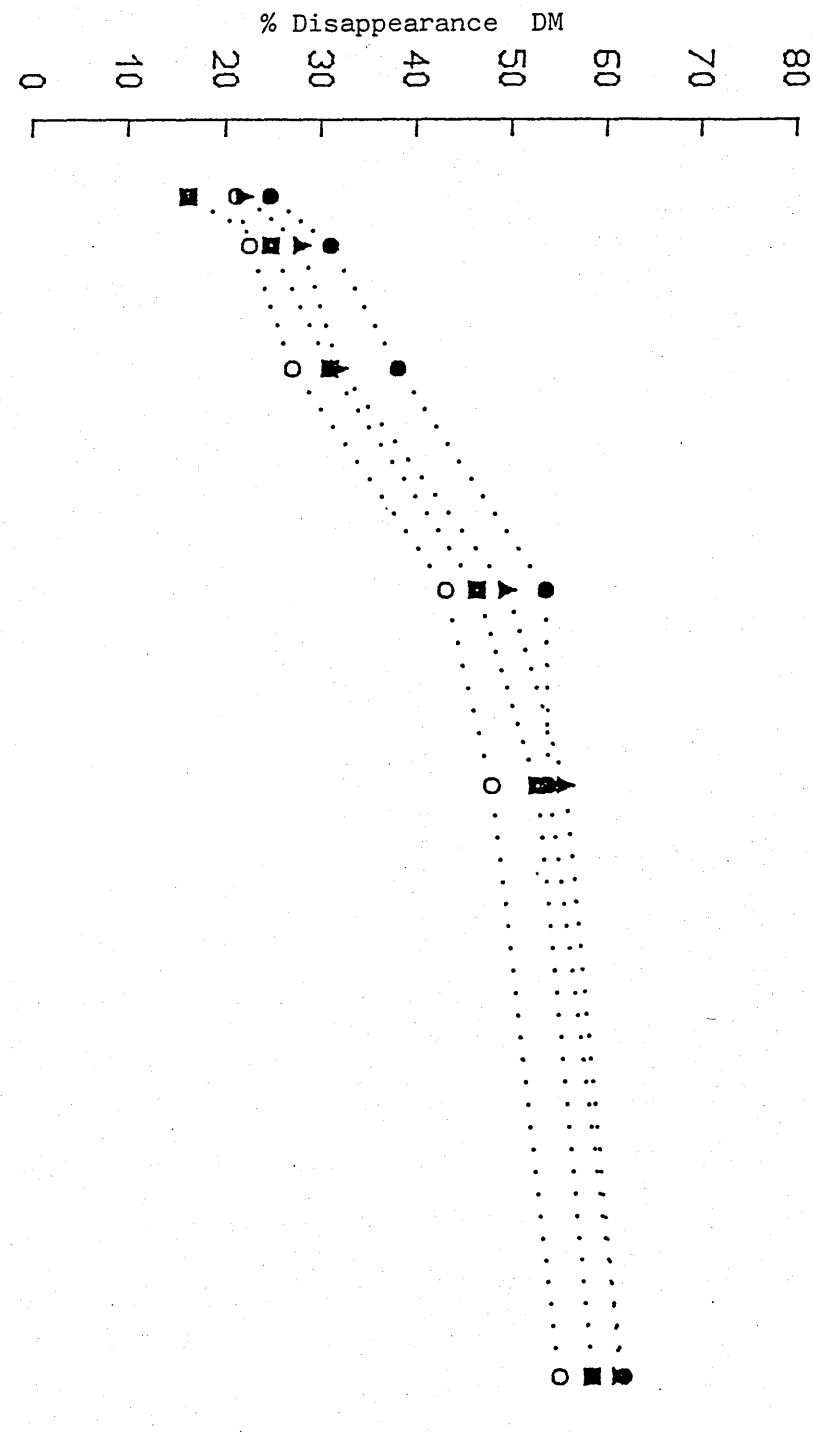


FIGURE 17: The disappearance (%) of dry matter with time for lucerne silages incubated in dacron bags. Silages were prepared as follows: o Formic Acid (3lt^{-1}); ● ES1 (125 mlt^{-1}); ■ ES1/ES2 (250 mlt^{-1}); ▲ ES3 (10 mlt^{-1}).

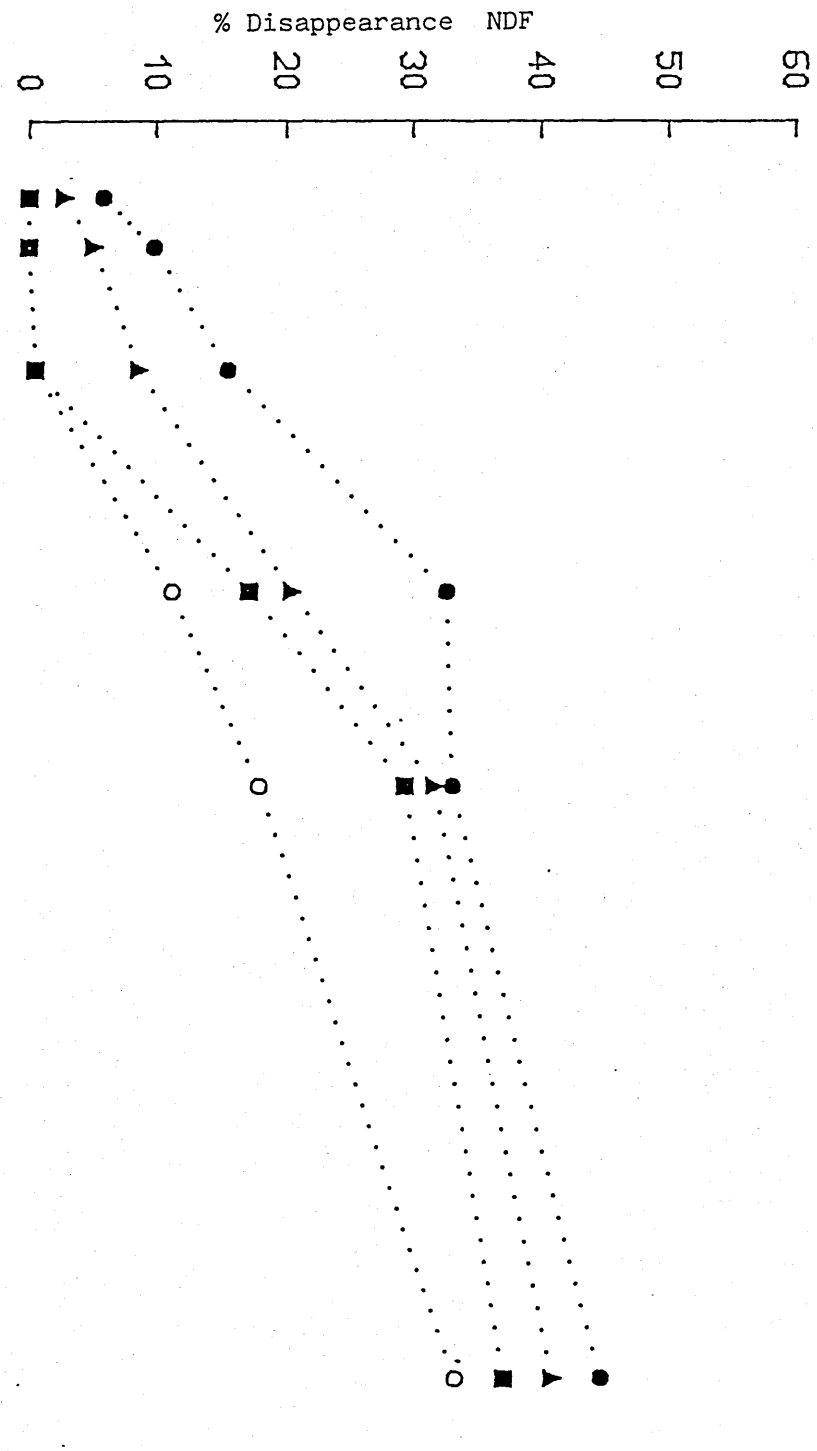


FIGURE 18: The disappearance (%) of NDF with time for lucerne silages incubated in dacron bags. Silages were prepared as follows: ○ Formic Acid (31 t^{-1}); ● ES1 (125 mlt^{-1}); ■ ES1/ES2 (250 mlt^{-1}); ▲ ES3 (10 mlt^{-1}).

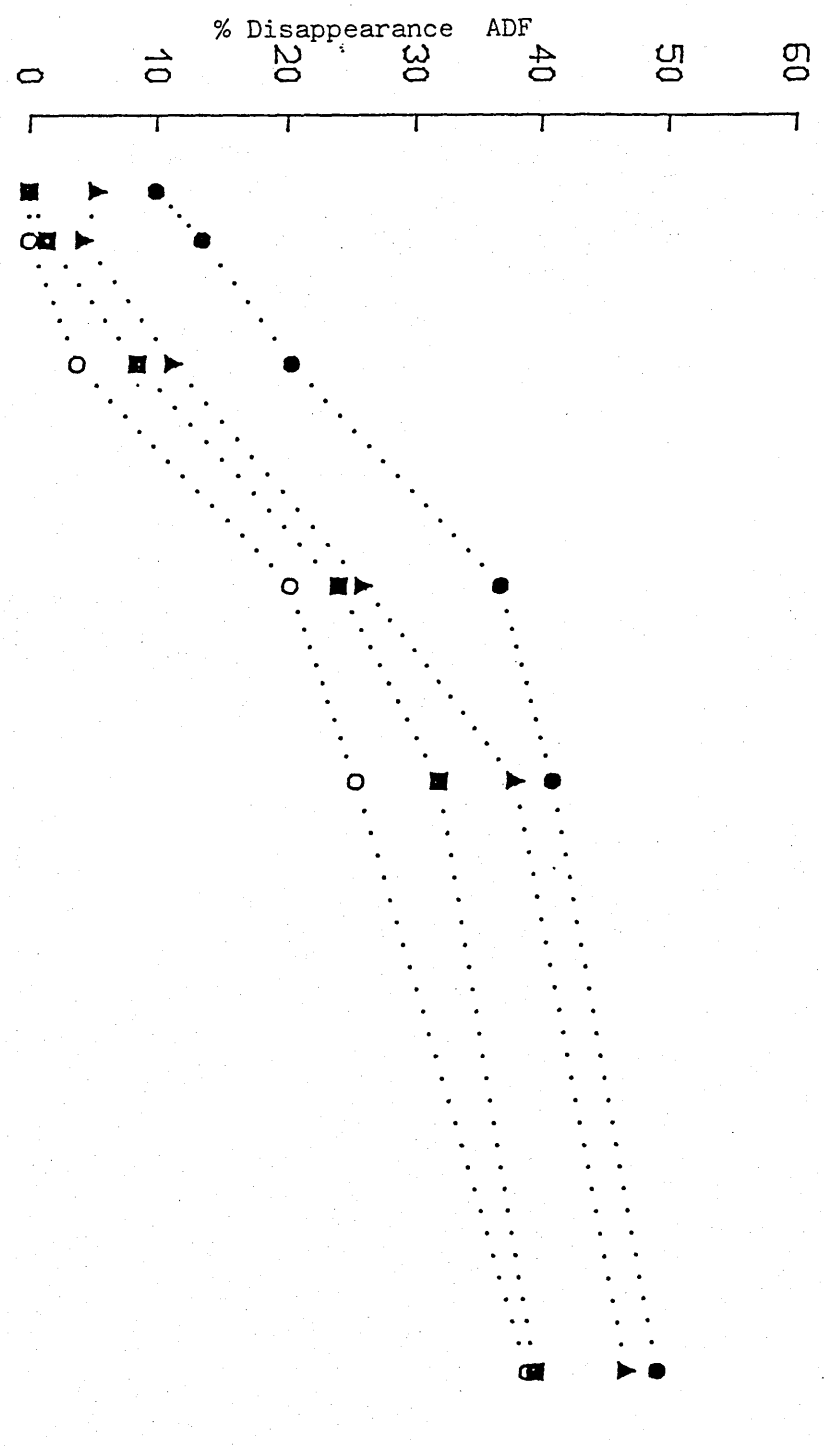


FIGURE 19: The disappearance (%) of ADF with time for lucerne silages incubated in dacron bags. Silages were prepared as follows: O Formic Acid; ● ES1 (125ml t^{-1}); ■ ES1/ES2 (250 ml t^{-1}); ▲ ES3 (100 ml t^{-1}).

The rates of disappearance in ADF for the grass silages followed a different pattern as the greatest rate of disappearance was obtained for the silage treated with formic acid along with 500 mlt^{-1} ES 1/ES 2. The differences between that treatment and that of formic acid alone were significant at all periods of incubation particularly after 2 h and 7 h ($P < 0.001$) and after 24 h ($P < 0.01$). However, after 48 h the differences were not significantly different ($P > 0.05$). The rates of ADF disappearance were lower still for the 100 mlt^{-1} and 300 mlt^{-1} levels of enzyme addition.

