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Biochemistry of Ensiling

John A. Rooke
SAC

Ronald D. Hatfield
USDA-ARS

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JOHN A. ROOKE

Research and Development Division
SAC
Aberdeen, United Kingdom

RONALD D. HATFIELD

USDA-ARS
U.S. Dairy Forage Research Center
Madison, Wisconsin

The biochemistry of ensiling is essentially a simple process, which, however, can become complex when interactions among plant enzymes and the activities of numerous microbial species become involved. The desired effect is the conversion of simple plant sugars such as glucose and fructose to lactic acid by lactic acid bacteria (LAB) in an anaerobic fermentation. When sufficient lactic acid has been produced, all microbial activity is suppressed, primarily through the effect of undissociated lactic acid, and the silage can then be stored anaerobically until required for feeding. Complications arise because:

1. There are always aerobic periods at the start and end of the ensiling process.
2. Simple sugars are not the only substrates metabolized.
3. Plant enzymes and other microbial species apart from LAB compete for substrate.

The complexity of ensilage is increased further when the difficulties of controlling large-scale processes like silage making on-farm are also considered.

OVERVIEW

An overview of ensiling will be presented, followed by a detailed discussion of the most important factors. A single chapter covering this subject necessitates some selection of topics, and the reader may find more detail on some topics in standard biochemistry textbooks and from extensive texts such as McDonald et al. (1991). The process of silage making can be divided into several stages: aerobic field, aerobic silo, anaerobic fermentation, anaerobic storage, and aerobic feed-out.

Aerobic Field Phase

The amount of substrate available for fermentation is determined by the status of the crop at cutting and by the length and conditions of the aerobic field phase (see Chapter 6, Muck et al., 2003). Plants maintain metabolic activity for extended periods after cutting, most notably the continued aerobic respiration of sugars to CO_2 and water to provide energy to the plant (see Chapter 6, Muck et al., 2003). Reserve carbohydrates (i.e., sucrose, starch, and fructans) may also be hydrolyzed and utilized during this phase. Carbohydrate loss may be partially offset by residual photosynthesis depending upon the field conditions and length of field exposure. Plant proteolytic activity is also responsible for hydrolysis of plant proteins to amino acids and peptides. The length of the aerobic field phase, therefore, can have a significant impact on the amount of fermentable substrate available for the ensiling process. Direct-cut ensiling minimizes the potential for field losses, but wilting has some advantages. Wilting forages prior to silage fermentation, to reduce moisture content, inhibits the growth of clostridia and enterobacteria, resulting in less spoilage (see Chapter 6, Muck et al., 2003).

Aerobic In-Silo Phase

When crops are ensiled, some O_2 is trapped in the silo with the forage. The amount of O_2 trapped depends on silo design, crop structure and chop length, and the extent of compaction of the silage. If the silo is well sealed, the O_2 is rapidly consumed by continuing plant respiration (see Chapter 6, Muck et al., 2003). A major difference from the aerobic field phase is that heat produced by respiration is trapped within the silo. Increased silo temperature can have an undesirable effect on the ensiling process. Forage protein degradation is stimulated due to increased rates of proteolysis. In addition, clostridia and enterobacteria have higher optimum growth temperatures than LAB, giving them a competitive edge at higher temperatures. Although the microflora in the ensiled material play little part in biochemical changes at this stage, the microbial population profile and numbers may change from that present on the standing herbage.

Anaerobic Fermentation

The removal of O_2 results in a shift to anaerobic metabolism. There is a rapid proliferation of microorganisms with the production of neutral and acidic end products. The most active bacteria are the enterobacteria and LAB. The acidic end products reduce silage pH and favor growth of the more acid-tolerant LAB. When substrate is not limiting, LAB dominate the fermentation, producing lactic acid and acidify the silage until a pH is attained which suppresses LAB growth and results in a stable silage. However, if substrate becomes limiting or the fermentation rate is slow, enterobacteria may not be suppressed and clostridia may also grow. Products other than lactic acid are produced, and silage pH may not decrease sufficiently to produce a stable silage. As a result of continued proteolysis, substantial amounts (250–750 g kg^{-1}) of forage proteins are converted to amino acids and peptides. In silages where enterobacteria and clostridia proliferate, amino acids are deaminated

to NH_3 and decarboxylated to amines, further reducing the nutritive value of the silage.

Anaerobic Storage

If a pH low enough to inhibit the growth of LAB is achieved, then a silage is normally considered stable. However, changes may still take place. Yeasts may ferment excess sugars to ethanol; LAB may convert lactate to other products if the sugar supply is exhausted. In addition, no silo is completely leak-proof, and aerobic metabolism will take place at sites of O_2 entry, allowing microorganisms, including potential pathogens, to survive.

Aerobic Feed-Out Phase

When the silo is opened for feeding, an inevitable aerobic phase ensues. After a variable time period, aerobic microbial growth takes place. Organisms, mainly yeasts and acetic acid bacteria that tolerate acid conditions, proliferate. These organisms metabolize sugars, lactic acid, and other acids to CO_2, water, and heat. In fact a rise in silo temperature is the most reliable indicator of aerobic deterioration. Utilization of acids also leads to a rise in silage pH, allowing a succession of other microbial species such as molds and bacilli to develop. Protein is catabolized to NH_3 that also contributes to the rise in pH. Molds and other organisms can degrade cell-wall carbohydrates, leading to decreased nutritional value as well as producing toxic substances that can render silage inedible.

Plant Carbohydrates

Carbohydrates are the major source of energy for ruminants feeding on forages. From a nutritional point of view, this potential energy is typically categorized into soluble or insoluble carbohydrates, implying their availability for utilization. Although this is a suitable operational definition, it lacks specificity and is dependent upon the reagents used to solubilize the carbohydrates in question. Gross solubility is an appropriate way to evaluate available carbohydrates for primary fermentation during the initial stages of the ensiling process, in that one has little concern over the actual source of the carbohydrate, just their availability to microbes. However, this does not provide clear information as to the pools the carbohydrates were derived from within the plant. To understand the relationships of available carbohydrates to specific processes (e.g., primary fermentation to produce high quality silage, rumen degradation for energy), it is helpful to consider the relationships among carbohydrate pools within the plant.

Carbohydrate Pools within the Plant

Plant carbohydrates can be categorized on the basis of their roles within the plant (Fig. 3–1), independent of the physical and chemical properties of the individual carbohydrates. The simplest method divides carbohydrates into structural and nonstructural functions. Structural carbohydrates are those polysaccharides that

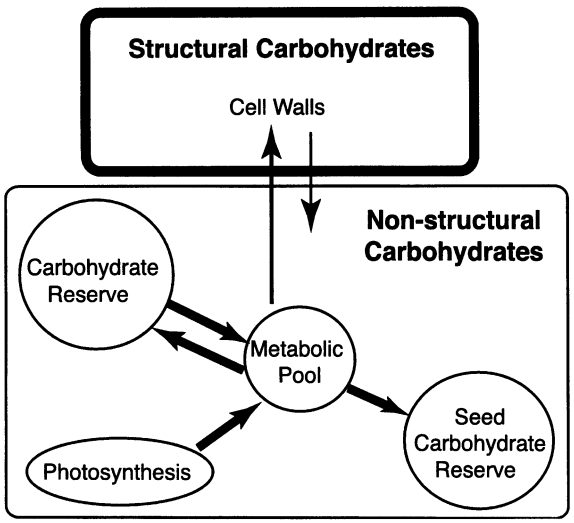


Fig. 3–1. Potential carbohydrate pools available within forages at the time of harvest.

make up the total cell wall (matrix and cellulosic macrofibrils) and function in providing structural integrity to the plant. At the time of harvest, structural carbohydrates represent the largest pool within forages and are composed of polysaccharides. Nonstructural carbohydrates are all other carbohydrates, including activated monosaccharides (the building blocks for all other carbohydrates), transport sugars (usually disaccharides), reserve carbohydrates, and storage carbohydrates (polysaccharides) (Fig. 3–1). The flux to and through these pools at the time of harvest is critical for establishing the final potential benefit to the animal.

Monosaccharides—The Building Blocks for All Complex Carbohydrates

The smallest unit, outside of metabolic intermediates (tricarboxylic acid cycle and glycolysis), are the monosaccharides (Fig. 3–2), from which all other complex carbohydrates are derived. Biosynthesis of individual monosaccharides is predominantly through the interconversion of activated glucose (nucleoside diphosphates) derived directly from photosynthetic activity or from the hydrolysis of reserve carbohydrates (i.e., sucrose, starch, or fructans) (Feingold & Avigad, 1980). Limited amounts of monosaccharides are present within the plant at any one time and are generally found as activated sugars. Flux through this pool can be quite high, especially during times of high metabolic activity.

Plant monosaccharides are classified according to the type of functional group they contain. Most contain an aldehyde group and are referred to as aldoses (e.g., glucose, galactose, xylose), but there are important ketone-containing sugars or ketoses, most notably fructose. The monosaccharides exist almost exclusively as cyclic molecules through the formation of hemiacetals or hemiketals. This cyclization occurs through the reaction of the aldehyde group with an open hydroxyl. In the case of glucose, it is a reaction of C1 (aldehyde group) with the hydroxyl on C5 forming a stable six-member ring (pyranose form). In the case of fructose, a ke-

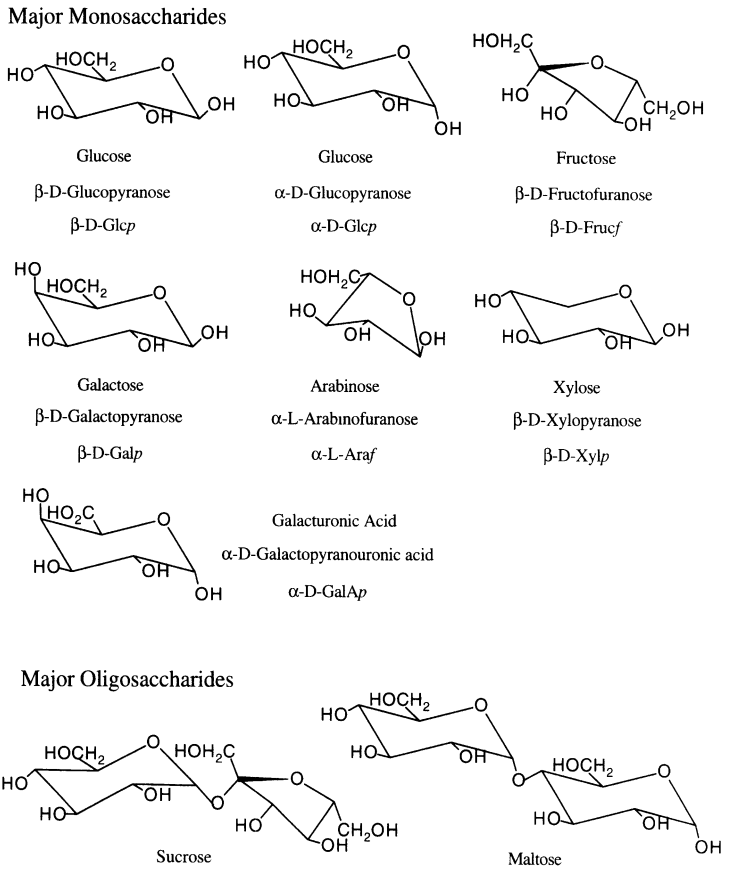


Fig. 3–2. Structural characteristics of the major monosaccharides and two of the predominate oligosaccharides found in forages.

tone sugar, the C2 (ketone group) reacts with the C5 hydroxyl to form a five-member ring (furanose form) (Kennedy & White, 1983). These ring structures provide an additional chiral center (C1) resulting in two distinct anomers (e.g., α-glucose and β-glucose differing only in configuration about C1; Fig. 3–2).

Monosaccharides are combined to produce more complex structures through the formation of glycosidic linkages, involving the C1 (C2 in the case of fructose) of one sugar and any hydroxyl on another sugar. The linkages formed will be anomeric specific (α or β) and result in the protection of the functional group from oxidation or reduction, hence the formation of a nonreducing end. Higher-order structures are made by the attachment of additional sugars. The potential for highly complex structures is enormous considering the number of potential glycosidic linkage sites (free hydroxyls) and the anomeric configuration at C1 or C2 (in the case of fructose) for each linkage type. In plants, this complexity is generally restricted to a few general linkage patterns for most oligosaccharides and polysaccharides, although pectic polysaccharides can be quite complex (Bacic et al., 1988; Carpita, 1997).

Table 3–1. Some of the common oligosaccharides identified in plants.

Trivial name	Composition	Function	Plant distribution
Sucrose	α-D-Glcp-(1→2)-β-D-Frucf	Storage/transport	Ubiquitous
Raffinose	α-D-Galp-(1→6)-α-D-Glcp-(1→2)-β-D-Frucf	Storage/transport	Widespread
Stachyose	α-D-Galp-(1→6)-α-D-Galp-(1→6)-α-D-Glcp-(1→2)-β-D-Frucf	Storage/transport	Widespread
Verbascose†	α-D-Galp-(1→6)-α-D-Galp-(1→6)-α-D-Galp-(1→6)-α-D-Glcp-(1→2)-β-D-Frucf	Storage/transport	Limited
6-Kestose‡	α-D-Glcp-(1→2)-β-D-Frucf-(6→2)-β-D-Fruc	Storage	Limited
1-Kestose‡	α-D-Glcp-(1→2)-β-D-Frucf-(1→2)-β-D-Frucf	Storage	Limited
Neokestose‡	α-D-Frucf-(2→6)-α-D-Glcp-(1→2)-β-D-Frucf	Storage	Limited

† Higher homologs with additional Galp residues have been found in plants.
‡ These oligosaccharides are typically found in conjunction with polymeric fructans. They may represent degradation intermediates.

