1 INTRODUCTION

A longstanding issue in the feeding of performance horses has been achieving a balance between providing high-energy diets to meet their energy demands and avoiding nutrition related disorders that may be associated with the feeding of these diets. Equines evolved primarily as grazers, consuming grass and grass-like species as the major components of their diet (Hubbard *et al.*, 1976; Salter *et al.*, 1979; Waring, 1983). Whilst consuming this 'natural' diet, seeds of the grass species consumed would have supplied some dietary energy. However, a majority of their energy was obtained through the bacterial fermentation of the more fibrous plant material in the enlarged equine caecum and colon (Frape, 1998; Leek, 1993). As horses were domesticated and used for work and sport, cereal grains were incorporated into their diet to help meet their increased energy demands (Halnan *et al.*, 1953; Morrison, 1954). Horses in training in the Australian thoroughbred industry are currently being fed an average of 7.3 kg of grain concentrate/day with oats, corn and commercial premixed diets being the most common (Chapter 3).

Ideally, the starch from these grains will be digested by amylolytic enzymes in the small intestine (Gray, 1992) and absorbed as glucose from the lumen of the small intestine into the bloodstream of the animal (Bird *et al.*, 1996; Huntington, 1997; Thorens, 1993). However, there is rarely complete small intestinal digestion and starch that is not digested in the small intestine will be rapidly fermented in the hindgut by amylolytic bacteria. This hindgut fermentation of starch is a less energy efficient process than enzymatic digestion in the small intestine (Black, 1971) and commonly causes an accumulation of volatile fatty acids and lactic acid in the caecum and colon of equines. Acid accumulation can lead to hindgut lactic acidosis (Garner *et al.*, 1977), a metabolic condition commonly associated with reduced fibre fermentation (de Fombelle *et al.*, 1999), behavioural changes (Johnson *et al.*, 1998; Willard *et al.*, 1977) and the crippling and potentially fatal disease, laminitis (Pollitt, 2001a). Laminitis is currently the second most frequent cause of equine fatality in the world, behind only colic (Pollitt, 2001a).

Thus, from the viewpoint of feed conversion efficiency, animal performance and animal welfare, it is essential that cereal grain starch be digested in the equine small intestine. Two main factors influence the extent of starch digestion in a horse's small intestine:

1. the physical and chemical characteristics of the cereal grain being fed; and
2. the physiological and physical characteristics of the horse consuming the grain.

Characteristics of cereal grains, such as the seed coat (Rowe *et al.*, 1999), starch granule and biochemical structure (Kotarski, 1992), endosperm cell wall structure (Choct, 1995) and protein matrix structures (McAllister *et al.*, 1993; Rooney *et al.*, 1986) will all influence the digestion of cereal grain starch by amylolytic enzymes in the small intestine.

Likewise, physical and physiological attributes of the horse, such as a small stomach (Argenzio, 1993b), relatively fast passage rate through the small intestine (Frape, 1998) and

a possible deficiency of starch degrading enzymes in the small intestine (Comline *et al.*, 1969; Kienzle *et al.*, 1994; Roberts, 1974) may all contribute to the horse not being well equipped to digest starch pre-caecally.

Grain processing may be used to disrupt seed coat, starch granule, cell wall and protein matrix structures within cereal grains to improve starch digestion in the small intestine (Rowe *et al.*, 1999). Even with these barriers to starch digestion removed however, digestion in the equine small intestine may still be limited by the physical and physiological attributes of the horse and starch will reach the caecum and colon undigested. Bird *et al.* (1999) demonstrated that enzymatic starch digestion and starch fermentation characteristics of cereal grains are highly related. Therefore, if a horse's capacity for small intestinal starch digestion is overloaded and large quantities of processed grain starch reach the hindgut, the very rapid fermentation of this material may cause more severe problems with hindgut lactic acidosis than would be the case if unprocessed grains were fed.

The hypothesis for the work presented in this thesis is that the attributes of cereal grains that determine starch digestibility as well as the ability of an individual horse to digest starch in the small intestine will determine how much grain may be fed 'safely' before problems with hindgut starch fermentation will be experienced.

The safe feeding of grain to horses will therefore depend on matching the capacity of a horse to digest starch with the amount and digestibility of the dietary starch that is to be fed. The practical application of this simple principle has not previously been possible due to the difficulty of assessing differences between animals in their ability to digest starch and the lack of a meaningful and practical method to measure the digestibility of starch contained in the various cereal grains. These aspects of equine nutrition are the subjects of this thesis.

This thesis has three main sections:

(i) firstly a review of the literature focuses on of the processes of small intestinal starch digestion, factors affecting this process, including grain species and processing and the consequences that may be expected if starch reaches the hindgut undigested (Chapter 2);

(ii) following is the description of an industry survey that focuses on current grain feeding practices, the range of cereal grains fed and the risks involved with grain feeding in the Australian thoroughbred industry (Chapter 3); and

(iii) The main research component of this thesis is then presented in Chapters 4, 5, 6 and 7 where a series of experiments designed to examine the effect of grain species, grain processing and enzyme supplementation on the digestion of starch both *in vitro* and *in vivo* in the equine small intestine are described.

The significance of this research lies in its potential to contribute to developing balanced feeding regimes for performance horses so that their energy requirements may be fulfilled without the risk of adverse side effects such as hindgut acidosis.

2 LITERATURE REVIEW

2.1 INTRODUCTION

> *"There is probably no field of knowledge relating to animals in which there are more widespread misconceptions than in the feeding of horses; and also in which, unfortunately, there is so little direct scientific information"*
>
> - G.L. McClymont 1971 (*in* Anderson *et al.*, 1971)

In 2000/2001 the Australian horse industry contributed approximately $6.3 billion to Australian gross domestic product, of which the racing industry (including breeding, racing businesses and wagering) contributed 62% (Gordon, 2001). The thoroughbred industry ranks fourth in Australia for industry employment, behind only retail, manufacturing and health and human services. Commonly dubbed the sport of kings, horse racing is the second most popular sporting event to attend in Australia behind Australian Rules Football (ARB, 2001).

It is estimated that there are 1.2 million horses in Australia, of which 285,116 are 'named' thoroughbreds, including 1,396 stallions and 30,980 broodmares. In 2000/2001, Australia had 31,637 horses in training, which amounted to the greatest number of horses in training per capita in the world, ahead of Ireland and New Zealand (ARB, 2001). In Australia there are 5,609 thoroughbred trainers, 11,220 thoroughbred breeders and 133,089 thoroughbred owners. Thoroughbred horses compete in about 21,390 races throughout Australia for prize money in excess of $A305 million each year (ARB, 2001).

The following sections of this literature review outline and discuss the relevant theory and research regarding the physiology and starch digestion processes in the horse and factors affecting this process.

2.1.1 The Horse in History

It is estimated that 55 million years ago the domesticated horse, *Equus caballus,* began its evolutionary journey as the 'dog sized, squat, four toed creature' *Hyracotherium* (Budiansky, 1998). These early equids were primarily browsers. However as dramatic climatic changes took place around 19 million years ago, the world became drier and savannas and grasslands began to replace many of the forests. These changes allowed the equids to diversify, with many increasing in size and changing their eating habits to those of a forager (Budiansky, 1998). However the climate change also created new challenges for equids, including adaptation to a diet based largely on grasses and in particular the breakdown and digestion of plant cellulose. The horse evolved from having the compressive action of a browser's jaw to having the grinding jaw action of a forager. The jaw also increased dramatically in size to allow for stronger muscle development in the cheek area, while the teeth became cement-covered, higher crowned and ever-growing (hypsodonty). These latter changes in tooth growth were probably in response to the teeth being worn out through excessive chewing of grasses and also to combat the corrosive effect of mineral

particles being mixed with the food when grazing close to the ground (Budiansky, 1998; Duncan, 1992)

The change from protected forest browsing to open grassland grazing also meant that the equids were exposed to predators. Thus an increase in size and the development of a fast locomotion method became essential. However the large increase in size does not appear to be fully explained by the need for an increase in speed, as an increase in size does not result in a proportional increase in speed. Instead it is thought that the change in diet and the need for a large fermentation compartment within the gut may have been the main evolutionary factor behind the large increases in size with the resulting increase in speed of secondary importance (Budiansky, 1998).

With their diet changing from a highly digestible combination of fruits, berries, seeds and leaves to a lower energy, high fibre diet of pasture, the equids needed to develop a system whereby they could extract the energy held in the otherwise indigestible bonds of cellulose. Equids achieved this, as ruminants have, by forming a symbiotic relationship with bacteria that have the necessary enzymes to digest cellulose. A highly specialised enlargement of the horses' large intestine provided a suitable fermentation chamber, allowing the symbiotic relationship between the micro-organisms and equines to be effective. The problem however of low energy density feeds as the basis of the diet remained (Janis, 1976).

Equids were able to overcome the problem of low energy density foods through the capacity to consume large quantities of plant material. Unlike ruminants, whose fermentation chamber limits the quantity that they may eat, horses appear to have no limit to how much forage they can consume. Thus by eating large quantities of poor quality feeds, the equids were, and are still able to exist on low quality pasture. Consequently this mechanism provided the equids with a niche in which they were able to exist without competition from other species (Budiansky, 1998).

Natural Feeding Behaviour and Diet

Equids in their natural environment devote large periods of their time to grazing and also browsing during both the day and night, with the time spent grazing being affected by the quality and quantity of food available. Duncan (1980) reports that adult Camargue horses spent 50 – 60% of their time grazing during summer and 55 – 63% of their time grazing in winter. It was observed that the mares, who were at most times pregnant and/or suckling a foal, spent longer periods grazing, presumably due to their higher maintenance energy requirements than the male animals. Similarly, Tyler (1972) reports that during the winter season, New Forest ponies devoted their whole day and most of the night to grazing and browsing, with only very short periods allowed for rest. As summer approached, ponies tended to spend less time grazing and rest periods were extended during the day until, during the peak of summer when virtually no grazing occurred between 0900 h to 1400 h. Most of the night during summer was however spent foraging. Likewise Salter *et al.* (1979)

observed that horses in Western Alberta devoted 75% of their daylight hours to grazing in winter and spring.

Grazing during the hours of darkness appears to be a common activity for equines. Keiper *et al.* (1980) observed that wild ponies spend 55% of the night grazing. In this study, peak foraging periods extending from sunset until 2200 h and from 0400 h until after sunrise.

Equines are opportunistic feeders with their natural diet consisting of whatever is available and palatable for them at the time of grazing. They are primarily grazers, preferring grasses and grass-like forages, however browsing is a commonly observed behaviour (Waring, 1983). For example, New Forest ponies were observed to graze a variety of grasses, rushes and small flowering plants with *Molinia caerulea* (purple moor grass) forming the bulk of their diet throughout the summer months. In some cases, the ponies in this study would wade into water to eat emergent and submerged aquatic plants. The ponies were also commonly observed browsing on gorse, dwarf gorse, brambles, oak, beech, and holly with the leaves of holly and gorse providing almost the sole constituents of their diet during the winter when snow covered the ground (Tyler, 1972).

In the Piceance Basin, Colorado, USA, 85 – 98% of the diet of feral horses consisted of eight different grass and grass-like plant species, with the remainder of their diet being forbs and shrubs (Hubbard *et al.*, 1976). Similarly Salter *et al.* (1979) observed that the diet of feral horses in Western Alberta was primarily grasses and grass-like species (92.6%). The remainder of the diet was made up of forbs, browse and other miscellaneous species such as lichen and moss. In this study horses were observed to consume up to 43 different plant species and species groups throughout the year. Hansen (1976) reported that diets of horses in southern New Mexico, USA were made up of just 50% grass and grass-like species, with other plants such as russianthistle, dropseed and mesquite forming a large portion of the diet. These observations serve to highlight the opportunistic feeding practices adopted by equines in their natural environment and the fact that the natural diet of equines is one of grass and grass-like roughages.

Development of the Horse/Human Association

Over 6000 years ago, humans saw the horse only as a source of food and equids were hunted in the isolated regions of the world where they existed. There are many theories surrounding the domestication of the horse, but the modern day horse is thought to have arisen from the Przewalski's horse and was first ridden in the area of modern day Ukrainia (Levine, 1990).

> *"Domesticating the horse...would have been a daunting prospect, for the animals speed, alertness and capacity for inflicting injury made it qualitatively different from sheep, goats and cattle...Riding a horse was a total departure from anything previously known in human experience".*
>
> (Budiansky, 1998)

The mobility provided by the horse upon domestication was responsible for many economic, social and ecological changes in human society. It meant communities had use of greater areas of land around their settlements, allowing them to produce more food and thus support a larger population of people. Increased mobility facilitated trade and warfare (Levine, 1990) and horses were also used as a domestic supply of meat and milk (Anderson *et al.*, 1971).

For humans, horses became an indispensable means of transport and were also valuable in farming industries throughout the world. Horses were used to pull buggies and coaches, as delivery animals, stock horses and for heavy-haulage and farm work. With the advent of farm mechanisation and the development of the motorcar, commercial uses for horses declined. In the modern day many horses are used for stock-work, racing and various competitive equestrian sports, while a great number are kept purely for the pleasure of the owner.

Altered Feeding Behaviour and Early Feeding Practices

As horses were used increasingly for work, their energy requirements were raised markedly and the inclusion of high-energy supplements such as cereal grains in a horse's diet became popular. Horses were also commonly confined to stables and yards to make them easily accessible and also to protect them from the harsh winters experienced in many parts of the world (Anderson *et al.*, 1971; Halnan *et al.*, 1953; Hewitt, 1961; Morrison, 1954). Thus, the use of horses as work animals brought about two major changes to their natural feeding behaviour: their diet was changed from one of pure roughage, made up primarily of grass and grass-like plants, to one that had a substantial inclusion of high starch cereal grains; and their feeding pattern was altered. As previously discussed, a horse in a natural situation may devote up to 75% of daylight hours and 55% of darkness hours to grazing. On the other hand, a horse in a domesticated situation has a limited number of meals supplied to it throughout the day and spends less time eating.

During the mid 1900's, horse nutrition was focussed mainly on the feeding of draught or heavy horses used on farms. Feed costs were a major consideration, as the animals were essential working animals on farms and thus their feed costs affected profitability. Oats was the most popular cereal grain fed to horses, with many horse owners refusing to feed anything else (Anderson *et al.*, 1971; Halnan *et al.*, 1953; Hewitt, 1961). Oats was recognised as a safe and palatable grain, with the safety of oats being attributed to the high fibre content (Halnan *et al.*, 1953). The fibrous husk was thought to provide bulk and prevent horses from overeating (Hewitt, 1961). A further theory on the safety of oats was that the grain 'formed a loose mass in the horses stomach' presumably preventing diseases such as colic (Anderson *et al.*, 1971).

To reduce the cost of feeding horses, other grains were commonly used to replace part of, or the entire oat portion of the diet. Barley and corn were common substitutes, while rye and wheat were used with caution, as it was recognised that they were likely to cause digestive

disturbances (Halnan *et al.*, 1953). Uncontrolled feeding of wheat caused horses to become 'food-sick' or suffer from colic (Hewitt, 1961), and it was later observed to cause severe and crippling laminitis, digestive disturbances and kidney disorders (Anderson *et al.*, 1971). Sorghum was seen as a satisfactory source of energy for horses and, as long it was cracked prior to feeding, it could make up to 90% of the concentrate ration. Bran, pollard, linseed, cottonseed and soybean meals were also fed to horses in smaller quantities (Anderson *et al.*, 1971).

Views on the processing of cereal grains prior to feeding in the mid 20th century were varied among nutritionists. The physical processing of grains prior to feeding was strongly advocated by Halnan (1953) and Anderson *et al.* (1971) in order to prevent horse swallowing grains unmasticated, as the hulls of the grains were hard and thought to severely restrict the digestion of the energy rich components within the grain as they passed through the gastrointestinal tract. In contrast Morrison (1954) believed it was not necessary to crush oats or corn for horses with good teeth as it appeared to make very little improvement to the digestibility of these grains. It was however recommended that barley, wheat, rye and sorghum be soaked, crushed or ground for horses prior to feeding due to the small size of the grain kernels and the high probability that they would not be adequately chewed before being swallowed (Morrison, 1954). Interestingly, it is noted by Morrison (1954) that there is no advantage in cooking, fermenting or predigesting feeds for horses prior to feeding. This may have been primarily an economic consideration.

Chaff was a frequently used ingredient in horse diets and was commonly made from poor quality cereal hays or straws that would not otherwise be consumed. The chaff was mixed with the cereal portion of the diet for three main reasons; it encouraged chewing by the horse, ensuring that grains in the diet were adequately masticated prior to being swallowed; it lessened the wastage of poor quality hays, as horses, when fed long hay, tended to eat the heads of the cereal hays and leave behind the coarser lower sections of the plant; and it prevented hasty eaters from consuming grains too quickly (Anderson *et al.*, 1971; Halnan *et al.*, 1953; Hewitt, 1961; Morrison, 1954). Experiments in the mid 20th century found that the chaffing of hay did not increase the value of hay as a feed and due to the quality of chaff commonly used it was not thought that horses gained much nutritional benefit from the chaff (Halnan *et al.*, 1953; Morrison, 1954). When chaff was expensive, crushed wheat was often used as a substitute (Hewitt, 1961). Hay was also fed to horses, with 'hard hays', such as rye grass hay, that had relatively little leaf, used in preference to the leguminous hays, that tended to be dusty and thought to lead to a shortness of breath (Halnan *et al.*, 1953; Hewitt, 1961).

With cereal grains, chaff and hay forming the main components of diets fed to horses, the early theories on how a horse should be fed were numerous. Halnan *et al.* (1953) commented that the many ideas that are prevalent among grooms were too numerous to

document, but that once settled on a 'favourite' ration, people were reluctant to try different diets.

> *"There are many deviations that can be made in the feeding of horses; many horse keepers and grooms prefer, however, to use the old tried rations and are loath to use the newer ones".*
>
> - Halnan *et al.* (1953)

Hewitt (1961) suggested the following daily ration for a heavy horse in work; "four kerosene tins full of chaff, three 7-pound jam tins full of oats and one sheaf of manger hay". Horse owners were advised to feed little and often with feeding three times per day viewed as the minimum acceptable number of feeds. For horses in hard work on a high grain ration, several small feeds per day were recommended. Gradual introduction of grain concentrates and new ingredients to the ration was advised to prevent any digestive disturbances (Anderson *et al.*, 1971; Hewitt, 1961). Vitamin and mineral supplements were not viewed as essential components of a ration formulated for mature working horses, as it was believed that a horse's vitamin requirements were small and given plenty of good quality hay, they would be adequately provided in the ration (Morrison, 1954).

As most of the nutritional advice in the early 20th century was for heavy draught horses used for work, there is scarce information about the feeding of light horses and thoroughbreds. Halnan *et al* (1953) made the following comment about the differences between the feeding of heavy horses and the feeding of racehorses.

> *"Here, without a doubt, one passes from the spheres of science into the realms of art"*
>
> (Halnan *et al.*, 1953).

Trainers of racehorses were known to have all sorts of feeding formulas, however trainers also believed that there was 'no grain like oats for horses' and that oats was the 'best energy-producing grain for thoroughbreds'. Thus the grain was almost invariably fed to thoroughbreds. Trainers often paid exceptional prices for oats to ensure they got only the best quality 'milling' oats (as opposed to 'feed grade' oats) (Halnan *et al.*, 1953; Morrison, 1954). It is noted that racehorses were fed higher quantities of grain than the average working draught horse, with the ration for these horses commonly being 3.6 to 4.5 kg of oats and 2.7 to 3.6 kg of hay or chaff. On the other hand, the quantity of hay fed to thoroughbreds was much lower than that fed to draught horses and only prime quality hay was fed (Halnan *et al.*, 1953). Barley, maize, bran and linseed meal were also fed to racehorses. Limestone and salt were recommended supplements to add to a racehorse's feed (Hewitt, 1961).

Although, oats, chaff and hay are still major components of diets for working horses, feeding practices have changed somewhat since the latter part of last century. Modern feeding practices in the Australian thoroughbred industry are detailed in Chapter 3.

2.1.2 Gastrointestinal Physiology

The horse is a monogastric animal and based on digestive physiology is classified as a non-ruminant herbivore and more specifically as a hindgut fermenter (Jackson, 2000). The gastrointestinal tract consists of the mouth, oesophagus, stomach, small intestine and the highly developed large intestine composed of the caecum, proximal and distal colons, small colon and rectum (Figure 2.1, Argenzio, 1993c).