The pattern of digestion of DM and forage fibre from Dacron bags for the lucerne silages was very different from that of the grass silages. Only the control silage was treated with formic acid (3 lt^{-1}) and it had the lowest rate of disappearance of DM, NDF and ADF. The lucerne silage treated with an addition of 125 mlt^{-1} ES 1 had the greatest loss in DM at all periods of incubation, except at 24 h, and these differences were highly significant ($P < 0.001$), ($P < 0.01$) at 24 h. The disappearances of DM for the silage treated with 10 mlt^{-1} ES 3 were generally lower than those of the ES 1 treatment, except at 24 h, and the losses for the treatment with ES 1/ES 2 were slightly lower still than the other enzyme additions.

The rates of disappearance of NDF followed a similar pattern with the greatest losses being obtained for the ES 1 (125 mlt^{-1}) treated lucerne. There were quite substantial differences between the ES 1 and the other treatments up until the 24 h incubation period at which the differences between the enzyme-treated silages began to even out, although the losses in NDF for the formic acid treatment remained

considerably lower even after 48 h. The losses in NDF for the ES 1/ES 2 treated silage were very similar to those of the formic acid treatment up until the 7 h incubation after which the rate of loss increased substantially. The differences in NDF digestion were highly significant ($P < 0.001$) at all incubation periods.

The lucerne silage treated with 125 $ml\,t^{-1}$ ES 1 also had the greatest losses in ADF at all periods of incubation with a similar pattern to that of NDF being followed by the other treatments. After the 16 h incubation period the rates of disappearance in ADF for the enzyme-treated silages were more closely related with the losses obtained for the ES 3 treatment being quite similar to those of the ES 1 treatment. The rates of disappearance for the formic acid treated lucerne remained considerably lower than those of the enzyme treatments. The differences in the rate of digestion of ADF were highly significant ($P < 0.001$) at all incubation periods except after 24 h ($P < 0.01$) .

The pattern of ruminal digestion for the grass ensiled with the addition of cell-wall degrading enzymes differed markedly from that of lucerne. For the grass silages the greatest losses in DM, NDF and ADF were obtained for those treated with formic acid (3 $l\,t^{-1}$) alone or with formic acid and the highest level of addition (500 $ml\,t^{-1}$) of the ES 1 and ES 2 enzyme preparations, in combination. However, with lucerne there were substantially greater losses in DM and forage fibre obtained with additions of cell-wall degrading enzyme preparations over formic acid controls, with the enzyme preparations being applied alone and at levels of addition which were not particularly high, especially for ES 3.

Experiment 2. The intake and digestibility of grass and lucerne ensiled with the addition of cell-wall degrading enzymes.

The intake and digestibility of the grass and lucerne silages was measured in sheep in two 4 x 4 Latin Square experiments. The dietary treatments were the silages treated with formic acid and/or enzyme additives as follows:

Grass:
1. 3 lt^{-1} Formic Acid (control)
2. 3 lt^{-1} Formic Acid + 50 mlt^{-1} ES 1 + 50 mlt^{-1} ES 2
3. 3 lt^{-1} Formic Acid + 150 mlt^{-1} ES 1 + 150 mlt^{-1} ES 2
4. 3 lt^{-1} Formic Acid + 250 mlt^{-1} ES 1 + 250 mlt^{-1} ES 2

Lucerne:
1. 3 lt^{-1} Formic Acid (control)
2. 125 mlt^{-1} ES 1
3. 125 mlt^{-1} ES 1 + 125 mlt^{-1} ES 2
4. 10 mlt^{-1} ES 3

Each period was 21 days with a faecal collection over the final 7 days of each period. The intake was measured throughout. The silage was offered ad-libitum with the amount offered exceeding intake by at least 20%.

Results and Discussion

From the results of the Latin Square experiments with four sheep (Table 10) the digestibilities of the enzyme-treated grass silages were similar to those of the formic acid control with any differences between the silages being statistically non-significant ($P > 0.05$).

TABLE 10: The ad libitum intake and digestibility by sheep of grass silages. [Each value is a mean for 4 sheep.]

	FORMIC ACID	ES1/ES2 ENZYME SYSTEM (ml/t)			SED
		100	300	500	
DM Intake (g/d)	1316	1123	1177	1031	36.0**
OM Intake (g/d)	1229	1052	1117	971	34.0**
Digestibility of Dry Matter	0.673	0.661	0.698	0.649	0.02
Digestibility of Organic Matter	0.720	0.705	0.727	0.689	0.02
Digestibility of NDF	0.658	0.620	0.635	0.658	0.03
Digestibility of ADF	0.643	0.623	0.633	0.638	0.02

** ($P < 0.01$)

The digestibility of the dry matter of the silage treated with 300 mlt^{-1} ES 1/ES 2 was slightly greater than that of the formic acid control whilst that of the other two enzyme additions was lower and similarly with regard to the digestibility of the organic matter of the silages. All three enzyme-treated silages had slightly lower digestibilities of NDF and ADF than that treated with formic acid alone.

There were, however, significant differences ($P<0.01$) in the ad-libitum intakes of the silages. Both the dry matter and organic matter intakes of the enzyme-treated silages were significantly lower ($P<0.01$) than those of the silage treated with formic acid only. The intake of DM and OM was lowest in each case for the silage treated with the addition of 500 mlt^{-1} ES 1/ES 2. The intakes of digestible OM were 16%, 8% and 24% lower for the silage treated with 100, 300 and 500 mlt^{-1} ES 1/ES 2 respectively than the intake of digestible OM for the silage treated with formic acid alone.

From the results of the digestibilities and ad-libitum intakes (Table 11) it can be seen that the effects obtained with the addition of the enzymes to the lucerne silages were again different from those obtained with the grass silages. The digestibility of dry matter was slightly higher for the silages treated with the addition of ES 3 (10 mlt^{-1}) and ES 1/ES 2 (250 mlt^{-1}) than that of the formic acid control treatment, whilst the DM digestibility of the silage treated with ES 1 (125 mlt^{-1}) was slightly lower. This pattern was similar when considering the digestibility of organic matter of the silages and any differences were not statistically significant ($P>0.05$).

The digestibility of NDF for the control silage was slightly higher than that of any of the enzyme-treated silages, whilst the

TABLE 11 : The ad libitum intake and digestibility by sheep of lucerne silages. [Each value is a mean for 4 sheep.]

	FORMIC ACID $3lt^{-1}$	ENZYME SYSTEM			SED
		ES1 $125mlt^{-1}$	ES1 $125mlt^{-1}$ + ES2 $125mlt^{-1}$	ES3 $10mlt^{-1}$	
DM Intake (g/d)	1233	1213	1411	1263	79.0
OM Intake (g/d)	1109	1069	1230	1115	76.0
Digestibility of Dry Matter	0.490	0.460	0.510	0.520	0.025
Digestibility of Organic Matter	0.545	0.522	0.585	0.589	0.028
Digestibility of NDF	0.407	0.366	0.401	0.391	0.030
Digestibility of ADF	0.453	0.415	0.490	0.475	0.029
Digestibility of Nitrogen	0.711	0.678	0.714	0.773	0.022

digestibility of ADF was slightly greater for the silages treated with ES 3 and ES 1/ES 2 than for the control, with that of the ES 1 treatment being slightly lower. However, again these differences were not statistically significant ($P > 0.05$).

There was an increase in the apparent digestibility of nitrogen of the lucerne silage treated with ES 3 over the formic acid control treatment. There was also a slight increase in the digestibility of nitrogen of the ES 1/ES 2 treatment whilst that of the ES 1 treated silage was lower than for the control silage. These differences in nitrogen digestibility were not statistically significant ($P > 0.05$).

The intakes of dry matter and organic matter for the lucerne silages were similar. The intakes of the silage treated with ES 1/ES 2 were highest with the DM and OM intakes of the silage treated with ES 3 being slightly higher than those of the formic acid treatment, whilst the intakes of the silage treated with ES 1 were slightly lower than those of the control. Although none of these differences were statistically significant ($P > 0.05$), when calculated the intake of digestible OM for the silage treated with ES 1/ES 2 was 19% higher than that of the formic acid control. There was a 9% increase in the intake of digestible OM for the silage treated with ES 3, whilst that of the ES 1 treatment was 8% less than the control.

EXPERIMENTAL SECTION 3: Farm Scale Trial with Dairy Cows

Experiment 1. The evaluation of silages made without additive, with formic acid and with the addition of cell-wall degrading enzymes.

A further experiment was designed to investigate the feeding value of silage made in farm-scale bunker silos (50t). All three silages were made from a corresponding late cut of ryegrass but were ensiled

(a) without additive

(b) with formic acid additive (2.6 lt^{-1})

(c) with ES 1/ES 2 additive (400 mlt^{-1})

The feeding value of the silages was estimated using 12 lactating cows in an experiment of a cyclical design with 2 blocks of 6 animals and 4 4-week periods per block.

There were 6 dietary treatments consisting of each of the silages offered ad-libitum with concentrate supplements given at flat rates of either 6 kg fresh weight d^{-1} or 9 kg fresh weight d^{-1}. The composition of the concentrates was as follows:

	DM(%)
Barley	85
Soyabean meal	10
Fishmeal	5
Minerals	1.5

	DIET COMPOSITION (g Kg DM^{-1})		
	Ash	CP	ADF
Barley	24	119	62
Soya	63	513	98
Fishmeal	196	728	34
(Mix	37	189	64)

Milk yield and food intake were recorded daily and body weights were measured on two days at the beginning and end of each experimental period. Samples of silage for oven DM were taken at daily weighings

with period bulk samples of silages and concentrate feeds being taken for analysis in the last week of each period. Milk samples were taken for analysis at am and pm milkings on two consecutive days in the last week of each period and used to give a weighted bulk sample for analysis.

The digestibility of each of the silages was determined using three sheep given each of the silages at a maintenance level of feeding for a 4-week period. Each period consisted of 14 days introduction followed by a 7-day period of faecal collection.

Results and Discussion

The chemical compositions and digestibilities of the bunker silages prepared without additive or with the addition of formic acid or cell-wall degrading enzymes are given in Table 12. The total DM intakes for the concentrates and the silages at the two levels of concentrate feeding are given in Table 13. Table 14 contains the total milk yield and % composition of the milk constituents produced on the diet of ad-libitum silage with the two levels of concentrate supplements.

The silages were well preserved (Table 12) with low pHs, low NH_3-N concentrations and negligible butyric acid levels. Since the silages were made from late cut grass the buffering capacity of the grass would be low which would allow for the high lactic acid levels obtained. These were reflected in the pHs which were lower than would normally be expected. There was a reduction in the content of NDF of the enzyme-treated silage in comparison to the untreated and formic

TABLE 12: Chemical composition (g kg DM^{-1}) of the grass bunker silages.

	UNTREATED	FORMIC ACID $2.6lt^{-1}$	ENZYME $400mlt^{-1}$
DM g kg^{-1}	226	216	238
pH	3.6	3.6	3.5
Total N	21	23	22
NH_3 -N (g kgTN^{-1})	140	110	120
NPN (g kgTN^{-1})	790	790	800
WSC	21	25	37
Lactic Acid	161	127	154
Acetic Acid	39	27	31
Butyric Acid	0	0	0
NDF	487	525	475
ADF	308	316	287
(NDF - ADF)	179	209	188
In vivo DOMD*	0.65 $\pm$ 0.01 (n = 3)	0.67 $\pm$ 0.01 (n = 3)	0.64 $\pm$ 0.01 (n = 3)

* As measured in sheep

acid treated silages of 12 and 50 gNDF $kgDM^{-1}$ respectively. Similarly the loss in ADF content was greatest for the enzyme-treated silage although the difference between the untreated and formic acid treated silage was not as great. The loss in ADF for the enzyme-treated silage of 21 and 29 gADF $kgDM^{-1}$ over the untreated and formic acid treated silage respectively was a greater proportion of the total loss in fibre constituents than that of NDF.

From the results for the concentrations of fibre components and, to some extent, WSC there are further indications of an increased degradation of plant cell walls and a release of fermentable substrates in response to the addition of the enzymes.

There was little difference in the digestibility (DOMD) of the silages prepared without additive or with the addition of formic acid or cell-wall degrading enzymes (Table 12). The DOMD value, as determined from in vivo studies using sheep, of the formic acid treated silage was slightly higher than that of the other two treatments. Although the grass was of low D-value being late cut there was no apparent improvement in digestibility with treatment with cell-wall degrading enzymes.