Oligosaccharides

Two or more (up to 10) monosaccharides linked together are referred to as oligosaccharides (Kandler & Hopf, 1980). This is a bit of an arbitrary definition to draw a distinction between oligosaccharides and polysaccharides. In most cases naturally occurring oligosaccharides that are not intermediates of polysaccharide degradation are generally two to five residues in length. Although there are a number of different oligosaccharides that have been isolated from various plants (Table 3–1), sucrose is by far the most abundant and important in forage plants.

Sucrose is a disaccharide formed by a glycosidic linkage between anomeric carbons on glucose (C1) and fructose (C2), thus forming a nonreducing oligosaccharide. Sucrose has three critical roles in plants as: (i) the principal product of photosynthesis, (ii) the major form of carbohydrate translocation within plants, and (iii) a major form of carbohydrate storage (Kruger, 1997). As the major form of carbohydrate translocation within plants, sucrose also functions as the primary donor for the synthesis of starch and fructans. Recent evidence also suggests it is a direct donor to cellulose synthesis through the action of a membrane-bound sucrose synthase that feeds UDP-glucose directly to the cellulose synthase complex (Amor et al., 1995). Many of the other oligosaccharides found in forage plants, particularly the temperate grasses, are fructan oligosaccharides and may represent intermediates in the synthesis of higher degree of polymerization (DP) fructans or degradation products from the hydrolysis of fructan polymers (Livingston et al., 1993).

Polysaccharides

Polysaccharides generally refer to carbohydrates that are products of one or more monosaccharides with a high DP (>100 monomers). The types of polysaccharides found in plants include both structural and nonstructural carbohydrates that are sources of nutritional energy for ruminants. They may also be a source of energy to fermentative bacteria during the ensiling process. All polysaccharides can be divided into two general groups, homo- and heteropolymers. There are reports of various true homopolymers (one type of monosaccharide and one type of gly-

cosidic linkage) in plants, but these are rare (Stephen, 1983). The exception is cellulose (glucose-based polymer linked β-1,4), the most abundant plant polysaccharide which constitutes 30 to 70% of the total cell wall (Stephen, 1983).

By far the majority of polysaccharides fall into the heteropolymer group (Fig. 3–3 and 3–4). Unlike homopolymers, heteropolymers contain combinations of different monosaccharides and/or glycosidic linkages. For example, starch is similar to cellulose being composed completely of glucose units and primarily linked 1,4 with branch points on the C6 of glucose in the main backbone (Fig. 3–4). The critical difference between cellulose and starch, aside from the occasional branching of the backbone, is the anomeric configuration about the glycosidic linkages. Starch always contains α anomers that confer a concentric (bending) conformation, while cellulose contains β anomers that confer a straight microfibrillar conformation. All structural polysaccharides (Fig. 3–3) that make up the matrix of the cell wall fall into the heteropolymer group. Therefore, enzymatic degradation of polysaccharides to monosaccharides requires a wide range of hydrolytic activities. Hydrolases are monosaccharide and linkage specific, and therefore the complexity of the structure being degraded must be matched by the complexity of the hydrolytic activities.

Structural polysaccharides are generally divided into three classes: pectic, hemicellulosic, and cellulose. Matrix polysaccharides (pectic and hemicellulosic) are traditionally classified according to the extraction procedures used during their isolation. It should be stressed that this type of classification provides a general framework for reference but should not be considered to represent discrete and finite groups of polysaccharides. Such schemes reflect commonality in physical properties of polysaccharides, not necessarily common chemical compositions (Wilkie, 1985; Bacic et al., 1988). Classification requires a combination of extraction, fractionation, and compositional analysis. This is particularly important when comparing polysaccharide groups before and after partial enzyme degradation or prolonged storage (ensiled forages). Partial degradation of a particular class of polysaccharides may alter their physical properties, including solubility in a given extraction medium. All forages contain polysaccharides that fall into these classes, although the proportion of each varies, particularly when comparing legumes and grasses (Table 3–2).

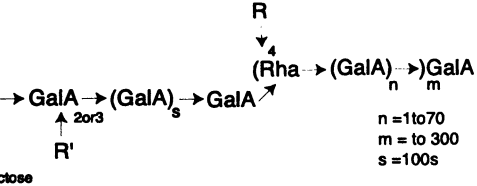
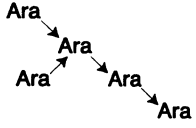
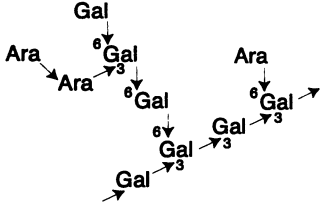
Role of Carbohydrates in Ensiling Process

The subject of carbohydrate metabolism as forages go from the field to the silo has been extensively reviewed elsewhere (McDonald et al., 1991) and will be covered briefly later. In this section, our attention will focus on the potential to improve the utilization of carbohydrate pools within the plant in order to produce a better silage, in the sense of improved preservation and improved nutritional value to the animal.

Available Carbohydrates

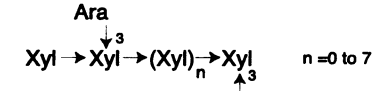
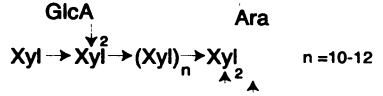
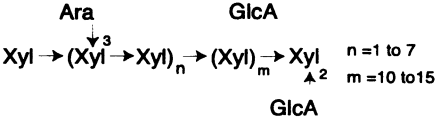
In terms of the ensiling process, it is best to partition carbohydrates according to their water solubility (from the nonstructural carbohydrate pool). Carbohy-

Structural Polysaccharides

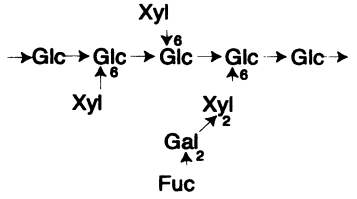
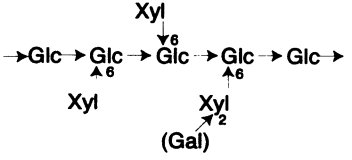
	Backbone Linkages	Major Branch Point Linkages	Major Linkage Patterns
Pectic Polysaccharides			
Rhamnogalacturonans	α-D-Gal*p* A (1→4)-α-D-Gal*p* A **and** α-D-Gal*p* A (1→2)-β-L-Rha*p* (1→4)-α-D-Gal*p* A	α-L-Ara*f* (1→4)-β-L-Rha*p* β-D-Gal*p* (1→4)-β-L-Rha*p*	
Arabinan	α-L-Ara*f* (1→5)-α-L-Ara*f*	α-L-Ara*f* (1→3 or 2)-α-L-Ara*f*	
Arabinogalactans			
Type I	β-D-Gal*p* (1→4)-β-D-Gal*p*	α-L-Ara*f* (1→3 or 2)-β-D-Gal*p* α-L-Ara*f* (1→5)-α-L-Ara*f* (1	
Type II	β-D-Gal*p* (1→3)-β-D-Gal*p* β-D-Gal*p* (1→6)-β-D-Gal*p*	α-L-Ara*f* (1→6)-β-D-Gal*p* α-L-Ara*f* (1→3)-β-D-Gal*p*	

Hemicellulosic Polysaccharides

Xylans

Arabinoxylans	β-D-Xyl*p* -(1→4)-β-D-Xyl*p*	α-L-Ara*f* - (1→2or3)-β-D-Xyl*p*	 n =0 to 7
Glucuronoxylans	"	α-D-Glc*p* A-(1→2)-β-D-Xyl*p*	 n =10-12
Glucuronoarabinoxylans	"	Both Ara*f* and GlcA*p* A	 n =1 to 7 m =10 to15

Xyloglucans

Dicot	β-D-Glc*p* -(1→4)-β-D-Glc*p*	α-D-Xyl*p* -(1→6)-β-D-Glc*p* β-D-Gal*p* -(1→2)-β-D-Xyl*p* α-L-Fuc*p* -(1→2)-β-D-Gal*p*	
Monocot	β-D-Glc*p* -(1→4)-β-D-Glc*p*	α-D-Xyl*p* -(1→6)-β-D-Glc*p* β-D-Gal*p* -(1→2)-β-D-Xyl*p* (minor branch sugar)	

Mixed linkage β-D-Glucans

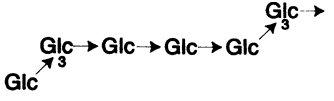
β-D-Glc*p* -(1→4)-β-D-Glc*p* and β-D-Glc*p* -(1→3)-β-D-Glc*p*	None	

Fig. 3–3. Major structural polysaccharides, including the composition and linkage characteristics.

Non-Structural Polysaccharides

	Backbone Linkages	Major Branch Point Linkages	Major Linkage Patterns
Starch			
	α-D-Glc*p* -(1→4)-α-D-Glc*p*	α-D-Glc*p* -(1→6)-α-D-Glc*p*	amylose Glc→Glc→(Glc)$_n$→Glc, n = 200 to 300
			amylopectin Glc→Glc(→Glc)→(Glc)$_n$→Glc, n = 10 to 30
Fructans			
	α-D-Glc*p* -(1→2)-β-D-Fruc*f* (Initiation point of fructan synthesis)		Fruc(→Fruc)(→Fruc)(→(Fruc)$_n$)→Fruc←→Glc, n = highly variable
	β-D-Fruc*f* -(2→1)-β-D-Fruc*f*	β-D-Fruc*f* -(2→6)-β-D-Fruc*f*	
	β-D-Fruc*f* -(2→6)-β-D-Fruc*f*	β-D-Fruc*f* -(2→1)-β-D-Fruc*f*	(Fruc)$_n$→Fruc(→Fruc)→Fruc←→Glc←→Fruc, n = highly variable
			(Fruc)$_n$→Fruc(→Fruc)→Fruc←→Glc←→Fruc(→Fruc(→(Fruc)$_n$)→Fruc), n = may or may not be branched

Fig. 3–4. Nonstructural polysaccharides, including the composition and linkage characteristics.

Table 3–2. Typical concentration ranges for structural polysaccharides found in herbage species ensiled.†

Crop	Pectic polysaccharides	Hemicellulosic polysaccharides	Cellulose
	g kg^{-1} DM‡		
Perennial ryegrass	10–20	160–240	190–260
Barley	20–60	195–210	180–200
Corn	10–20	145–180	206–232
Alfalfa	90–100	60–120	120–330

† From Bailey (1973), Galliard (1966).
‡ DM, dry matter.

drates within the water soluble carbohydrate (WSC) group are the most critical in providing sufficient fermentable material to the LAB to produce a high quality silage. Although WSC is a practical operational definition describing carbohydrates important for ensiling, not only is the water solubility of carbohydrates important, but also their availability (i.e., are they readily metabolized by the microbes). The lack of hydrolytic activity towards complex carbohydrates by common LAB, therefore, requires the presence of substantial amounts of readily fermentable sugars (i.e., monosaccharides and small oligosaccharides).

The availability of monosaccharides and oligosaccharides for LAB depends on environmental influences on the plant at the time of harvest and immediately thereafter. Utilization of available monosaccharides and oligosaccharides (really only sucrose in most forages) will continue through plant respiration. Depending on the environmental conditions and harvest practices, the fermentable carbohydrate available to LAB for fermentation may be insufficient to produce a high quality silage. Opportunities for improving the fermentation process are dependent upon the carbohydrate in other pools that can be rendered available for rapid fermentation. Nonstructural polysaccharides (both carbohydrate reserves and seed storage carbohydrates) and structural polysaccharides can potentially be used to provide quantities of monosaccharides to increase fermentation. The challenge is to convert these pools, at least partially, to fermentable substrates in a timely fashion to produce desirable fermentation characteristics.

Hydrolytic Enzymes Necessary to Degrade Complex Carbohydrates

Enzymes are required to convert polysaccharides or complex oligosaccharides to fermentable sugars. The type of enzyme activity needed depends on the specific polysaccharides and/or oligosaccharides that are available for degradation. A general discussion of hydrolytic enzyme activity may provide a common ground from which to view the problems and potential solutions. There are four general classes of enzymes—endo-, exo-, glycosidase, and debranching hydrolases—that are important in the degradation of complex carbohydrates (Fig. 3–5) (Matheson & McCleary, 1985). Although some hydrolases have similar outcomes (e.g., exo-β-1,4-D-glucanase and exo-α-1,4-D-glucanase both release glucose monomers), every hydrolase has a high specificity for the anomeric configuration of the glycosidic linkage being cleaved. In some cases the enzymes have similar hydrolytic patterns. For example, exoglycanases and glycosidases both release nonreducing terminal sug-

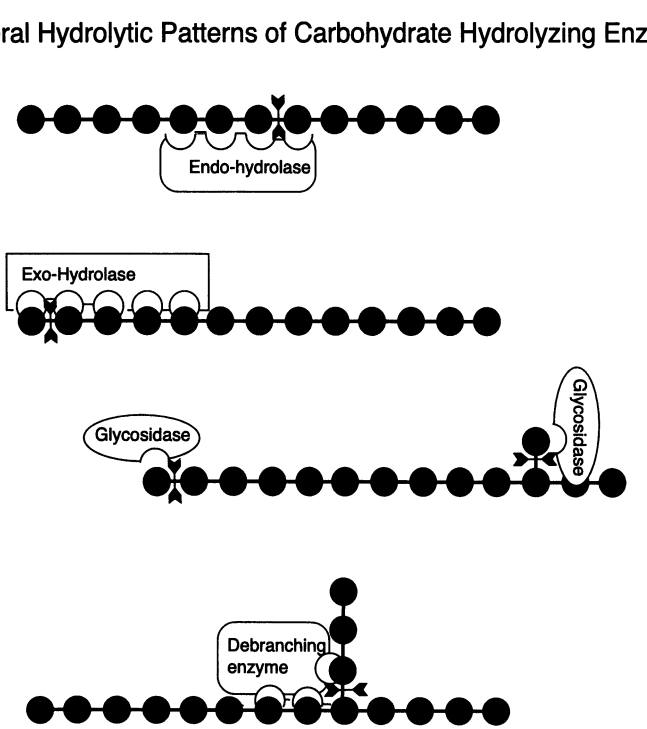


Fig. 3–5. Major types of hydrolytic enzymes involved in the degradation of complex carbohydrates.

ars from polysaccharides and oligosaccharides. They differ in their binding and hydrolytic kinetics. Exo-type enzymes prefer to bind to four- to six-sugar residues, but only the terminal glycosidic linkage is hydrolyzed. Glycosidases also release only the terminal sugar but do not need to bind to the remaining unhydrolyzed sugars. Therefore, glycosidases have a preference for small oligosaccharides, while exohydrolases prefer polysaccharides. Glycosidases can also hydrolyze single branch sugars from polysaccharide backbones (e.g., β-D-galactosidase will remove the single β-D-galactose residues that are branches on oligosaccharides or polysaccharides). There are important exoglycanases that are capable of releasing oligosaccharides, notably the common enzymes β-amylase and exocellobiohydrolase. In the case of β-amylase, hydrolytic activity is a stepwise process starting at the nonreducing end of starch and hydrolyzing alternate linkages releasing maltose due to anomeric conversion during hydrolysis. Exocellobiohydrolase releases cellobiose units.