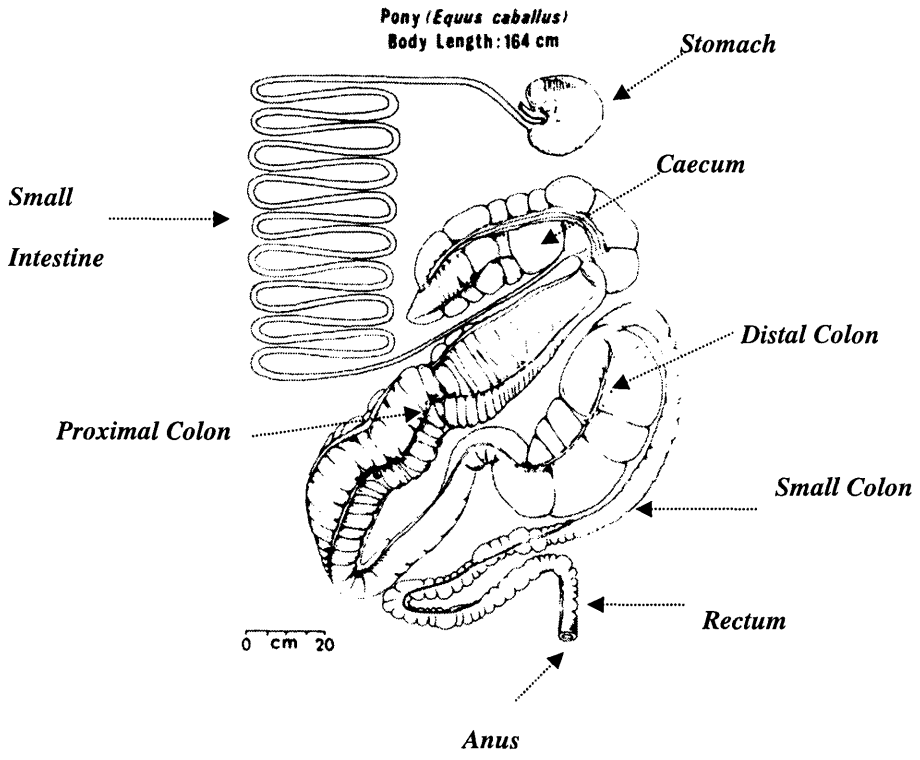

Figure 2.1: The structure of the gastrointestinal tract of *Equus caballus* (adapted from Argenzio, 1993c).

The Mouth

The digestive process begins with food entering the mouth. The lips and teeth of the horse are specifically adapted for grazing forages. The upper lip is used to place forage between the teeth, and the teeth are used, in the first instance, to shear the forage from its base. In contrast to sheep and cattle, the horse has incisors on both the top and bottom jaws. The tongue pushes forage to the molars and premolars where it is intensely chewed reducing its size to a few millimetres in length. The horses jaw moves in a lateral motion allowing the effective 'mechanical grinding' of coarse plant material (Argenzio, 1993b).

Due to the inability of the horse to regurgitate and re-chew feeds as in the case of ruminants, mastication needs to be thorough resulting in longer chewing times and slower ingestion rates than ruminants. Thus the horse in general needs more time to eat than the ruminant (Budiansky, 1998). Whilst in the mouth, food is mixed with saliva. Horses may secrete 10 – 12 L of saliva/day with secretion increasing during periods of feeding. There is no evidence that equine saliva has any enzymatic function. Rather it acts as a lubricant and can contain up to 50 mEq/L of bicarbonate ions and some NaCl to help buffer the proximal region of the stomach (Anderson *et al.*, 1971; Frape, 1998; McDonald *et al.*, 2002).

Stomach

Once food is swallowed, it is moved via peristaltic movements down the oesophagus and through the cardiac sphincter into the stomach. The stomach of the horse has a capacity of approximately 5 – 15 L and comprises around 10% of the total volume of the digestive tract. The stomach is a 'J' shaped organ, situated under the diaphragm and has two major functions: the mixing, storage and controlled release of digesta into the small intestine; and the initiation of protein digestion (Argenzio, 1993b; Budras *et al.*, 2001; Frape, 1998)

The stomach is divided into four main regions (Figure 2.2), with each area having a specific function. The regions and their specific functions are:

1. stratified squamous area - the large region where the oesophagus enters the stomach. This region is lined with stratified squamous tissue and is non-glandular. This area has no digestive function and acts only as a storage area;
2. the cardiac region – this region contains cardiac glands that produce mucus, which is thought to be their only function;
3. the proper gastric mucosa - secretes hydrochloric acid, from parietal cells and pepsinogen, the inactive form of pepsin, from peptic cells, into the lumen to be mixed with digesta. Parietal cells also secrete 'intrinsic factor', a mucoprotein essential for vitamin B_{12} absorption (Argenzio, 1993e); and
4. the pyloric region - secretes mucous and some pepsinogen and also contains the 'gastrin cell', which, when stimulated, releases the hormone gastrin into the blood (Argenzio, 1993e). Gastrin then acts on parietal cells to promote further gastric secretion (Argenzio, 1993c). The pH of the pyloric region of the equine stomach is around 2.6 for horses on a roughage diet (Kern *et al.*, 1974).

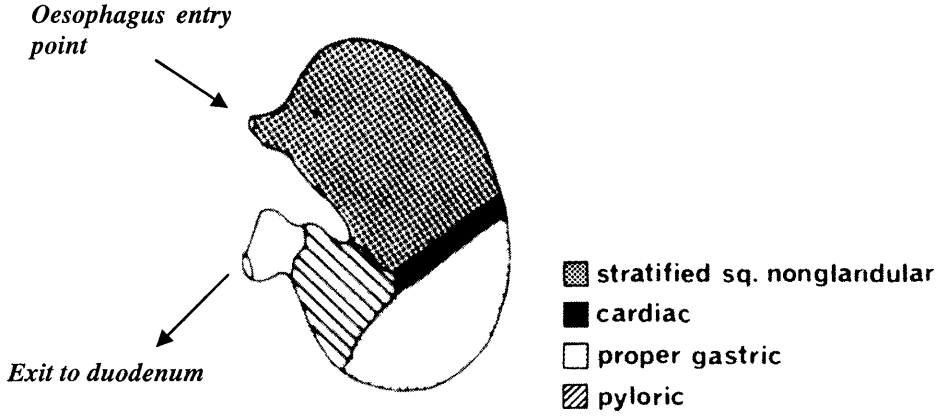

Figure 2.2: Structure of the horse stomach, showing the type and distribution of gastric mucosa (adapted from Argenzio, 1993b).

Digesta commonly makes a rapid passage through the horse stomach. However, the stomach is rarely empty, with expulsion of digesta into the duodenum being arrested when

feeding stops (Frape, 1998). It has been observed that 99% of ingested dry matter will be emptied from the equine stomach within 18 hours and that the stomach will be completely empty 24 hours after a roughage meal (Healy *et al.*, 1993). Kern *et al.* (1974) observed that only 20% of ingested food remained in the stomach 2 hours postprandially. Passage of liquid from the stomach is commonly more rapid than that of solid digesta. Following consumption of a 7.5 g/kg body weight (BW) meal, 75% of liquid marker (polyethylene glycol-4000) administered during the meal had left the equine stomach within half an hour, however 75% of a particulate marker (2 mm radiopaque polyethylene tubing) remained in the stomach after the same time period (Argenzio *et al.*, 1974).

The pyloric sphincter regulates the exit of chyme from the stomach and entry to the small intestine. Regulation of gastric emptying is important to ensure thorough digestion of feedstuffs in the small intestine. Gastric emptying may be slowed if hypertonic, acidic or irritating contents enter the small intestine, or when digesta contains high levels of lipid (Argenzio, 1993b; Cooke, 1975; Cooke *et al.*, 1976; Pagan, 2001).

Small Intestine

The equine small intestine is approximately 20 – 27 metres long with a capacity of 55 – 70 litres and is composed of three sections: the duodenum, approximately 1 m long; the jejunum, approximately 25 m long and; the ileum, a 50 cm section at the end of the small intestine (Budras *et al.*, 2001). It is in the small intestine that a majority of non-structural carbohydrate, protein and fat digestion and absorption takes place. The inner surface of the small intestine has a massive surface area in order to allow efficient digestion and absorption of carbohydrates, fats and proteins. The internal surface is lined by visible folds of Kerckring, which increase the surface area by a factor of three. These folds are in turn covered by microscopic villi, which are themselves lined with microvilli, increasing the surface area by a factor of 10 and 600, respectively (Argenzio, 1993a). The microvilli, also known as the 'brush border', are composed of epithelial cells (enterocytes) and it is here that the brush border saccharidase and proteinase enzymes are located. The brush border also houses specialised transport mechanisms that facilitate movement of digestion end products from the lumen of the small intestine into the cytoplasm of brush border cells (Argenzio, 1993a; Gray, 1992; Wright, 1993).

A majority of enzymes involved in the digestion of non-structural carbohydrate, fat and protein are produced in the pancreas of the horse and secreted into the duodenum via the pancreatic duct (Budras *et al.*, 2001). Pancreatic secretion in the horse is unusual in that the concentration of enzymes is low. However, the quantity is profuse, with up to 12 L being secreted per day by a 100 kg pony, in comparison to 0.5 – 1.0 L/100 kg/day for sheep (Argenzio, 1993e).

Passage of chyme through the horse's small intestine is rapid, moving at approximately 30 cm/min and delivering digesta to the caecum in as little as 45 minutes after a meal (Frape, 1998). Argenzio (1993a) estimated that the mean transit time from the stomach to the

caecum in an adult pony is 2 hours, while de Fombelle *et al.* (2001) observed a mean retention time of nylon bags in the foregut (stomach and small intestine) of two 400 kg horses to be 5.7 hours. As observed in the stomach, liquids move more rapidly along the small intestine than particulate matter (Argenzio *et al.*, 1974).

The digestion and absorption of non-structural carbohydrates, including starch, occurs in the small intestine and the specifics of starch digestion will be covered in more detail in Section 2.2. Protein and fat digestion in the equine small intestine will be briefly covered here.

Although protein digestion is initiated in the stomach with the enzyme pepsin, most activity occurs in the duodenum and jejunum of the small intestine. The pancreatic enzymes trypsinogen, chymotrypsinogen, elastase and carboxypeptidases, as well as brush border oligopeptidases cleave protein into tripeptides, dipeptides and free amino acid units that are absorbed from the small intestine (Argenzio, 1993a; Fox, 1991). The NRC (1989) recommends that weanling and yearling horses receive 11.95 g and 10.76 g of crude protein/MJ of digestible energy respectively. Mature horses at rest and at work have a protein requirement of 9.56 g of crude protein/MJ of digestible energy (NRC, 1989). No specific amino acid requirements for mature horses are provided by the NRC, on the basis that horses fed 'typical ingredients' are likely to receive adequate amounts of essential amino acids.

The exclusive site for fat digestion in the horse is in the small intestine. When fats enter the small intestine, they are emulsified by bile salts to form minute emulsified droplets of triglyceride. Pancreatic lipase degrades the triglycerides, removing two of the three fatty acids to form free fatty acid and monoglyceride units. The fatty acids and monoglycerides are then incorporated into micelles, a mixture of bile salts, lecithin and cholesterol and absorbed from the small intestine (Argenzio, 1993a; Fox, 1991).

Fats and carbohydrates are major suppliers of energy in a horse's diet. There are many theories regarding the amount of energy a horse requires (Anderson *et al.*, 1983; Hintz *et al.*, 1966; NRC, 1989). The NRC (1989) publication states that:

> *"Numerous factors such as individuality, body composition of the animal, environmental temperature and humidity, intensity and duration of work, weight and ability of the rider, conditions of the running surface, and degree of fatigue can influence the energy requirements of a horse"*
>
> (NRC, 1989)

Thus the calculation of a horse's energy requirement based on a single 'standard' equation should be treated as a guide only, as many of the above factors will not be taken into account. The NRC (1989) estimates that the daily maintenance energy requirement for a horse 600kg or less can be calculated using the following equation:

$$DE\ (MJ/day) = 4.184(1.4 + 0.03BW)$$

Where: DE = digestible energy; BW = body weight in kg.

It is recommended that daily digestible energy allowance be increased by 25, 50 and 100 percent above maintenance for horses in light, medium and intense work with *light* work being defined as pleasure and equitation, *medium* work defined as ranch work, barrel racing and show jumping, and *intense* work being racing and polo.

Large Intestine

The large intestine of the horse comprises the caecum, colon, small colon and rectum and is commonly referred to as the 'hindgut'. The major functions of the hindgut are to:

(1) provide a facility for microbial digestion of feedstuffs not already digested in the stomach and small intestine; and

(2) reabsorb electrolytes and water.

It is not clear whether the hindgut has the ability to absorb amino acids (Judson *et al.*, 1975; Reitnour *et al.*, 1969; Slade *et al.*, 1971; Slade *et al.*, 1970), however, given that equines evolved to survive on poor quality roughage, it would make sense that protein absorption occurs from the hindgut, in order to allow horses to utilise the rich source of bacterial protein present there. This would also explain how the protein requirements, particularly of pregnant or lactating mares, are satisfied when consuming low protein roughages.

The caecum is approximately one meter long with a capacity of roughly 35 litres. The colon, which may be divided into the proximal and distal colons, is approximately four meters long with a capacity of 80 litres. The small colon is around three meters long and links the distal colon and rectum (30 cm), which terminates at the horses anus (Budras *et al.*, 2001; Frape, 1998). Mucous secreting cells line the internal surface of the hindgut (Frape, 1998).

Carbohydrates and proteins that remain undigested after passing through the small intestine are fermented by the populations of micro-organisms that live in the hindgut of the horse. There are approximately 0.5×10^9 to 5×10^9 micro-organisms/g of digesta resident in the hindgut of the horse (Frape, 1998). The end products of fermentation are primarily volatile fatty acids (VFA) and ammonia. Both products are absorbed from the hindgut via passive diffusion mechanisms (Leek, 1993).

Several species of cellulolytic bacteria in the hindgut possess the enzymes necessary to digest β-linked carbohydrates such as cellulose, hemicelluloses (pentosans, β-glucans, xyloglucans, arabinans and galactans), fructans and pectin. Such species include the rod shaped *Fibrobacter (Bacteroides) succinogenes* (gram –ve), *Butyrivibrio fibrisolvens* (gram –ve) and *Clostridium locheadii* (gram +ve) and the cocci *Ruminococcus albus* (gram –ve) and *Ruminococcus flavefaciens* (gram +ve) (Hungate, 1966). The VFAs produced during β-linked carbohydrate fermentation are primarily acetate (70%), propionate (15%) and butyrate (10%). These VFAs constitute an important energy source for the horse, particularly if the diet contains large quantities of roughage. Cellulolytic bacteria have a low metabolic rate and take approximately 18 hours to double their population (Leek, 1993).

The optimum pH that these bacteria need to degrade cellulose is between 6.2 and 6.8. Kern *et al.* (1974) report an average pH of 6.6 in the caecum and colon of horses maintained on a roughage diet.

There are several steps in the fermentation of carbohydrate to produce VFAs. The first stage involves hydrolysis of carbohydrates to their constituent monosaccharides. The monosaccharides are then converted to fructose-1,6-bisphosphate, which is anaerobically oxidised to form pyruvate, via phosphoenolpyruvate (Leek, 1993). The final stages involve various reactions to form VFAs. The pathways for the production of the major VFAs, acetate, propionate and butyrate are outlined in Figure 2.3.

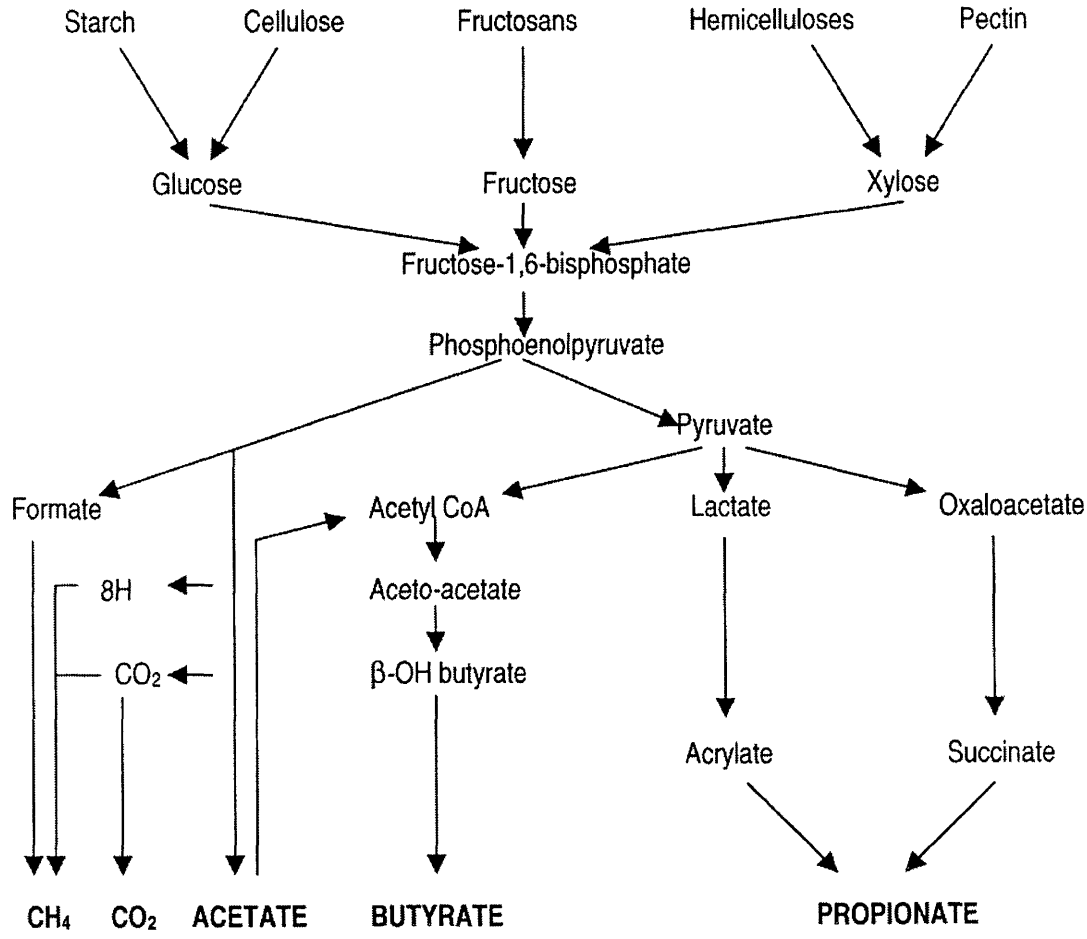

Figure 2.3: The pathways for the fermentation of dietary carbohydrates to form the volatile fatty acids acetate, propionate and butyrate (adapted from Leek, 1993).

Acetate, propionate and butyrate are passively absorbed from the hindgut of the horse, with production and absorption of VFAs being in equilibrium (DeGregorio *et al.*, 1984; Schwabenbauer *et al.*, 1982). Acetate is absorbed from the hindgut and converted to acetyl CoA in body tissue. Acetyl CoA is then used in the citric acid cycle for energy generation. Propionate is converted to oxaloacetate by the liver and is then subsequently used in the citric acid cycle. Propionate is also the only VFA, which may be converted to glucose by the liver. Butyrate is converted to β-hydroxybutyrate by the liver or the gastrointestinal

wall. β-hydroxybutyrate is readily metabolised and used as an energy source by most body tissues (Leek, 1993).

Bacteria in the hindgut also ferment proteins that escape digestion in the small intestine. Proteins are adsorbed to the outer surface of bacteria where they are broken down by microbial protease enzymes to peptides and free amino acids. The peptides and amino acids can be utilised by microbial cells. Using energy in the form of adenosine 5'-triphosphate (ATP) or guanosine 5'-triphosphate (GTP) to form peptide bonds, the microbial cells reassimilate the free amino acids into microbial protein (Hungate, 1966; J. Nolan *pers. comm.*). Amino acids can also be fermented to VFAs and ammonia. Ammonia may be utilised by micro-organisms for the synthesis of microbial protein. Urea recycled from body tissues can also enter the hindgut and provide a satisfactory source of nitrogen for microbial protein synthesis. Given an adequate supply of energy and protein or nitrogen, micro-organisms in the rumen or hindgut are able to multiply rapidly (Hungate, 1966; J. Nolan *pers. comm.*).