Increasing the level of intake of a concentrate supplement will normally be accompanied by a decrease in the intake of silage DM. The intake of silage DM was slightly greater for the untreated silage (Table 13) than for the other two silages at the 6 kgd^{-1} level of concentrate feeding. In increasing the level of concentrate supplement to 9 kgd^{-1} the greatest reduction in silage DM intake of 0.8 kgd^{-1} was

TABLE 13 Total DM intakes and the DM intakes for the concentrates and the grass bunker silages at the two levels of concentrate feeding.

	UNTREATED		FORMIC ACID $2.6lt^{-1}$		ENZYME $400ml\,t^{-1}$		SED
	6kg concs.	9kg concs.	6kg concs.	9kg concs.	6kg concs.	9kg concs.	
Concentrate DM1	4.82	7.19	4.73	7.09	4.70	6.88	
Silage DM1	9.28	8.49	9.05	8.74	9.21	9.29	0.33
Total DM1	14.10	15.68	13.78	15.83	13.91	16.17	0.39***

***($P < 0.001$)

TABLE 14: Milk yield and yield and % composition of milk constituents from feeding bunker grass silages with two levels of concentrate supplement.

	UNTREATED		FORMIC ACID $2.6lt^{-1}$		ENZYME $400mlt^{-1}$		SED
	6kg concs.	9kg concs.	6kg concs.	9kg concs.	6kg concs.	9kg concs.	
Milk Yield (kgd^{-1})	19.73	21.33	19.53	20.78	19.65	21.46	0.59**
Fat %	3.63	3.80	3.87	3.93	3.61	3.80	0.12
Fat Yield (gd^{-1})	713	798	758	811	710	805	23.19***
Crude Protein %	3.07	3.25	3.06	3.32	3.07	3.33	0.05***
Crude Protein Yield (gd^{-1})	603	692	592	685	600	705	18.67***
Lactose %	4.74	4.77	4.69	4.70	4.73	4.69	0.03
Lactose Yield (gd^{-1})	933	1017	917	978	932	1006	30.12*
Body Weight (kg)	514	531	527	537	523	539	4.81***

*** ($P < 0.001$)

in the intake of the untreated silage with a lower reduction (0.3 kgd^{-1}) in the intake of the formic acid treated silage. However, there was no corresponding decrease in silage DM intake obtained with the enzyme-treated silage from increasing the level of concentrate feeding and, in fact, a very slight increase in silage DM intake was recorded.

The total DM intakes were significantly greater ($P<0.001$) at the 9 kg level of concentrate supplement than at the 6 kg level. The greatest increase in total DM intake of 2.26 kgd^{-1} was obtained with the enzyme-treated silage whilst the lowest increase of 1.58kgd^{-1} was obtained with the untreated silage.

Generally the increases obtained for the silages in milk yield, % composition and yield of the milk constituents (Table 14) were significantly, at least ($P<0.05$) except for fat % and lactose % ($P>0.05$) when the level of concentrate supplement was increased from 6 kgd^{-1} to 9 kgd^{-1}. The differences observed between the silages showed little effect of enzyme treatment on milk yield and composition. On all the silage treatments there was a disappointing response to the higher level of concentrate intake and subsequent energy intake in terms of milk yield. However, the cows did show a significant increase ($P<0.001$) in body weight at the higher concentrate level but they were not of a physiological state to translate this increase into milk yield.

SECTION IV GENERAL DISCUSSION

Introduction

The aim of the work was to examine the use of cell-wall degrading enzymes as silage additives. The main concern was to investigate their effects on the nutritional value of the silage, it being recognised that such improvements might become apparent via increases in digestibility and/or voluntary intake. It was also important to obtain information on the effects of the enzyme additions on the silage fermentation. There was a need to establish that the enzymes had no adverse effects on fermentation and there was a need to establish whether the enzymes had any potentially beneficial effects on fermentation via increases in the supply of fermentable substrates.

Some potential obstacles to the use of the enzyme preparations were evident from the start. There were uncertainties over the activiity of the enzymes in conditions existing in the silo. The optimum conditions for cellulases are 40°C and a pH of 4.5-5.0 (Toyama, 1968; Bemiller *et al*, 1968) whilst the corresponding optimum for hemicellulases is 37°C and pH 6.0 (Dewar *et al*, 1968). Temperatures in well-preserved silages would be lower that these optimum values and the typical pH range of 3.7-4.5 is also well below optimum. However, against these disadvantages had to be weighed the advantage of length of time spent in the silo which might compensate for pH and temperature conditions which are far from optimum.

The series of experiments with laboratory silos was set up to answer these questions and also to provide information on the activities of the two separate enzyme mixtures supplied and the

dose rate required. The laboratory ensilage experiments showed that the enzyme mixtures had no adverse effects on fermentation. After two weeks of ensilage, which was the shortest time response studied, the enzymes were found to have increased the rate of production of lactic acid. This may have resulted from an increased supply of fermentable carbohydrate by way of a breakdown of plant fibre as suggested from reduced NDF/lignin and ADF/lignin ratios obtained with the enzyme-treated silages. With further laboratory experiments it was the ES 1 preparation which was found to be capable of degrading plant cell walls with increased reductions in NDF and ADF concentrations as the concentration of the enzyme was increased. With regard to the activity of the enzyme preparations containing predominantly hemicellulase enzymes, ES 2, when applied on its own, as in the laboratory ensilage Experiments 1 and 2 and not in combination with ES 1, it was not associated with a reduction in the NDF and ADF. However, reduced concentrations of WSC and increased production of lactic acid were recorded following the addition of ES 2 and also the crude hemicellulase preparation obtained from Sigma Chemicals. Thus, although the hemicellulase preparations do not give a breakdown of fibre they would seem to be stimulating the fermentation of WSC to lactic acid despite the inhibitory effects on fermenation of formic acid (Chamberlain and Quig, 1986), applied in conjunction with the enzymes in these experiments. It would appear that the hemicellulase preparations are converting WSC to fermentable substrates. It may be that the apparent inhibition of formic acid on fermentation is the result of a block in WSC being converted to fermentable sugars and, presumably, hemicellulase is relieving this block. It is not

clear how this may be coming about but it may be that it is simply a case of the hemicellulase being added in sufficient amounts to overcome the formic acid inhibition of these enzymes that are naturally present in the silage. Therefore, in combinations of ES 1 and ES 2 there may be the effect of cellulases causing a breakdown of cell walls and hemicellulases stimulating further conversion of intermediate products to fermentable substrates and increased lactic acid production.

Mechanism of Enzyme Action

Probably the simplest view of the enzyme action would be to expect reductions in NDF and ADF to be reflected in increased concentrations of WSC and/or fermentation products.

Ensiled forage crops treated with cellulolytic enzyme preparations alone have generally been reported to have lower pH values than their corresponding untreated control silages. However, treatment with cell-wall degrading enzymes applied in conjunction with formic acid may have no effect on the pH value of treated silages (Henderson et al, 1982) presumably because of the inhibitory effect of formic acid. With alfalfa silage, Olson and Voelker (1961) found that the pH of the enzyme-treated silage declined more rapidly during the first week of preservation and remained lower for five weeks than that of the untreated silage. Similarly, with alfalfa, Leatherwood et al (1961) reported lower pHs for silages treated with cellulolytic enzymes over untreated controls. The pH of ensiled corn silage treated with fungal cellulase was found to vary inversely with cellulase concentration (Autrey et al, 1974). Henderson and McDonald (1977), studying the

effects of cellulase preparations on grass treated with a range of chemical additives and ensiled in laboratory silos, reported that only where the fermentation was not inhibited by the additions of organic acids or at high levels of enzyme application (4 g cellulase/ kg fresh herbage) were lower pH values obtained.

Several workers have reported increased residual WSC levels in silages treated with cellulase preparations over formic acid treated silages (Henderson and McDonald, 1977; Whittemore and Henderson, 1977; Henderson et al, 1982) and untreated silages (Huhtanan, Hissa, Jaakkola and Poutiainen, 1985). Further encouragement of metabolism of these residual sugars in enzyme-treated silages to lactic acid by homo-fermentative lactic acid bacteria has also been suggested (Huhtanan et al, 1985). It is possible, however, that higher levels of residual WSC, than those present in the original herbage, are found in silages treated with acid alone (McDonald and Henderson, 1974; Ohyama and McDonald, 1975). It has been suggested that the source of sugar in these experiments, where no cellulase enzymes have been added, has been the breakdown of hemicellulose or a glucan other than cellulose (Henderson, 1975; Ohyama, 1977). In the normal ensilage process the breakdown of hemicellulose is caused by plant enzymes and, at low pH, by acid hydrolysis (McDonald, 1981).

It was in the reduction in the concentration of cell-wall polysaccharides, particularly cellulose, that the enzymes had the greatest effect. The enzyme preparations would appear to contain the necessary initialising enzymes required to start off the breakdown of fibre. Also, from the results of reduced contents of fibre constituents, the enzyme

preparations, particularly ES 1, would seem to contain enzymes capable of hydrolysing the cell-wall polysaccharides. However, it is clear that in some cases with the enzyme mixtures used here, it is difficult to reconcile the measured reductions in NDF/ADF with the measured changes in the concentration of WSC and fermentation products.

The WSC method used (Somogyi, 1945) measures total reducing sugars after hydrolysis with dilute acid (McDonald and Henderson, 1964). It is possible that oligosaccharides are present that would require more severe conditions for complete hydrolysis to reducing sugars. The total CHO method (Dubois, Gilles, Hamilton, Rebers and Smith, 1956) was used as a check against this possibility for a limited number of samples but the results agreed closely with the estimates based on total reducing sugars.

This discrepancy is illustrated in Table 15 which shows the results of attempting to account for the NDF/ADF breakdown in terms of measured changes in silage composition. The assumptions made in these calculations are:

1. Reductions in ADF content signify a breakdown of cellulose.
2. The reduction in NDF - ADF is a measure of the breakdown in hemicellulose.
3. In terms of fermentable products, cellulose yields only glucose and hemicellulose yields only pentoses.
4. All fermentation occurs via lactic acid bacteria; hence acetic acid is produced only from pentoses.
5. Fermentation of glucose occurs 50% via the homofermentative pathway and 50% via the heterofermentative pathway. It is assumed that

TABLE 15: The breakdown of NDF/ADF in terms of measured changes in silage composition of laboratory silages treated with cell-wall degrading enzymes. All concentrations are measured in g $kgDM^{-1}$.

TREATMENT AND EXPERIMENT No.	REDUCTION IN		INCREASE IN			PRODUCTS OF FIBRE BREAKDOWN NOT ACCOUNTED FOR.†
	NDF	ADF	WSC	HLa	HAc	
Expt. 2						
ES1 mlt^{-1}						
100	0	0				0*
200	27	22	25	12	2	0
300	56	32	0	26	6	22
400	69	35	16	20	0	40
500	107	58	0	17	8	89
Expt. 3						
1000	111	61	-11	57	5	42
1500	131	78	-22	3	3	160
2000	139	71	-32	11	6	166

† Degraded NDF and ADF (as hexose and pentose) not accounted for either in WSC or HLa or HAc.

* Increase in WSC accounted for by a decrease in lactic acid production.

TABLE 16: The breakdown of NDF/ADF in terms of measured changes in silage composition of 1-tonne^{-1} silages treated with cell-wall degrading enzymes. All concentrations are measured in g kgDM^{-1}.

TREATMENT AND EXPERIMENT No.		REDUCTION IN		INCREASE IN			PRODUCTS OF FIBRE BREAKDOWN NOT ACCOUNTED FOR.†
		NDF	ADF	WSC	HLa	HAc	
Expt. 2							
Grass							
ES1/ES2	mlt^{-1}						
	50	26	1	-21	12	0	34
	150	41	0	-55	3	0	97
Lucerne							
ES1	mlt^{-1}						
	125	30	14	-2	17	11	5
ES1/ES2	250	55	34	-1	15	10	32

† Degraded (NDF and ADF (as hexose and pentose) not accounted for either in WSC or HLa or HAc.

once produced, lactic acid is not further metabolised.

6. Products of fibre breakdown unaccounted for are expressed as the degraded cellulose and hemicellulose (expressed as hexose and pentose) not accounted for either in WSC or lactic and acetic acids.

An example of the calculation is as follows and was repeated for the laboratory silo treatments illustrated in Table 15 and for the pilot-scale silo treatments illustrated in Table 16.