Endoglycanases generally require stretches of unsubstituted sugar residues for the enzyme to bind and hydrolyze the appropriate glycosidic linkage. Some enzymes are capable of cleaving polysaccharides to relatively small oligosaccharides (DP 2-4), while others have a minimum binding requirement of greater than six residues. The rate of hydrolytic activity decreases with the size of binding domain (i.e., the smaller the oligosaccharide the slower the reaction). Debranching enzymes are special in that they are capable of removing whole oligosaccharides that are attached to polysaccharide backbones (e.g., α-1,6-hydrolases that cleave the 1,6 linkage in amylopectin).

Nonstructural Carbohydrates

It would appear that LAB can only metabolize simple sugars and a few disaccharides (sucrose and maltose). Therefore, to use the complex carbohydrates in other pools (Fig. 3–1) requires appropriate and sufficient hydrolytic activity to provide suitable substrates. The reserve complex carbohydrates, starch and fructans, are subject to metabolic turnover during plant development, indicating that the necessary enzymatic machinery should be available within the plant to hydrolyze these carbohydrates to suitable substrates. Degradation of starch in vegetative parts of plants is not as well understood as the metabolism of seed storage starch during germination (Kruger, 1997). In vegetative tissues, starch degradation apparently requires coordination of hydrolytic and phosphorolytic pathways (Kruger, 1997). No work has investigated the degradation of starch, at the enzyme level, during the initial stages of forage ensiling. It is clear that starch metabolism does occur, as levels in fresh herbage are higher than after ensiling. It is also apparent that in most cases there are significant amounts of vegetative starch remaining in silages at the time of feed-out, indicating that normal degradation has been curtailed. These results suggest that at least in those forages that store starch (warm-season grasses and legumes) vegetative starch represents a viable pool that could provide additional simple carbohydrates to assist in adequate fermentation.

If the plant hydrolytic activity is insufficient to degrade this starch, the alternative is to supply the necessary enzymes at the time of harvest or ensiling. Addition of commercial enzymes is always a possibility. However, this is usually not an economically favorable alternative. Common species of *Lactobacillus* available at ensiling (natural and added inoculations) lack amylolytic activity. However, recent work (Fitzsimons et al., 1994) has demonstrated that the α-amylase gene from *L. amylovorus* could be successfully cloned into a competitive inoculant strain of *L. plantarum*. The transformed *L. plantarum* could survive on starch-based media and expressed high levels of amylase activity. Such organisms should provide benefits when ensiling forage legumes or warm-season grasses as the metabolic pool of usable carbohydrates would be expanded beyond the simple sugars and sucrose (i.e., WSC).

For temperate grasses the situation can be approached in a similar manner. However, the reserve carbohydrates are fructans (Pollock & Cairns, 1991). Plant degradation of fructans appears to be limited to a single type of enzyme, fructan exohydrolase, that cleaves the terminal fructosyl residue from fructans. Although most species of *Lactobacillus* found in silage lack fructan hydrolyzing activity, there are some species that are capable of degrading fructans.

Merry et al. (1995) conducted experiments to test the advantage of added fructan degradation capabilities, found in *L. casei* subsp. *paracasei*, during the ensiling process for perennial ryegrass (*Lolium perenne* L.). Their results clearly show that microbial fructan hydrolases increased the degradation of fructans compared with sterile herbage ensiled without inoculation or which were inoculated with LAB strains that lacked fructan hydrolases. There was a small improvement in the initial rate of fermentation over normal plants that relied on native populations of LAB. Interestingly, the rate of fructan degradation in normal plants was the same as the material inoculated with *L. casei*. Sterile plants alone (sterilized by irradiation to

preserve plant enzymatic activity) had a much slower rate of fructan degradation. The fructan exohydrolase activity was not determined in the plants before and after irradiation to determine if there had been adverse effects of the sterilization procedure. This would seem to indicate that there are enzyme mechanisms in place to degrade fructans to fructose, whether from the plant or native strains of LAB. The advantages of additional fructan degradation may only be realized under conditions of limited readily fermentable carbohydrate (monosaccharides and disaccharides).

Structural Carbohydrates

Structural carbohydrates represent a potential, but much less accessible, source of fermentable carbohydrate. Although plants produce cell-wall hydrolases, they are generally restricted to specific tissues or organs (e.g., leaf abscission zones, fruits) or to the primary wall and functional only during wall expansion (Carpita, 1997; Fry, 1985). There is little turnover of cell-wall polysaccharides in plant leaves and stems. To utilize this pool of carbohydrate for LAB fermentation requires the addition of hydrolytic enzymes. Because of the complexity of the polysaccharides within the wall matrix (Fig. 3–3), it is difficult for a single enzyme to provide sufficient monosaccharides to benefit LAB fermentation. The complexity within the wall also creates a problem with enzyme accessibility (i.e., a readily degradable substrate cannot be degraded if the enzyme does not have access to it). This cell-wall complexity, both in polysaccharide composition and organization, hampers the development of approaches that are successful for reserve polysaccharides such as starch and fructans.

Studies in which cell-wall hydrolytic enzymes were added at ensiling to increase the WSC pool during ensiling have produced mixed results (El Hag et al., 1982; Huhtanen et al., 1985; Rauramaa, et al., 1987; Muck, 1993; Stokes & Chen, 1994; see also Chapter 7, Kung et al., 2003). If the forage is of good quality going into the silo, little advantage seems to be gained from additional enzyme treatment. In other cases there does seem to be some improvement in silage quality, particularly in forages with limited WSC. The variable results obtained from the addition of hydrolases may be due in part to the types of enzyme activities added to the ensiled material. A complex mixture of hydrolases or ones that contain amylolytic activity would be more likely to provide the necessary sugars for fermentation in a timely fashion.

Changes in Carbohydrate Pools (Mostly Cell Walls) During Long-Term Ensiling

The long-term impact of ensiling on carbohydrate pools depends upon the type of forage and ensiling conditions. From a nutritional point of view, it is important to conserve as much of the carbohydrate as possible to provide the ruminant with energy. Realistically, only the reserve, storage, and wall polysaccharides remain as major potential energy sources. Small amounts of monosaccharides and oligosaccharides measured after long-term ensiling are due to continued hydrolytic activity either from low levels of enzymatic activity or due to the acidic conditions within the silo. Degradation of fructans would be most notable as the fructosyl linkages

are highly susceptible to acid hydrolysis. An exception to the above is the considerable amounts of fermentable sugar, measured as WSC, which can be present in silage resulting from the preservation of wilted temperate grass in which the extent of fermentation is restricted (McDonald et al., 1991). There is little information concerning the composition of this residual WSC, although given the acid lability of fructans noted above and the activity of plant fructan hydrolases at pH 5 and above in silage (Winters et al., 1998), it is likely that a substantial proportion of fructan will have been hydrolyzed to hexose sugars. Changes have also been noted in the cell-wall fraction of ensiled grasses and legumes (Jones et al., 1992; Morrison, 1979; Morrison, 1988). Most of the significant changes that occurred in the pectic and hemicellulosic fractions could be traced to changes in arabinosyl residues. Arabinosyl side branches are predominately in the furanose form, and therefore their linkage to the main backbone (e.g., xylan or galactan) is susceptible to acid hydrolysis even under weak acid conditions (Jones et al., 1992). Cell-wall hydrolases used as silage additives will result in significant changes in the structural polysaccharide pools.

SUGAR METABOLISM

Introduction

Essentially, ensiling is the anaerobic fermentation of crop sugars by the microflora present on the ensiled crop. The microflora metabolize sugars to obtain energy for growth and release acid and neutral end products. In the majority of silage fermentations, the substrates are fructose and glucose. Concentrations of these sugars at the time of harvest are quite variable and depend on environmental conditions and the type of forage (Chapter 5, Buxton & O'Kiely, 2003). Once cut, however, total carbohydrate concentration tends to decrease. Fructose and glucose concentrations can only be increased by hydrolysis of polysaccharides (see above) and to a limited amount by photosynthesis. There is usually a pronounced decrease in these sugars because of continued plant respiration and microbial fermentation. Alternative energy sources for the silage microflora may be pentose sugars produced by the hydrolysis of matrix polysaccharides (pectic and hemicellulosic) by endogenous or exogenous enzymes, organic acids present in the ensiled crop, or amino acids resulting from the hydrolysis of crop proteins. The end products arising from fermentation of hexoses, pentoses, and organic acids by the different classes of bacteria and by yeasts will be outlined in this section. Factors that contribute to variations in the type and amount of end products formed will then be discussed. Changes in the nitrogenous components of ensiled crops will be discussed later.

Sugar Concentrations in Forage Crops

The monosaccharides glucose and fructose and the disaccharide sucrose are present in all forage crops. In addition there are small amounts of oligosaccharides present in aqueous extracts made from most forages. Temperate grasses and barley (*Hordeum vulgare* L.) among the cereals contain fructans. Quantitatively, the

Table 3–3. Constituents of nonstructural carbohydrate with typical concentration ranges found in herbage species ensiled.†

Crop	Total	Glucose	Fructose	Sucrose	Fructan	Starch
	g kg^{-1} DM‡					
Perennial ryegrass	60–250	10–30	10–30	50–70	0–200	0
Barley	60–300	20–60	20–60	5–20	20–170	3–42
Corn§	188–418	50–60		70–80	--	68–278
Alfalfa	40–100	10–30	10–30	15–40	--	48–80

† Adapted from Smith (1973), McDonald et al. (1991).
‡ DM, dry matter.
§ For corn, glucose and fructose are together.

oligosaccharides raffinose, stachyose, and verbascose contribute only small amounts of substrate to the silage fermentation, whereas fructans can contribute a large proportion of the fermentable carbohydrate where they occur. Fermentable substrate in forage crops is routinely measured as WSC. This measure includes fructans, which are hydrolyzed by plant enzymes and the silage microflora, but excludes starch, which is largely non-water soluble and is not hydrolyzed by most silage bacteria. Water soluble carbohydrates are therefore usually a reliable index of substrate availability.

The composition of the WSC of forage crops commonly ensiled is shown in Table 3–3. Two points are apparent. First, all di-, oligo-, and polysaccharides measured, as WSC contain quantities of fructose equal to or greater than glucose. Hence, in most crops fructose is the principal source of hexose substrate. Secondly, there is more sucrose in most crops than the sum of glucose and fructose, the exception to this being barley. There is little evidence, however, that the rate of breakdown of sucrose to glucose and fructose is ever rate-limiting in the silage fermentation (Merry et al., 1995). Finally warm season grasses differ from temperate grasses in that they store their carbohydrate reserves as starch and not fructan and therefore have correspondingly lower WSC concentrations.

Respiration

Respiration and photosynthesis in the intact plant are mainly responsible for the synthesis and utilization of hexose sugars. When a forage crop is cut, the surface area exposed to light is reduced. When the crop is mown into a swath, only a small fraction of the area exposed to light in the standing crop remains and is potentially capable of photosynthesis. Rotz (1995), modeling losses during forage harvesting, summarized the literature on photosynthesis and concluded that while photosynthesis may continue for a few hours after mowing, only small increases in dry matter (DM) were likely. The extent of any increases in DM was related to weather conditions and the extent of exposure to light.

In plants, two different types of respiration exist (Stryer, 1988). In addition to its carbon-fixing carboxylase reaction, the chloroplast enzyme, ribulose 1,5-bisphosphate carboxylase (rubisco), has an oxygenase activity. The carboxylase (light dependent) and oxygenase (light independent) activities compete for the same active site, and the rates of the two reactions depend on the concentrations of CO_2 and

O_2 in the active site. The carboxylase results in the synthesis of two molecules of 3-phosphoglycerate from ribulose 1,5-bisphosphate from which one molecule of a hexose sugar can be synthesized via gluconeogenesis. However, the oxygenase reaction generates from ribulose 1,5-bisphosphate one molecule of 3-phosphoglycerate and one molecule of phosphoglycolate. The carbon skeletons of two phosphoglycolate molecules are scavenged via conversion to glyoxylate and eventual synthesis of one molecule of serine with the loss of one molecule of CO_2. This CO_2 production is termed *photorespiration*. Although energetically a seemingly wasteful process, photorespiration has been shown recently to act as a means of protecting plants from photooxidation (Kozaki & Takeba, 1996). In relation to wilting and ensiling, photorespiration is probably of little importance since it is a light-dependent process.