Efficient fermentation within the hindgut depends on a well-buffered environment and a retention time sufficient to allow cellulose fermentation. The buffering system of the large intestine is dominated by HCO_3^- and PO_4^- (Alexander, 1962; Argenzio, 1993a). Pancreatic HCO_3^-, released to neutralise digesta moving from the stomach to the small intestine, may contribute to the buffering system of the large intestine. However, HCO_3^- secreted into the ileum and mixed with digesta as it passes into the hindgut is the major source of buffering. The hindgut is also capable of secreting HCO_3^- (Argenzio, 1993a). Phosphate buffers are also important in hindgut buffering. However, unlike ruminants, where PO_4^- is primarily derived from saliva, in the equine hindgut it is derived from the diet. The hindgut buffering system normally achieves precise control of hindgut pH, maintaining it within the range optimum for cellulolytic bacteria (6.2 – 6.8, Argenzio, 1993a)

Transit time through the large intestine of an adult pony is approximately 50 hours. However digesta may remain in the hindgut for as long as 10 days (Argenzio, 1993b; Argenzio *et al.*, 1974). Markers administered directly to the caecum have been observed to move quickly into the proximal colon. Thus it is assumed that digesta entering the hindgut from the small intestine, first enters the caecum, before being moved relatively quickly to the proximal colon. Peak concentrations of marker in the proximal colon were observed 12 hours after direct administration to the caecum (Argenzio *et al.*, 1974). The caecal-colonic orifice, the proximal colon-distal colon and the distal colon-small colonic orifices provide resistance to digesta flow in the hindgut, presumably to prolong the length of time digesta is held in the hindgut to allow the relatively slow process of cellulose fermentation to occur (Argenzio, 1993b; Argenzio *et al.*, 1974). Larger particles tend to be retained in the horse's hindgut for longer periods of time than liquids and small particles. It has been observed that 60% of 2 cm pieces of radiopaque polyethylene tubing markers remained in the hindgut 10 days after administration (Argenzio *et al.*, 1974).

The second major function of the hindgut is to absorb water of dietary origin and reabsorb water and electrolytes secreted into the upper digestive tract. Water and solutes may be actively transported via intracellular pathways or passively transported via extracellular pathways (Argenzio, 1993d). In an adult 160 kg pony, the hindgut absorbed/reabsorbed 30 litres of water/day, a volume equivalent to the animal's extracellular fluid space (Argenzio *et al.*, 1974).

2.2 Digestion of Starch and Absorption of Glucose in the Equine Small Intestine

2.2.1 Introduction

The main component of dry matter in cereal grains is starch. Cereal grains are common ingredients in the diets of performance horses, with the average Australian thoroughbred being fed 7.3 kg of grain/day (Chapter 3). Cereal grains have 12 – 16 MJ of digestible energy/kg DM and are a more concentrated source of energy than good quality roughages that have approximately 8-9 MJ of digestible energy/kg DM. Cereal grains are thus fed to performance horses with high energy requirements to increase the energy density of the diet, allowing large quantities of energy to be consumed in smaller meals (Frape, 1998; McDonald *et al.*, 2002; NRC, 1989). Starch provides a majority of the energy present in cereal grains and is primarily digested in the small intestine by pancreatic and small intestinal brush border glycanases. The glucose that is produced is absorbed from the small intestine and used as a source of energy in the animal's body. An explanation of the structure of starch follows. The importance of this for the present study rests in the part starch structure plays in its digestion in the equine small intestine.

2.2.2 Starch

Starch is the primary storage polysaccharide used by plants and is deposited in granular storage bodies in the seeds, roots and tubers of many plant species. Two forms of starch exist in nature, amylose and amylopectin. Amylose consists of long, unbranched chains of D-glucose units connected by (α 1→ 4) linkages with an average molecular weight of up to 100 kDa (Figure 2.4a). Amylopectin is a highly branched polysaccharide consisting of linear (α 1→ 4) linked D-glucose molecule chains and (α 1→ 6) linked branch points that occur approximately every 20 residues (Figure 2.4b). Amylopectin has a molecular weight of 1000 – 6000 kDa (Lehninger *et al.*, 1993; Zobel, 1988).

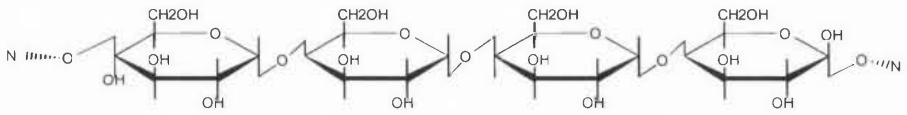

Figure 2.4a: The chemical structure of amylose

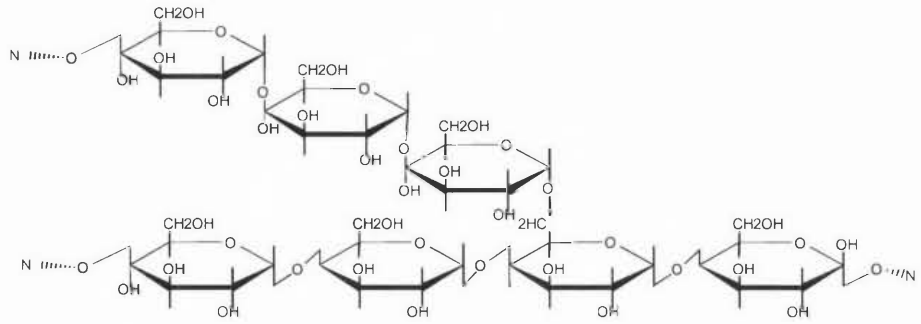

Figure 2.4b: The chemical structure of amylopectin

2.2.3 Enzymatic degradation of Starch

Starch digestion in the small intestine of the monogastric occurs via a three-step process, beginning with the degradation of starch by α-amylase (Figure 2.5). α-Amylase breaks the two principle forms of starch, amylose and amylopectin (Figures 2.4a and 2.4b) into disaccharide (maltose), trisaccharide (maltotriose) and α- limit dextrin units (Fig 2.6a, 2.6b and 2.6c) (Gray, 1992). The pH-optimum for amylase taken from horse pancreatic tissue and jejunal chyme is pH 7. Activity is decreased by 28% and 26% when the pH is reduced to 6 and raised to 8 respectively (Kienzle *et al.*, 1994).

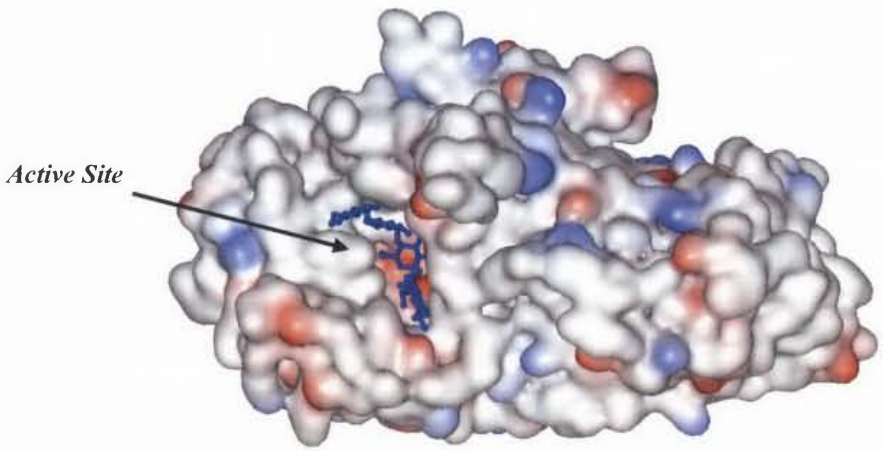

Figure 2.5: The structure of porcine α-amylase, with an α-amylase inhibitor bound to the active site (courtesy of A. Shaw, Genencor International).

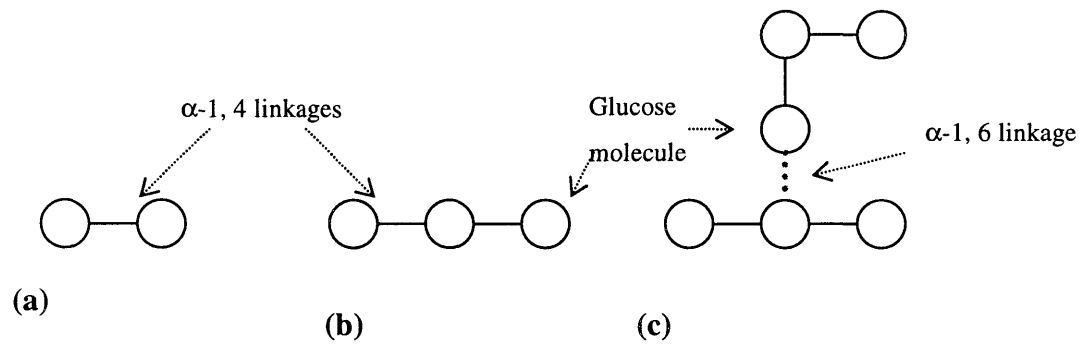

Figure 2.6: The structure of (a) maltose, (b) maltotriose and (c) α- dextrin units, adapted from Gray (1992).

The second phase of starch digestion involves the hydrolysis of maltose, maltotriose and α-dextrin units by small intestinal brush border glycanases, to form free glucose units (Argenzio, 1993a; Gray, 1992). Brush border glycanases, namely maltase, α-dextrinase and amyloglucosidase (AMG), are synthesised within intestinal enterocytes before being transferred to the intestinal brush border, where they remain anchored to the surface via a short terminal hydrophobic section of their protein chain (Gray, 1992). The specific substrates and action of each brush border glycanase are summarised in Table 2.1. Once single glucose units are liberated, they are transported across the brush border surface of the small intestine and into the animal's bloodstream

Table 2.1: The preferred substrates and specific activity for brush border glycanase enzymes, adapted from Gray (1992)

Brush Border Glycanase	Substrate	Action
Amyloglucosidase (AMG)	α-1,4 glycans α-dextrins (α-1,4 links)	Removes single glucose residues sequentially from the non-reducing end of the α-1,4 chain. Is blocked when an α-1,6 linked glucose is situated at the terminal end of the oligosaccharide (Gray, 1992).
α-Dextrinase	α-dextrins	Cleaves the non-reducing terminal α-1,6 link of α-dextrins, once uncovered by AMG (Gray, 1992).
Maltase	Maltose and Maltotriose	Breaks α-1,4 links to liberate glucose. Prefers shorter chain α-1,4 glycans, particularly maltose and maltotriose (Gray, 1992).

2.2.4 Glucose Absorption

Two transcellular transport systems are used to move glucose from the lumen of the small intestine, through intestinal epithelial cells (enterocytes) and into the bloodstream of the animal; Na^+-dependant active transport and Na^+-independent facilitated diffusion (Bird *et al.*, 1996; Hediger *et al.*, 1994; Huntington, 1997; Thorens, 1993). Glucose is absorbed by mature intestinal enterocytes, which line the upper third of intestinal villi (Hediger *et al.*, 1994; Wright, 1993). Na^+-dependant active transport is carried out by sodium-glucose transporters (SGLT1), which are embedded in the apical pole of the intestinal enterocytes. The SGLT1 perform the first step in glucose transport by carrying glucose against its

concentration gradient from the intestinal lumen into the enterocytes (Figure 2.7). Na^+ ions are transported down their electrochemical gradient by the SGLT1 to provide the energy needed to co-transport glucose (Hediger *et al.*, 1994; Thorens, 1993, Wright, 199). The SGLT1 transport one molecule of glucose and two molecules of sodium every cycle and in the human and mouse small intestine they have the capacity to carry out approximately 50 to 170 cycles per second respectively (Ferraris *et al.*, 1989; Hediger *et al.*, 1994)

The glucose accumulated in the enterocytes is then transported across the basolateral membrane (basal pole of enterocyte) and into the extracellular space adjacent to blood capillaries via Na^+-independent facilitated diffusion that is effected by GLUT2 transporters (Figure 2.7, Thorens, 1993; Cheeseman *et al.*, 1992).

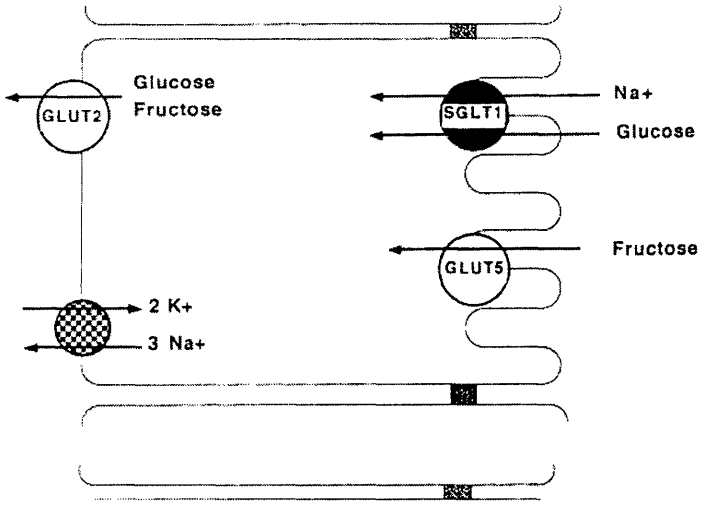

Figure 2.7: The epithelial cell of the small intestine, with SGLT1 transporters moving glucose from the intestinal lumen into the cell and GLUT2 transporters moving glucose from within the cell into the extracellular space adjacent to blood capillaries. Adapted from Thorens (1993)

Passive paracellular diffusion also plays a role in glucose transport and is driven by differing solvent concentration gradients between the intestinal lumen and the interstitial fluid underlying the epithelial layer (Bird *et al.*, 1996; Huntington, 1997). Pappenheimer *et al.* (1987), who estimate luminal glucose concentrations to range between 50 mM- 500 mM, argue that at luminal glucose concentrations of >250 mM, the contribution of passive diffusion to glucose absorption outweighs that of active transport. Ferraris *et al.* (1990) however found luminal glucose concentrations normally ranged between 0.2 mM and 48 mM, and did not exceed 100 mM. Thus Bird *et al.* (1996) and Ferraris *et al.* (1990) disagree with Pappenheimer *et al.* (1987) stating that the contribution paracellular diffusion makes to overall glucose transport is likely to be minimal. This is due to the small glucose concentration differential between the gut lumen and mesenteric blood, which in the horse, is estimated to be 4.4 mM at basal levels (Ruckebusch *et al.*, 1991).

2.2.5 Insulin

The transport of glucose from the small intestine into the bloodstream and the resulting rise in blood glucose concentrations stimulates the release of the hormone insulin from the

pancreas. Insulin is a small 51-amino acid protein with a molecular weight of approximately 5700 (Dickson, 1993), thus when released it is metabolised quickly with a half-life of 5-10 minutes (Sperelakis *et al.*, 1996). Insulin is stored in secretory granules in the beta cells of the pancreas and the normal stimulus for insulin secretion is the increasing concentration of glucose perfusing the pancreatic islets of Langerhans and the metabolism of this glucose in the insulin-producing beta cells (Dickson, 1993; Reiser, 1967; Sperelakis *et al.*, 1996). When stimulated by increasing blood glucose concentrations, secretory granules that contain insulin, fuse with the plasmalemma of the beta cells in the pancreas, releasing insulin from storage. Insulin is released in two phases, the first being the rapid release of stored insulin and the second, a much slower release of newly synthesised insulin (Sperelakis *et al.*, 1996).

The primary effect of insulin on carbohydrate metabolism is to increase the uptake and storage of glucose in muscle, adipose and organ tissue, thus reducing blood glucose concentrations. Insulin achieves this by promoting the active transport of glucose via GLUT 4 transporters, into muscle and adipose cells that are otherwise virtually impermeable to glucose in the absence of insulin. Within these cells, and in the cells of the liver, which is freely permeable to glucose, insulin promotes the production of ATP from glucose and inhibits the production of glucose via gluconeogenesis (Figure 2.8) (Dickson, 1993; Lehninger *et al.*, 1993). When cell requirements for ATP are satisfied, insulin promotes the storage of glucose within the body by increasing the activity of enzymes responsible for glycogenesis and lipogenesis (Figure 2.8, Sperelakis *et al.*, 1996; Dickson, 1993; Lehninger *et al.*, 1993).

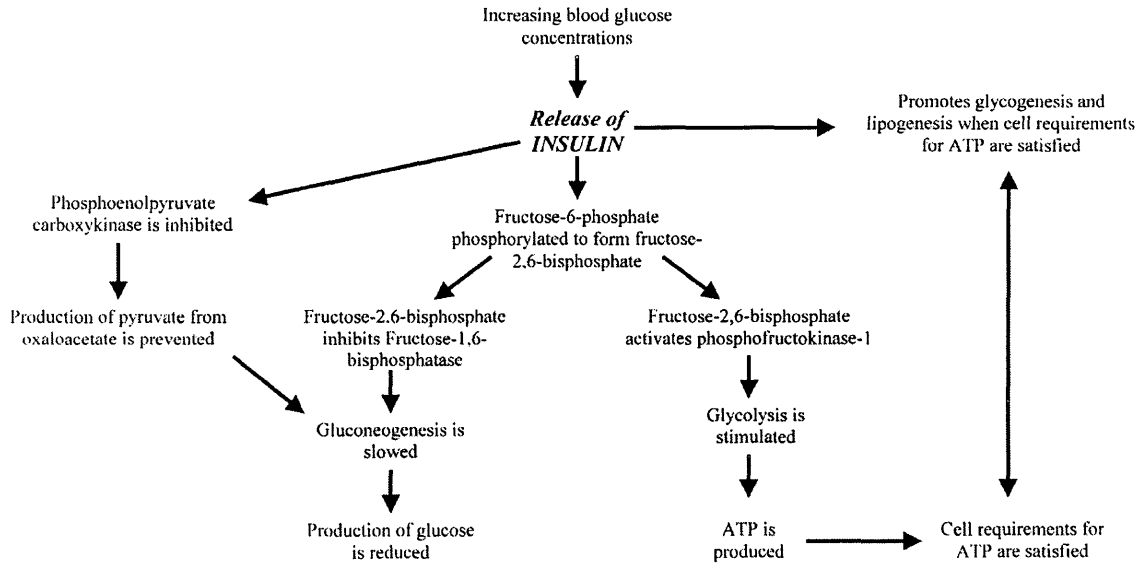

Figure 2.8: The functions of insulin in the control of blood glucose concentration and utilisation of glucose as an energy source within body tissue (Dickson, 1993; Lehninger *et al.*, 1993; Sperelakis *et al.*, 1996).

Glucagon, insulin's antagonistic hormone is released when hypoglycaemia is detected in the blood. Glucagon activates the enzymes glycogen phosphorylase and fructose-1,6-bisphosphatase, stimulating the production of glucose via glycogenolysis and

gluconeogenesis respectively. The enzymes phosphofructokinase and glycogen synthase are inhibited, thus preventing glycolysis and glycogenesis and thereby stopping the use of glucose as fuel and the storage of glucose as glycogen (Dickson, 1993; Lehninger *et al.*, 1993). With the intimately controlled release of insulin and glucagon, blood glucose concentrations are maintained at a 'normal baseline' level, which is approximately 4.4 mmol/L in the horse (Ruckebusch *et al.*, 1991).

2.3 Factors Affecting Small Intestinal Starch Digestion in the Horse

As this study sought to examine the effect of grain species, grain processing and dietary enzyme supplementation on the digestion of starch in the equine small intestine, it is useful to overview theory and research regarding factors that affect pre-caecal starch digestion in horses. There are many factors that affect the rate and extent of starch digestion and glucose absorption in the horses' small intestine. These factors may be grouped into three broad categories:

(i) grain and starch characteristics;

(ii) physiological and physical differences between horses; and

(iii) feeding management which controls parameters such as meal size, diet composition and feeding frequency (Figure 2.9).