Calculation:

Lucerne 1-tonne pilot-scale silo

Treatment of 125 $ml\,t^{-1}$ ES 1 + 125 $ml\,t^{-1}$ ES 2

Reduction in ADF = (444-410) g $kgDM^{-1}$

= $\underline{34\ g\ kgDM^{-1}}$

Reduction in NDF = (529-474) g $kgDM^{-1}$

= $\underline{55\ g\ kgDM^{-1}}$

Assuming all cell-wall polysaccharides are broken down to glucose and pentoses:

glucose supplied by ADF = $34 \times \frac{180}{162}$

= $\underline{37.8\ g\ kgDM^{-1}}$

Hemicellulase degraded = 55-34 g $kgDM^{-1}$

= $\underline{21\ g\ kgDM^{-1}}$

Assuming the hemicellulose consists of anhydropentose chains:

pentose supplied by hemicellulose hydrolysis

= $21 \times \frac{168}{150}$

= $\underline{23.5\ g\ kgDM^{-1}}$

So if there is no fermentation of glucose and pentoses the WSC content should increase by about 60 g $kgDM^{-1}$.

However the observed increase in WSC content was only 1 g $kgDM^{-1}$

$\therefore$ It is concluded that about 60 g fermentable sugar $kgDM^{-1}$ was further metabolised.

Metabolism of glucose

Assuming metabolism is by lactic acid bacteria, then two pathways are possible.

(a) 1 mol glucose $\longrightarrow$ 2 mol lactic acid (homofermentative)

(b) 1 mol glucose $\longrightarrow$ 1 mol lactic acid + ethanol + CO_2 (heterofermentative)

Hence the maximum yield of lactic acid if pathway (a) operates

$$38 \text{ g glucose} \longrightarrow \frac{38}{180} \times 180 \text{ g lactic acid}$$

ie. 38 g lactic acid $kgDM^{-1}$

Minimum yield of lactic acid if pathway (b) operates

38 g glucose $\longrightarrow$ 19 g lactic acid $kgDM^{-1}$

Assuming 50% of glucose metabolism via each pathway,

lactic acid yield = 28.5 g $kgDM^{-1}$

Metabolism of pentoses

One pathway in lactic acid bacteria

pentose $\longrightarrow$ lactic acid + acetic acid

$$23.5 \text{ g pentose} \longrightarrow \frac{23.5}{168} \times 90 \text{ g lactic acid}$$

$$+ \frac{23.5}{168} \times 68 \text{ g acetic acid}$$

ie. 12.6 g lactic acid $kgDM^{-1}$

plus 8.4 g acetic acid $kgDM^{-1}$

Hence we would predict:

at least 41 g lactic acid $kgDM^{-1}$

and 8 g acetic acid $kgDM^{-1}$

Observed 15 g lactic acid $kgDM^{-1}$

and 10 g acetic acid $kgDM^{-1}$

The good agreement between predicted and observed increases in acetic acid content suggest that the predicted fermentation of pentoses is a reasonable estimate. So if the asumptions about hemicellulose breakdown and fermentation are correct then

yield of lactic acid from 38 g glucose = 2 g lactic acid $kgDM^{-1}$ (13 g lactic acid from pentoses)

$\therefore$ The products of cell wall breakdown unaccounted for

= 32 g $kgDM^{-1}$

These calculations for the laboratory silages show a general tendency for the "unaccounted" fraction to increase with increasing dosage of the enzyme (Fig 20). Although the position is more complicated to interpret with respect to the 1-tonne silos because of the effluent loss, a similar result in seen (Table 16).

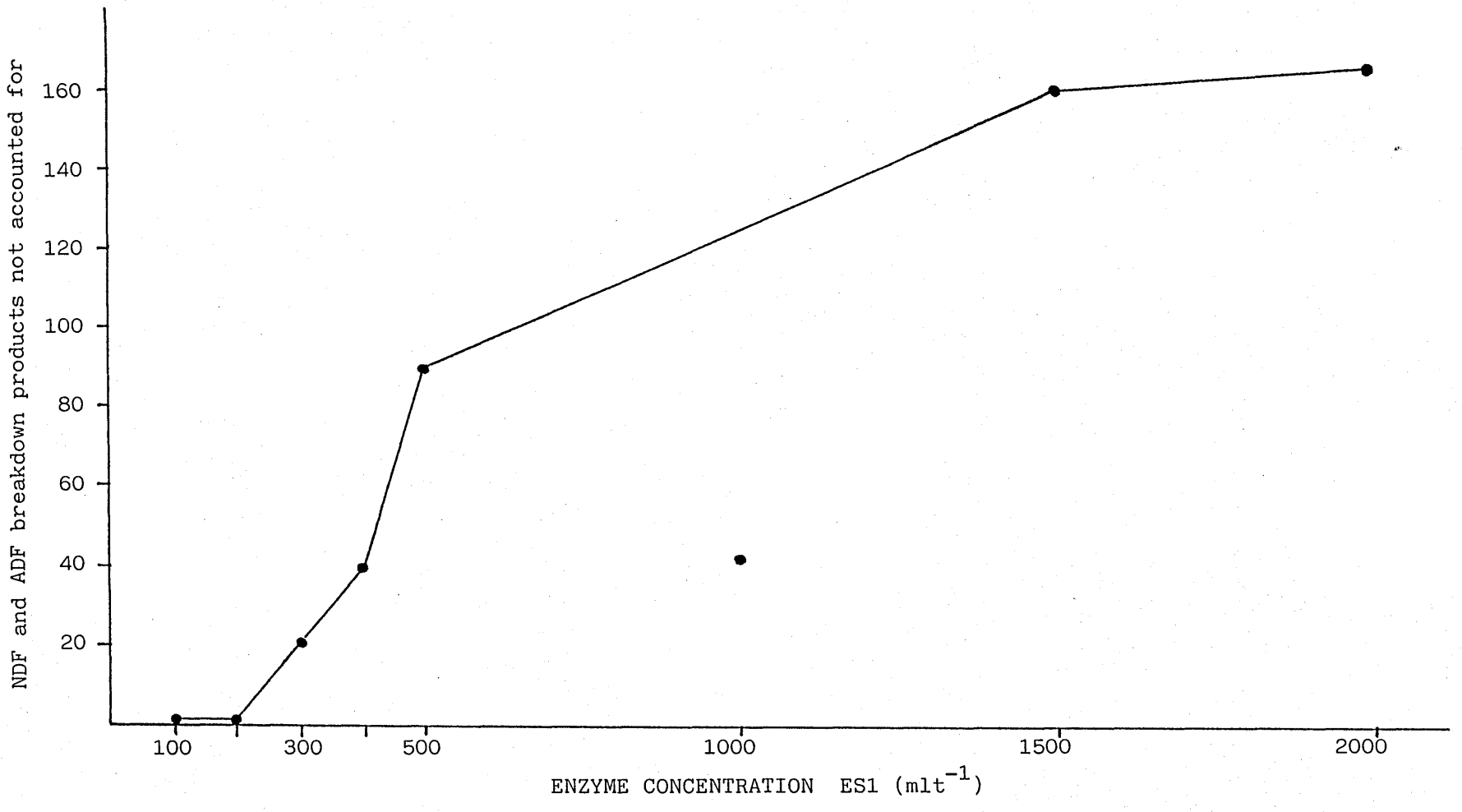


FIGURE 20: The effect of increasing concentration (mlt^{-1}) of cell-wall degrading enzymes on the concentration (g $kgDM^{-1}$) of NDF and ADF breakdown products not accounted for by changes in silage composition.

Clearly a more detailed chemical analysis is required to gain a better picture of the way in which the enzymes are acting, but within the constraints of the analytical system used here, some attempt can be made. It appears that cell walls are being degraded (as judged by NDF and ADF measurements), but that the products of this breakdown are not being measured as increases in WSC or fermentation products.

This suggests that the breakdown of fibre is not proceeding as far as WSC (this is particularly so as the dose rate of the enzyme is increased). One interpretation is shown schematically in Figure 21. Here the action of the enzymes is considered very crudely to consist of three main series of reactions:

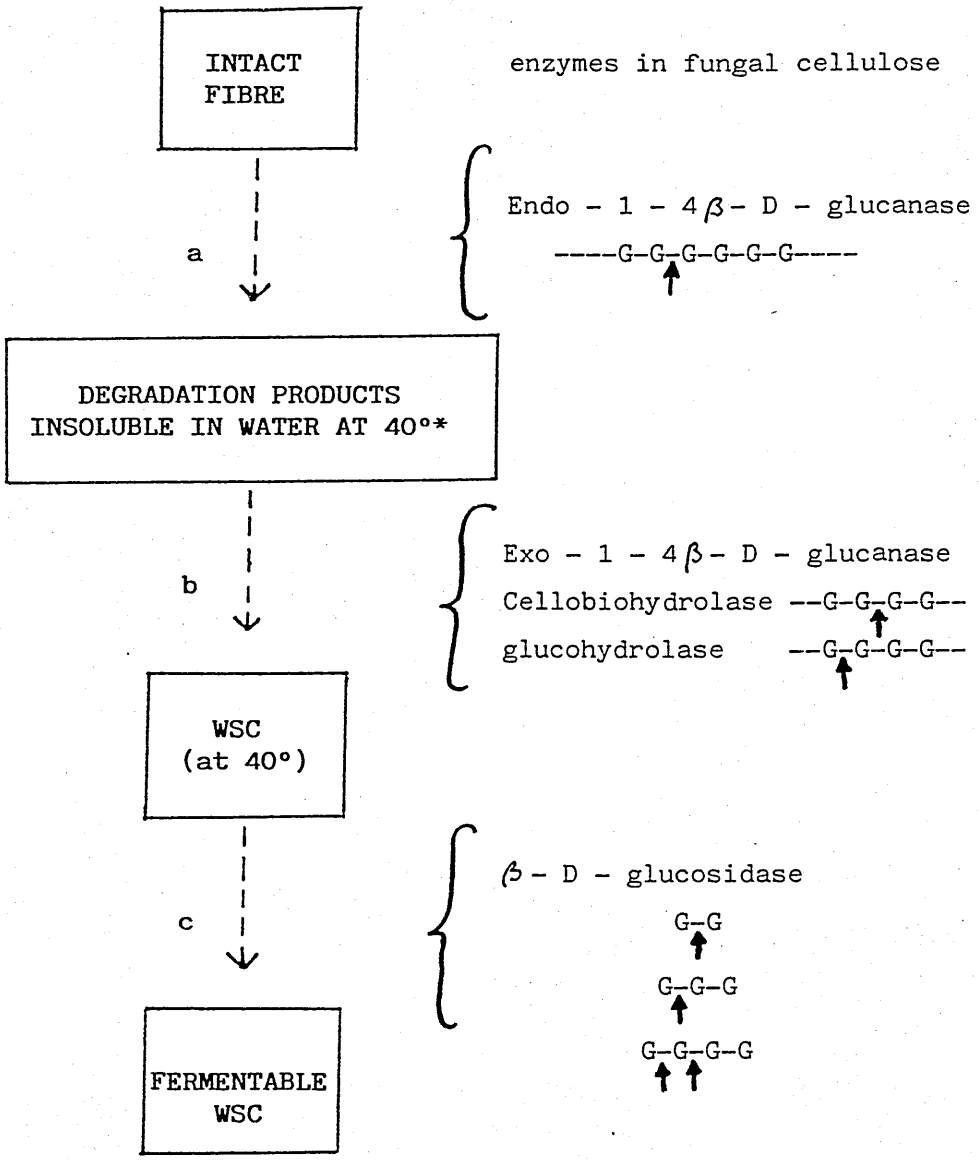
(a) The production of insoluble (in water at 40°C) degradation products;

(b) The conversion of insoluble products to WSC;

(c) The conversion of WSC to fermentable substrates.

Clearly this is an oversimplified view and such well-defined stages probably do not occur. However, studies on fungal cellulases (Wood, 1985) show three types of enzymes in the complex; these are included in the Figure 21 but it is recognised that a degree of overlap exists between them in the production of end products.

The results of the experiments described here show that the enzyme mixtures contained the enzymes corresponding to the three stages of breakdown. However, as the dose rate of the mixture is increased, the activity of the enzyme(s) responsible for step b cannot keep pace with the activity of the enzyme(s) responsible



*These products are soluble in boling detergent solutions and hence breakdown to this stage is reflected in reduced contents of NDF and ADF.

FIGURE 21: Schematic representation of the stages in the breakdown of cell walls by the enzymes. (Also shown are the enzymes isolated from fungal cellulases (Wood, 1985)).

for step a. The result is an accumulation of insoluble degradation products. However, these substances are soluble in boiling detergent solutions and so their production is signified by reduced NDF and ADF concentrations. From evidence available compounds of cellulose degradation of five glucose units and greater have a very low solubility in water even at elevated temperatures (Purves, 1943).

Effects of the Enzyme Treatments on Intake and Digestibility

(a) Dacron bag incubations

Because of the uncertainties over the interpretation of detergent extractions referred to earlier, it is probably more meaningful to consider the disappearance of dry matter from the Dacron bags.

For the grass silage experiment the enzyme treatments appear to have a negative effect. This is clearly evident in the "zero-time wash-out" values (Appendix Table 1) where the disappearance (%) of dry matter was much greater for the formic acid alone treatment than for the enzyme-treated silages. The most likely explanation of this would be that there has been a loss of soluble DM in the effluent with all the enzyme treatments.