Dark or mitochondrial respiration in plants is similar to other species. In the presence of O_2, substrate, primarily hexose sugar, is converted first to pyruvate by the glycolytic pathway and then to CO_2 via the tricarboxylic acid cycle. The energy released is captured as ATP either directly by substrate level phosphorylation or via the electron transport chain. The energy not captured as ATP is released as heat. Heat is easily dissipated from wilting crops but is trapped in ensiled forage when the silo is sealed. In addition, when the crop is harvested, few biosynthetic reactions take place, and therefore ATP formed by respiration will be used by the plant in attempts to maintain homeostasis. This energy is also released as heat. In the presence of O_2, the results of plant respiration for the wilting and ensiled crop can be summarized as:

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O + 0.69 \text{ Mcal mol}^{-1} \text{ glucose}$$

The consequences for the ensiling process are a decrease in the amount of substrate available for fermentation and, in the silo, a rise in temperature. The extent of temperature increases in the silo has been described by Pitt et al. (1985). Temperatures of up to 40°C have been noted in grass silage (McDonald et al., 1966). The extent to which temperature is increased in the silo has consequences for the bacteria that become dominant during the silage fermentation as clostridia and enterobacteria have a higher optimum temperature for growth than LAB. McDonald et al. (1966) found that ryegrass silage incubated at 42°C produced a clostridial fermentation, whereas at 27°C silage dominated by LAB was the result.

Factors Affecting the Rate of Respiration

Factors that influence aerobic plant cell respiration are temperature, moisture content, and pH. In the wilting crop, the temperature in the swath will remain close to ambient. Any increases in temperature will increase the rate of respiration but not its extent, as temperature is unlikely to become high enough to inactivate enzymes. Similarly the pH of wilting material does not vary markedly and consequently will have little effect upon respiration. The moisture content of the wilting crop thus has the greatest effect on respiration rate. There is agreement that respiration rate decreases with decreasing moisture content (Greenhill, 1959; Meidner, 1967) but does not cease until moisture contents below those at which crops are normally ensiled are reached (less than 400 g water kg^{-1}; see Chapter 6, Muck et al.,

2003). In practical terms, therefore, the more rapid the wilt, the lower are the respiration losses.

In the silo, the amount of O_2 trapped in the ensiled mass, its temperature, and its pH influence the extent of respiration. The amount of O_2 trapped in the silo is the most important factor. Heat produced by respiration increases the rate of respiration, but the extent of heat production is limited by O_2 availability. The acidity of the material also has an effect on the rate and extent of respiration. Acidification of the crop at ensiling with, for example, formic acid (Henderson et al., 1972) reduces the amount of respiration and therefore reduces temperature rise. This may in part be due to disruption of plant membranes.

Anaerobic Plant Cell Respiration

When the O_2 in the silo is exhausted, plant respiration can continue anaerobically. Since the respiratory chain is inhibited in the absence of O_2, NAD reduced to NADH during glycolysis must be regenerated using alternative H acceptors (Davies, 1980). The initial reaction is conversion of pyruvate to lactate, which lowers silage pH. At low pH, ethanol production then becomes important in the ensiled forage. Although lactic acid production is beneficial to the silage fermentation, the quantitative significance of its generation by anaerobic plant cell respiration is not clear. It is probable that cell rupture, associated first with chopping herbage and then with acidification, limits the amount of anaerobic respiration.

In summary, some plant cell respiration is inevitable during ensiling and consists of the utilization of hexose sugars and generation of heat. Prolonged wilting results in the greatest losses of fermentable hexose, while the effects of heat can be minimized by good compaction and rapid sealing of the silo to remove O_2.

Utilization of Hexoses by Silage Microorganisms

Production of acid in an anaerobic environment is critical for successful preservation by ensiling. However, as far as the microflora encountered in ensiling are concerned, acid production is simply a means of ensuring that oxidation and reduction (redox) are balanced during anaerobic metabolism of substrates to generate energy (ATP) for growth and carbon skeletons for biosynthesis. In practice redox balance is achieved by reoxidation of NADH. The genetic makeup of bacteria provides them with a wide variety of enzymes and therefore metabolic pathways with which to achieve redox balance. Indeed the ability to use different substrates and produce different end products is an important tool in bacterial classification. It must also be stressed that adaptive mechanisms allow individual strains of bacteria to utilize a variety of substrates and to produce different end products depending upon the environment in which they find themselves (e.g., pH, moisture content, redox potential, O_2 tension).

Glucose Metabolism

Four metabolic pathways can be used to catabolize glucose and other substrates under anaerobic conditions. The most important pathways, the Embden–Meyerhof and 6-phosphogluconate/phosphoketolase pathways, are illustrated

in Fig. 3–6. The pentose phosphate and Entner–Doudoroff pathways will be briefly described; the reader should consult standard biochemistry texts for a more complete description.

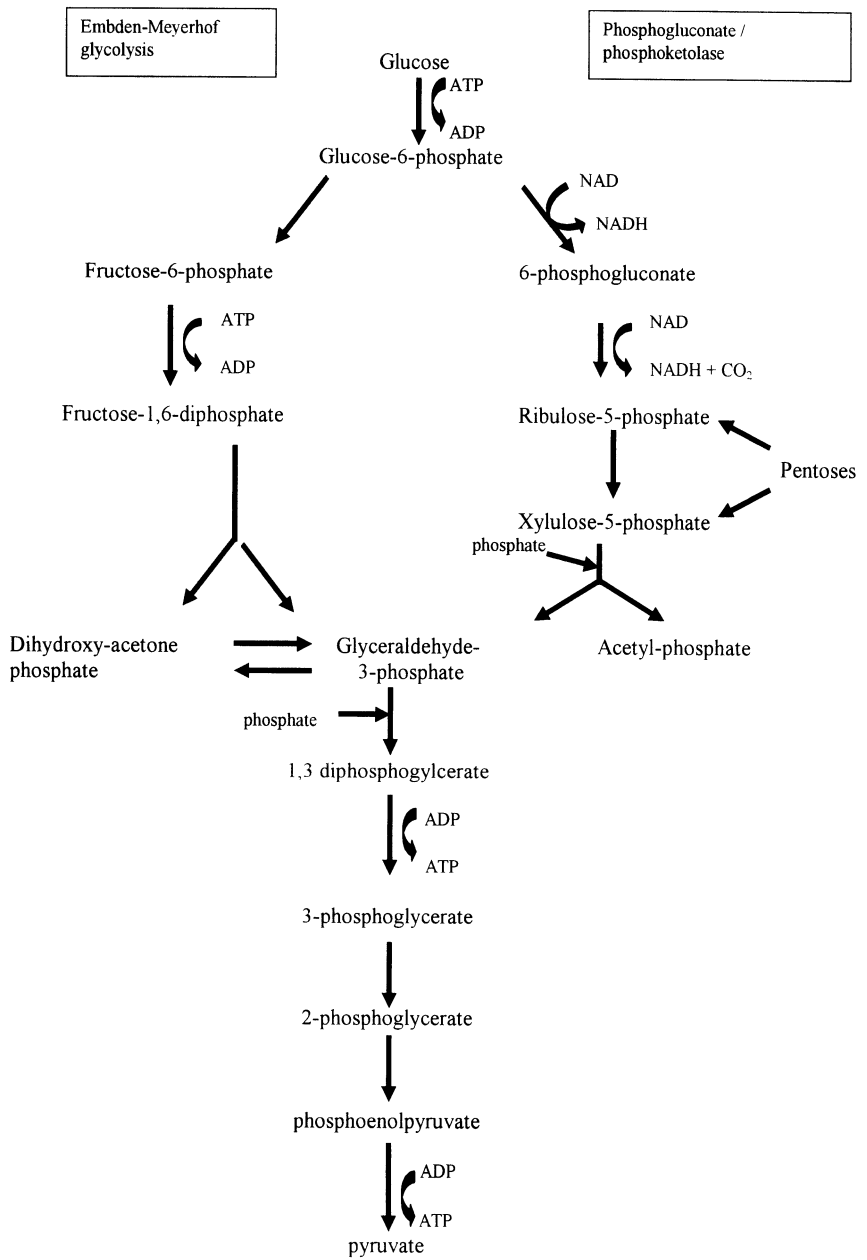


Fig. 3–6. Embden–Meyerhof glycolysis and phosphogluconate/phosphoketolase pathways for anaerobic metabolism of glucose, fructose, and pentoses. Glycolysis gives rise to two molecules of pyruvate from glucose, while one molecule of pyruvate and one molecule of acetyl phosphate are produced by the phosphogluconate/phosphoketolase pathway.

Embden–Meyerhof Glycolytic Pathway. In this, the classical glycolytic pathway, one molecule of glucose is converted to two molecules of pyruvate with the net synthesis of 2 mol of ATP (by substrate level phosphorylation) and 2 mol of NADH. The key enzyme is fructose bisphosphate aldolase, which catalyzes the hydrolysis of fructose 1,6-bisphosphate into two molecules of triose phosphate. In the presence of O_2, the pyruvate formed enters the tricarboxylic acid and is completely oxidized to CO_2 and water with the generation of further ATP via the electron transport chain. In anaerobic conditions, the pyruvate formed must be reduced to reoxidize NADH and thus maintain redox balance.

Phosphogluconate/Phosphoketolase Pathway. Metabolism of glucose via the phosphogluconate/phosphoketolase pathway yields one molecule of pyruvate, one molecule of acetyl phosphate, and one molecule of CO_2. After Embden–Meyerhof glycolysis this pathway is the most important in silage making and is the primary route for the catabolism of pentose sugars. Glucose 6-phosphate is reduced to 6-phosphogluconic acid and then decarboxylated to ribulose 5-phosphate, which is then cleaved by phosphoketolase to glyceraldehyde 3-phosphate and acetyl phosphate. Glyceraldehyde 3-phosphate metabolism proceeds as in glycolysis to yield ATP and pyruvate with the consequent need for reduction of pyruvate to regenerate NAD. The fate of the acetyl phosphate depends on the redox state of the cell. If no alternative electron acceptor is available, then NAD has to be regenerated, and ethanol is the end product. Otherwise an extra molecule of ATP can be generated by donation of the phosphate group from acetyl phosphate to ADP, and acetate is the end product. In bacteria where both glycolysis and phosphogluconate/phosphoketolase are functional, the phosphogluconate/phosphoketolase pathway is normally repressed. Thus pyruvate and acetyl phosphate are the main substrates that are reduced in order to regenerate NAD under anaerobic conditions.

Pentose Phosphate Pathway. The primary function of the pentose phosphate pathway is generation of NADPH for use in biosynthetic reactions. The key reaction is the first reaction catalyzed by glucose 6-phosphate dehydrogenase, which when coupled with lactonase and 6-phosphogluconate dehydrogenase gives rise to a molecule of ribulose 5-phosphate, CO_2, and two molecules of NADPH. Depending on the metabolic requirements of the cell, a series of interconversions catalyzed by transaldolase and transketolase enzymes give rise to a range of end products including pyruvate. As far as the end products of anaerobic fermentation are concerned, fermentation via pentose phosphate gives rise to pyruvate and CO_2.

Entner–Douderoff Pathway. The pathway is similar to the classical glycolytic pathway as it produces two 3-carbon sugars from glucose. However, it differs in that glucose 6-phosphate is reduced to 6-phosphogluconic acid and cleaved by a specific aldolase to glyceraldehyde 3-phosphate and pyruvic acid. In contrast to glycolysis, only one molecule of ATP is generated by substrate level phosphorylation and only one molecule of NADPH has to be reoxidized. The pathway was first described in *Pseudomonas* species and is probably of little consequence in bacteria present in during silage making. However, it is present in *Escherichia coli* (Conway, 1992) and may be the pathway for glucosamine metabolism in bacteria (Iwamoto & Imanaga, 1991).

Utilization of Pyruvate and Two Carbon Compounds

The microorganisms active during ensiling use a variety of different characteristic metabolic routes to reduce pyruvate in order to reoxidize NADH under anaerobic conditions. There are four main pathways used by the majority of bacteria. These pathways and their use by different microorganisms will be described. Finally, alternative metabolic pathways used by silage microflora when substrates apart from pyruvate are available for reduction will be discussed.