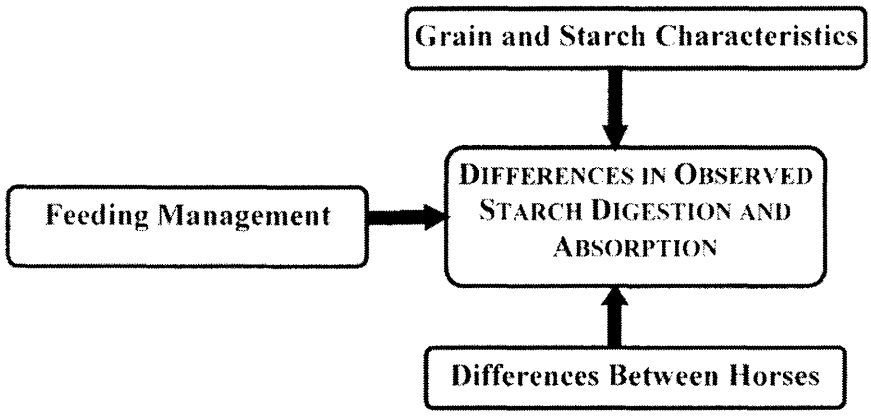

Figure 2.9: The factors affecting small intestinal starch digestion in horses.

A detailed discussion of these factors and how they affect small intestinal starch digestion in the equine follows.

2.3.1 Grain and Starch Characteristics

It is well recognised that the extent of starch digestion in the small intestine varies between grain species when consumed by horses, cattle, sheep, pigs and poultry (de Fombelle *et al.*, 2001; Huntington, 1997; Meyer *et al.*, 1993; Meyer *et al.*, 1995; Potter *et al.*, 1992; Rowe *et al.*, 1999; Theurer, 1986; Weurding *et al.*, 2001). Grain and starch characteristics that affect starch digestion in equines are presented diagrammatically in Figure 2.10. Differences in

rate and extent of starch digestion between grains may be due to starch granule structure, the biochemical structure of the starch polymers incorporated into the starch granule and the method by which enzymes degrade the starch granule. The seed coat, endosperm cell walls and protein matrix structures of the grain may also affect starch digestion, as can the growth and storage conditions and the method of processing used prior to feeding (Figure 2.10).

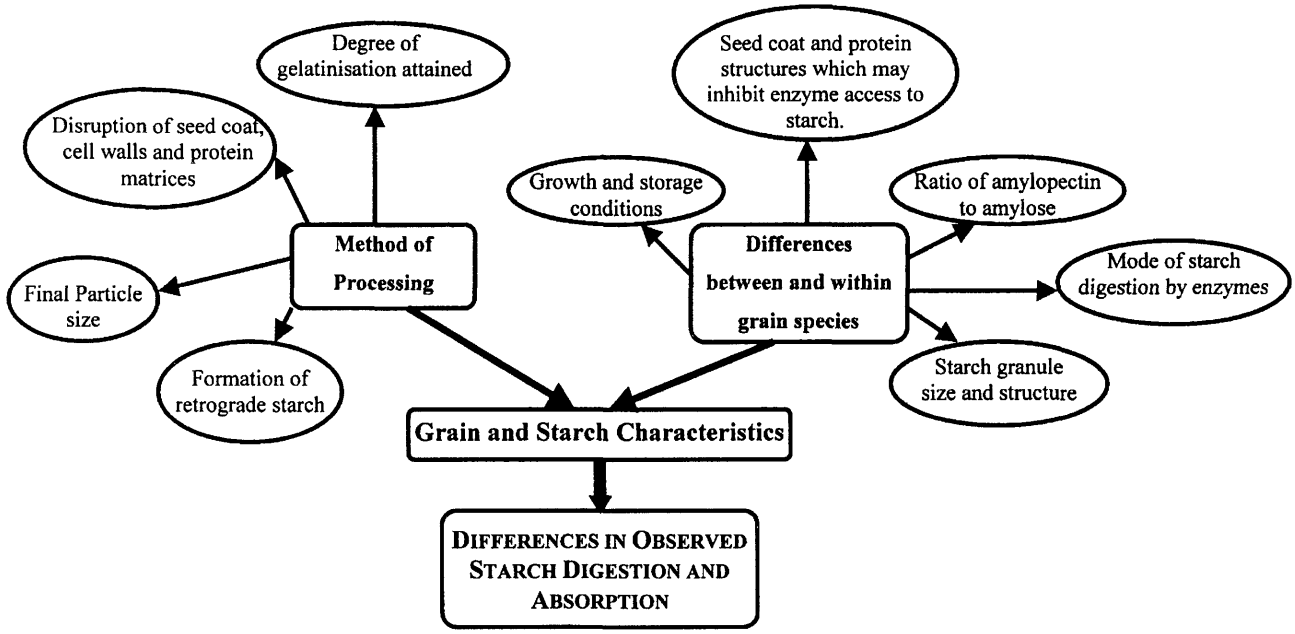

Figure 2.10: Grain and starch characteristics that may affect the extent of digestion and absorption of starch in the equine small intestine.

These grain and starch related factors that affect starch digestion in the small intestine of equines are discussed in more detail below.

Seed Coat

Cereal grains are comprised of the embryo, scutellum, starchy endosperm, aleurone, nucellus, testa and finally the pericarp. The pericarp is the outermost layer of a cereal grain and is composed of several complete and incomplete layers of dried and mostly empty cells, when the grain is mature (Evers *et al.*, 1999; Rowe *et al.*, 1999). The pericarp protects the seed from moisture, insect and fungal attack and provides an effective barrier to digestive enzymes, restricting their access to the starch contained within the endosperm of the seed. Thus either chewing, or mechanical processing must crack the pericarp to allow digestive enzymes access to starch granules. Once this physical barrier to starch digestion is removed, the pericarp has little further influence on the digestion of starch (Rowe *et al.*, 1999).

Starch Granule and Biochemical Structure

Starch is a polysaccharide composed of the two α-linked glucose polymers, amylose and amylopectin (Figure 2.4). These starch polymers are deposited within grain endosperm cells in semi-crystalline granules (Figure 2.11). Within these granules, amylopectin and amylose molecules are held together via hydrogen bonding (Banks *et al.*, 1980; Kainuma, 1988; Kienzle, 1994; MacMasters *et al.*, 1959; Rooney *et al.*, 1986). Starch granules are insoluble in cold water, have the ability to swell reversibly and contain both crystalline and amorphous regions (Rooney *et al.*, 1986). It is estimated that 15 – 45% of starch within native starch granules is crystalline in structure (Gallant *et al.*, 1997; Zobel, 1988).

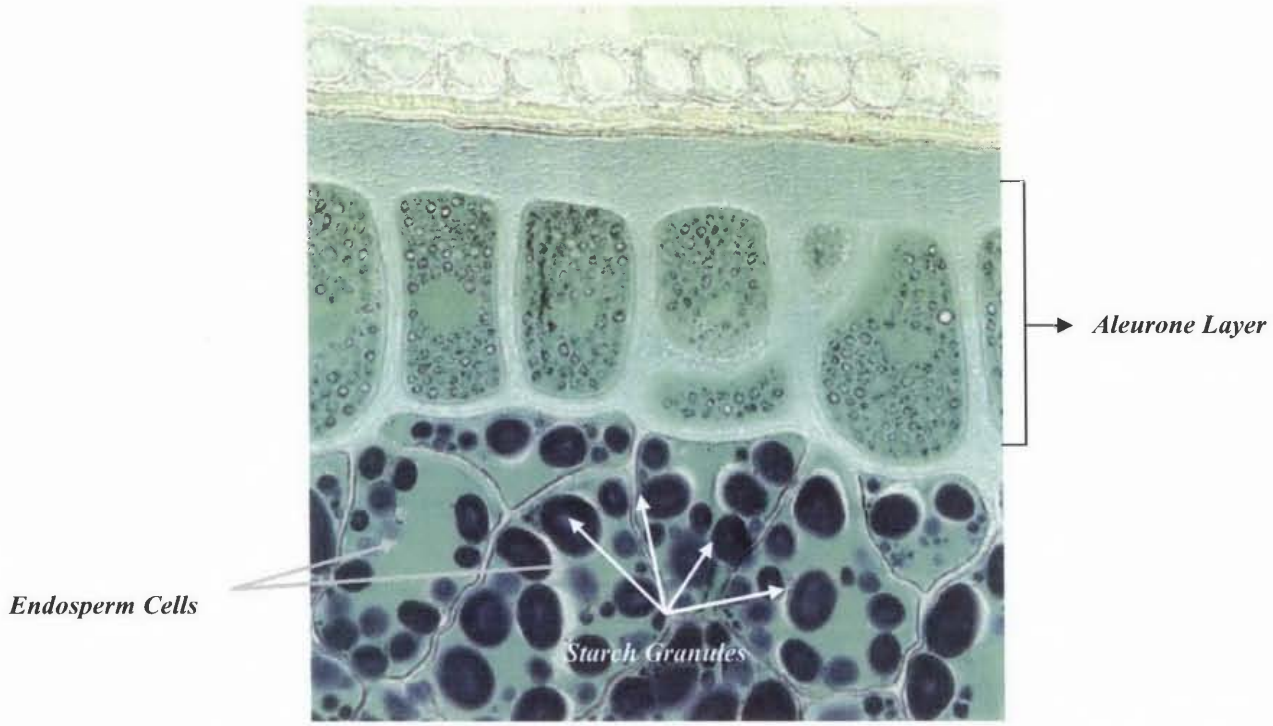

Figure 2.11: The location of starch granules (stained black) within the endosperm cells of barley grain. Note the highly variable starch granule size. Protein (stained green) surrounds the starch granules within the endosperm cells (images produced by P. Littlefield, University of New England).

The relative ratios of amylose to amylopectin within starch granules vary between cereal grains and have a large influence on starch digestibility (Banks *et al.*, 1980; Kainuma, 1988; Kienzle, 1994; MacMasters *et al.*, 1959; Zobel, 1988). Cereal grain starch granules primarily contain the branched chain polymer amylopectin (70 – 80% of starch) with smaller quantities of the mainly linear polymer amylose (20 – 30% of starch) (Rooney *et al.*, 1986). Within the starch granule it appears that amylopectin provides the crystalline skeleton, while amylose tends to be non-crystalline or amorphous in structure (Banks *et al.*, 1980; Rooney *et al.*, 1986). However, when the starch is isolated and placed in solution, amylose can be crystallised from solution, while amylopectin is amorphous and easily solubilised in water (Banks *et al.*, 1980). Thus amylopectin tends to be more susceptible to enzymatic attack within the small intestine (Kienzle, 1994; Rowe *et al.*, 1999). A review by Kotarski *et al.* (1992) outlines various *in vitro* studies that have shown that high

amylopectin grains (waxy varieties) have a higher starch digestibility in comparison to non-waxy grains, which characteristically have high amylose contents. Huntington (1997) was also able to demonstrate an improvement in animal performance when steers were fed waxy grains in comparison to non-waxy grains of the same cereal grain species. The improved performance was presumably due to the superior starch digestibility characteristics of the waxy grains with their nearly 100% amylopectin.

Likewise, starch granule shape and size that determine the surface area available for enzyme attack, are thought to be a major determinants of starch degradability (Annison *et al.*, 1994). Planchot *et al.* (1995) noted, however, that potato starch and high amylose starch with granule sizes of 42 μm and 10 μm respectively, were equally resistant to enzyme attack, leading these researchers to conclude that specific surface area, which includes the surface area of internal pores, may be more relevant criteria for predicting susceptibility to enzyme digestion than surface area alone.

Starch Granule Structures of Common Cereal Grains

Cereal grain starch granules are generally considered to be spherical or ovoid and composed of concentric layers (lamellae) around a hilum (air space). The cereal grains however all have subtle differences in the structure of their starch granules.

Corn starch granules are spherical to polygonal ranging from 2 – 30 μm in diameter. The lamellae of the corn starch granules are indistinct to invisible and the granules themselves are highly birefringent (MacMasters *et al.*, 1959). Corn starch granules are fused together within a protein matrix structure in a mosaic stone like fashion (Kienzle *et al.*, 1997).

Sorghum starch granules are similar in appearance to corn, however they can reach up to 35 μm in diameter and have a greater number of large starch granules than are characteristic in corn (MacMasters *et al.*, 1959).

Wheat grains display a bimodal distribution of starch granules with both small spherical granules and large lens-shaped granules up to 38 μm in diameter. Wheat starch granules are only moderately birefringent (MacMasters *et al.*, 1959).

Barley and rye starch display the same bimodal distribution of starch granule characteristics as wheat, however, there are generally fewer small granules in barley and rye starches and the large granules may reach up to 48 μm and 60 μm in diameter respectively.

Oats starch granules are small in comparison to the starch granules of sorghum, wheat, barley and rye, having a diameter of just 2 – 10 μm. Individual oat starch granules occur in compact spherical clusters called compound granules that are up to 60 μm in diameter. Individual starch granules are polygonal or round in shape on one side only, as a result of the pressure exerted on them by other granules within the compound granule. Birefringence in oat starch is weak (MacMasters *et al.*, 1959).

Rice displays a microscopic appearance similar to oats, with individual starch granules ranging from 2 – 12 μm in diameter being incorporated into compound granules of 2 – 150 individual starch granules (MacMasters *et al.*, 1959).

Endosperm Cell Shape and Cell Wall Structure

It is possible that endosperm cell shape may affect starch digestion in the small intestine. Longer shaped endosperm cells (Figure 2.12a) are likely to be more prone to destruction via chewing or mechanical processing than small or round shaped endosperm cells (Figure 2.12b). Thus starch granules within longer endosperm cells are more likely be exposed to digestive enzymes in the small intestine (Simon Bird *pers. comm.*).

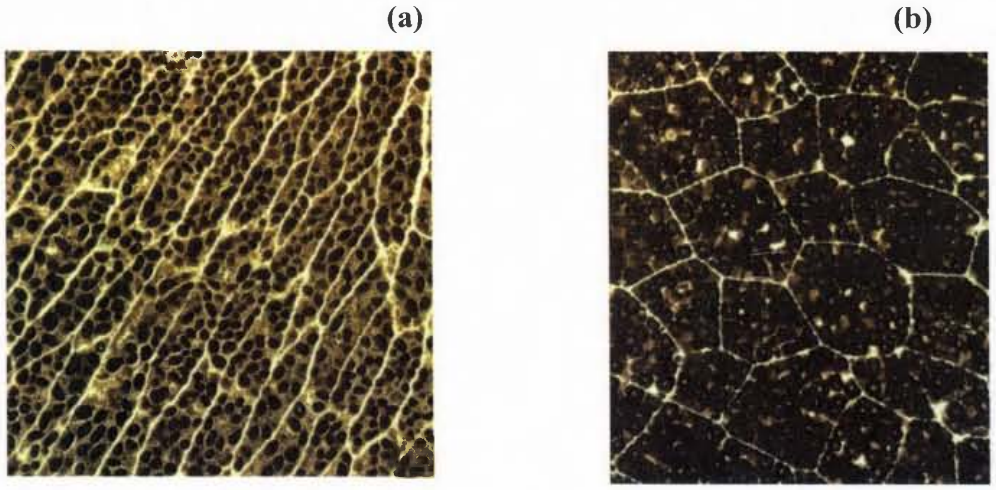

Figure 2.12: An example of (**a**) long thin endosperm cells found in barley and (**b**) short, round endosperm cells found in waxy sorghum (cell wall stained yellow, starch granules stained black/brown; images produced by P. Littlefield and S. Bird, University of New England).

The thickness of endosperm cell walls may also affect the digestion of starch in the small intestine, by restricting access of glycanases to starch contained within these cells. Choct (1995) noted that wheat, with low metabolisable energy in poultry diets (Figure 2.13a), had significantly higher levels of cell wall material (non-starch polysaccharides, NSP) than wheats of higher metabolisable energy (Figure 2.13b).

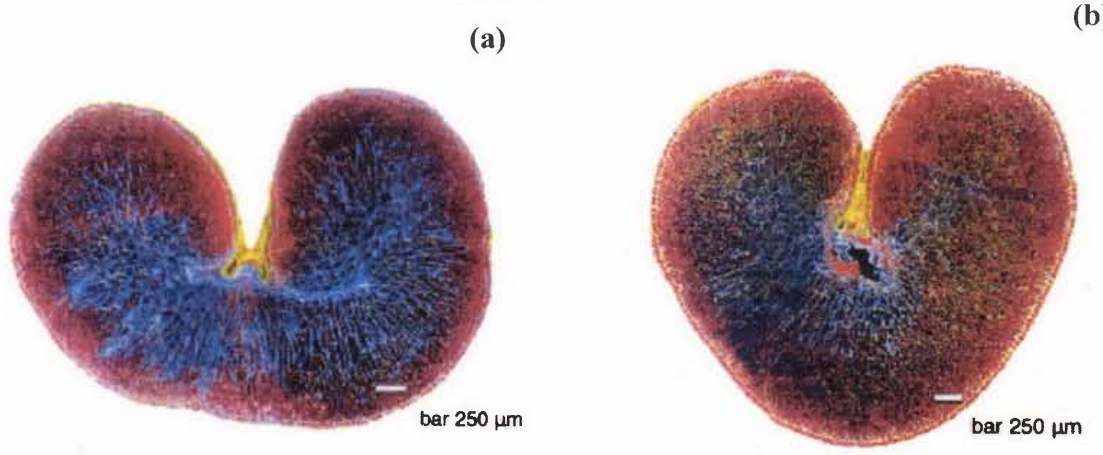

Figure 2.13: Cross sections of (**a**) low metabolisable energy wheat and (**b**) normal wheat. Cell walls are stained fluorescent blue (adapted from Choct, 1995, images produced by Dr Karin Autio, Finland)

Protein Matrix Structures

Starch granules, within the cereal grain endosperm, are embedded within a protein matrix composed of small protein bodies (Figure 2.14). There is evidence that this structure restricts the access of starch degrading enzymes and/or bacteria to the starch granules, thus reducing starch digestibility (McAllister *et al.*, 1993; Rooney *et al.*, 1986).

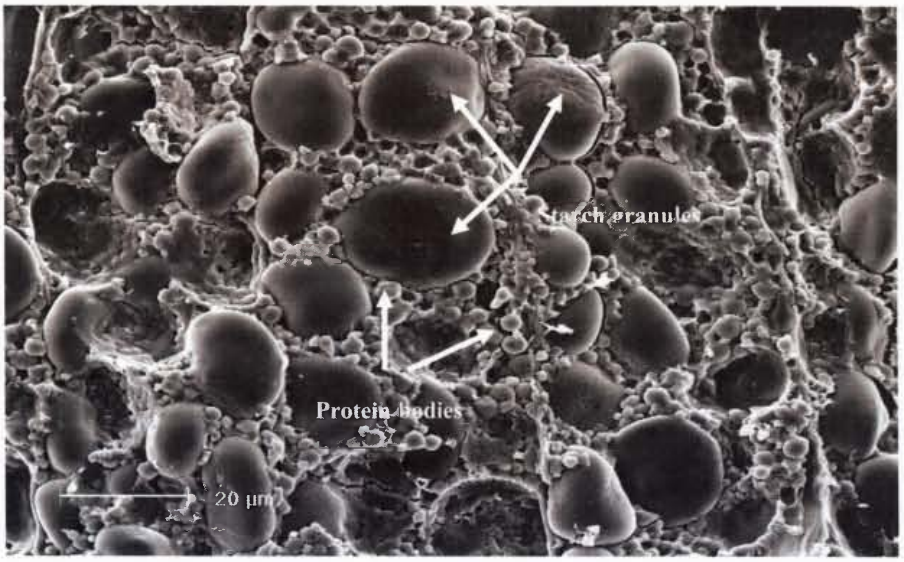

Figure 2.14: A scanning electron micrograph of the endosperm of barley showing the starch granules embedded within the protein matrix, adapted from Black (2001)

Jenkins *et al.* (1987) observed that by removing gluten from wheat starch, *in vitro* rate and extent of starch digestion was increased. In agreement with the *in vitro* results, *in vivo* starch digestibility, measured in human subjects using the glycaemic response, was greater when subjects consumed gluten free white bread, compared to untreated white bread, suggesting that gluten, when present as a protein matrix within the wheat endosperm, restricts enzyme access and thus starch digestibility.

Similarly McAllister *et al.* (1993) found that the digestion of barley by ruminal micro-organisms was more extensive than the digestion of corn, however, the digestion of isolated barley and corn starch granules by ruminal micro-organisms was similar, suggesting that structural components within the endosperm affect starch digestion. Treatment of barley and corn with protease prior to *in vitro* fermentation, significantly increased the microbial digestion of both starch sources and removed differences in starch digestibility observed prior to protease treatment, leading these researchers to suggest that the protein matrix surrounding starch granules, within the intact endosperm, restricted starch digestion.