For the lucerne silages the zero time losses of DM were broadly similar across treatments (Appendix Table 4) but there were increased rates of disappearance of DM with increased time interval from all the silages treated with enzymes. These differences between grass and lucerne silages and between control and enzyme treatments are seen in Figs 14-19 and in Appendix Figs 1-6 and Appendix Tables 1-6

where for all the grass silages the rates of disappearance of DM and fibre were greater for the formic control treated silage than for the enzyme treatments, whereas for the lucerne silages, treatment with the enzymes gave increased rates of DM and fibre disappearance than for formic acid treatment.

Under the appropriate conditions (seen with lucerne at the dose rate used but not with grass at the dose rate used) the enzyme treatments clearly increased the rate of ruminal digestion of silage as judged from the disappearance of DM from Dacron bags.

The suggestion is that the dose rate of enzyme applied needs to take account of the nature of the crop in terms of species, digestibility and possibly also dry matter content. If the enzymes are to have a role increasing the rate of ruminal digestion of highly digestible grass silage then the indication is that much lower rates of addition of the enzyme may be required if excessive cell wall breakdown, with the consequent loss of nutrients in effluent, is to be avoided. An alternative, or additional, approach might be to wilt the crop quite heavily in order to reduce effluent loss; this strategy highlights the need for further work to establish the critical moisture levels required for the enzymes to function.

(b) Intake/digestibility trials

Overall digestibility (DOMD) There were suggestions with the lucerne silage of increases in DOMD amounting to about 20 gkg^{-1} with enzyme treatment (particularly with the addition of 250 mlt^{-1} ES 1/ES 2. There were also suggestions of increases of a similar magnitude in the 1-tonne grass silage experiment (enzyme level 300 mlt^{-1} ES1/ES 2 v control). Neither of the effects was statistically

significant ($P > 0.05$). However, whereas for the lucerne silage the increase in digestibility was associated with an increased intake, leading to a substantial increase in the intake of digestible organic matter (19% at the quoted enzyme level), with the grass silage the increased digestibility was accompanied by a decreased intake. With the farm-scale (50 t) grass silage there was no suggestion of an improved DOMD with enzyme treatment. It would have to be concluded that from the experiments here there is no clear-cut evidence of an improvement in DOMD in response to enzyme treatment. It would therefore seem that for the silages used in these experiments any improvement in nutritional value would be expressed as increased voluntary intakes arising from the increased rates of ruminal digestion indicated by the Dacron bag studies.

Voluntary intake In the two intake trials with sheep the silages were fed unsupplemented. Only with the lucerne silage treated with 250 $ml\,t^{-1}$ ES 1/ES 2 was there a clear suggestion of an increased voluntary intake. For the grass silage the dacron bag incubations would indicate that no increase in VFI would be seen; indeed they would suggest a reduction in VFI which is in line with the observations.

However, as mentioned earlier, all three enzyme-treated lucerne silages showed increased rates of ruminal digestion and yet for only one of the silages was this translated into an increased intake. This illustrates the difficulty in predicting intake effects from Dacron bag measurements.

Assuming that the increased losses of DM and fibre from the bag are indicative of an increased rate of ruminal digestion of fibre,

these findings raise issues fundamental to the understanding of the physical control of intake. Clearly, with the lucerne silage used here (DOMD $\sim$ 500 gkg^{-1}) the conventional view would be that intake was limited "physically" and that an increase in the rate of ruminal digestion might be expected to result in a reduced residence time in the rumen and an associated increase in voluntary intake. However, this interpretation is true for only one of the silages; moreover, from the disappearance curves (Figs 17-19) the 250 mlt^{-1} ES 1/ES 2 treatment which showed the intake response had a lower disappearance rate than the other two treatments.

There is no explanation for the discrepancy although it should be remembered that the enzyme treatment could have effects on rumen digestion more generally and these could confound the interpretation. In this context, it is noteworthy that enzyme treatment can result in a substantial increase in the content of water-soluble nitrogen (M.R. Stokes, personal communication) and also of interest here is the effect of enzyme treatment of the lucerne on the apparent digestion of nitrogen. The nitrogen digestibilities of the lucerne-treated silages were 0.711, 0.678, 0.714 and 0.773 (Table 11) for the treatments of formic acid (3 mlt^{-1}), 125 mlt^{-1} ES 1, 250 mlt^{-1} ES 1/ES 2 and 10 mlt^{-1} ES 3 respectively. The ES 3 treatment had the largest effect on nitrogen digestibility and similarly gave increased rates of disappearance from Dacron bags (Appendix Tables 4-6) and yet had little effect on intake (Table 11). It is possible that enzyme treatment may alter markedly the pattern of release of available nitrogen for the rumen micro organisms. In some circumstances this

could lead to limitations on microbial growth which could in turn result in adverse effects on the rate of fibre digestion. In other words, although enzyme treatment may produce an increase in the potential rate of fibre digestion (which would be indicated by Dacron bag measurements) more general effects on rumen digestion may prevent the potential benefits from being realised.

In the milk production trial with cows the silages were given supplemented with concentrate at two levels, 6 kgd^{-1} and 9 kgd^{-1}. The results of this experiment again illustrate the complexity of the control of voluntary intake. Although the silages were consumed in similar amounts when given with 6 kgd^{-1} concentrates, there was a clear advantage to the enzyme-treated silage when the concentrate intake was increased to 9 kgd^{-1}. Dacron bag incubations were not made with these silages so no direct comment can be made on the rate of ruminal digestion of the silages. With this limitation there seemed to be two interpretations of the results.

Addition of starchy concentrate to the diet is associated with a reduced rate of ruminal digestion of fibre (Ben-Ghedalia and Miron, 1984) and the extent of depression would be expected to increase with the level of concentrate addition . This may indicate that the enzyme treatment led to an increase in the rate of degradation of fibre only when the amount of cellulolytic activity in the rumen was depressed to some critical level which was exceeded when 9 kgd^{-1} concentrates were fed. An alternative explanation is that the rate of digestion of fibre was increased at both levels of concentrate addition but that this increased ruminal digestion was translated into an effect on the intake of silage only at the higher level of

concentrate intake.

As well as raising important questions about the concept of the physical control of intake these results also highlight the need for caution in the interpretation of data from Dacron bag incubations. Clearly, from the foregoing discussion much more attention needs to be given to the choice of basal diet used in the incubations, an observation also supported by the results of studies on the ruminal digestion of starch and nitrogen (Kassem, 1986). It is also clear that having established differences in the rate of ruminal digestion of fibre, whether or not these differences are translated into effects on voluntary intake will again depend on the dietary circumstances.

In conclusion, the enzyme preparations used in the experiments have the capacity to degrade intact forage cell walls. They can also result in improved nutritional value of ensiled forage although there is no clear evidence from the experiments here that overall digestibility is increased. Further work with a range of crop types and digestibility levels would be required to reach a firm conclusion. The improvements seen in the experiments reported here appear to derive from increases in the rate of ruminal digestion leading to increases in voluntary food intake. However, it is clear that whether or not the enzyme treatment results in improvements in voluntary intake will depend very much on the dietary circumstances. The studies indicate that the addition of cell-wall degrading enzymes at ensiling is a promising technique, but they also expose the need for further work in certain key areas. There is a need to obtain further basic information on the mode of action of the enzymes since if indeed they can lead to

the formation of water-insoluble products of degradation then this would be of very clear benefit in terms of avoiding losses of nutrients in effluent. The whole question of the dose rates required needs to be examined in relation to the type of crop and the conditions of ensilage.

APPENDIX

TABLE 1 : The disappearance (%) of dry matter for grass silages incubated in dacron bags in the rumen of a cow for varying lengths of time. Values are means of 12 sets of observations for grass silages prepared with formic acid ($3lt^{-1}$) or formic acid and various rates of a 50:50 mixture of the ES1 and ES2 enzyme systems.

INCUB. TIME (hours)	FORMIC ACID	ES1/ES2 (mlt^{-1})			SED
		100	300	500	
0	40.1	30.6	31.9	33.2	2.10*
2	42.0	32.3	34.1	35.9	3.20*
7	44.1	35.9	35.5	40.4	1.90***
16	54.7	47.1	47.1	49.1	2.3**
24	61.4	58.6	55.2	61.4	2.7
48	78.4	74.4	73.5	75.5	1.2**

*** ($P < 0.001$)

TABLE 2 : The disappearance (%) of NDF for grass silages incubated in dacron bags in the rumen of a cow for varying lengths of time. Vales are means of 12 sets of observations for grass silages prepared with formic acid ($3lt^{-1}$) or formic acid and various rates of a 50:50 mixture of the ES1 and ES2 enzyme systems .

INCUB. TIME (hours)	FORMIC ACID	ES1/ES2 (mlt^{-1})			SED
		100	300	500	
0	18.3	7.7	11.0	12.8	2.2**
2	24.3	10.6	15.5	18.7	2.2***
7	25.9	14.4	15.5	22.4	2.6**
16	29.5	29.3	31.3	35.1	2.9**
24	47.6	43.8	40.6	47.7	3.6
48	70.9	64.9	65.9	67.8	1.6**

*** ($P<0.001$)

TABLE 3 : The disappearance (%) of ADF for grass silages incubated in dacron bags in the rumen of a cow for varying lengths of time. Values are means of 12 sets of observations for grass silages prepared with formic acid ($3lt^{-1}$) or formic acid and various rates of a 50:50 mixture of the ES1 and ES2 enzyme systems.

INCUB. TIME (hours)	FORMIC ACID	ES1/ES2 (mlt^{-1})			SED
		100	300	500	
0	15.2	11.7	11.9	19.4	2.2*
2	17.4	11.7	11.2	21.4	2.7***
7	19.7	15.9	14.7	25.8	2.3***
16	35.6	31.7	33.7	37.7	3.4*
24	46.3	48.3	41.4	53.1	3.6**
48	71.2	69.2	67.2	71.3	1.5

*** ($P < 0.001$)

TABLE 4 : The disappearance (%) of dry matter for lucerne silages incubated in dacron bags in the rumen of a cow for varying lengths of time. Values are means of 12 sets of observations for lucerne silages prepared with formic acid ($3lt^{-1}$) or various rates of the enzyme systems ES1, ES2, and ES3 .

INCUB. TIME (hours)	FORMIC ACID	ENZYME SYSTEM (mlt^{-1})			SED
		ES1 125	ES1/ES2 250	ES3 10	
0	21.2	24.7	16.2	22.0	2.2***
2	22.6	31.0	24.8	28.1	1.0***
7	27.1	38.2	31.0	32.0	2.6***
16	43.2	53.6	46.5	49.6	1.8***
24	48.0	53.9	52.9	55.7	2.1**
48	54.9	61.7	58.3	61.4	0.8***

*** ($P < 0.001$)

TABLE 5 : The disappearance (%) of NDF for lucerne silages incubated in dacron bags in the rumen of a cow for varying lengths of time. Values are means of 12 sets of observations for lucerne silages prepared with formic acid ($3lt^{-1}$) or various rates of the enzyme systems ES1, ES2, and ES3 .

INCUB. TIME (hours)	FORMIC ACID	ENZYME SYSTEM (mlt^{-1})			SED
		ES1 125	ES1/ES2 250	ES3 10	
0	0	5.8	0	2.8	2.8***
2	0	9.8	0	5.1	1.9***
7	0.6	15.6	0.6	8.7	3.3***
16	11.3	32.7	17.3	20.6	2.8***
24	18.1	33.2	29.5	31.8	3.4***
48	33.3	44.6	37.1	40.9	1.3***

*** ($P < 0.001$)

TABLE 6 : The disappearance (%) of ADF for lucerne silages incubated in dacron bags in the rumen of a cow for varying lengths of time. Values are means of 12 sets of observations for lucerne silages prepared with formic acid ($3lt^{-1}$) or various rates of the enzyme systems ES1, ES2 and ES3 .