Lactate Dehydrogenase

Lactate dehydrogenase catalyzes the reduction of pyruvate to lactate with the reoxidation of one molecule of NADH to NAD (Fig. 3–7). In glycolysis, therefore, the reduction of the two molecules of pyruvate formed from glucose leads to redox balance. Lactate is an optically active compound and is present as the D+ form in

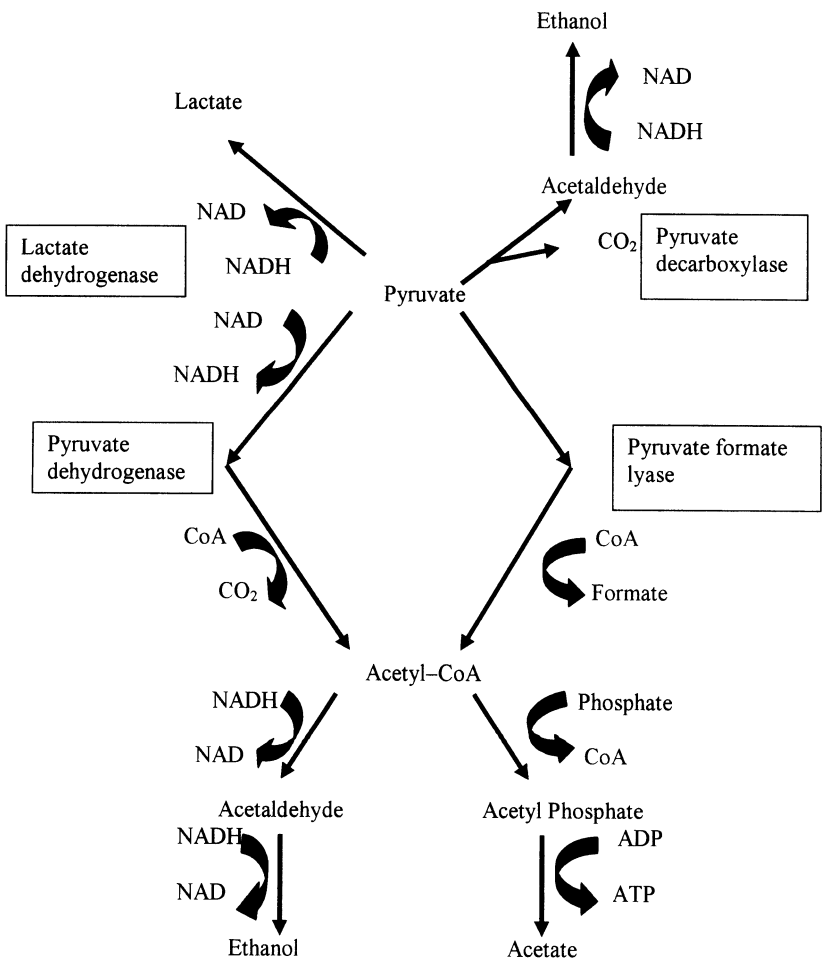


Fig. 3–7. Pathways for anaerobic metabolism of pyruvate during ensiling (adapted from Axelsson, 1998).

mammalian cells. This is not true for bacteria. Instead, the isomer of lactate formed is a characteristic of the bacterial species involved. For example, among the *Lactobacilli*, *L. plantarum* produces a mixture of D+ and L– lactate; *L. casei* produces L– lactate and *L. delbrueckii*, D+ lactate (Hammes & Vogel, 1995). The heterogeneity of lactate isomer production arises for two reasons; first, differences in the specificities of lactate dehydrogenases from different species and secondly the presence of a lactate racemase, which interconverts D+ and L– lactate (Hammes & Vogel, 1995). Since Gill et al. (1986) found that rumen bacteria could metabolize both isomers of lactic acid equally efficiently, the isomer of lactate produced in silage is unlikely to have nutritional consequences.

Production of Acetate and Ethanol from Pyruvate

Pyruvate may be metabolized by three independent mechanisms based on the key enzymes, pyruvate decarboxylase, pyruvate formate lyase, and pyruvate dehydrogenase.

Pyruvate Decarboxylase. In yeasts and some bacteria, pyruvate decarboxylase converts pyruvate to acetaldehyde via decarboxylation. The acetaldehyde is reduced to ethanol by alcohol dehydrogenase. Oxidation–reduction balance is maintained in catabolism of glucose to two molecules of ethanol.

Pyruvate Formate Lyase. Pyruvate formate lyase catalyses the formation of formate and acetyl phosphate from pyruvate using CoA. Formate produced by this mechanism usually dismutates to CO_2 and water. The acetyl phosphate may be further metabolized to yield ethanol or acetate as the final end product depending on the redox balance of the cell; if there is a need for NADH to be reoxidized, then acetyl CoA is first converted to acetyl phosphate and then reduced to acetaldehyde. Acetaldehyde is further reduced to ethanol by alcohol dehydrogenase. If redox balance permits, acetate is the end product with formation of an extra mole of ATP.

Pyruvate Dehydrogenase. Pyruvate dehydrogenase oxidizes pyruvate to acetyl CoA with the formation of an extra molecule of NADH and CO_2. The acetyl CoA can yield either acetate or ethanol as the end product of metabolism, depending on the redox balance of the fermentation.

Table 3–4 summarizes the end products from the main reactions involving pyruvate. The end products are dependent on substrate and pathway, but lactate, ac-

Table 3–4. Summary of anaerobic fermentation pathways encountered during ensiling and end products utilizing glucose as substrate.

Substrate	Pathway	NAD reduced	End products
		mol	
Glucose	glycolysis + pyruvate decarboxylase	2	2 ethanol + 2 CO_2
Glucose	glycolysis + pyruvate formate lyase	2	1 acetate + 1 ethanol + 2 CO_2
Glucose	glycolysis + pyruvate dehydrogenase	4	2 ethanol + 2 CO_2
Glucose	phosphogluconate/phosphoketolase	3	1 lactate + 1 ethanol + 1 CO_2
Glucose	glycolysis + lactate dehydrogenase	2	2 lactate
Pentose	phosphogluconate/phosphoketolase	1	1 lactate + 1 acetate

etate, and ethanol dominate. The pathways in which ethanol and acetate are produced from pyruvate involve loss of C as CO_2 and production of end products, which are either not acidic or have a higher pK_a than that of lactic acid (3.8 vs. 4.8 for acetic acid) and therefore do not acidify silage to the same extent.

Reactions Involving Condensation of Two Molecules of Pyruvate

Production of Butyric Acid Particularly by Clostridia. One molecule of butyrate is produced from two molecules of pyruvate (Fig. 3–8). The pyruvate may be derived either from glucose or lactate. The pyruvate is first decarboxylated to acetyl CoA with the production of H rather than NADH. Then two molecules of acetyl CoA condense to form acetoacetyl CoA, which is progressively reduced to butyrate. The reactions consume a molecule of NADH for each molecule of pyruvate and therefore achieve oxidation–reduction balance with one molecule of glucose or two molecules of lactate. The net result of butyrate production is one molecule of a weak acid from glucose. If lactate is the substrate for clostridia, then two molecules of a strong acid are converted into one molecule of butyric acid, and the pH in the ensiled mass will rise.

Other possible end products (Fig. 3–8) can arise from the acetoacetyl CoA produced from the condensation of two molecules of acetyl CoA. Acetoacetyl CoA may be decarboxylated to yield acetone, which may also be reduced to isopropanol. Alternatively, butyryl CoA can be further reduced to butanol, oxidizing a further two molecules of NADH if the redox state of the system allows it.

Production of Acetoin, Diacetyl, and 2,3-butanediol. Production of diacetyl is an important feature of dairy starter cultures as diacetyl is a flavor compound in cheese manufacture. The pathway is a feature of several species of LAB and of some enterobacteria. In lactococci (Hugenholz, 1993), acetolactate synthase catalyses the synthesis of acetolactate from two molecules of pyruvate (Fig. 3–8). Thiamine pyrophosphate (TPP) is a necessary cofactor for the reaction, which involves the formation of a hydroxyethyl-TPP intermediate. Acetolactate is decarboxylated to acetoin by acetolactate decarboxylase. Acetoin may be the end product of the reaction, or it may be reduced to 2,3-butanediol by acetoin reductase. It is now accepted that diacetyl is only formed under aerobic conditions when acetolactate spontaneously decarboxylates to diacetyl.

Lactic Acid Bacteria

The dominant pathways used by LAB are Embden–Meyerhoff glycolysis and phosphogluconate/phosphoketolase, which produce pyruvate and acetyl phosphate (from phosphogluconate/phosphoketolase) as end products. The bulk of pyruvate is reduced to lactate by lactate dehydrogenase, whereas the fate of acetyl phosphate depends on the initial substrate and redox state. If hexose is fermented, the acetyl phosphate is reduced to ethanol to maintain redox balance if no alternative electron acceptor is available. Fermentation of pentose sugars, however, yields acetate. Indeed, glucose metabolism by LAB is a major means of classifying LAB (Axelsson, 1998):

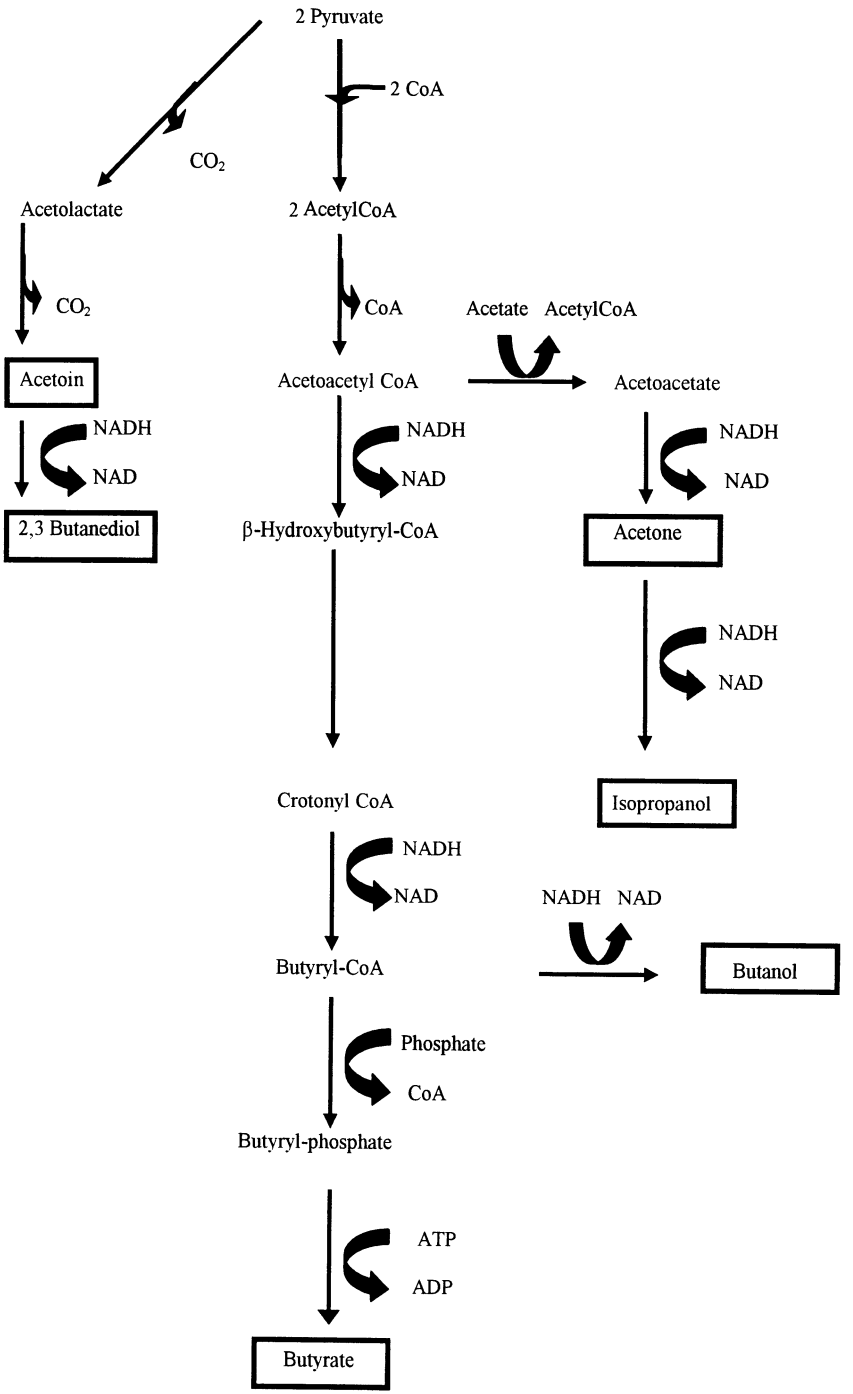


Fig. 3–8. Production of four-carbon and related compounds from pyruvate during ensiling.

1. Obligate homofermentative LAB ferment glucose via the glycolytic pathway and lack the ability to ferment pentoses; that is, they do not possess the phosphogluconate/phosphoketolase pathway.
2. Facultative homofermentative LAB ferment glucose primarily via the glycolytic pathway but also possess the ability to ferment pentoses; that is, they possess the phosphogluconate/phosphoketolase pathway.
3. Obligate heterofermentative LAB lack the glycolytic pathway and therefore ferment both glucose and pentose sugars by the phosphogluconate/phosphoketolase pathway.

Other Reactions of Lactic Acid Bacteria

The reactions described above cover quantitatively the most important reactions used by the LAB. However, there are other biochemical pathways that can have an important influence on the end products produced by LAB during the silage fermentation. They are:

1. Minor pathways for the fermentation of hexose by some species of LAB.
2. Metabolism of organic acids.
3. Metabolism of protein and amino acids (see section below on nitrogenous constituents).
4. Changes induced in fermentation pathways by the availability of alternative H acceptors.

Minor Pathways

In temperate grasses, the dominant hexose available for fermentation is not glucose but fructose because of the presence of fructans. Certain LAB, particularly the leuconostocs (Dellaglio et al., 1995), can reoxidize NADPH by reducing fructose to mannitol. The quantitative significance of this pathway is unclear as mannitol is rarely measured when silages are analyzed.

Isobutyric acid in silage has normally been ascribed to deamination of valine by clostridia. However, Suzzi et al. (1990) showed that a *L. brevis* strain isolated from corn (*Zea mays* L.) silage produced isobutyric acid under anaerobic conditions. The isobutyrate was not derived from valine; however, the biochemistry of this reaction has not been described.

Organic Acids

All forage species normally ensiled contain organic acids. The types and amounts of these acids vary between species (Table 3–5). Jones and Barnes (1967) compared several grass species and found the most common acids were malic, citric, quinic, and succinic, with lesser amounts of fumaric and shikimic acids. More recently, Muck et al. (1991) measured the concentrations of organic acids in permanent pastures and found an acid profile similar to that found by Jones and Barnes (1967) but at lower concentrations. In legumes, concentrations of acids are

Table 3–5. Organic acids in grasses and legumes.