Mode of Digestion by Enzymes

Starch granules may be digested by α-amylase either via exo-corrosion or endo-corrosion and starches from different botanical origins are digested by a specific means. Kienzle *et al.* (1997) reported that corn starch granules, collected from the jejunal chyme of horses, had 'pinholes' (1 – 5 µm in diameter) on a considerable number of the granules. These pinholes functioned to allow digestive enzymes into the starch granule, digesting it from the inside

out. Several corn starch granules, observed in this study, were practically hollow, while the original circumference remained the same as prior to when the grains were fed. Barley starch displayed a similar mode of digestion, however the number of pin holes on the surface of the barley starch granules were considerably fewer than those present on corn starch granules and correspondingly the barley starch granules were not digested to the same extent as corn starch granules.

In contrast to corn and barley, oats was observed to be digested via exo-corrosion. Oat starch granules viewed via scanning electron microscope showed no sign of any pinholes, however the surface of the starch granules was extensively corroded, giving the appearance that they were 'melting' under the influence of α-amylase. The average particle size of starch granules had decreased from 10 – 50 μm to 10 – 19 μm (Kienzle *et al.*, 1997). Starch digestibility, calculated for these starches using jejuno-fistulated horses, was 29% for corn, 22% for barley and 80% for oats (Kienzle *et al.*, 1997; Meyer *et al.*, 1995). Thus it would appear that grains, in which starch is digested via exo-corrosion, are more extensively digested in the equine small intestine.

Grain Processing

As noted in Figure 2.10, grain processing methods affect characteristics of cereal grains and starch and aim to improve pre-caecal starch digestion. Grain processing can be separated into two major categories: mechanical processing such as cracking and grinding; and processing methods that involve the use of heat, moisture and pressure. These methods usually also involve some form of mechanical processing, with examples including extruding, steam rolling/flaking, expanding, micronising and popping. Grain processing methods are extensively reviewed elsewhere (McLean, 2001; Rowe *et al.*, 1999) and will not be covered here where only the outcomes of the different methods are outlined.

Mechanical grain processing methods aim to change the macrostructure of the grain, breaking the seed coat to allow starch digesting enzymes access to the starchy endosperm and increasing the surface area available for enzymatic and microbial attack (Kienzle *et al.*, 1997; Rowe *et al.*, 1999). Processing methods utilising heat, moisture and pressure on the other hand aim to maximise starch digestion by changing the chemical structure, as well as the physical structure, of grains. Many such processing methods disrupt starch granule structure, destroy crystalline starch formations, increase the water solubility of starch and physically expose starch to digestive enzymes (Kienzle *et al.*, 1997; Rowe *et al.*, 1999). Grain processing methods involving heat, moisture and pressure also destroy protein matrix and cell wall structures within the starchy endosperm, allowing digestive enzymes easier access to the starch granules contained there (Rowe *et al.*, 1999).

The loss of crystalline structure and birefringence within the starch granule is termed gelatinisation. Each individual starch type has its own specific gelatinisation temperature, at which point all starch granules within the grain will have lost their crystalline structure. The gelatinisation temperatures for common cereal grains are: corn 62 - 72°C; Oats 53 - 59°C;

barley 57 - 64°C; and triticale 55 - 62°C (Evers *et al.* 1999). If starch granules have gelatinised but not swollen, it is thought that they retain their ability to return to a crystalline state during cooling, in a process known as starch retrogradation. The double-helical crystalline structure with a B-type X-ray diffraction pattern, formed during retrogradation, is highly resistant to enzyme degradation and in many cases is known as 'resistant starch'. Thus for true gelatinisation to occur, starch granules must be heated in a moist environment, until irreversible swelling occurs and starch granules lose their ability to return to a crystalline state (Annison *et al.*, 1994; Banks *et al.*, 1980; Englyst *et al.*, 1987; MacMasters *et al.*, 1959).

The effectiveness of different processing methods, in achieving starch gelatinisation and destruction of starch/protein interactions, will vary depending on the water content, pressure, temperature and shear forces employed during processing. Generally, grains processed at lower moisture contents, temperatures, pressures or shear forces will experience less starch gelatinisation and will thus display lower enzyme digestibilities (Holm *et al.*, 1988).

The effects of grain processing on starch digestibility and production performance in ruminants (Gaebe *et al.*, 1998; Joy *et al.*, 1997; Knowlton *et al.*, 1998; Rowe *et al.*, 1999; Theurer, 1986; Theurer *et al.*, 1999; Zinn, 1993; Zinn, 1995), and grain processing effects on small intestinal starch digestion in humans (Brand *et al.*, 1985; Holm *et al.*, 1989; Holt *et al.*, 1994; Muir, 1995; Snow *et al.*, 1981; Thorne *et al.*, 1983) have been extensively studied and show that starch digestion in these species is enhanced via grain processing. However, research into the effects of grain processing on pre-caecal starch digestion in equines is limited. In an experiment conducted to determine the effect of grain processing on the digestibility of corn in the equine small intestine, it was observed that as the magnitude of processing progressed from minor mechanical processing (cracking) to processing that involved the use of heat (popping), the extent of starch digestion in the small intestine increased (Table 2.2, Meyer *et al.*, 1993).

Table 2.2: The effect of varying grain processing methods on the pre-caecal starch digestion of corn. Digestibility determined using jejuno-fistulated ponies and chromic oxide as an external marker (*from* Meyer *et al.*, 1993).

Grain	Pre-caecal Digestibility
Whole corn	28.9
Cracked corn	29.9
Ground corn	45.6
Popped corn	90.1

Microscopy work was conducted on these grains (Kienzle *et al.*, 1997) to determine the effect of the processing on starch granule structure. While cracking altered the structure of the grain, it did not have any effect on the starch granules themselves. Grinding destroyed some maize starch granules, however, the starch remained insoluble in water (remained in a crystalline state). In contrast, popping, a processing method involving heat, moisture and pressure, led to extensive destruction of the maize starch granules and a major proportion of

them became soluble in water, indicating that gelatinisation of the starch granules had occurred (Kienzle *et al.*, 1997).

Similar benefits of grain processing and starch gelatinisation were reported by Hoekstra *et al.* (1999). Using the glycaemic response as an indirect measure of small intestinal starch digestion, these authors reported that horses consuming steam-flaked corn displayed significantly higher mean and peak plasma glucose concentrations and a significantly larger area under the glycaemic response curve than when consuming cracked or ground corn. Unlike Meyer *et al.* (1993), Hoekstra *et al.* (1999) did not observe improvement in starch digestion when corn was ground rather than cracked. In fact cracked corn produced a significantly higher peak glucose response than ground corn (Hoekstra *et al.*, 1999).

Oats responds to processing in a similar way to corn with improvements in starch digestibility increasing as the severity of processing is raised. Meyer *et al.* (1993) found that rolling oats only increased pre-caecal oat starch digestibility from 83.5% to 85.2%. Householder *et al.* (1977) observed however, that micronising oats improved oat starch digestibility from 48.0% for crimped oats to 62.4% when micronised. The pre-caecal oat starch digestibilities reported by these authors vary considerably, even though both used small intestinal cannulae and chromic oxide as a marker to estimate digestibility. The differences between these results may be partially attributed to the use of chromic oxide as an external marker. Owens *et al.* (1992) report that chromic oxide readily separates from the specific fractions, such as starch, that it should closely adhere to and travel with through the gastrointestinal tract. Likewise, Haenlein *et al.* (1966) notes that chromic oxide displays a different rate of passage, depending on the form of roughage fed in the diet.

McLean (2001) reports pre-caecal starch digestibility values for unprocessed, extruded and micronised corn and barley close to 100% (Table 2.3). In this situation it appears that grain processing has little effect on small intestinal starch digestibility, however, these digestibilities for unprocessed barley and corn are higher than those reported by other researchers (Arnold *et al.*, 1981; de Fombelle *et al.*, 2001; Meyer *et al.*, 1995; Radicke *et al.*, 1991) and should be interpreted with caution. McLean (2001) reported slow passage rates of grains in nylon bags through the ponies' stomach and small intestine (mean 3.6 h for barley, mean 5.1 h for corn). It is possible that the size and shape of the nylon bags may restrict flow rate and contribute to the unusually high estimates of starch digestion. McLean (2001) also notes that the small mean particle size of feedstuffs placed in the nylon bags may have allowed loss of feed particles through the 41 μm pores, which would consequently lead to an overestimation of pre-caecal digestibility.

Table 2.3: The effect of various grain processing methods on the pre-caecal starch digestion of barley and corn. Digestibility determined using the mobile bag technique (*from* McLean, 2001).

	Unprocessed	Extruded	Micronised
Barley	97.7	98.3	99.0
Corn	94.1	96.2	99.2

Digestion of Common Cereal Grains in the Equine Small Intestine

There is little information on the digestibility of starch from different grains in the horse's small intestine. Differences in level of feeding and method of measurement, makes it difficult to compare values. Methods previously used to measure small intestinal starch digestion include the mobile bag technique and external markers combined with small intestinal or caecal fistulation. A summary of estimated digestibilities for grains commonly used in equine diets, and the method by which the digestibilities were calculated, are presented in Table 2.4.

Table 2.4: Estimated pre-caecal digestibilities for oats, corn, barley and sorghum in the horse.

		% Pre-caecal Starch Digestion			
	Method Used	**Oats**	**Corn**	**Barley**	**Sorghum**
de Fombelle *et al.* (2001)	Measured using horses. Mobile bag technique with collection occurring via caecal fistula.	99.3	66.2	81.4	-
Meyer *et al* (1995)	Measured using ponies. Cr_2O_3 used as marker. Digesta collected from jejunal fistula for 15 mins every hr for 11 hrs following feeding. Mean meal size 3.2 g/kg BW/meal.	83.5	28.8	21.5	-
Arnold *et al.* (1981)	Measured using ponies. Cr_2O_3 used as marker. Digesta grab samples collected from posterior ileal cannulae every 30 min for 1 hr, every 15 min for 2 hrs and then each 30 mins for a further 1 hr following feeding. Meal size 0.4 g/kg BW/meal.	91.1	78.2	-	94.3
Radicke *et al.* (1991)	Measured using ponies. Cr_2O_3 used as marker. Digesta grab samples collected from posterior jejunal cannulae for 15 mins in the 3rd, 4th, 5th, 6th and 7th hours after morning meal. Starch intake 1.8 g/kg BW/meal	98.0	70.8	-	-
McLean (2001)	Measured using ponies. Mobile bag technique with collection occurring via caecal fistula.	-	94.1	97.7	-
Householder *et al.* (1977)	Measured using horses. Cr_2O_3 used as marker. Samples collected from posterior ileal cannulae every 15 min for 6 hr following feeding.	48.0	-	-	36.0
Hinkle *et al.* (1983)	Measured using ponies. Cr_2O_3 used as marker. Samples collected from ileal cannulae every 30 min for 3 hrs after feeding then every hour for a following 9 h.		60.7		

It is clear that there are large differences in starch digestibility between grain species, when measured by the same researcher, with oats commonly observed to be more digestible than corn and barley. There is also substantial variation in measured starch digestibility within a grain species. For example, estimates of starch digestibility for corn range from 29% to 94%. It is likely that this variation is due to differences in sampling and measurement procedures used by various researchers. So, while it is difficult to draw any firm conclusions regarding the digestibility of cereal starches in the small intestine of the horse, the results serve the purpose of highlighting the lack of knowledge and information

available with regards to the digestion of starch from cereal grains within the small intestine of the equine.

2.3.2 Physiological and Physical Differences between Horses

As well as the digestibility of the starch in grains fed to horses, intrinsic factors within and between horses in their ability to digest starch, will also affect the extent of starch digestion in the equine small intestine. Starch digestion and absorption may be affected by: α-amylase activity; brush border glycanase activity; protease activity; glucose absorption capacity; and the rate of eating, extent of chewing and digesta passage rate through the small intestine of individual horses (Figure 2.15). These potentially limiting factors are discussed in more detail below.

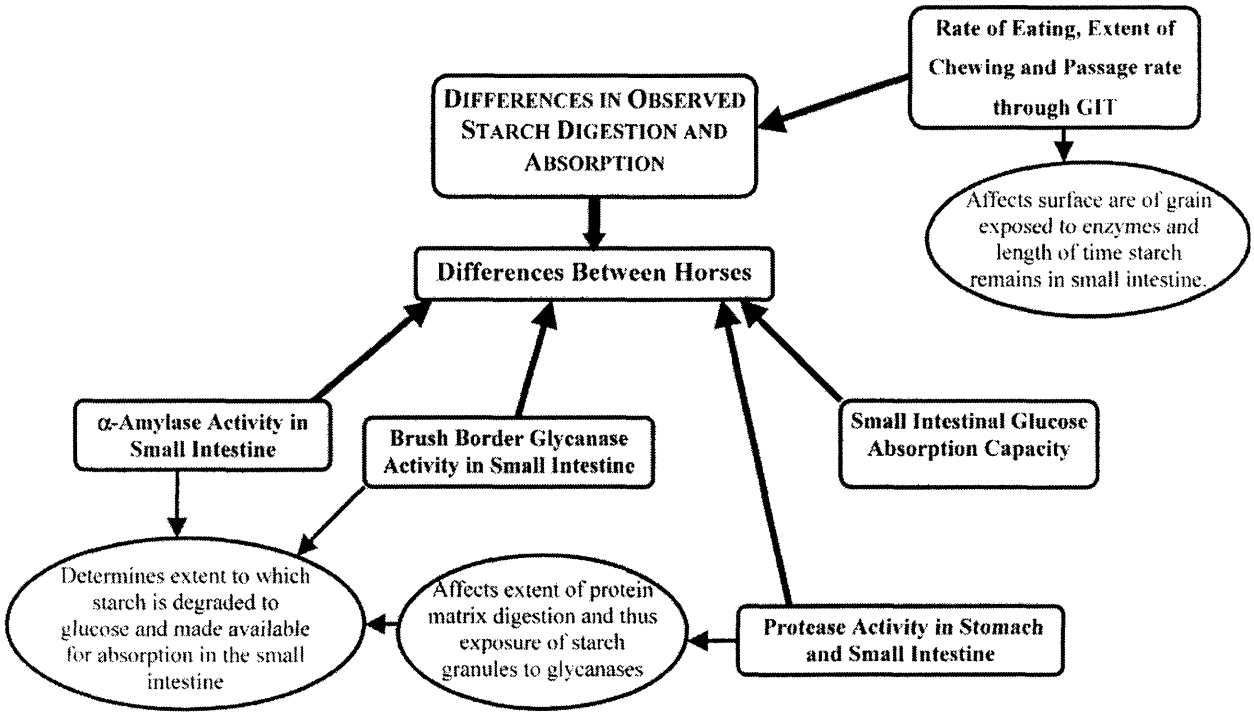

Figure 2.15: Physiological and physical differences between horses that may affect the extent of digestion and absorption of starch in the equine small intestine.

α-Amylase Activity

Observations of low concentrations of digestive enzymes in the horses' gastrointestinal tract were made as far back as 1958 (Alexander *et al.*, 1958). Although equine pancreatic secretion is profuse, with up to 10% of BW secreted in 24 hours, the concentration of enzymes is low (Comline *et al.*, 1969). The onset of feeding initiates an increase in pancreatic secretion and enzyme concentration, with Comline *et al.* (1969) reporting a four-fold increase from resting values of pancreas secretion within 10-15 minutes after feeding commenced. During this period of increased flow, the concentration of enzymes also rose. Similarly, it was observed in horses fitted with jejunal cannulae that α-amylase activity increased over time following a meal, peaking at 4-5 hours postprandial (Kienzle *et al.*, 1994).

Limited literature detailing α-amylase activities in the small intestine of the horse is available (Kienzle *et al.*, 1994; Roberts, 1974), and differences in the definitions for units (U) of enzyme activity make comparisons between the studies difficult. Roberts (1974) measured α-amylase activities in the small intestines of 15 horses and found a range from 0 to 7 U/g wet mucosa/min, with the activity calculated using the auto analyser method of Kidder *et al.* (1972) for measuring the concentration of reducing sugars. Roberts (1974) noted that activity was highest in the duodenum and extremely variable between animals, with α-amylase barely detectable at any site along the gastrointestinal tract in 40% of the horses. Roberts reports values for α-amylase activities in the small intestine of rabbits, dogs and pigs, also measured using the method of Kidder *et al.* (1972), with activities being 3.8, 24.9 and 28.4 times greater in the small intestines of these animals respectively, compared to the horse.

Similar findings are reported by Kienzle *et al.* (1994), with low and highly variable α-amylase activities observed in the equine small intestine. α-Amylase activity during this later study was determined using the Phadebas™ method and ranged from 1 to 70 U/g wet weight with a mean of 22.3 U/g wet weight. These α-amylase activities are lower than those activities, measured using the same method, in the small intestine of cats (classified as strict carnivores) adapted to carbohydrate free diets and less than half the activity recorded in cats adapted to a high carbohydrate diet (Kienzle, 1992).

With these findings in mind, it would be reasonable to assume that the breakdown of starch into maltose, maltotriose and α-dextrin units by α-amylase in the small intestine of the horse will be a rate limiting step in the starch digestion process. This assumption has been partially confirmed by Meyer *et al* (1993), who observed a significant increase in the small intestinal digestion of ground corn (from 45.6% to 57.7% of starch digested), when 50 g/ kg DM of powdered α-amylase (activity 800 000 U/g) was added to the diet. Kienzle *et al.* (1994) report that α-amylase activities in the jejunal chyme of horses on the ground corn + α-amylase diet described by Meyer *et al* (1993), were significantly higher than activities measured in the jejunal chyme of horses on the ground corn only diet. Any significant differences in small intestinal α-amylase activity between horses, that were present prior to the addition of α-amylase to the diet, were also removed when the exogenous enzyme was added (Kienzle *et al.*, 1994).

Thus by increasing α-amylase activity in the small intestine of horses', through the addition of exogenous enzyme to the diet, the extent of starch digestion in the small intestine may be increased and variation between horses may be decreased by overcoming deficiencies in some animals. It is therefore likely that the addition of exogenous enzymes to equine diets could reduce some of the need for individual diet formulation and decrease the amount of starch passing undigested through the small intestine for fermentation in the hindgut (Section 2.4). This possibility influenced the decision to examine the effect of supplementing equine diets with amylolytic enzymes (Chapter 6).

With respect to small intestinal α-amylase concentrations, the length of time a horse is allowed to adapt to a high starch diet will probably also affect its ability to digest starch pre-caecally. It is well documented that rats increase the activity of α-amylase in their small intestine in response to high non-structural carbohydrate diets. Rats, placed on a diet with a ratio of carbohydrate: fat of 95: 5, had an α-amylase activity in their small intestine, that was ten-fold greater than rats on an isoenergetic diet containing 5: 95 carbohydrate: fat (Snook, 1971).

More recent research has confirmed these findings for horses (Kienzle *et al.*, 1997; Radicke *et al.*, 1991), cattle (Clary *et al.*, 1969; Russell *et al.*, 1981) and pigs (Corring, 1977). In horses, diets containing cereal grains significantly increased the concentration of α-amylase in jejunal chyme from 15.0 U/g wet weight, when animals were maintained on a hay diet, to 30.8 U/g wet weight for horses on a high starch diet (Kienzle *et al.*, 1994). Grain processing and starch digestibility in the horses' small intestine did not affect small intestinal α-amylase activity, however, feedstuffs with negligible starch digestion (6-10%) in the small intestine, such as potato and tapioca did not induce an increase in intestinal α-amylase activity (Kienzle *et al.*, 1994). Radicke *et al.* (1991) noted however, that horses consuming oats (1.8 g starch/kg BW/meal), with a pre-caecal digestibility of 98% had a significantly higher α-amylase activity in the small intestine than horses consuming corn (1.8 g starch/kg BW/meal), with a lower pre-caecal digestibility of 71%.

In cattle, Clary *et al.* (1969) observed a 40% increase in the specific α-amylase activity in the pancreatic tissue of steers adapted to a corn (73.5%), soybean meal (26.5%) diet in comparison to steers receiving a legume-grass based pasture diet. Similarly, they also noted a 188% increase in specific α-amylase activity in pancreatic fluid collected via catheters from wethers, when placed on an 80% corn diet, in contrast to when the same animals were consuming a hay only diet. Likewise, Russell *et al.* (1981) observed a 129% increase in pancreatic α-amylase activity when steers were fed a predominantly corn diet in comparison to steers fed lucerne only, while Corring (1977) in a review of his own work, reports that a pig fitted with a pancreatic fistula, displayed a 30% increase in pancreatic α-amylase secretion when dietary starch content was increased from 20 to 60%. It took as little as 3-days on a 60% starch diet for noticeable increases in α-amylase concentrations to occur (Corring, 1977).