INCUB. TIME (hours)	FORMIC ACID	ENZYME SYSTEM (mlt^{-1})			SED
		ES1 125	ES1/ES2 250	ES3 10	
0	0	9.9	0	5.3	2.7***
2	0	13.5	1.4	4.3	1.3***
7	3.8	20.4	8.5	11.3	3.2***
16	20.3	36.8	24.1	26.0	2.4***
24	25.4	40.9	31.9	38.0	3.1**
48	38.8	48.9	39.4	46.6	1.3***

*** ($P < 0.001$)

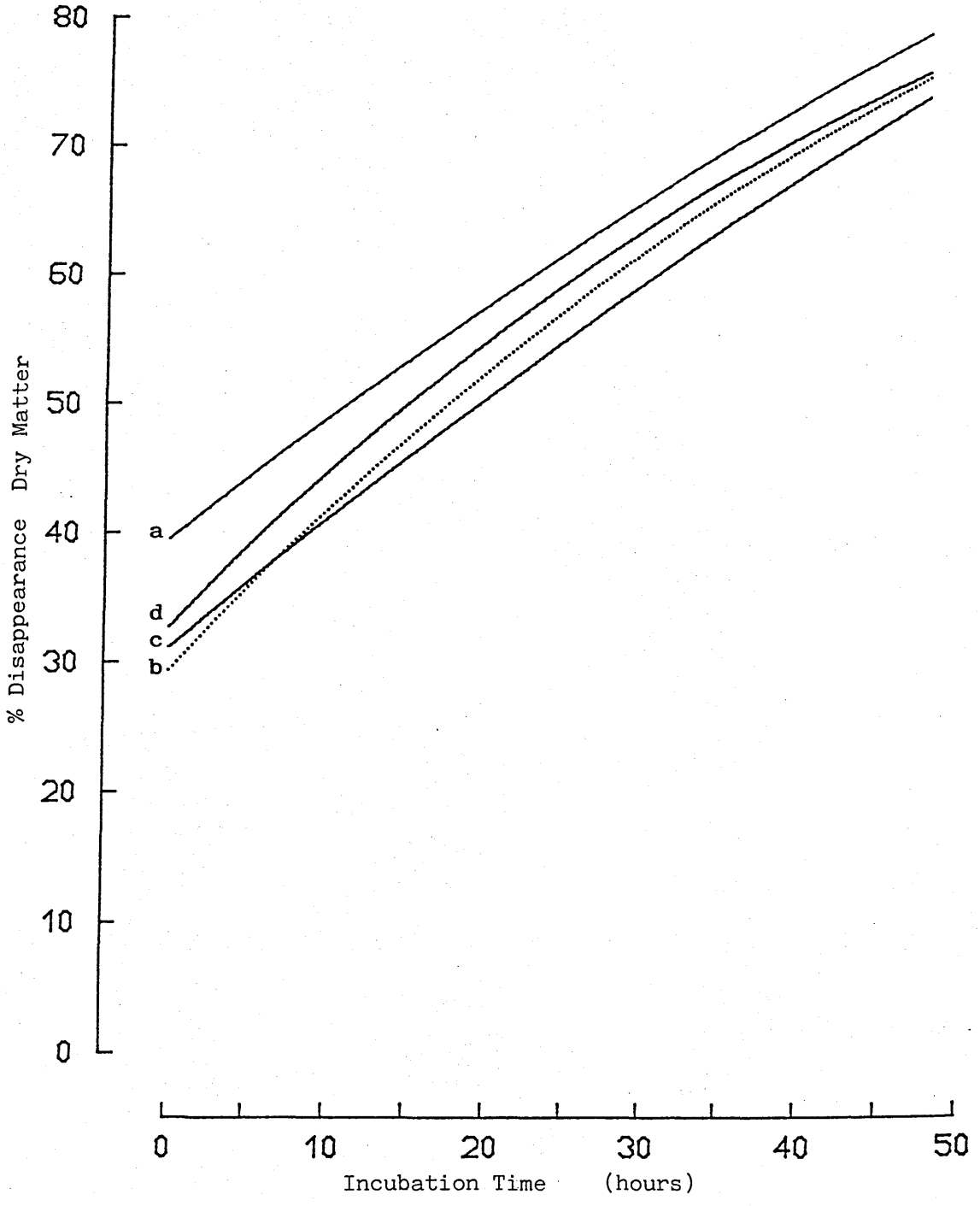


FIGURE 1 : Best fit curves for the disappearance (%) of dry matter for grass silages with time. **a** Formic Acid ($3lt^{-1}$); **b** Formic Acid + 100 mlt^{-1} ES1/ES2; **c** Formic Acid + 300 mlt^{-1} ES1/ES2; **d** Formic Acid + 500 mlt^{-1} ES1/ES2 .

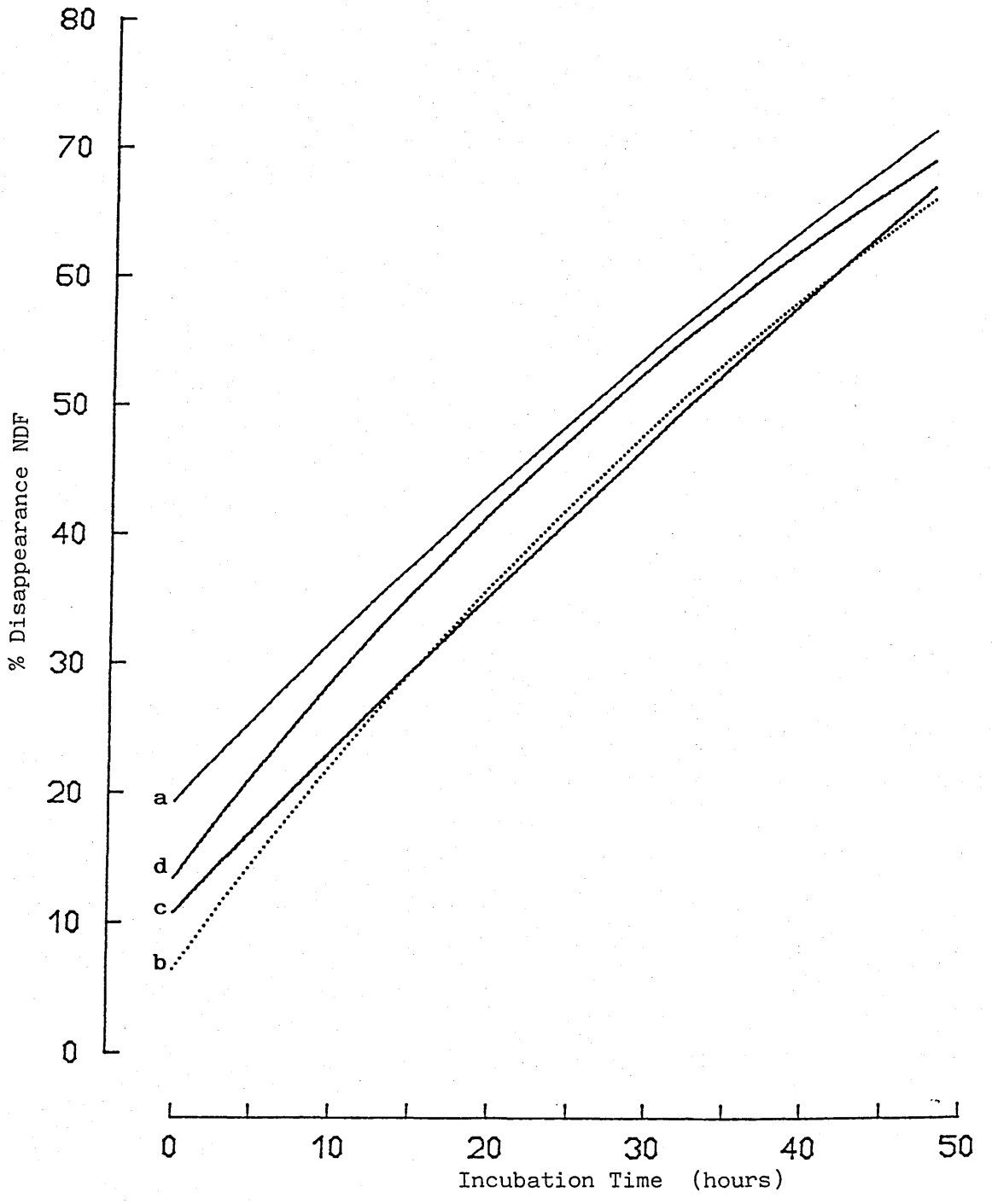


FIGURE 2 : Best fit curves for the disappearance (%) of NDF for grass silages with time **a** Formic Acid ($3lt^{-1}$); **b** Formic Acid + $100\ mlt^{-1}$ ES1/ES2; **c** Formic Acid + $300\ mlt^{-1}$ ES1/ES2; **d** Formic Acid + $500\ mlt^{-1}$ ES1/ES2 .

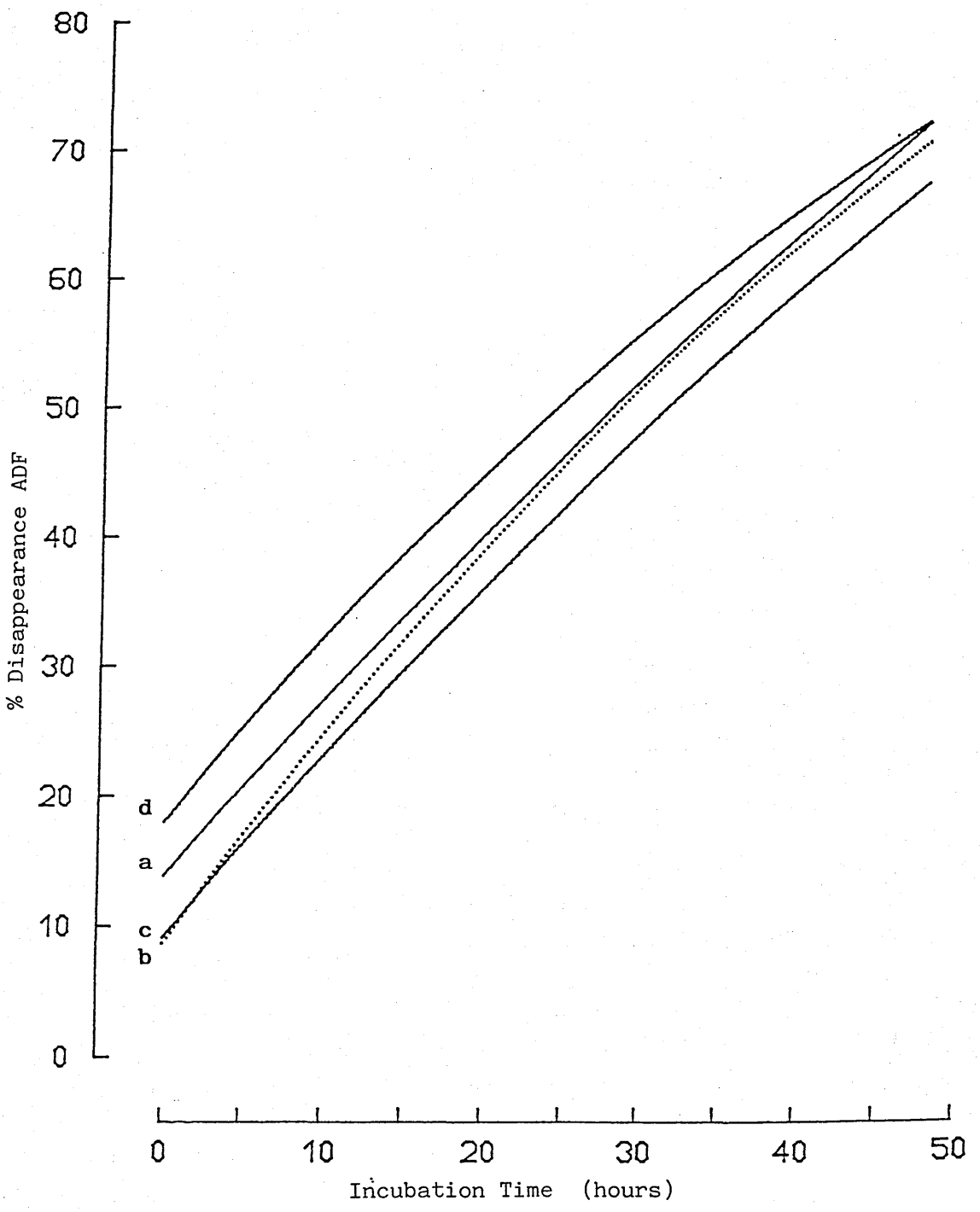


FIGURE 3 : Best fit curves for the disappearance (%) of ADF for grass silages with time. **a** Formic Acid ($3lt^{-1}$); **b** Formic Acid + $100\ mlt^{-1}$ ES1/ES2; **c** Formic Acid + $300\ mlt^{-1}$ ES1/ES2; **d** Formic Acid + $500\ mlt^{-1}$ ES1/ES2 .