Acid	Grass		Legumes	
	Perennial ryegrass†	Permanent pasture‡	Alfalfa§	Red clover¶
	g kg^{-1} DM#			
Malic	4–17	2–6	20–70	38
Citric	2–8	1–5	9–22	5
Quinic	1–15	--	3–27	--
Succinic	1–5	<1	--	<1
Malonic	--	<1	10–31	6
Glyceric	--	--	--	37
Total	13–45	4–13	42–150	101

† Jones & Barnes (1967).
‡ Muck et al. (1991).
§ Dijkshoorn (1973).
¶ Lessard & McDonald (1966).
# DM, dry matter.

substantially higher than in grasses. Dijkshoorn (1973) reported that malic, citric, malonic, and quinic acids were the most abundant acids in legumes, although glyceric acid may be abundant in red clover (*Trifolium pratense* L.) (Lessard & McDonald, 1966).

Since the pH of a solution of a free organic acid is less than 2, these acids are neutralized at forage pH (around pH 6.5). Furthermore, since the acids are weak acids, they function as biological buffers and resist acidification of the ensiled mass by the production of lactic acid. Thus, organic acids are a main contributor to the buffering capacity of forage crops. Muck et al. (1991) calculated that the organic acids in permanent pasture contributed 15 to 32% of the total buffering capacity.

Metabolism of Organic Acids

Malic Acid. Malic acid is metabolized predominantly to lactic acid during ensiling (Fig. 3–9). It can be decarboxylated in the malolactic fermentation (e.g., by lactococci) to yield lactic acid and CO_2 with ATP synthesis occurring indirectly via a hydrogen pump and a coupled ATPase (Teuber, 1995). Alternatively, malic acid may be oxidized to oxaloacetate followed by decarboxylation to pyruvate, which is further metabolized as described for citric acid. Since one molecule of a monocarboxylic acid is produced from a dicarboxylic acid, metabolism of malic acid leads to a rise rather than a decrease in pH.

Citric Acid. All metabolism of citric acid proceeds through the activity of citrate lyase, which converts citrate to acetate and oxaloacetate (Fig. 3–9). The oxaloacetate is then decarboxylated to pyruvate. The fate of the pyruvate then depends on the organism involved and the metabolic state of the cell using any of the routes of pyruvate metabolism described above. For example, in *Leuconostoc* species, the end products of fermentation change from lactate and ethanol when only glucose is present to lactate and acetate when citrate is added. The conversion of citrate to acetate and pyruvate does not require the reduction of NAD to NADH. Therefore, reoxidation of NADH produced from hexose metabolism alone can be switched from production of ethanol from acetyl phosphate to acetate, and so extra ATP is gener-

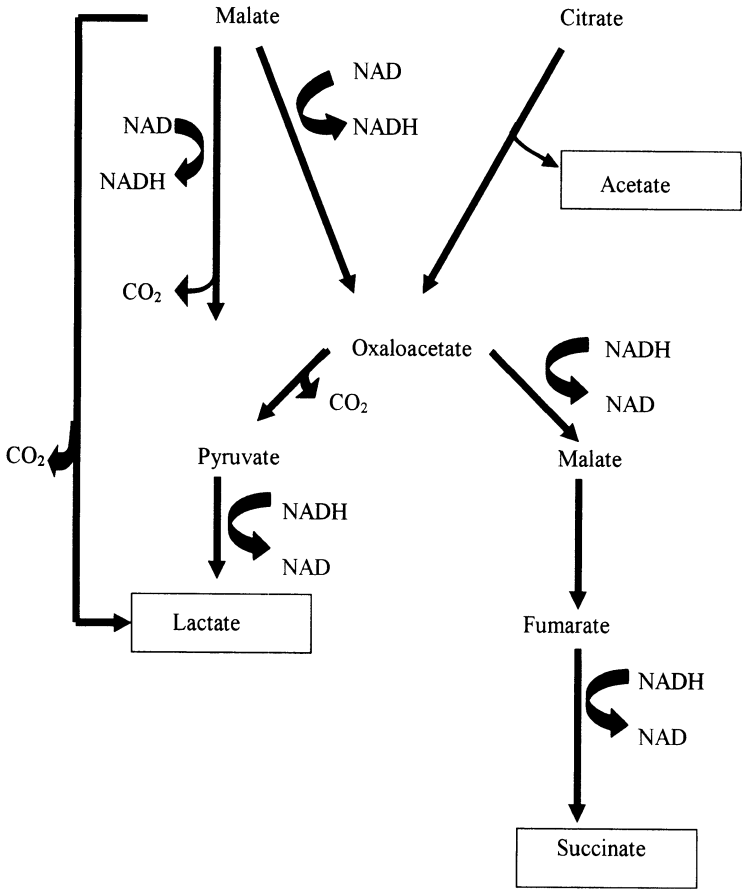


Fig. 3–9. Metabolism of organic acids during ensiling.

ated through the production of acetate. Growth yields of *Leuconostoc* species have been shown to increase (Cogan, 1987) when citrate was included in the growth medium.

In the absence of hexose, many LAB do not utilize citrate. However, in those that do, products of metabolism may be directed away from lactate. L-lactate dehydrogenase activity is low in the absence of glucose because the concentrations of its two activators, fructose 1,6-diphosphate and NADH, are low. Pyruvate may therefore accumulate in the cell due to rapid metabolism of citrate, and in this situation, the 4-carbon compounds acetoin and 2,3-butanediol accumulate.

Citrate metabolism may also be important in the anaerobic utilization of lactate in silage by *L. plantarum* (Lindgren et al., 1990; Fig. 3–9). Citrate is decarboxylated to oxaloacetate and then progressively reduced to malic and succinic acids using two molecules of NADH. The NAD so formed can then be used as a cofactor to allow the oxidation of lactate to pyruvate and the subsequent production of ATP by the formation of acetyl phosphate and then acetic acid. It is thought that this pathway may account, in part, for the gradual decline in lactate concentrations

seen in some silages over an extended storage period. Recently, Driehuis et al. (1999) have described an alternative pathway of anaerobic metabolism by *L. buchneri*, which results in the production of acetic acid and 1,2-propanediol from lactate. The details of the metabolic pathway involved have not been described, although lactaldehyde has been suggested as an intermediate in an analogous fashion to the production of 1,2-propanediol by mixed rumen microorganisms (Czerkawski, 1986).

Changes in Fermentation Pathways

Cofermentation of hexose with organic acids is one way of providing an alternative means of reoxidizing NADH. Two other pathways for reoxidizing NADH are reduction of NO_3 and utilization of O_2.

Nitrate

Several LAB, including *L. plantarum,* possess both NO_3 and NO_2 reductase activities (Wolf & Hammes, 1987; Dodds & Collins-Thomson, 1985). Glucose can serve as a hydrogen donor for this reaction, and therefore NO_3 and NO_2 reduction is a potential alternative means of regenerating NADH. Reduction of NO_3 leads either to the formation of NH_3 (Wolf & Hammes, 1987) or nitrous oxide gases (Dodds & Collins-Thompson, 1985) and the production of acetate rather than lactate from pyruvate, thus allowing the generation of an extra mole of ATP from glucose.

Oxygen

The LAB are facultative anaerobes and are therefore tolerant of a certain amount of environmental O_2. They utilize O_2 in a number of enzyme catalyzed reactions (Table 3–6; Condon, 1987). Of the reactions, NADH oxidases (Reactions 1 and 2) are present in many LAB, but NADH peroxidase (Reaction 6) is rare (Condon, 1987). The activity of these enzymes allows metabolism of pyruvate to be switched from production of lactate to production of acetate and generation of an extra mole of ATP through acetyl phosphate. This has been demonstrated in *Leuconostoc* spp. (Lucey & Condon, 1986). In facultatively homofermentative lactobacilli, a switch in metabolism from homofermentative to heterofermentative is normally repressed when glucose availability is high. Rapid glucose utilization will

Table 3–6. Enzymes used in aerobic metabolism by lactic acid bacteria.

No.	Substrates	Enzyme	Products
1	$NADH + H^+ + O_2$	$NADH:H_2O_2$ oxidase	$NAD^+ + H_2O_2$
2	$2\ NADH + 2\ H^+ + O_2$	$NADH:H_2O$ oxidase	$2NAD^+ + 2\ H_2O$
3	pyruvate + phosphate + O_2	pyruvate oxidase	acetyl phosphate + CO_2 + H_2O_2
4	α-glycerophosphate + O_2	α-glycerophosphate oxidase	dihydroxyacetone phosphate + H_2O_2
5	$2\ O_2^- + 2\ H^+$	superoxide dismutase	$H_2O_2 + O_2$
6	$NADH + H^+ + H_2O_2$	NADH peroxidase	$2\ H_2O$

generate fructose 1,6-bisphosphate, an activator of lactate dehydrogenase leading to lactate production (Thomas et al., 1979). However, when glucose concentrations fall, the fermentation becomes heterofermentative, with acetate being produced as an end product in part because of pyruvate oxidase (Reaction 3, Table 3–6). Indeed, lactate itself may be oxidized to pyruvate and then to acetate to allow slow growth of LAB in the presence of O_2 (Murphy & Condon, 1984).

The hydrogen peroxide produced by NADH oxidases will inhibit aerobic growth if allowed to accumulate. Many LAB produce catalase and therefore can detoxify hydrogen peroxide. Superoxide dismutase (Reaction 5) may also be an important means of detoxifying free radicals generated in aerobic conditions. However, many LAB accumulate Mn ions from the environment and are therefore able to use a nonenzymic dismutation system to scavenge free radicals (Archibald & Fridovich, 1981).

Enterobacteria

Enterobacteria ferment carbohydrates by species and environment dependent pathways that typically give rise to a wide range of products. These products include lactate, acetate, ethanol, formate, and four-carbon compounds such as acetoin and butanediol. The general series of reactions for metabolism of hexose sugars and pyruvate presented above are used by enterobacteria. Lactate, however, is a relatively minor end product, and therefore the enterobacteria are not desirable in the silage fermentation. Two of the more typical overall stoichiometries of carbohydrate fermentation are the acid fermentation described for *Escherichia coli* and the neutral fermentation of glucose by organisms such as *Klebsiella* spp.

For acid conditions:

$$2 \text{ glucose} + 4 \text{ NAD} + 4 \text{ ADP} \rightarrow 4 \text{ pyruvate} + 4 \text{ NADH} + 4 \text{ ATP}$$

$$4 \text{ pyruvate} + 4 \text{ NADH} + \text{ADP} \rightarrow 2 \text{ lactate} + 1 \text{ acetate} + 1 \text{ ethanol} + 4 \text{ NAD} + 1 \text{ ATP} + 2 \text{ CO}_2$$

In the neutral fermentation, a variety of balances are possible, giving different acid/neutral product ratios, one of which is given below:

$$2 \text{ glucose} + 4 \text{ NAD} + 4 \text{ ADP} \rightarrow 4 \text{ pyruvate} + 4 \text{ NADH} + 4 \text{ ATP}$$

$$4 \text{ pyruvate} + 4 \text{ NADH} \rightarrow 1 \text{ (2,3-butanediol)} + 1 \text{ lactate} + 1 \text{ ethanol} + 4 \text{ NAD} + 3 \text{ CO}_2$$

While the LAB can reduce NO_3 to NO_2 and NO_2 to NH_3 and nitrous oxide gases, enterobacteria are probably responsible for a major proportion of the NO_3 reduction during ensiling (Spoelstra, 1985, 1987). Inoculation of forage with enterobacteria increases NO_3 reduction, and the time course of NO_3 utilization in silage follows the population profile of the enterobacteria. Thus, the majority of NO_3 reduction takes place during the early stages of silage making when the enterobac-

teria are active. Nitrate reduction can produce toxic nitrous oxide gases, and in some cases, in well-sealed silage clamps, the nitrous oxide gases can redissolve at the edges of the clamp to give locally extremely high concentrations of nitric acid with pH values of around 1 that render the silage in these areas brown and extremely corrosive.

Clostridia

The pathways involved in the production of butyric acid by clostridia from either hexoses or from lactate have been described (Fig. 3–8). These reactions are typical of the saccharolytic or sugar-fermenting clostridia. The pathway is of course undesirable in silage, as butyric acid is a weaker acid than lactic acid, and thus conversion of lactic to butyric acid will lead to an increase in silage pH. However, butyric acid is not the only possible product of sugar fermentation by clostridia; acetate, ethanol, propionate, and butanol can also be produced. In addition, the proteolytic clostridia ferment amino acids (see below) and give rise to a wide range of end products.

Yeasts and Molds

The dominant fermentation pathway in yeasts is the decarboxylation of pyruvate to acetaldehyde and subsequent reduction of the acetaldehyde to ethanol to regenerate NAD (Fig. 3–7). As with the clostridia, other end products of anaerobic fermentation are possible, including acetate, lactate, propionate, and higher alcohols such as propanol and butanol. The production of ethanol is undesirable because there is loss of DM as CO_2 and no acid produced during fermentation.

Unlike the bacteria discussed so far, the yeasts possess an electron transport chain. Therefore, in the presence of O_2, substrates such as sugars and lactic acid are completely oxidized to CO_2 and water via the glycolytic pathway and tricarboxylic acid cycle with a substantially enhanced ATP yield. Aerobic metabolism is the reason for deterioration of silage when it is exposed to O_2. Utilization of lactate aerobically by yeasts increases silage pH and temperature and allows a succession of aerobic organisms to become established in the silage.

Propionic Acid Bacteria

Since undissociated propionic acid is a potent inhibitor of yeasts and molds, there has been interest in using these bacteria as silage inoculants to increase the aerobic stability of silages. *Propionibacterium shermanii* has been tested as a silage additive (Rauramaa et al., 1996) as this organism produces propionic acid from lactic acid (see Chapter 7, Kung et al., 2003). In the production of propionic acid there is a penalty paid in reduced acidification of the silage; the stoichiometry of the reaction is as follows:

$$2 \text{ lactate} + 2 \text{ ADP} \rightarrow \text{acetate} + \text{propionate} + 2 \text{ ATP} + CO_2$$

The reaction proceeds through the carboxylation of one molecule of pyruvate to oxaloacetate, which is then reduced to succinate coupled with the oxidation of

another molecule of lactate to pyruvate. From the succinate, propionate and two moles of ATP are generated, while the pyruvate is decarboxylated to acetate with loss of molecular hydrogen.