From these observations, it seems likely that when feeding a high starch diet to horses, an appropriate adaptation period should occur to allow the gastrointestinal tract to up-regulate α-amylase activity, which should, in turn, contribute to more extensive digestion of starch in the small intestine.

Brush Border Glycanase Activity

It is considered unlikely that a deficiency in brush border glycanases will be a limiting factor in the small intestinal digestion of starch, since brush border glycanase activities observed in horses are comparable to those in omnivorous species (Kienzle *et al.*, 1993; Roberts, 1975; Roberts *et al.*, 1974).

Roberts *et al.* (1974) observed that sucrase and maltase (maltase was used to describe isomaltase, glucoamylase and maltase II and III) reached adult levels of activity in the horse at seven months of age, with the highest activity in the middle and lower jejunum. They also report that activities of brush border glycanases in the horse are comparable to that of humans, pigs and dogs and thus believe that horses should be capable of digesting substantial quantities of disaccharides. Using the glycaemic index Roberts (1975) found that the disaccharides maltose and sucrose produced glycaemic response curves similar to those associated with identical doses of the constituent monosaccharides. He concluded that disaccharidase activity is unlikely to limit the digestion of disaccharides and absorption of monosaccharides in the equine small intestine.

Kienzle *et al.* (1993) also measured substantial activities of brush border glycanases in the equine small intestine and hypothesised that horses have high activities of brush border glycanases to allow them to digest the large quantities of sugars that may be present in grass and grass-like plant species, which are considered to be their natural diet (Kienzle *et al.*, 1993).

As with α-amylase activity, the activity of brush border glycanases appears to up-regulate when equines consume a high carbohydrate diet, with Kienzle *et al.* (1993) reporting an increase in sucrase and maltase activity in the small intestine of ponies, placed on a maize or high sugar diet for 6-weeks, with higher brush border glycanase activity reported in these animals in comparison to ponies on a hay diet. Although Kienzle *et al.* (1993) reported an increase in brush border glycanase activity in horses adapted to a high carbohydrate diet, Janes *et al.* (1985) and Russell *et al.* (1981) report no increases in activity in the small intestine of sheep or cattle. In sheep adapted to either a grass or corn based diet, Janes *et al.* (1985) observed no increase in the activity of glucoamylase or the specific activity of maltase in the small intestine of sheep fed corn. In agreement with the findings of Janes *et al.* (1985), Russell *et al.* (1981) noted that steers, adapted to a high corn diet for 106 days, displayed no increase in the activity of maltase in their small intestine. Thus, there is no clear evidence to suggest that animals up regulate brush border glycanase activities when consuming a high starch diet.

Protease Activity in the Stomach and Small Intestine

The protein matrix, surrounding starch granules within endosperm cells, restricts the access of starch digesting enzymes to starch granules, thus reducing starch digestibility (Rooney *et al.*, 1986; McAllister *et al.*, 1993; Section 2.3.1). With Jenkins *et al.* (1987) and McAllister

et al. (1993) showing that removal or predigestion of the protein matrix from cereal grains improves starch digestion, it is reasonable to speculate that protease activities in the stomach and small intestine of equines may affect starch digestion, by determining the extent to which starch granules are exposed to glycanase enzymes.

Small Intestinal Glucose Absorption Capacity

Given the capacity of small intestinal glucose transporters to transport glucose from the small intestine into the blood stream (Ferraris *et al.*, 1989; Hediger *et al.*, 1994), it is unlikely that glucose absorption capacity will limit small intestinal starch digestion and absorption in equines. Small intestinal enterocytes, where glucose transporters are located, are a cell population that turn over every two to four days. Thus response to changes in the dietary supply of glucose can be rapid (Hediger *et al.*, 1994), and Diamond *et al.* (1984) have shown that the mouse small intestine is able to rapidly adjust to increased supplies of dietary starch, with the capacity to actively transport glucose potentially being able to increase twofold over a two to four day period.

Both sodium-glucose transporters (SGLT1) in the apical membrane of the epithelial cell and GLUT2 transporters in the basolateral membrane have the capacity to adjust to increased supplies of glucose (Cheeseman *et al.*, 1991; Ferraris *et al.*, 1992; Maenz *et al.*, 1987). Glucose transporters may increase their capacity to transport glucose by two methods: by increasing the activity of the transporters; or by increasing the density, or number of transporters available. Ferraris *et al.* (1992) indicate that the glucose transporters in the apical membrane adapt to changes in the supply of specific nutrients in the diet by altering the density of transporters. In the basolateral membrane, it has been hypothesised that acute exposure to a high glucose diet may result in intrinsic changes in basolateral glucose transporters, increasing their activity for a short period of time (Maenz *et al.*, 1987), while it is thought that chronic exposure to a high glucose diet may stimulate the formation of additional basolateral glucose transporters (Cheeseman *et al.*, 1991).

The ability to up regulate the transport of glucose from the small intestine is largely related to the presence of glucose substrate in the gastrointestinal tract. Shirazi-Beechey *et al.* (1991) observed that lambs maintained on milk diet, which delayed the development of a rumen and delivered glucose and galactose to the small intestine, maintained their population of SGLT1 and their ability to absorb hexoses from the small intestine. Conversely, sheep, that were permitted to forage and develop a rumen, displayed a significant decline in glucose transport activity. In support of the hypothesis that the presence of glucose in the small intestine is the stimulus required for maintenance and/or up regulation of glucose transport, adult sheep, that received continuous infusions of 30 mM D-glucose into the duodenum (1.5 L/day), restored their ability to transport glucose within four days (Shirazi-Beechey *et al.*, 1991).

Likewise, Cheeseman *et al.* (1991) found that the exposure of rats to a high carbohydrate diet increased the number of basolateral membrane active glucose transporters. The

increased capacity for glucose transport occurred within a minimum of three days and they suggested that the metabolism of glucose (or fructose) within the epithelial cell provides the signal for the changes in transport observed across the basolateral membrane.

Glucose malabsorption from the small intestine has been observed in equines and may be caused by a variety of pathological lesions, including lymphosarcoma, villous atrophy, granulomatous enteritis and eosinophilic gastroenteritis (Mair *et al.*, 1991). In horses with these conditions, small intestinal starch digestion may be limited by the capacity to absorb glucose. Affected horses characteristically display 'total' malabsorption responses when given an oral glucose tolerance test (Mair *et al.*, 1991). For example, Church *et al.* (1997) reported flat plasma glucose response curves following oral glucose absorption tests in two horses suffering chronic weight loss, despite normal to increased feed intake. The flat glycaemic response curves and weight loss in these cases suggests that the digestion and absorption of feed nutrients, including starch, was being limited by their compromised small intestinal glucose absorption capacity.

Physical Animal Factors

In addition to differences between animals due to variations in enzyme activities and glucose absorption capacities, there are a number of physical factors that can influence starch digestion. These are outlined below.

Rate of Eating and Extent of Chewing

Animals that eat quickly tend to chew their food less, and thus the particle size of the food ingested by these animals will be larger. Larger particles have less surface area exposed to digestive enzymes, which consequently reduces enzymic attack on starch and allows more starch to escape digestion in the small intestine (Owens *et al.*, 1986). This effect of rapid ingestion, limited mastication and large particle size, on starch digestion, has been observed in equines by Meyer *et al.* (1995), who found that a horse who was a slow eater and thorough chewer exhibited a pre-caecal starch digestibility for whole corn of 50%, while a horse in the same trial who ate quickly, had an apparent digestibility of 1% for the same diet. The particle size of the corn in the jejunal chyme of these two horses varied considerably, depending on their chewing behaviour, with the hasty eater having a larger mean particle size (Kienzle *et al.*, 1997).

Similar effects have been reported in humans, with the consumption of thoroughly chewed corn, potato, rice and apple producing glycaemic responses that were 60%, 34%, 21% and 16% higher respectively than glycaemic responses produced, when the subjects swallowed the same meals whole. This apparent improvement in starch digestion when food was chewed may be due to: enhanced salivation stimulated by chewing, which, in humans, would enhance starch digestion by the amylase present in human saliva; or it may be an effect of reduced particle size and increased exposure of starch to glycanases in the small intestine (Read *et al.*, 1986).

Digesta Passage Rate

The efficiency of small intestinal starch digestion and glucose absorption in animals is highly dependant on the rate at which digesta moves through the gastrointestinal tract (Argenzio, 1993c). Chapman *et al.* (1985) showed that in human ileostomates, the administration of loperamide to delay small intestinal passage rate, significantly reduced the quantity of starch escaping digestion in the small intestine. On the other hand, administration of magnesium citrate, to increase the rate of small intestinal passage, significantly increased the amount of starch escaping digestion in the small intestine.

Pearson *et al.* (1991) also found that donkeys, with significantly longer mean retention times of fluid and particulate markers in the gastrointestinal tract, displayed significantly higher apparent digestibilities for dry matter, organic matter and acid detergent fibre than ponies with a shorter retention time. Thus it is likely that digesta retained for longer periods in the small intestine and hindgut will be more extensively digested. However, Loeb *et al.* (1971) found that glycaemic response following a carbohydrate meal, was not affected by increasing passage rate through the gastrointestinal tract in equines using injections of neostigmine or oral administration of dihydroxyanthroquinone (Istizin).

Frape (1998) estimates that digesta moves through the small intestine of equines at a mean rate of 30 cm/minute. However, many factors including: meal composition (Section 2.3.3); frequency of feeding (Section 2.3.3); and individual differences between horses may change this estimated rate (Argenzio, 1993b; Debas *et al.*, 1975; Frape, 1998; Vander Noot *et al.*, 1967).

Vander Noot *et al.* (1967) noted large differences in cumulative mean recovery of chromium oxide marker between horses, indicating differences in whole tract passage rate. Likewise, McLean (2001) reported retention times in the pre-caecal segment of the equine digestive tract to vary, both between experiments and between horses. Pre-caecal transit time ranged between horses from 1 to 8 hours, with mean retention times of 2.3 hours, 3.6 hours and 5.1 hours recorded for three different experiments. Similarly, total tract retention time was found to vary from 9 – 250 hours in two separate experiments where the mobile bag technique was used (McLean, 2001).

Condition Score and Insulin Response

Although it is unlikely that a horse's condition score and insulin response affect starch digestion in the small intestine, these parameters are likely to influence glycaemic and insulin response curves, which can be used as indicators of pre-caecal starch digestion.

Insulin resistance is shown when normal concentrations of insulin initiate a less than normal biological response (Kahn, 1978) and animals that suffer this condition commonly display higher than normal baseline insulin values and have exaggerated insulin responses following a carbohydrate challenge (Freestone *et al.*, 1992). Corke (1986) suggests that insulin resistance may be due to the presence of elevated concentrations of hormones that

antagonise the actions of insulin, including growth hormone, cortisol, adrenalin, progesterone and glucagon, and in the horse, stress, pregnancy and obesity are physiological factors that have also been associated with insulin resistance (Fowden *et al.*, 1980; Freestone *et al.*, 1992; Guyton, 1971; Jeffcott *et al.*, 1986).

Jeffcott *et al.* (1986) illustrated the effect of obesity on insulin response when fat ponies displayed exaggerated glycaemic and insulin response curves when given an oral glucose tolerance test (1g/kg BW of glucose) in comparison to 'normal' ponies and standardbred horses. The blood glucose levels in the fat ponies also failed to return to baseline levels 6 hours after the glucose challenge. These observations suggest that fat animals suffer insulin resistance, which in turn causes inflated glucose and insulin responses. Insulin resistance in these fat animals was subsequently confirmed when administration of 0.4 iu of insulin/kg BW intravenously, failed to cause a fall in blood glucose which was comparable to the normal ponies and standardbred horses (Jeffcott *et al.*, 1986). In a similar study, Freestone *et al.* (1992), using overweight hyper-insulinaemic ponies observed an improvement in insulin sensitivity following six-weeks of controlled feeding combined with an exercise regime to significantly reduce bodyweight.

Likewise, in dogs Mattheeuws *et al.* (1984) reports that obesity was the most important factor determining variations in insulin response to intra-venous glucose tolerance tests, with obese dogs characteristically having exaggerated insulin responses. Thus, although individual animal factors, such as condition score may have no actual effect on starch digestion in the small intestine, the interpretation of indicators of small intestinal starch digestion, such as the glycaemic and insulin responses, may change due to individual body condition scores.

2.3.3 Meal Size, Feeding Frequency and Meal Composition

Feeding management factors such as the feeding frequency, meal size and meal composition may also affect starch digestion and absorption in the equine small intestine by altering rates of feed intake, the intensity of mastication and small intestinal passage rates. These factors are discussed in more detail below.

Meal Size and Feeding Frequency

Feeding frequency and meal size are important factors to consider when formulating diets for horses. As discussed in Chapter 1, equines in their natural environment spend up to 75% of their daylight hours and 55% of darkness hours eating. Thus the equine digestive tract is designed to accommodate small meals, which are taken frequently. Although there is no conclusive evidence to suggest that large meal sizes increase the rate of passage through the gastrointestinal tract in either equines or humans (Argenzio *et al.*, 1974; Chapman *et al.*, 1985), negative effects of increasing meal size on starch digestibility have been observed in horses, humans and ruminants (Chapman *et al.*, 1985; DeGregorio *et al.*, 1982; Karr *et al.*,

1966; Meyer *et al.*, 1995). These authors reported that the quantity of starch escaping pre-caecal digestion increased with meal size and level of starch intake.

Potter *et al.* (1992) estimates that the critical upper limit for small intestinal starch digestion in the horse to be 3.5 – 4 g of starch/kg BW/meal. It appears that beyond this point, the capacity for pre-caecal starch digestion is limited and that the feeding of larger meals will result in increasing quantities of starch entering the hindgut. It is hypothesised that this effect of low frequency feeding and large meal sizes is due to an increased passage rate through the small intestine.

Massey *et al.* (1985) found that feeding a grain mix containing oats and corn with 36% starch, at maintenance levels (maximum intake 1.5% BW/day), in two, three or four meals/day, had no effect on apparent pre-caecal starch digestibility. Likewise Brown (1987, *see* Potter *et al.*, 1992 found no improvement to pre-caecal starch digestion when a 70% corn/oat concentrate, 30% lucerne diet, fed at 1.5% BW/day was fed in two, three or four meals/day. Thus it appears that when feeding grain-based meals at maintenance levels, high starch concentrates may be fed safely to horses in two meals/day. However, further research needs to be conducted to determine the effect of feeding frequency and meal size on pre-caecal starch digestion for performance horses fed above maintenance levels.

Diet Composition

The roughage component of the diet, may affect the digestion of starch in the small intestine (Frape, 1998). In the mid 1900's, chaff was widely used in equine diets for three primary reasons: it encouraged chewing by the horse; it lessened the wastage of poor quality long hays; and it prevented horses from consuming grains too quickly (Section 2.1.1). A recent survey of thoroughbred trainers (Chapter 3), found that 95.8% of trainers mixed chaff with their grain diets. An average of 0.77 kg of lucerne chaff and 0.85 kg of oaten or wheaten chaff was added to thoroughbred horse diets, confirming that roughage is still a major component of modern equine diets. The effects of chaff and hay on starch digestion in the equine small intestine are poorly understood however and there are numerous conflicting reports.

Pre-caecal starch digestion may be enhanced by the inclusion of chaff in equine diets, as chaff can function to substantially increase the intensity of feed mastication and reduce the rate of feed intake, with poor quality chaffs tending to reduce rate of feed intake to a greater degree than more palatable chaffs (Meyer *et al.* 1975 *in* Frape, 1998). It has also been reported that poor quality roughages reduce the rate of passage of fluid and particulate markers through the gastrointestinal tract in ponies and donkeys (Pearson *et al.*, 1991) and thus may enhance pre-caecal starch digestion by reducing small intestinal passage rate.

However, Meyer *et al.* (1993) have reported a negative effect of roughage on starch digestion. They found that the small intestinal digestion of starch within ground corn was reduced from 46 ±10.6% to 16 ± 19.5%, when grass hay was fed in place of lucerne meal.

These authors suggested that the decreased starch digestibility was due to increased passage rate of digesta through the small intestine and increasing secretions of 'digestive juices', which may have functioned to dilute the concentration of digestive enzymes in the gastrointestinal tract, thus decreasing starch digestion when hay was fed in place of lucerne meal. Kleffken (1993 *in* Kienzle, 1994) also showed that feeding hay in combination with a concentrate diet decreased pre-ileal starch digestion. When the hay was replaced with a green meal however, Kleffken (1993 *in* Kienzle, 1994) reported that small intestinal passage rate slowed and pre-ileal starch digestion increased.

Therefore, although hay and chaff have been found to reduce rate of intake, increase chewing activity and slow passage rate through the small intestine, the addition of different forms of roughages to grain based diets have also been found to increase passage rate through the gastrointestinal tract and significantly reduce the digestion of starch in the small intestine. The addition of chaff to a grain concentrate diet will also increase meal size. Thus, no clear conclusions regarding the feeding of chaff and hay with grain concentrates and their effect on starch digestion may be drawn, except that this is an area of horse nutrition requiring extensive research.

Feeding frequency, meal size and diet composition are perhaps the most easily modified parameters affecting starch digestion in the small intestine of the horse. However, with limited data and conflicting evidence available to confirm the effects of these parameters on starch digestion, no firm recommendations or conclusions may be drawn.

If, due to any of the factors discussed above, starch remains undigested as it passes through the small intestine, it will be delivered to the equine hindgut and rapidly fermented by the massive populations of bacteria that reside there.

2.4 HINDGUT FERMENTATION OF STARCH

Starch is fermented in the hindgut by amylolytic bacteria. These bacteria are capable of producing large quantities of VFAs and lactic acid. This production and accumulation of organic acids in the equine hindgut can cause a condition known as hindgut lactic acidosis, which can have severe consequences for the horse, including; reduced feed conversion efficiency, behavioural changes and diseases such as endotoxemia, metabolic acidosis and laminitis. The processes of hindgut starch fermentation and the negative consequences associated with it are discussed in detail in the following section of this literature review.

2.4.1 Bacteria of the Hindgut

There are three major groups of bacteria in the equine hindgut:

(i) cellulolytic bacteria, who, as discussed in Section 2.1.2, are responsible for the fermentation of fibre in the hindgut;

(ii) amylolytic bacteria that ferment starch when it is an available substrate in the hindgut. Species of amylolytic bacteria include *Streptococcus bovis, Streptococcus equinus, Bacteroides amylophilus, Bacteroides ruminicola,*

Succinimonas amylolytica, Selenomonas ruminantium and several species of *Lactobacillus* (Hungate, 1966; Kern *et al.*, 1974). *S. bovis, S. equinus* and *Lactobacillus spp.* are all known to be present in the equine hindgut (Alexander *et al.*, 1963; de Fombelle *et al.*, 1999; Higginbottom *et al.*, 1954; Kern *et al.*, 1973); and

(iii) lactate utilising bacteria that function to convert lactic acid, when it is present in the hindgut, to propionate. Lactate utilising bacteria species such as *Propionibacterium shermanii, Megasphera elsdenii, Selenomonas lactilytica, Selenomonas ruminantium* and *Veillonella alcalescens* commonly found in ruminants (Nocek, 1997; Hungate, 1966) have been isolated from the gastrointestinal tract of the horse (Alexander *et al.*, 1963; Kern *et al.*, 1973).

The cellulolytic and lactate utilising bacteria have vastly different metabolic rates, requirements for growth, fermentation and survival and end products of fermentation in comparison to amylolytic bacteria. The major characteristics of each group are presented in Table 2.5.

Table 2.5: The individual characteristics of the three major groupings of bacteria that may be found in the equine hindgut (Leek, 1993).

	Cellulolytic Bacteria	Amylolytic Bacteria	Lactate Utilising Bacteria
Metabolic rate	Slow	Fast	Slow
Population doubling time	18 hours	0.25 – 4 hours	16 hours
Optimum pH	6.2 – 6.8	5.5 – 6.6	6.2 – 6.8
End products	Volatile fatty acids	Volatile fatty acids and lactic acid	Volatile fatty acids
Characteristic VFA profile	55% acetate: 25% propionate:15% butyrate	70% acetate: 15% propionate:10% butyrate	-

It is these vastly different growth rates, requirements for survival and fermentation end products of amylolytic versus cellulolytic and lactate utilising bacteria that contribute to predisposing the equine hindgut to acidosis when fermentable starch substrate become available.