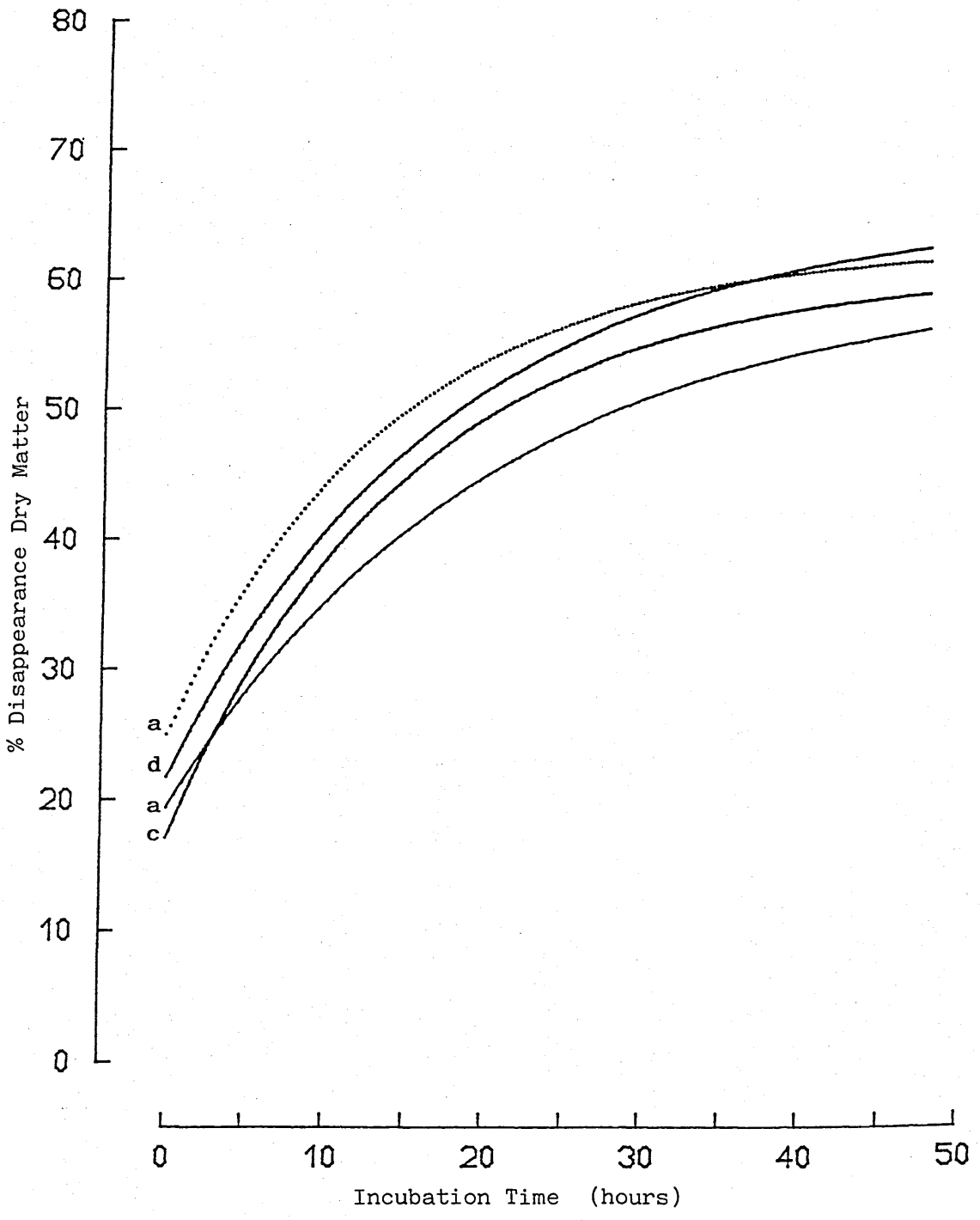


FIGURE 4 : Best fit curves for the disappearance (%) of dry matter for lucerne silages with time. **a** Formic Acid ($3lt^{-1}$). **b** ES1 ($125\ ml\,t^{-1}$); **c** ES1/ES2 ($250\ ml\,t^{-1}$); **d** ES3 ($10\ ml\,t^{-1}$) .

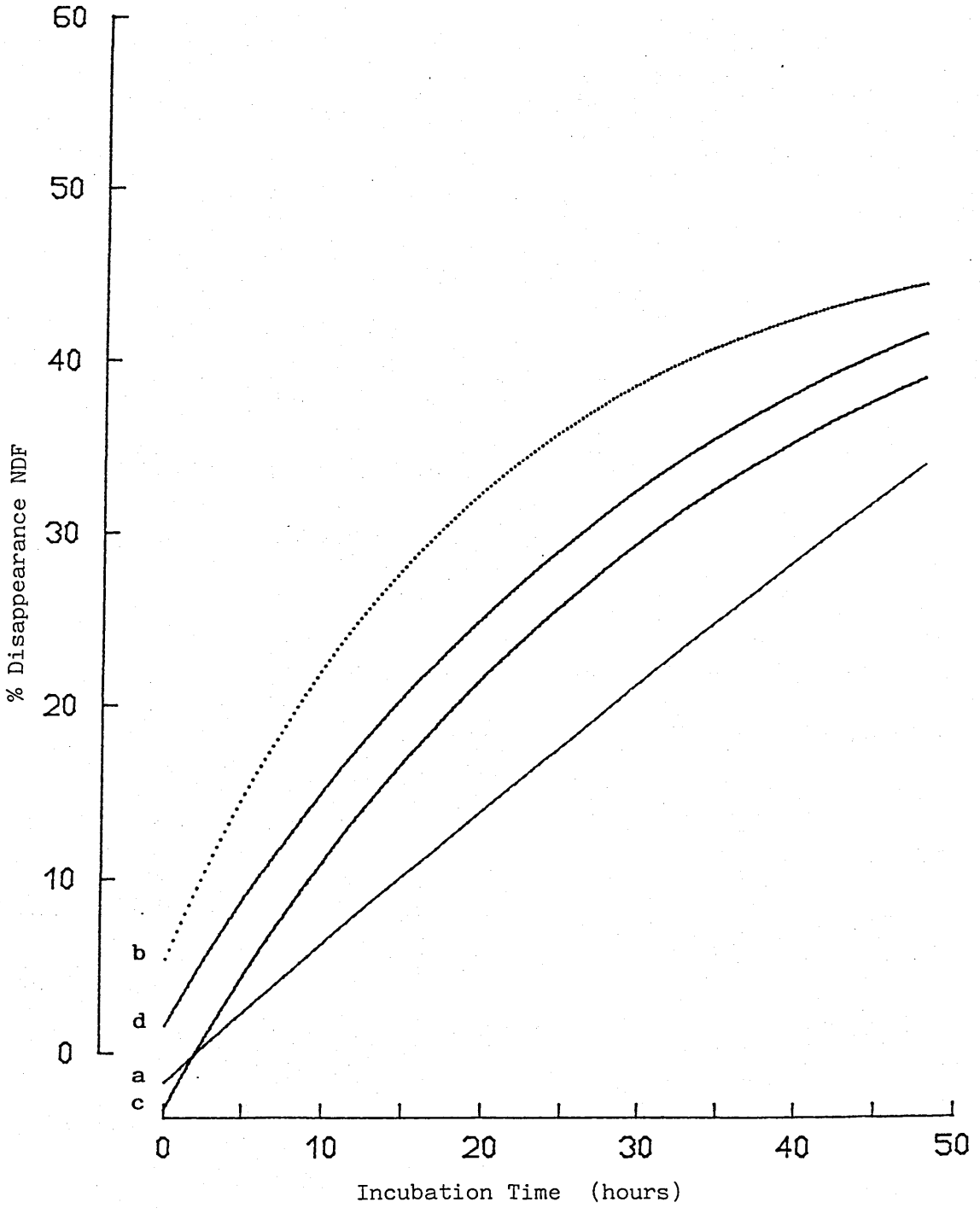


FIGURE 5: Best fit curves for the disappearance (%) of NDF for lucerne silages with time. **a** Formic Acid (3 lt^{-1}); **b** ES1 (125 mlt^{-1}); **c** ES1/ES2 (250 mlt^{-1}); **d** ES3 (10 mlt^{-1}) .

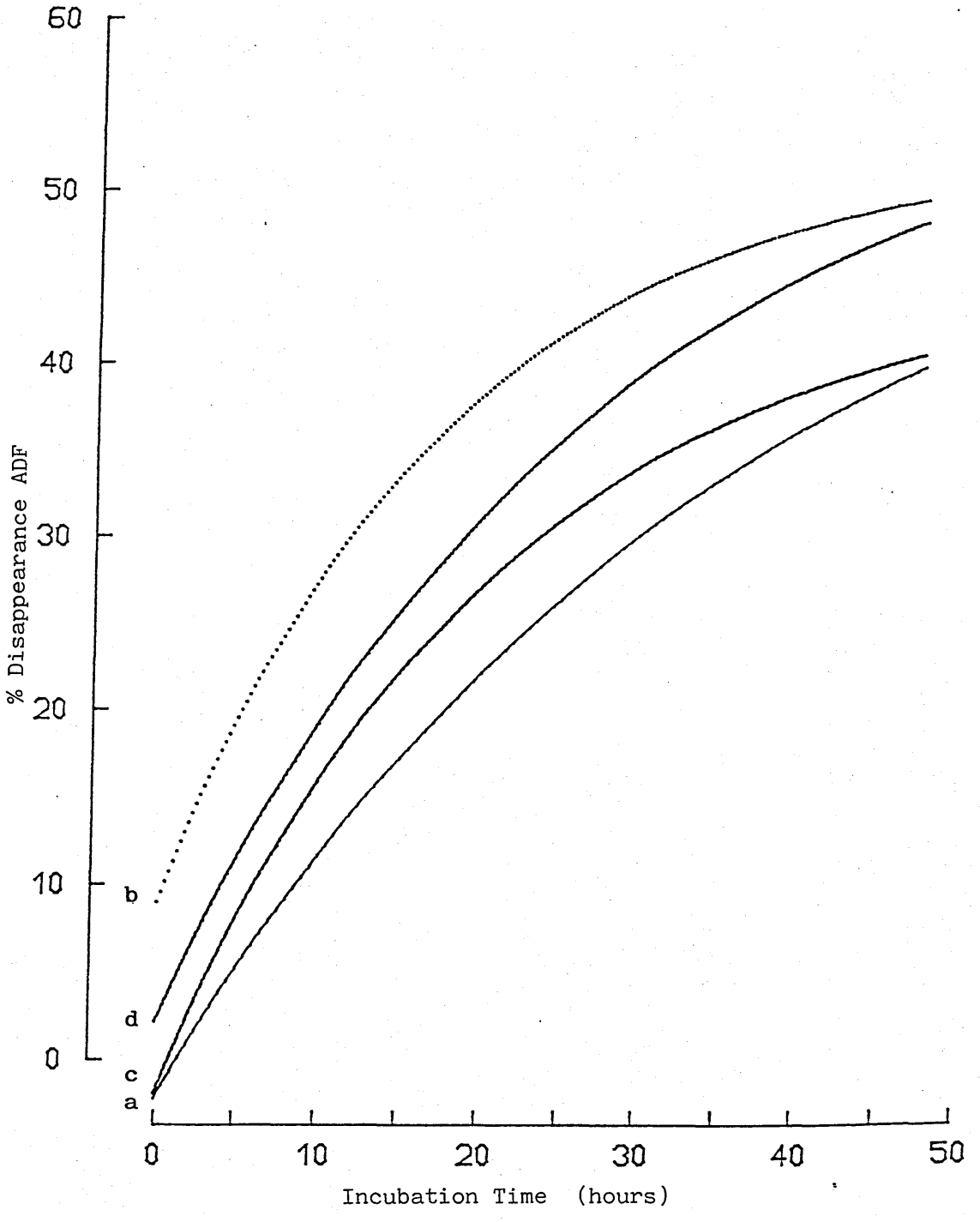


FIGURE 6 : Best fit curves for the disappearance (%) of ADF for lucerne silages with time. **a** Formic Acid (3 lt^{-1}); **b** ES1 (125 mlt^{-1}); **c** ES1/ES2 (250 mlt^{-1}); **d** ES3 (10 mlt^{-1}) .

REFERENCES

A.R.C. (1984) *The Nutrient Requirements of Ruminant Livestock.* Supplement No. 1. Commonwealth Agricultural Bureaux, Slough.

Autrey, K.M., McCaskey, T.A. and Little, J.A. (1974) *J. Dairy Sci.* ***58***: 67–71.

Ayers, A.R., Ayers, S.B. and Eriksson, K.-E. (1978) *Eur. J. Biochem.* ***90***: 171–181.

Bacon, J.S.D., Gordon, A.H., Morris, E.J. and Farmer, V.C. (1975) *Biochem. J.* ***149***: 485–487.

Baile, C.A. and Mayer, J. (1969) *Am. J. Physiol.* ***217***: 1830–1836.

Balch, C.C., Broster, W.H., Rook, J.A.F. and Tuck, V.J. (1965) *J. Dairy Res.* ***32***: 1–11.

Baldwin, R.L. and Allison, M.J. (1983) *J. Anim. Sci.* ***57***: Suppl. 2, 461–477.