Other Bacteria

The other bacteria that have varying roles during ensiling are the bacilli, acetic acid bacteria, and listeria. Both the acetic acid bacteria and listeria are aerobic bacteria and require at least a micro-aerophilic niche in silage to survive.

Listeria spp. metabolize sugars to CO_2 and water by the respiratory chain. These bacteria are important as pathogenic organisms particularly in big bale silage (Fenlon et al., 1989). Acetic acid bacteria have been implicated in initiating aerobic deterioration in corn silage, since they can utilize lactic acid, acetic acid, and ethanol at low pH. Ethanol is the preferred substrate of the acetic acid bacteria (Spoelstra et al., 1988) and is oxidized in preference to acetic and lactic acids.

The role of bacillus species in silage is not well established. They are able to ferment WSC and have been reported to produce a spectrum of end products ranging from homofermentative production of lactic acid to 4-carbon neutral compounds such as butanediol and acetoin (Woolford, 1977). Similarly it has been suggested that bacilli may be implicated in initiating aerobic deterioration in some silages as they possess an electron transport chain and can therefore metabolize lactic acid aerobically.

Fermentation Balance and Conservation Efficiency

Table 3–7 summarizes the conservation efficiencies of typical silage fermentation pathways. Since preservation is achieved by production of sufficient acid to inhibit microbial activity, the conservation efficiency of any pathway should be considered as a combination of the amount of acid produced and the losses of energy incurred. Using these criteria, homofermentative LAB are the most efficient, producing two molecules of lactic acid (pK_a 3.86) with minimal energy loss. While the fermentation of hexose to ethanol by yeasts is energetically efficient, no acid is produced; therefore, yeasts divert substrate away from potential acid production. Clostridia have a low acidification potential and large energy losses. Neither of these microorganisms is therefore desirable if efficient preservation is to be achieved. The

Table 3–7. Acidification and fermentation efficiencies of main fermentation pathways of silage bacteria.

				Recovery	
Organism	Pathway	Substrate	Product	Energy	DM
				—— % ——	
LAB	Homofermentative	Glucose	2 lactate	96.9	100
LAB	Heterofermentative	Glucose	1 lactate + 1 acetate	79.6	83
LAB	Heterofermentative	Glucose	1 lactate + 1 ethanol	97.2	83
Yeasts		Glucose	2 ethanol	97.4	51
Clostridia		Glucose	1 butyrate	77.9	66
Enterobacteria		2 glucose	2 lactate + 1 acetate + 1 ethanol	88.9	83

heterofermentative LAB and the enterobacteria rank between the extremes of the homofermentative LAB and the yeasts and clostridia.

NITROGENOUS CONSTITUENTS

Forage Composition

The nitrogenous constituents of different forages have many similarities. Therefore, the N constituents of temperate grasses will be described and then comments made on qualitative and quantitative differences between temperate and tropical grasses and legumes.

The protein in grasses has historically been classified into three fractions, based on solubility in ammonium sulfate (Mangan, 1982): Fraction 1 leaf protein, Fraction 2 leaf protein, and chloroplast membrane proteins. Fraction 1 leaf protein accounts for approximately 50% of total protein and is a single soluble protein. This protein is the enzyme rubisco, the key enzyme in the Calvin photosynthetic pathway responsible for fixation of CO_2. Rubisco has a molecular weight of 560 kD and is comprised of eight large (56 kD) and eight small (14 kD) subunits. The amino acid pattern of rubisco is broadly similar across species. Variations are largely caused by differences in amino acid composition of the small subunit. Not surprisingly, this homogeneity of amino acid content confers a similar degree of homogeneity to the amino acid pattern of total herbage protein. Rubisco has been calculated to be the most abundant protein in the biosphere.

In contrast to Fraction 1 leaf protein, Fraction 2 leaf protein is a heterogeneous mixture of proteins derived from both the chloroplast and cytoplasm of the plant cell. Fraction 2 protein comprises about 25% of total protein containing six to nine major protein species, separable by gel electrophoresis. These have been identified as structural proteins, such as actin, or enzymes, such as ATP synthetase.

The chloroplast membrane proteins are insoluble and are derived from the thylakoid membranes of the chloroplast. These membrane proteins have been estimated to contribute 40% of the chloroplast protein or 15 to 20% total protein. The remaining leaf protein is derived from nuclear (1–2%), mitochondrial (5%, of which 60% is soluble), and cell-wall proteins.

Herbage protein is therefore characterized as being predominately soluble (around 75% of total true protein) and dominated by rubisco. The protein is susceptible to proteolysis, as can be demonstrated by its high rumen degradability (Nugent & Mangan, 1981; Nugent et al., 1983).

Free amino acids and NO_3 account for a variable proportion of herbage total N (3–20% of total N) with the quantity and composition being determined by herbage species, fertilizer inputs, stage of botanical growth, and climatic conditions. The free amino acid pool probably represents a pool of newly synthesized amino acids and amino acids that are being transported about the plant. The metabolic route for N assimilation in plants is shown in Fig. 3–10. Formation of asparagine by transamination from glutamine is important in legumes, as asparagine rather than glutamine is the main form of amide transport within the plant. Other amino acids are formed from glutamic acid by transamination. Examples of the free amino acid distribution in perennial ryegrass and alfalfa (*Medicago sativa* L.) are given in Table

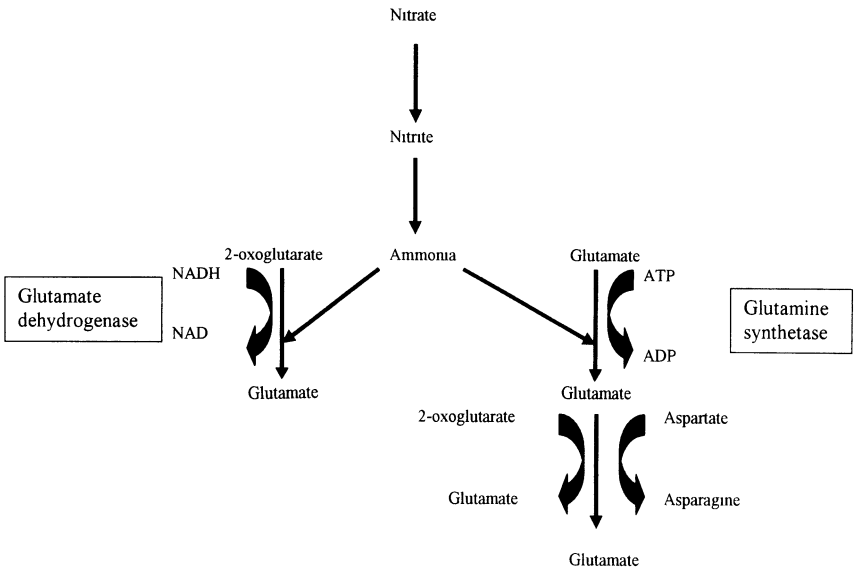


Fig. 3–10. Assimilation of NO_3 by plants.

3–8 (Bathurst & Mitchell, 1958; Fauconneau, 1960). The dicarboxylic acids, their amides, and δ-amino butyric acid, the decarboxylation product of glutamic acid, dominate the free amino acids. The main difference between legumes and grasses is the higher concentration of asparagine in legumes.

Of the remaining nonprotein N, NO_3 is quantitatively most important. The amount of NO_3 in an herbage sample represents the balance between uptake of NO_3 by the plant and its assimilation into amino acids. Nitrate can accumulate to levels in excess of 10% of total N or 1% of herbage DM in grass shortly after fertilizer application. These concentrations present several risks to the success of silage making and to the health of the animal. In the silo there is the potential for production of toxic nitrous oxide gases and increases in silage NH_3–N concentrations. In the rumen reduction of NO_3 to NO_2 and NH_3 increases risks of NH_3 toxicity in the animal.

Table 3–8. Free amino acid composition of forage crops.†

Amino acid	Perennial ryegrass	Alfalfa
	— g kg^{-1} total amino acids —	
Asparagine	50	350
Glutamine	110	13
Aspartic acid	140	110
Glutamic acid	135	33
δ-amino butyric acid	235	150
Alanine	160	63
Others	165	200

† From Bathurst & Mitchell (1958) and Fauconneau (1960).

Low concentrations of amines have also been reported in fresh forage. Recently Van Os et al. (1996) found 100 to 200 mg amines kg^{-1} grass DM. This amine fraction was comprised mainly of putrescine, spermidine, and tyramine. The presence of amines in silage is undesirable because their presence has been correlated with reductions in silage intake (Buchanan-Smith & Philip, 1986) and at high concentration amines may be toxic.

Characteristically, legumes have higher total crude protein concentrations (150–300 g kg^{-1} DM) than temperate grasses (100–200 g kg^{-1} DM), which in turn have higher N concentrations than tropical grasses (60–150 g kg^{-1} DM). Legume and temperate grass differences are largely a result of different leaf/stem ratios. The lower N concentrations in tropical grasses, including corn, when measured at a similar stage of growth to temperate grasses, arise because the Fraction 1 leaf protein in the mesophyll chloroplasts of temperate grasses is absent from tropical grasses. Therefore, tropical grasses have an inherently lower soluble protein content than temperate grasses (Mangan, 1982).

Proteolysis

Degradation of plant protein during wilting and ensiling is inevitable and results in changes in the N constituents of ensiled herbage which reduce the nutritive value of the crude protein in the ensiled crop compared with fresh herbage. Degradation of protein can be considered a two-stage process. First, peptide bond hydrolysis (proteolysis) takes place, which results in the formation of free amino acids and peptides. Second, amino acids are degraded to a variety of end products, including NH_3, organic acids, and amines. During wilting and in well-fermented silages dominated by LAB, proteolysis is largely the result of plant enzymes. The evidence for involvement of plant enzymes is well established, and the clearest examples come from experiments in which the crop has been grown under aseptic conditions (Kemble, 1956), treated with antibiotics (Brady, 1960), or irradiated (Heron et al., 1986). Sterilization of the crop by any of these methods usually does not reduce proteolysis compared with the unsterilized crop but does prevent fermentation, clearly indicating the role of plant enzymes in protein breakdown during ensiling (see Table 3–9; Heron et al., 1986).

Proteolysis during Wilting

A considerable quantity of proteolysis can take place during wilting. With ryegrass, Kemble and MacPherson (1954) reported that 20% of protein N was degraded

Table 3–9. Effect of sterilization by irradiation on proteolysis of perennial ryegrass during ensiling.†

		Silage	
	Grass	Untreated	Irradiated
Protein, g N kg^{-1} total N	858	293	359
NH_3, g N kg^{-1} total N	11	136	82
pH	--	3.8	5.2
Lactic acid, g kg^{-1} DM	--	119	11

† Heron et al. (1986).

into nonprotein N in 3 d. Henderson et al. (1972) found a similar reduction in a 31-h wilt, while Ohyama (1970) found losses of up to 0.5 of protein N in a 5-d wilt. The end products of proteolysis during wilting are peptides, amino acids, and amides. The amino acid composition of the wilted herbage can also undergo changes (see below).

Length of wilting period and climatic conditions during wilting influence the extent of proteolysis. Proteolysis is minimized with a rapid wilt under dry conditions, whereas a long wilt under humid conditions increases proteolysis (Brady, 1960; Carpintero et al., 1979). The effects of aerobic/anaerobic conditions during wilting are less clear. Ohyama (1970) found that the rate of proteolysis was more rapid in the absence of O_2 and ascribed the increase in proteolysis under anaerobic conditions to activation of thiol dependent enzymes. Recently Makoni et al. (1997) observed a reduction in proteolysis when ensiling alfalfa in a controlled atmosphere containing only 30 mL L^{-1} O_2. Differences between these two studies may relate to the amounts of CO_2 present and to differences between wilting and ensiling.

Changes are observed in the amino acid composition of herbage during wilting. Proline has been shown to be synthesized from glutamate and ornithine (Kemble & MacPherson, 1954; Ashbell et al., 1986; Delauney et al., 1993), and this increase has been suggested to be a response to osmotic stress to detoxify NH_3 as the proline is synthesized from glutamate (Boggess et al., 1976), the main end product of NH_3 assimilation (Fig. 3–10). Arginine may also disappear during wilting largely through metabolism to ornithine. This decrease contributes to part of the overall decrease in arginine concentration observed during silage making.

Proteolysis during Silo Phase

Proteolysis continues when herbage is ensiled. As little as 250 g protein N kg^{-1} total N may remain at the end of silage making, little of which is soluble (Gouet et al., 1970). Indeed, in grass silage, protein precipitants such as trichloroacetic acid do not usually precipitate protein from aqueous silage extracts. Most available evidence suggests that the proteins that resist degradation during ensiling are the insoluble membrane-bound proteins. Grum et al. (1991) found that soluble buffer-extractable alfalfa proteins rapidly disappeared from silage with time. Messmann et al. (1994) found that a 54-kD protein (presumably the heavy chain of rubisco) was rapidly degraded during ensiling of alfalfa and ryegrass. Makoni et al. (1994) further found that alfalfa chloroplast membrane proteins were resistant to protein degradation in the silo. A similar conclusion was made in studies on perennial ryegrass by Aufrere et al. (1994). Davies et al. (1997) used liquid chromatographic techniques to measure rubisco concentrations during ensiling and found that variable quantities remained after silage making was complete. An apparent discrepancy exists between results obtained using electrophoretic measurement of forage protein and liquid chromatographic techniques. Davies et al. (1997) measured rubisco concentrations up to 400 g kg^{-1} of the amount present in herbage, while no soluble protein is frequently recovered after silage making when measured using electrophoresis. The discrepancy may be caused by differences in extraction techniques that may resolubilize rubisco precipitated by the acid conditions in the silo.