2.4.2 Hindgut Lactic Acidosis in the Horse

Hindgut lactic acidosis occurs when large quantities of starch pass through the upper gastrointestinal tract without being digested by enzymes in the small intestine and are consequently delivered to the hindgut. Amylolytic bacteria within the hindgut ferment the starch substrate rapidly. With access to starch, the population growth of amylolytic bacteria accelerates, while cellulolytic and lactate utilising bacterial populations grow slowly. VFA production from all classes of bacteria is increased and fermentation end products switch from VFAs to lactic acid in response to the accelerated growth rates for certain amylolytic bacteria such as *Streptococcus bovis* (Leek, 1993; Nocek, 1997; Russell *et al.*, 1985; Russell *et al.*, 1979).

Lactic acid is usually only present in the fermentation media transiently, as lactate utilising bacteria transform it to propionate (Figure 2.3, Leek, 1993; Phillipson, 1952). With the increased production of VFAs and lactic acid however, the pH of the fermentation medium begins to drop. As pH declines, the growth rates of lactic acid utilising bacteria are inhibited and the rate of conversion of lactic acid to propionate by these bacteria is slowed down, allowing a gradual accumulation of lactic acid and a further decline of pH.

Eventually the pH drops below that of the optimum for lactate utilising and cellulolytic bacteria (Table 2.5) and they begin to die as the pH gradient maintained across the cell membrane by the bacteria allows lipid soluble VFAs to pass through the cell membrane and dissociate within the cell causing anion toxicity (Russell *et al.*, 1995). Amylolytic bacteria are able to survive under these conditions due to their ability to prevent anion toxicity by allowing their intracellular pH to fall with the declining pH of the fermentation medium (Russell *et al.*, 1985; Russell *et al.*, 1995). This falling intracellular pH within amylolytic bacteria also inhibits the enzyme pyruvate formate lyase that is essential for the conversion of pyruvate to formate, acetate and ethanol and thus pyruvate metabolism within amylolytic bacteria is further diverted to the production of lactic acid (Russell *et al.*, 1985).

The low pH now being experienced in the hindgut also creates an environmental niche for lactate producing populations of *Lactobacilli.* With lactate utilising bacteria no longer present in the fermentation medium, and lactate producing bacterial populations rapidly increasing, lactic acid concentrations continue to rise and the pH within the hindgut spirals rapidly downward (Figure 2.16) (Leek, 1993; Nocek, 1997; Russell *et al.*, 1985; Russell *et al.*, 1979).

Clinical outcomes linked to the occurrence of hindgut lactic acidosis are outlined in Section 2.4.3. Research has provided physiological evidence of the occurrence of hindgut lactic acidosis in horses and the following paragraphs outline the key relevant studies.

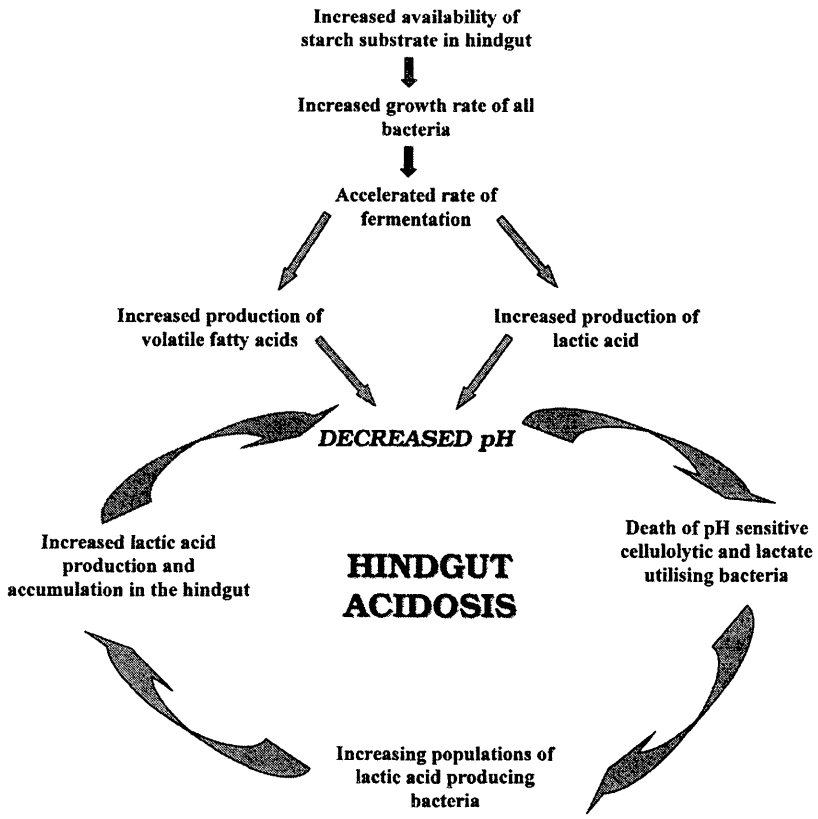

Figure 2.16: The processes involved in the development of hindgut acidosis in the horse.

Hindgut acid accumulation in horses consuming cereal grains is frequently reported. Zeyner *et al.* (1992) reported that in equines, as the quantity of grain consumed increased, the pH of faeces declined, indicating the fermentation of starch and increased acidity in the hindgut. Similarly Willard *et al.* (1977) observed that horses on a grain concentrate diet had higher caecal lactic acid concentrations (23.9 mmol/L) in comparison to horses on a hay diet (0.96 mmol/L).

Changing bacterial populations in the hindgut of the horse and declining pH have been clearly demonstrated by Garner *et al.* (1978). When acidosis was induced using the method of Garner *et al.* (1975), it was observed that over a period of 24 hours post carbohydrate overload, caecal pH significantly declined (Figure 2.17). Coupled with the decline in pH was an increase in the lactic acid producing bacteria species L*actobacilli spp* and *Streptococci spp* (Figure 2.17a) while the pH sensitive *Enterobacteriaceae spp* and *Bacilli spp* numbers declined (Figure 2.17b).

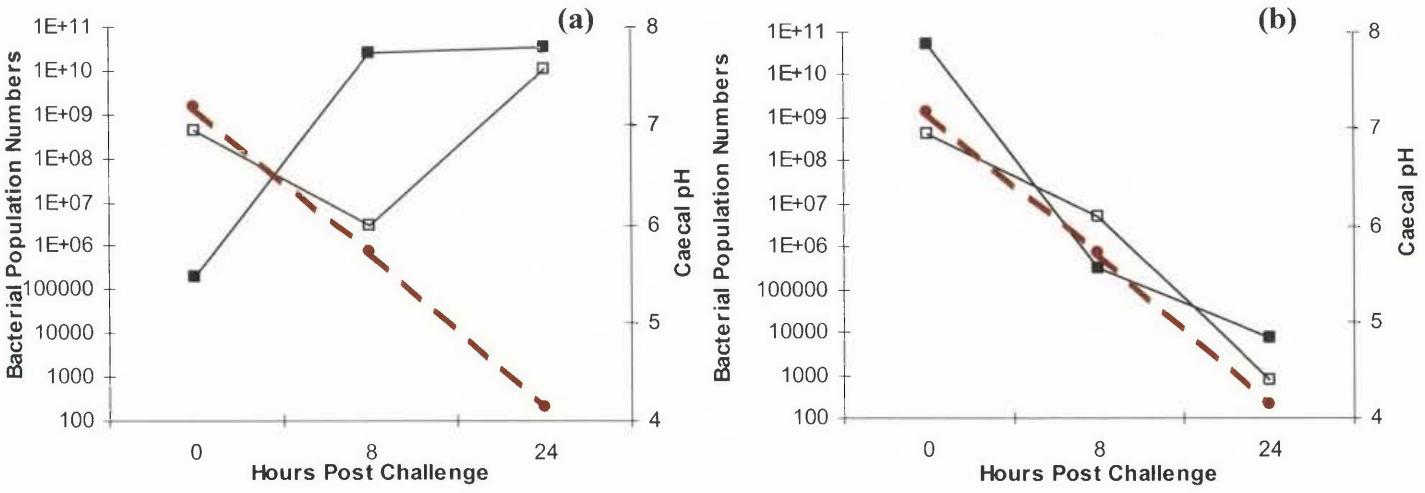

Table 2.17: The (**a**) lactic acid producing bacterial species (■) *Lactobacilli spp.* and (□) *Streptococci spp* and (**b**) the pH sensitive species (■) *Enterobacteriaceae spp* and (□) *Bacilli spp* cultured from horse (n=6) caecal fluid at time 0 (control), 8 hours and 24 hours post carbohydrate overload and the (--•--) caecal pH measured at these time intervals (Garner *et al.*, 1978).

Similarly Kern *et al.* (1973) observed that the inclusion of 25% oats in equine diets and the resultant delivery of small quantities of starch to the hindgut was enough to causes changes in the resident microbial populations. The inclusion of 25% oats in the diet initiated a 66% increase in the number of lactic acid producing *Streptococcus equinus* and a 91% increase in the total number of *Lactobacillus* bacteria in the hindgut (Figure 2.18). The *Propionibacterium* population numbers increased 400%, presumably in response to a raised availability of lactic acid for conversion to propionate and the cellulolytic *Bacteroides spp.* declined in number from 8.1% of the total bacterial population to 3.5% (Figure 2.18).

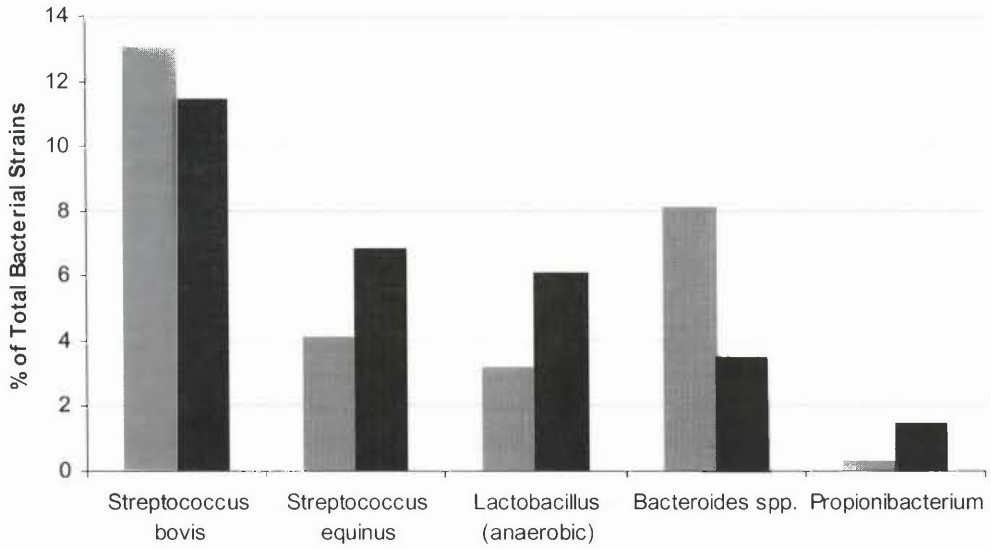

Figure 2.18: The change in the population distribution of bacteria in the hindgut of the horse when changed from being fed a 100% roughage diet (■) to a 75% roughage, 25% oat diet (■), (Kern *et al.*, 1973).

Susceptibility to hindgut acidosis varies between horses. In a trial designed to specifically induce hindgut lactic acidosis and laminitis, plasma L-lactate concentrations were used to gauge the severity of the acidosis (Garner *et al.*, 1977). Five horses, who subsequently died from circulatory collapse, reached an average plasma L-lactate concentration of 3.5 mmol/L prior to their death. Twenty one horses that developed Obel grade 3 laminitis reached average plasma L-lactate concentrations of 2.35 mmol/L and the remaining five horses, who displayed very few, if any ill effects, barely had an increase in plasma L-lactate concentrations (Garner *et al.*, 1977).

Variations in hindgut bacterial populations may help to explain the variability in susceptibility to hindgut acidosis amongst horses. For example, Alexander *et al.* (1963), while working with digesta samples collected from horses at abattoirs, were only able to isolate *Lactobacilli* from the colon of 6 out of 16 horses and were unable to isolate lactic acid producing *Streptococci* spp from the hindgut of some of these horses. In similar work de Fombelle *et al.* (1999) determined that by suddenly introducing grain to the diet, the number of lactate producing bacteria in the hindgut of the horse increased significantly, simultaneous with an increase in lactate utilising bacteria populations and a decrease in the number of cellulolytic bacteria. Five and 30 hours post introduction of grain to the diet, lactate concentrations had increased significantly while pH had declined (de Fombelle *et al.*, 1999). As observed by Alexander *et al.* (1963) there appeared to be large variation between horses in the change in microbial pattern that their intestinal microflora took on. de Fombelle *et al.* (1999) suggested this difference may explain why some horses are particularly susceptible to acidosis and related illnesses while others are not.

2.4.3 Consequences of Hindgut Starch Fermentation and Acidosis

The fermentation of starch in the hindgut may result in three major consequences for the horse:

(i) a reduced feed conversion efficiency;

(ii) behavioural changes; and

(iii) disease.

These three factors are discussed in detail below.

Reduced Feed Conversion Efficiency

There are two ways in which hindgut starch fermentation may reduce feed conversion efficiency in horses. These are:

(i) through a loss of energy in the forms of heat, gases and bacterial protoplasm when starch is fermented in the hindgut as opposed to digested in the small intestine; and

(ii) through a reduction in fibre fermentation in the hindgut.

Energy Losses During Fermentation

In the ruminant, during the process of fermentation, energy is lost as methane and heat during the process of carbohydrate fermentation. Wolin (1981) estimates that the production and eructation of CH_4 during rumen fermentation results in a loss of about 10% of the energy intake of ruminants. A further 6% of energy is lost through the heat of fermentation and 10% of energy is incorporated in microbial protoplasm (Hungate 1965 and Blaxter 1962 *in* Black, 1971). Black (1971), using calculations based on these apparent energy losses, estimates that a ruminant lamb digesting 100% of its diet in the rumen, will receive 25% less net energy from the diet than a theoretical non-ruminant lamb digesting feedstuffs in the small intestine. Energy losses in the equine hindgut may be even higher than those in the rumen, as in ruminants, a majority of energy incorporated in microbial protoplasm will become available to the ruminant when these microbes pass from the rumen to the small intestine to be digested (Black, 1971). However in the equine, where fermentation takes place in the hindgut and micro-organisms are excreted in the faeces (Frape, 1998), this incorporation of 10% of energy into microbial protoplasm must also be counted as a loss of energy.

Livingstone and Fowler (1987 *in* Kienzle, 1994) also estimate that organic matter which is fermented by micro-organisms in the hindgut will yield only 60% of the metabolisable energy which may be yielded from the same material if digested by small intestinal enzymes and absorbed pre-caecally.

Reduced Fibre Fermentation

The changes that take place in the rumen and hindgut during lactic acidosis, particularly the lowered pH and inactivation or death of many of the cellulolytic bacteria have been commonly observed to reduce the fermentation of fibre within these sites of the digestive tract. Kane *et al.* (1959) observed that a gradual introduction of 2.7 kg of starch to a 100% lucerne hay diet in dairy cattle had no effect on the total tract digestibility of dry matter. However a sudden introduction of 2.7 kg of starch to the diet significantly reduced the dry matter digestibility of the lucerne hay. Likewise Orskov *et al.* (1975) observed that when sheep were consuming whole barley, that had a minimal effect on ruminal pH, dry matter digestion of dried grass incubated in the rumen in nylon bags was 625 mg/g. Dry matter digestion was however reduced to 423 mg/g when dried grass was incubated in the rumen of sheep fed pelleted barley that reduced rumen pH to 5.3 within 2 to 3 hours after feeding. These sudden introductions of starch, or the incorporation of a highly fermentable processed starch source into the diet, have probably instigated changes to the rumen which have disadvantaged cellulolytic bacteria, subsequently causing the observed reductions in the fermentation of fibre throughout the gastrointestinal tract.

In agreement with work conducted in ruminants, de Fombelle *et al.* (1999) observed that by increasing the grain to forage ratio in diets fed to ponies, the extent of fibre digestion in the gastrointestinal tract decreased, even though the mean retention time increased as grain

inclusion was raised (diets were fed to maintenance). Thus it was concluded that, as the amount of grain fed increased, the quantity of starch reaching and fermenting in the hindgut rose, presumably disrupting the microbial ecosystem present in the hindgut and causing the inactivation or death of cellulolytic bacteria and therefore reducing fibrolytic activity.

Subsequent studies (de Fombelle *et al.*, 1999) showed that cellulolytic populations of bacteria decreased in number from 1.87×10^6 to 6.17×10^4 and lactate producing bacteria increased in number from 5.00×10^5 to 8.94×10^6 for *Streptococcus spp.* and from 4.17×10^5 to 7.34×10^6 for *Lactobacilli spp.* when the horses were changed from a 100% roughage diet to a 50: 50 roughage to grain diet. Thus the fermentation of starch in the hindgut appears to depress the efficiency of roughage digestion and utilisation in the caecum and colon of the horse.

Behavioural Changes

Horses suffering hindgut acid accumulation have been observed to change their behaviour. Willard *et al.* (1977) observed that horses consuming a 6 kg concentrate diet, with caecal pH values significantly lower and caecal lactate concentrations 24% higher than horses consuming a 100% hay diet, spent significantly less time eating feed and significantly more time chewing wood, practicing coprophagy and searching for feed. These changes in behaviour may be due to the changes observed in the hindgut and hindgut acid accumulation or possibly to boredom, as grain fed horses were only able to spend 3.4% of their time eating, whereas horses provided with hay spent an average 39.5% of their time eating. In this study, the addition of Na_2CO_3 to the concentrate diet significantly increased caecal pH 3, 4 and 5 hours after consumption of the meal and reduced the observed incidence of wood chewing and coprophagy in these horses, indicating that abnormal behaviours are partially instigated by hindgut acid accumulation.

Likewise, in work conducted by Johnson *et al.* (1998), it appeared that abnormal behaviours exhibited by stabled horses were due to hindgut starch fermentation and acid accumulation. It was observed by these researchers that as grain intakes increased over a 4-week period, faecal pH decreased and the horses increased the frequency of abnormal behavioural events (not exhibited by animals in their natural environment), including grasping, wood chewing, the eating of bedding and to a lesser extent, stall licking, with faecal pHs of 6.2 – 6.3 resulting in a rapid rise in these behavioural events. Abnormal behavioural events were not observed in horses receiving a hay diet only, even after 4-weeks of confinement in stables. The addition of virginiamycin (Founderguard®) to the diet to reduce lactic acid production and maintain hindgut pH above 6.5 (Rowe *et al.*, 1994; Rowe *et al.*, 1995), prevented the occurrence of grasping and the eating of bedding and made a definite reduction in the incidence of wood chewing (Johnson *et al.*, 1998). Thus it appears that there are definite links between hindgut acid accumulation, faecal pH and behavioural changes in horses.

Disease

The adverse effects that cereal grains can have on the health of horses have been recognised for centuries.

> *Grass is the first nourishment of colts after they are weaned...whereas when they are fed with corn and hay, but especially with the first (corn)...it exposes them to unspeakable injuries.*
>
> - Gibson (1726 *in* Frape 1998)

Endotoxemia and Metabolic Acidosis

The changes in bacterial populations and the resulting acid accumulation and low pH conditions experienced in the equine hindgut during starch fermentation have been observed to destroy the epithelial layer of the hindgut, with Krueger *et al.* (1986) reporting that within 48 hours of carbohydrate overload, the epithelial cells of the caecum were showing areas of desquamation. At 72 hours post carbohydrate overload widespread sloughing of these cells was apparent (Krueger *et al.*, 1986). With the surface of the hindgut so severely affected, the penetration of

(i) endotoxins (vasoactive bacterial lipopolysaccharides) that are released from the pH sensitive gram –ve *Enterobacteriaceae* bacteria upon their death and lysis in low pH conditions (Figure 2.17b); and

(ii) lactic acid that has been produced by amylolytic bacteria,

through the epithelial layer into the portal circulation, lymphatic system or peritoneal cavity of the horse is possible (Krueger *et al.*, 1986; Radostits *et al.*, 2000).