Barker, S.D. and Summerson, W.H. (1941) *J. Biol. Chem.* ***138***: 535–554.

Barry, T.N., Cook, J.E. and Wilkins, R.J. (1978) *J. agric. Sci., Camb.* ***91***: 701–715.

Bemiller, J.N., Tegtmeier, D.O. and Pappelis, A.J. (1969) *Cellulases and Their Application* (ed. C.J. Hajny and E.T. Riese), p 188. Advances in Chemistry, Series 95. American Chemical Society, Wash. D.C.

Ben-Ghadalia, D. and Miron, J. (1984) *J. Nutr.* ***114***: 880–887.

Bickerstaffe, R., Annison, E.F. and Linzell, J.L. (1974) *J. agric. Sci., Camb.* ***82***: 71–85.

Blaxter, K.L., Wainman, F.W. and Wilson, R.S. (1961) *Anim. Prod.* ***3***: 51.

Brady, C.J. (1960) *J. Sci. Fd Agric.* ***11***: 276–284.

Brice, R.E. and Morrison, I.M. (1982) *Carbohyd. Res.* ***101***: 93–100.

Brobeck, J.R. (1948) *Yale J. Biol. Med.* ***20***: 545–552.

Bryant, M.P. and Robinson, I.M. (1962) *J. Bacteriol.* **84**: 605-614.

Chamberlain, D.G. and Quig, J. (1986) *J. Sci. Fd Agric.* (in press).

Dewar, W.A. and McDonald, P. (1961) *J. Sci. Fd Agric.* **12**: 790-795.

Dewar, W.A., McDonald, P. and Whittenbury, R. (1963) *J. Sci. Fd Agric.* **14**: 411-417.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) *Analyt. Chem.* **28**: 350-356.

Egan, A.R. (1966) *Aust. J. agric. Res.* **17**: 741-755.

Egan, A.R. and Moir, R.J. (1965) *Aust. J. agric. Res.* **16**: 437-449.

Emmanuel, B. (1974) *Biochim. Biophys. Acta* **337**: 404-413.

Emmanuel, B. (1978) *Biochim. Biophys. Acta* **328**: 239-246.

Flynn, A.V. and O'Kiely, P. (1984) In *Proc. 7th Silage Conf.* (ed. F.J. Gordon and E.F. Unsworth), pp 45-46 (Abstr.). Queen's University, Belfast.

Garton, G.A. (1969) *Proc. Nutr. Soc.* **23**: 131-139.

Garton, G.A., Lough, A.K. and Vioque, E. (1961) *J. Gen. Microbiol.* **25**: 215-225.

Gascoigne, J.A. and Gascoigne, M.M. (1960) *J. Gen. Microbiol.* **22**: 242-248.

Gibson, T. (1965) *J. appl. Bact.* **28**: 56-62.

Gilligan, W. and Reese, E.T. (1954) *Can. J. Microbiol.* **1**: 90-107.

Goering, H.K. and Van Soest, P.J. (1970) *Agricultural Handbook*, no. 379.

Greenhalgh, J.F.D., Pirie, R. and Reid, G.W. (1976) *Anim. Prod.* **22**: 159 (Abstr.).

Greenhalgh, J.F.D. and Reid, G.W. (1967) *Nature, Lond.* **214**: 744.

Harrison, F.A. and Leat, W.M.F. (1975) *Proc. Nutr. Soc.* **34**: 203-209.

Hartley, R.D. and Jones, E.C. (1977) *Phytochemistry* **16**: 1531-1534.

Heath, T.J. and Morris, B. (1963) *Br. J. Nutr.* **17**: 465-474.

Henderson, A.R. (1975) *Edinburgh School of Agriculture, Ann. Rep.*, p 90.

Henderson, A.R. and McDonald, P. (1977) *J. Sci. Fd Agric.* **28**: 486–490.

Henderson, A.R., McDonald, P. and Anderson, D. 1982) *J. Sci. Fd Agric.* **33**: 16–20.

Henderson, A.R., McDonald, P. and Woolford, M.K. (1972) *J. Sci. Fd Agric.* **23**: 1079–1087.

Hervey, G.R. (1969) *Nature, Lond.* **222**: 629–631.

Hobson, P.N. and Mann, S.O. (1961) *J. Gen. Microbiol.* **25**: 227–240.

Huhtanen, P., Hissa, K., Jaakkola, S. and Poutianen, E. (1985) *Finn. J. agric. Sci.* **57**: 284–292.

Hungate, R.E. (1966) In *The Rumen and its Microbes*. Academic Press, New York.

Jackson, M.G. (1977) *Anim. Feed Sci. & Technol.* **2**: 105–130.

Jones, D.I.H. (1970) *J. agric. Sci., Camb.* **75**: 293–300.

Jones, D.I.H. and Hayward, M.V. (1975) *J. Sci. Fd Agric.* **26**: 711–718.

Karr, A.L., Jr. and Albersheim. P. (1970) *Plant Physiol.* **46**: 69–80.

Kassem, M.M. (1986) *Ph.D. Thesis, Univ. Glasg.*

Kemble, A.R. (1956) *J. Sci. Fd Agric.* **7**: 125–130.

Laidlaw, R.A. and Reid, S.G. (1952) *J. Sci Fd Agric.* **3**: 19–25.

Leatherwood, J.M., Mochrie, R.D. and Thomas, W.E. (1961) *J. Anim. Sci.* **20**: 948 (Abstr.).

Lewis, D. and Hill, K.J. (1983) In *Nutritional Physiology of Farm Animals* (ed. J.A.F. Rook and P.C. Thomas), pp 3–40. Longman, London.

MacRae, J.C. and Armstrong, D.G. (1969) *Br. J. Nutr.* **23**: 377–387.

McAllan, A.B. and Smith, R.H. (1974) *Br. J. Nutr.* **31**: 77–88.

McCullough, M.E. (1964) *J. Dairy Sci.* **44**: 342 (Abstr.).

McCullough, M.E. and Neville, W.E. (1960) *J. Dairy Sci.* **43**: 444 (Abstr.).

McDonald, I.M. (1952) *Biochem. J.* **51**: 86–90.

McDonald, P. (1981) In *The Biochemistry of Silage*. John Wiley & Sons,Chichester.

McDonald, P., Edwards, R.A. and Greenhalgh, J.F.D. (1966) In *Animal Nutrition*, pp 342–352. Longman, London and New York.

McDonald, P. and Henderson, A.R. (1964) *J. Sci. Fd Agric.* **15**: 395–398.

McDonald, P. and Henderson, A.R. (1974) *J. Sci. Fd Agric.* **25**: 791–795.

McDonald, P., Stirling, A.C., Henderson, A.R. and Whittenbury, R. (1962) *J. Sci Fd Agric.* **13**: 581–590.

McDonald, P., Stirling, A.C., Henderson, A.R. and Whittenbury, R. (1964) *J. Sci. Fd Agric.* **15**: 429–486.

Mangan, J.L. (1972) *Br. J. Nutr.* **27**: 261–283.

Mayer, J. (1955) *Ann. N.Y. Acad. Sci.* **63**: 15–43.

Mayes, R.W. and Orskov, E.R. (1974) *Br. J. Nutr.* **32**: 143–153.

Mehrez, A.Z. and Orskov, E.R. (1977) *J. agric. Sci., Camb.* **88**: 645–650.

Montgomery, M.J., Schultz, L.H. and Baumgardt, B.R. (1963) *J. Dairy Sci.* **46**: 1380–1384.

Morrison, I.M. (1972) *J. Sci. Fd Agric.* **23**: 455–463.

Morrison, I.M. (1974a) *Carbohydr. Res.* **36**: 45.

Morrison, I.M. (1974b) *Biochem. J.* **139**: 197–204.

Morrison, I.M. (1975) *Biochem. Soc. Trans.* **3**: 992–994.

Morrison, I.M. (1979) *Proc. Nutr. Soc.* **38**: 269–274.

Morrison, I.M. (1980) *J. Sci. Fd Agric.* **31**: 639–645.

Nolan, J.V., Norton, B.W. and Leng, R.A. (1973) *Proc. Nutr. Soc.* **32**: 93–98.

Ohyama, Y. and McDonald, P. (1975) *J. Sci Fd Agric.* **26**: 941–948.

Ohyama, Y. and Masaki, S. (1977) *J. Sci. Fd Agric.* **28**: 78–84.

Okorie, A.U., Buttery, P.J. and Lewis, D. (1977) *Proc. Nutr. Soc.* **36**: 38A (Abstr.).

Olson, M. and Voelker, H.H. (1961) *J. Dairy Sci.* **44**: 1204 (Abstr.).

Osbourn, D.F. (1980) In *Grass, Its Production and Utilization* (ed. W. Holmes), p 70. Blackwell, London.

Owen, F.G. (1962) *J. Dairy Sci.* **45**: 934–936.

Pilgrim, A.F., Gray, F.V., Weller, R.A. and Belling, C.B. (1970) *Br. J. Nutr.* **24**: 589–598.

Purves, C.B. (1943) In *Cellulose and Cellulose Derivatives* (ed. E. Ott), pp 63–69. Interscience Publishers Inc., New York, N.Y.

Reese, E.T., Sui, R.G.H. and Levinson, H.S. (1950) *J. Bacteriol.* **59**: 485–497.

Smith, G.P., Gibbs, J. and Young, R.C. (1974) *Fedn. Proc. Fedn. Am. Socs. exp. Biol.* **33**: 1146–1149.

Somers, M. (1961) *Aust. J. exp. Biol. Med. Sci.* **39**: 111–145.

Somogyi, M. (1945) *J. Biol. Chem.* **160**: 69–73.

Stirling, A.C. and Whittenbury, R. (1963) *J. appl. Bact.*, **26**: 86–90.

Storry, J.E. and Rook, J.A.F. (1966) *Br. J. Nutr.* **20**: 217–228.

Thomas, C. and Thomas, P.C. (1985) In *Recent Advances in Animal Nutrition* (ed. W. Haresign and D.J.A. Cole), p 223. Butterworths, London.

Thomas, P.C. (1973) *Proc. Nutr. Soc.* **32**: 85–91.

Thomas, P.C. and Chamberlain, D.G. (1982) In *Silage for Milk Production* (ed. J.A.F. Rook and P.C. Thomas), pp 63–101. National Institute for Research in Dairying, Reading.

Thomas, P.C. and Clapperton, J.L. (1972) *Proc. Nutr. Soc. 31*: 165–170.

Thomas, P.C., Kelly, N.C., Chamberlain, D.G. and Chalmers, J.S. (1980) In *Energy Metabolism* (ed. L.E. Mount), pp 357–362. Butterworths, London.

Thomas, P.C., Kelly, N.C. and Wait, M.K. (1976) *J. Br. Grassld. Soc. 31*: 19–22.

Thomas, P.C. and Rook, J.A.F. (1977) In *Recent Advances in Animal Nutrition* (ed. J.A.F. Rook and P.C. Thomas), pp 83–109. Butterworths, London.

Toyama, N. (1969) *Cellulases and their Application* (ed. C.J. Hajny and E.T. Riese), p 359. Advances in Chemistry, Series 95. American Chemical Society, Wash. D.C.

Tracey, M.V. (1948) *Biochem. J., 42*: 281–287.

Waite, R., Johnstone, M.J. and Armstrong, D.G. (1964) *J. agric. Sci., Camb. 62*: 391–398.

Waldo, D.R. (1973) *J. Anim. Sci. 37*: 1062–1074.

Walker, P.J. and Hopgood, M.F. (1961) *Aust. J. agric. Res. 12*: 651–660.

Weller, R.A. and Pilgrim, A.F. (1974) *Br. J. Nutr. 32*: 341–351.

Whistler, R.L. and Masak, E. (1955) *J. Amer. Chem. Soc. 77*: 1241.

Whittemore, C.T. and Henderson, A.R. (1977) *J. Sci. Fd Agric. 28*: 506–510.

Whittenbury, R., McDonald, P. and Bryan-Jones, D.G. (1967) *J. Sci Fd Agric. 18*: 441–444.

Wilkinson, J.M., Wilson, K.F. and Barry, T.N. (1976) *Outlook on Agric.* *9*: 3–8.

Wilson, R.F. and Wilkins, R.J. (1973) *J. agric. Sci., Camb.* ***80***: 225–231.

Wood, T.M. (1985) In *Cellulose and its Derivatives* (ed. J.F. Kennedy, G.O. Phillips, D.J. Wedlock and P.A. Williams), pp 173–188. Ellis Horwood Ltd., Chichester.

Woolford, M.K. (1975) *J. Sci. Fd Agric.* *26*: 219–228.

Woolford, M.K. (1984) *The Silage Fermentation.* Microbiology Series, Vol. 14. Marcel Dekker Inc., New York.