Products of Proteolysis

The end products of proteolysis are usually considered to be free amino acids and peptides. Ammonia and amines are largely end products of microbial activity rather than plant enzyme activity. For example, in studies using irradiated ryegrass (Heron et al., 1986), little NH_3 was formed when grass was irradiated (Table 3–9). However, little attention has been devoted to the occurrence of peptides as an end product of protein breakdown in silage. Peptide N has usually been determined as the difference between total soluble and free amino acid N in silages. In ryegrass peptide N has been estimated to range from 11 g kg^{-1} (Hughes, 1970) to 207 g kg^{-1} total protein (Heron et al., 1986). Makoni et al. (1997) recently reported values in alfalfa ranging from 60 to 300 g kg^{-1} N. There is, therefore, a substantial range in peptide concentrations. Little is known about the chain length or amino acid composition of silage peptides. Nsereko and Rooke (2000) found that most peptides in ryegrass silages were di- and tripeptides and had a variable amino acid composition.

Proteinase Enzymes

Protein breakdown is usually initiated by endopeptidases, which cleave internal peptide bonds in the polypeptide chain. The number of free amino and carboxyl groups is thus amplified and allows exopeptidases to attack terminal peptide bonds in the polypeptides. Endopeptidases are classified according to the active amino acid residue at the catalytic site of the enzyme (Barrett, 1994). There are four types: cysteine, aspartate, serine, and metallo endopeptidases. Identification of an endopeptidase is made using specific inhibitors of each class of endopeptidase. In plants, cysteine and aspartic endopeptidases are plentiful, while reports of metallopeptidases are more rare, and serine peptidases are not normally found. Few studies of the types of endopeptidases in forage crops have been conducted. In alfalfa, McKersie (1980) found enzymes with acid pH optima suggestive of cysteine and aspartate enzymes and perhaps a serine protease at neutral pH. However, the inhibitors used to classify serine protease were not specific to serine proteases. Wetherall et al. (1995) reduced proteolysis in ryegrass using specific inhibitors of cysteine and aspartate endopeptidases. More recently, Nsereko and Rooke (1999) found that inhibitors of metallo-endopeptidases also inhibited proteolysis in ryegrass. Thus, several different endopeptidases are involved in proteolysis during ensiling. Little is known about the nature of exopeptidases active during silage making. Nsereko and Rooke (1999) noted an accumulation of peptides in silages treated with metalloprotease inhibitors suggesting that metallo exopeptidases were involved in peptide degradation.

Factors Affecting Proteolysis

Crop

In general, legumes undergo more proteolysis than grasses (Papadopoulos & McKersie, 1983). However, within the legumes, red clover consistently undergoes less proteolysis than alfalfa (Papadopoulos & McKersie, 1983). When extracts of red clover and alfalfa were assayed, similar rates of proteolysis were observed (Jones

et al., 1995a). Jones et al. (1995b) noted that the reduced proteolysis observed with red clover did not occur when the red clover was allowed to brown before ensiling. Browning in red clover is attributed to a soluble polyphenol oxidase, and, thus, the differences between the two species in susceptibility to proteolysis are now attributed to inhibition of proteinases by polyphenol oxidase in red clover (Jones et al., 1995b,c).

pH

The pH optimum of proteases can only be truly determined using synthetic substrates because the pH optimum determined using protein substrates reflects not only the pH optimum of the enzyme but also the effect of pH on the conformation of the substrate. The effect of pH on protein substrates relates to the isoelectric point of the protein, that is, the pH at which the protein carries no net charge and at which its solubility is lowest. Both the net charge and the solubility of a protein will influence its availability as a substrate for proteases. Therefore two methods of determining the effect of pH on proteolysis are relevant as the appropriate substrate is used: autolysis of herbage or herbage extracts and proteolysis of rubisco. Heron et al. (1989) measured autolysis in ryegrass extracts and found the optimum pH for autolysis was between 5 and 6; these authors also found evidence for plant enzyme activity below pH 4 and chemical hydrolysis below pH 3.0. Wetherall (1993) found the same pH range and optimum using partially purified ryegrass protease preparations. In legumes, Jones et al. (1995a) measured the pH optimum for rubisco hydrolysis by alfalfa and red clover proteases to be 5.5. Again, activity was measurable for the entire pH range encountered during ensiling. The observations are consistent with additive studies in which acids or inoculants have been used to increase the rate of pH decline in the silo. In general, rapid acidification reduces proteolysis in the silo (e.g., Carpintero et al., 1979). There is little information on the effect of pH on peptide hydrolysis during ensiling. Nsereko and Rooke (1999) found that treatment of ryegrass with formic acid not only reduced proteolysis but also caused an increase in peptide-N concentrations without changes in chain length, suggesting the di- and tripeptidases in herbage were also inhibited by acidification and hence had a pH optimum between 4.5 and 6.0.

Protease activity is unstable at acid pH. Jones et al. (1995a) found that 0.67 of activity was lost after 24 h at pHs 4 and 5. Thus, reductions in proteolysis below pH 5.0 are a result of both reduced activity and enzyme inactivation.

Temperature

Most studies have shown that activity of herbage proteases increases progressively to 50°C (McKersie, 1980; Jones et al., 1995a) above which temperature the enzymes are inactivated. High temperature treatment of herbage has indeed been used as an experimental treatment to successfully reduce proteolysis (Charmley & Veira, 1990; Carpintero & Suarez, 1992).

Moisture

The effects of moisture on proteolysis are inconsistent (McDonald et al., 1991). There is evidence that reducing moisture content can reduce proteolytic ac-

tivity (Makoni et al., 1997). Inconsistencies in experimental results may be related to the observation that reducing moisture content also slows the rate of fermentation. Therefore any effects of reduced proteolytic activity at low moisture levels may be offset by extended exposure to pHs at which the proteases are most active.

Other Factors

The tannin content of forages may influence the extent of proteolysis as tannin-protein complexes are less susceptible to hydrolysis. Albrecht and Muck (1991) measured tannin concentrations in a variety of legumes and found a significant negative correlation between tannin content and the amount of soluble N formed in silage. Recently, Salawu and Acamovic (1997) found that addition of quebracho or mimosa tannins to perennial ryegrass before ensiling reduced proteolysis in the silo.

Makoni et al. (1997) found that ensiling alfalfa under a modified atmosphere (30 O_2, 150 CO_2, and 820 N_2 mL L^{-1}) reduced proteolysis. The authors attributed the reduction in proteolysis to a decrease in cell lysis, and in this there are probably similarities between proteolysis during silage making and senescence in plants.

Microorganisms

Lactic Acid Bacteria

Most research indicates that LAB are effectively nonproteolytic during ensiling (McDonald et al., 1991). However, it should be noted that some species (e.g., lactococci) are proteolytic and can hydrolyze peptides; in the dairy starter industry LAB are actively selected for their proteolytic ability (Pritchard & Coolbear, 1993; Teuber, 1995; Christensen et al., 1999). It might be more accurate to say that the contribution of LAB to total proteolysis is small in relation to plant proteolysis during silage making.

LAB depend on a supply of free amino acids to meet their requirements for growth but also can obtain energy from a limited catabolism of amino acids. In particular, arginine is fermented to ornithine (Fig. 3–11) and serine deaminated to pyruvate by some species (Poolman, 1993). The fermentation of arginine by LAB together with its catabolism by plant enzymes is the reason for the low concentration of arginine in silage N. It is also possible that metabolism of amino acids can be coupled with oxidation of lactate to acetate by LAB when glucose is not available. Rooke (1991) found that lysine and serine were utilized anaerobically at pH 4.0 by homofermentative LAB and arginine, glutamate, and serine by heterofermentative LAB when no glucose was available, although no evidence concerning the metabolic pathways involved was obtained.

Enterobacteria

The role of enterobacteria in reducing NO_3 has been discussed above. Enterobacteria are also proteolytic, but their role in protein breakdown in the silo has not been investigated to any great extent. However, studies in which grass was inoculated with enterobacteria before ensiling have shown that these bacteria can produce high concentrations of NH_3 in silage (Henderson, 1991). Thus, part of the in-

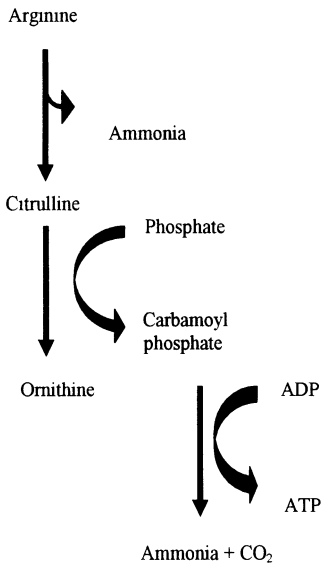


Fig. 3–11. Metabolism of ornithine (adapted from Christensen et al., 1999).

creased NH_3 concentrations observed in silages derived from slurry-treated herbage may be attributable to the enterobacteria.

Clostridia

The clostridia are divided into two classes, the saccharolytic or carbohydrate-fermenting clostridia and the proteolytic clostridia. As the name suggests, if the conditions in the silo are conducive to growth of clostridia, then proteolysis over and above that caused by plant proteases occurs. Of more importance, the proteolytic clostridia ferment the free amino acids arising from proteolysis to a variety of end products. The catabolism of amino acids by clostridia proceeds by three general reaction types: deamination, decarboxylation, and oxidation–reduction reactions (Table 3–10). The products of deamination (Table 3–10) are organic acids and NH_3.

Table 3–10. Amino acid catabolism by clostridia.

Reaction	Substrate	Product
Deamination	glutamic acid	acetic acid + pyruvic acid + NH_3
	lysine	acetic acid + butyric acid + 2 NH_3
	serine	pyruvic acid + NH_3.
Decarboxylation	arginine	putrescine + CO_2
	histidine	histamine + CO_2
	lysine	cadaverine + CO_2
	tyrosine	tyramine + CO_2
Stickland reactions		
Oxidation	alanine + 2 H_2O	acetic acid + NH_3 + CO_2
	leucine + 2 H_2O	isovaleric acid + NH_3 + CO_2
Reduction	glycine	acetic acid + NH_3
	ornithine	δ amino valeric acid + NH_3

The organic acids are further fermented to yield energy, while the NH_3 buffers the acidifying effects of the acids. The products of decarboxylation are CO_2 and amines (Table 3–10). Amines are basic, and as NH_3, tend to buffer or increase silage pH. Furthermore, many amines have bioactive properties and may reduce feed intake and in high concentrations can be toxic. Amine production is normally associated with clostridia; however, Van Os et al. (1996) found that considerable amounts of amines are formed in well-preserved silages during the first 10 d of ensiling, which could not be attributed to clostridia. Enterobacteria, which proliferate in the early stages of the stages of fermentation, may be responsible for this amine production. Oxidation–reduction or Stickland reactions involve a coupled reaction of pairs of amino acid, thus maintaining redox balance (Table 3–10). The net results of the Stickland reactions are to produce NH_3, CO_2, and two molecules of organic acids from two amino acids.

From the above reactions the products are bases (NH_3 and amines) and organic acids (with acetic acid a major component). Therefore, the production of butyric and acetic acids and NH_3 with a resulting high pH is usually taken as a reliable index of clostridial activity in silage. It should be noted that enterobacteria have been shown to produce NH_3 and acetic acid. Thus, butyric acid is probably the most reliable indicator of clostridial activity.

CONCLUDING REMARKS

In general, the reactions involved in production of acid from carbohydrate during ensiling are qualitatively well understood. As farmers demand a more consistent product in order to more accurately feed silage, the quantitative interactions between plant and microbial enzymes will become more important. In part, product consistency may be achieved by the use of additives. However, more knowledge is likely to be required in the biochemistry of acid production at low crop moisture concentrations, since a large proportion of silage research has been carried out with high moisture crops.

The biochemistry of proteolysis during silage making is less well characterized. In particular, little is known about the enzymes involved and which factors control the range of end products produced. As many countries become more aware of the costs of importing protein to feed ruminant livestock, a detailed knowledge of proteolysis during ensiling will aid the selection of improved forage cultivars by plant breeders and perhaps selection of appropriate silage additives to enhance the feeding value of forage protein. One potential change in the nitrogenous constituents of ensiled forage, the potential for formation of indigestible Maillard products, has not been discussed to date. Silage, particularly wilted silages, can contain substantial concentrations of soluble carbohydrate that could become involved in the formation of Maillard products (Goering et al., 1973) if temperature in the silo rises because of respiration. Nelson and Bozich (1996) measured increases in acid detergent fiber-N in ensiled alfalfa when incubated at 60°C and attributed the increases to formation of Maillard products. However, the quantitative significance of Maillard product formation in silages made at ambient temperature has not been investigated.

This chapter has concentrated on biochemical events involving crop carbohydrates and protein. Changes in vitamins and provitamins during ensiling have not been discussed. The length of any field wilting phase affects three vitamins and their provitamins (Sullivan, 1973). Beta-carotene (provitamin A) is progressively inactivated by a lipoxygenase during drying. Certain plant sterols can develop vitamin D activity during exposure to sunlight and therefore increase vitamin D activity in the conserved crop. Finally, vitamin E activity of a drying crop may be reduced by exposure to sunlight. After ensiling, beta-carotene activity can be further reduced by lipoxygenase (Kalac & McDonald, 1981), but rapid acidification minimizes any losses (Shahane & Mungikar, 1991). Jukola et al. (1996) described higher vitamin E concentrations in silage than in hay, suggesting that losses of vitamin E during ensiling were not of great importance, possibly because of the reducing environment of silage. However, the effects of ensiling on these vitamins have not been well documented.

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