Once endotoxins penetrate the epithelia of the hindgut, the horse may enter into a state of endotoxemia, with which depression, hyperthermia followed by hypothermia, decreased systemic blood pressure, cool skin and extremities, diarrhoea and muscle weakness and recumbency are associated. In severe cases the horse will suffer cardiovascular collapse and death (Radostits *et al.*, 2000). Likewise, the absorption of lactic acid from the hindgut can have severe complications for the equine, disrupting the body's acid-base balance and causing a state of metabolic acidosis. Mental depression and muscular weakness are common clinical signs associated with this metabolic condition (Radostits *et al.*, 2000).

Laminitis

It is well established that the fermentation of large quantities of starch in the horse's hindgut can lead to the development of laminitis. The administration of high starch gruel to horses is an established experimental model for inducing laminitis in horses (Garner *et al.*, 1975), however, until recently the disease was poorly understood. For example Moyer in 1990 commented that since the exact pathophysiology was still unclear a 'myriad of therapies have been employed' in attempts to cure horses of the disease, with limited success. Due to this poor understanding of laminitis until recent times, there is limited literature detailing

the pathophysiology of laminitis. Dr Chris Pollitt of the Australian Equine Laminitis Research Unit (AELRU) has recently made significant progress towards understanding the pathophysiology of laminitis and is thus the major source of up-to-date information concerning the disease.

Laminitis (*lamellae,* the section of the hoof which is affected and *itis* meaning inflammation) is the second biggest killer of horses in the world, after colic (Pollitt, 2001a). Severe damage to the internal hoof structures can occur within a few hours and in many cases euthanasia is the only humane and sensible option. In the normal hoof, the distal phalanx (which is the equivalent of the human toe, also called the P3 bone, pedal bone or the coffin bone) is suspended from the hoof wall by a tough, flexible suspensory apparatus called the lamellae, with the upper surface of the distal phalanx parallel to the hoof wall (Figure 2.19). During the development of laminitis these lamellae fail, allowing the distal phalanx to shift downward into the hoof capsule, damaging arteries, veins, the corium of the coronet and the sole of the hoof, causing horrific pain and a lameness characteristic of laminitic horses (Pollitt, 2001a).

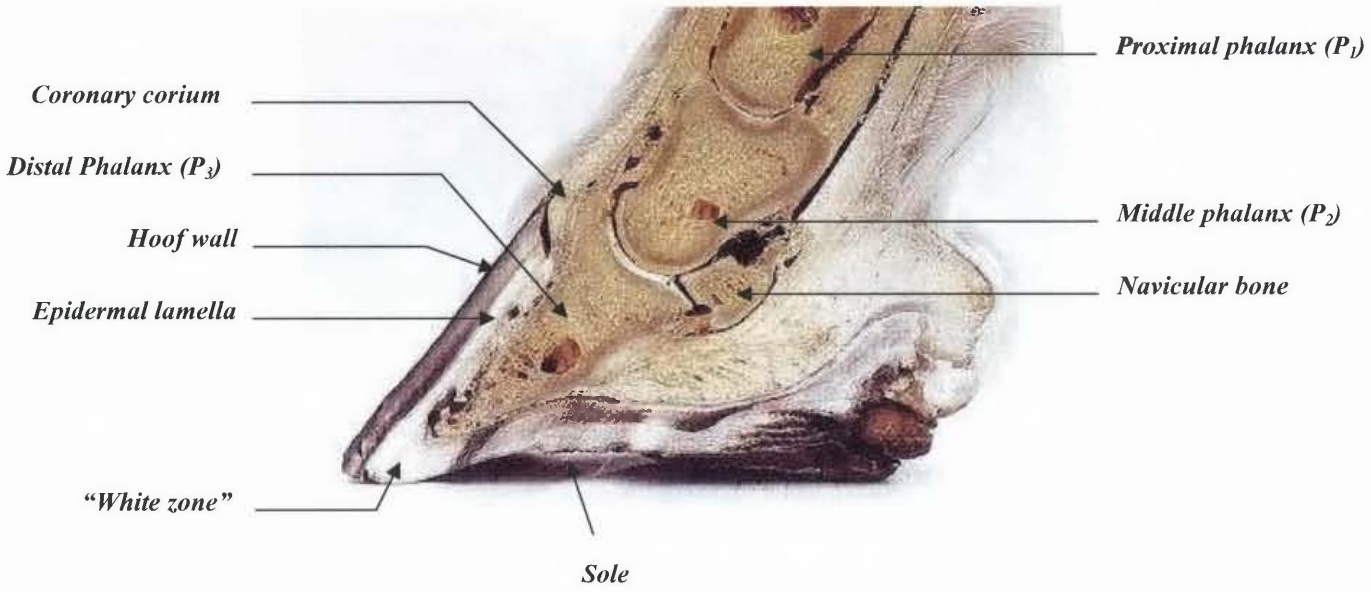

Figure 2.19: The distal phalanx in this normal hoof is securely attached and parallel to the hoof wall (Source: www.naturalhorsetrim.com).

There are many theories surrounding the causes of laminitis and the processes that take place in the hoof. The current theory developed at the Australian Equine Laminitis Research Unit is that two enzymes, matrix-metalloproteinase-2 and matrix-metalloproteinase-9 (MMP-2 and MMP-9), which are normally involved in the controlled remodelling and growth of the hoof wall, are activated beyond control, destroying the lamellae apparatus (French *et al.*, 2001; Pollitt, 2001a; Pollitt, 2001b). MMP-2 and MMP-9 are under tight regulation in the normal hoof and are used to release cell to cell and cell to basement membrane attachments as required, in order to allow the continually proliferating hoof wall to move past the stationary distal phalanx. Controlled release of MMP inhibitors keeps the growth and remodelling process in equilibrium. The hoof lamellae and the hoof itself move

slowly past the basement membrane, which remains firmly attached to connective tissue covering the upper surface of the distal phalanx (Pollitt, 2001a).

Laminitis is essentially a lesion of this basement membrane. Anchoring filaments that attach the basement membrane to the lamellae disappear, probably due to the action of the MMP enzymes. With the basement membrane no longer attached to the laminae, and thus the distal phalanx no longer suspended from the hoof wall, the weight of the horse drives the bone downward until the change in distance between the hoof wall and distal phalanx may be measured in mm's (Figure 2.20) (French *et al.*, 2001; Pollitt, 2001a; Pollitt, 2001b).

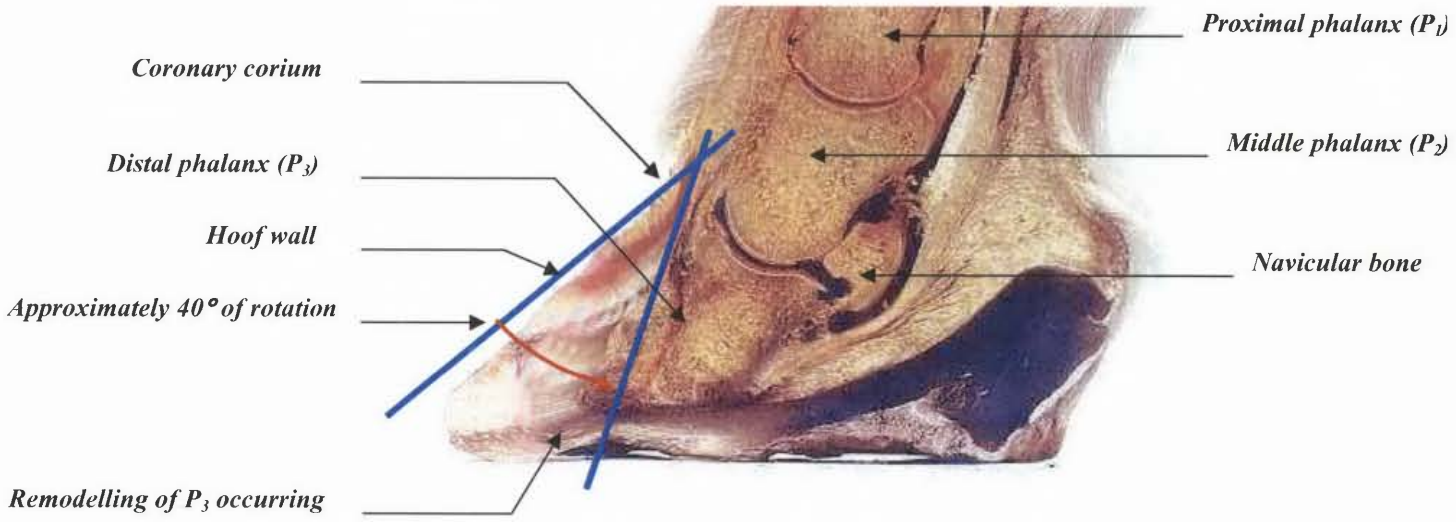

Figure 2.20: The distal phalanx in this hoof has rotated downward away from the hoof wall approximately 40° during laminitis (Source: www.naturalhorsetrim.com).

In vitro laminitis may be induced by adding known MMP activators to incubation medium containing hoof tissue explants (Pollitt, 2001a). The question still remains how processes in the gastrointestinal tract, and more specifically in the hindgut, can cause such devastating processes to occur in the hoof. Previous theory suggested that laminitis following 'grain overload' was caused by endotoxins (vasoactive bacterial lipopolysaccharides) released from the cell walls of the gram –ve bacteria *Enterobacteriaceae,* when they die in the high acid, low pH conditions experienced in the gut following the fermentation of large quantities of starch (Figure 2.17b). It was believed that when these endotoxins leaked from the gut and into the bloodstream, the resulting endotoxemia caused laminitis (Moore *et al.*, 1979). However, the experimental administration of endotoxin has never been shown to induce laminitis in horses and similarly drugs that are effective against endotoxins are unable to prevent laminitis in endotoxemic horses (Pollitt, 2001a). Thus it appears that other factors 'leaking' from the hindgut following the hindgut fermentation of starch may be the cause. Garner *et al.* (1977) observed a strong relationship between levels of plasma L-lactate concentrations and the severity of laminitis which developed in 31 horses that had been administered a high starch gruel to experimentally induce the disease. This

relationship led these researchers to conclude that L-lactate may 'contribute to the onset of laminitis', with the relationship between L-lactate and laminitis appearing to be 'more than coincidental'. Recent findings however have shown that a 'factor' present in the supernatant of *Streptococcus bovis*, isolated from the hindgut of horses activates MMP-2 and causes *in vitro* laminitis (Pollitt, 2001a; Pollitt, 2001b). *Streptococcus bovis* plays a major role in the rapid fermentation of starch substrate and production of lactic acid in the horses hindgut during 'grain overload' (Russell *et al.*, 1985). Work is continuing to determine if this factor is able to cross the mucosal barrier of the hindgut to enter the bloodstream and activate MMP in the hoof *in vivo* (Pollitt, 2001a; Pollitt, 2001b).

Hindgut starch fermentation and acidosis can thus have severe implications for horses, with effects ranging from reduced feed conversion efficiency to death. With variations between grains, variations between horses and feeding management practices (Section 2.3) determining how much starch enters the hindgut of the horse, the occurrence of hindgut starch fermentation and acid accumulation will be spasmodic and difficult to predict. Determining the site of digestion of specific cereal grain starches will allow precise feeding management practices that will help to prevent the delivery of fermentable starch substrate to the equine small intestine.

2.5 Non-Invasive Methods for the Determination of Site of Starch Digestion

Non-invasive experimental methods for the measurement of site of starch digestion in horses are attractive for two main reasons:

1. they are more acceptable from an animal welfare perspective and allow the re-use of experimental animals; and
2. they allow the use of experimental designs such as the latin square, which help to remove between animal variation effects and reduce the number of experimental animals required. They thus reduce the costs involved with conducting statistically valid experiments.

In addition, since this study had the most application in performance horse stables, invasive methodology would have had a limited usefulness. This consideration influenced the choice of research reported later in this thesis. Following is a discussion of non-invasive parameters that may be used to:

(i) determine the extent of small intestinal starch digestion occurring. Such methods include measurement of the glycaemic and insulin responses and *in vitro* starch digestion assays; and

(ii) monitor the extent of hindgut starch fermentation in horses. Methods include measurement of faecal volatile fatty acid and lactic acid concentrations; caecal and faecal pH; faecal starch concentrations; faecal nitrogen concentrations; and faecal dry matter.

2.5.1 *In Vivo* Determination of Small Intestinal Starch Digestion Using the Postprandial Glycaemic and Insulin Responses

Changes in blood glucose and insulin concentrations following a high starch meal have been used to quantify the digestibility of starchy foodstuffs in the small intestine of human and equine subjects (Brand Miller *et al.*, 1996; Pagan, 1999). The measurement of glycaemic and insulin responses following a carbohydrate meal, and determination of digestibility, is based on the premise that a starch source, susceptible to digestion by α-amylase and brush border glycanases will release more glucose into the bloodstream than a starch that is less susceptible to enzyme degradation. Thus digestible starches will cause larger rises and falls in blood glucose and insulin concentrations than poorly digested starches. A detailed discussion of the glycaemic and insulin responses as predictors of small intestinal starch digestibility follows.

Glycaemic Response

The glycaemic response is the measured postprandial effect of carbohydrates on blood glucose concentration (Brand Miller *et al.*, 1996). It is measured by feeding a chosen subject a specific portion of a carbohydrate containing foodstuff and measuring the change in blood glucose concentration over time. The glycaemic response curve consists typically of three phases:

1. the initial phase is characterised by a rise in blood glucose concentration, during which time glucose is entering the blood stream at a rate faster than it may be removed;
2. phase two occurs when this rise in blood glucose stimulates the release of insulin and thus the second phase of the glycaemic response curve is a lowering of blood glucose concentration, as glucose under the influence of insulin, enters cells where it is used for energy or converted to glycogen for storage;
3. a third phase on the glycaemic response curve, known as the 'overshoot', may occur when a delay in the cessation of insulin action results in blood glucose concentrations that drop below baseline blood glucose levels (Figure 2.21; Loeb, 1971).

Carbohydrates that are easily broken down to glucose by enzymes in the small intestine and absorbed into the bloodstream will display a high glycaemic response. Carbohydrates that are much less susceptible to enzyme degradation in the small intestine will initiate much lower postprandial glycaemic responses (Brand Miller *et al.*, 1996; Wolever *et al.*, 1991). There are many variables that may affect the observed glycaemic response including. These include the method of blood sampling and site at which blood is collected; the length of blood sampling time; subject characteristics including age; condition score or body fatness; glucose tolerance status and insulin sensitivity; fat, protein and roughage content of the foodstuff; and the meal feeding frequency. Other factors such as the method of food preparation and degree of processing using heat, moisture and pressure may also affect the observed glycaemic response, as will the particle size of the foodstuff as it enters the small

intestine (Brand Miller *et al.*, 1996; Heaton *et al.*, 1988; Holm *et al.*, 1989; Holt *et al.*, 1994; Jeffcott *et al.*, 1986; Jenkins *et al.*, 1981; Pagan *et al.*, 2001; Ross *et al.*, 1987; Stull, 1988). Likewise, because glucose is a normal metabolite within the horse's body, plasma concentrations of glucose following a meal will be affected by not only the rate of uptake from the small intestine, but also by the metabolic status of the horse. Metabolic status will, in turn be affected by the horse's endocrine system (Mair *et al.*, 1991).

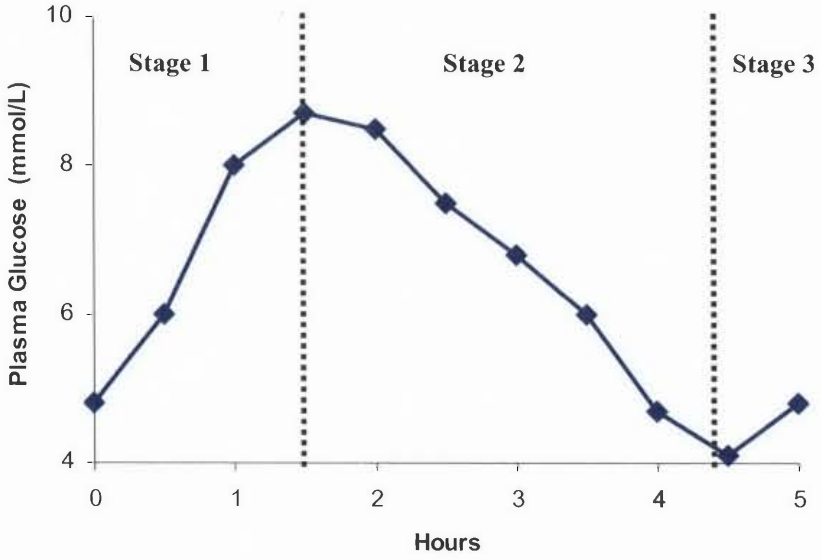

Figure 2.21: The three stages of the equine glycaemic response as described by Loeb (1971).

Throughout the development of the glycaemic response as a tool for predicting the immediate postprandial effect on blood glucose concentrations in humans, it was commonly observed that meal size also had a large, but unexplained effect on observed blood glucose responses. In humans, it appears that postprandial glycaemic responses increase linearly with meal size up to 50 g of carbohydrate/meal, beyond which the response curve tends to flatten and increasing quantities of carbohydrate no longer produce a proportional increase in blood glucose concentration (Jenkins *et al.*, 1981; Lee *et al.*, 1998; Wolever *et al.*, 1991).

Similar observations regarding the effect of meal size on the glycaemic response have been made in equines. Radicke *et al.* (1994) observed that an increase in meal size from 2g/kg BW of starch to 4 g/kg BW of starch resulted only in significantly higher blood glucose concentrations 30 min after consumption of the meal with peak glucose concentrations reached not being significantly different. Blood glucose concentrations did however remain elevated for a longer period of time following consumption of the larger meal. As observed in human subjects, the increase in meal size did not result in a proportional increase in blood glucose concentration and Radicke *et al.* (1994) proposed that this may be due to the regulation of blood glucose by insulin.

Similarly Pagan (1999) investigated the effect of increasing various grain meal sizes from 0.75 kg grain/meal (approximately 0.85 g starch/kg BW) to 1.5 kg grain/meal

(approximately 1.65 g starch/kg BW) and 2.5 kg grain/meal (approximately 2.8 g starch/kg BW) on the equine glycaemic response. Increasing the quantity of a commercial sweetfeed meal (sweetfeed's are a grain mix blended with molasses that may or may not contain chaff) resulted in step like increases in glycaemic response, however the increases in response were not proportional to increases in meal size. Increasing the quantity of corn from the medium to high feeding level resulted in no increase in the observed glycaemic response, while the increase from the medium to high meal size for the oat diet actually decreased the observed glycaemic response below that observed for oats at the lowest feeding level (Pagan, 1999). Thus the glycaemic response in equines appears to be sensitive to meal size.

Insulin Response

Insulin response following a carbohydrate meal has also been used as a predictor of small intestinal carbohydrate digestion and absorption and it has been observed that the insulin response in human subjects closely resembles that of the glycaemic response (Holt *et al.*, 1994; O'Dea *et al.*, 1980; Ross *et al.*, 1987). In some cases the insulin response appeared to be a more accurate predictor of small intestinal carbohydrate digestion and absorption in humans than the glycaemic response (Heaton *et al.*, 1988; Lee *et al.*, 1998).

Heaton *et al.* (1988), when measuring the effect of particle size on starch digestion in a variety of grains, observed that postprandial plasma insulin responses closely resembled postprandial glucose responses, with both responses increasing as grain particle size decreased. It was observed, however, that plasma glucose responses did not increase as expected when the particle size of corn decreased. The insulin response, however, showed a clear increase as corn particle size decreased and Heaton *et al.* (1988) concluded that the insulin response might in fact be a more accurate non-invasive *in vivo* predictor of small intestinal starch digestion than the glycaemic response.

Similarly, data from Lee *et al.* (1998) indicate that the insulin response may be able to predict the extent of starch digestion in the small intestine more accurately than the glycaemic response. When increasing carbohydrate meal portions from 25 to 50 to 100 g, plasma insulin concentrations increased linearly with the increase in meal size. Plasma glucose concentrations increased non-linearly however, increasing by 68% with the increase in meal size from 25 to 50 g and increasing only a further 37% when meal size increased from 50 to 100 g of carbohydrate/meal.

2.5.2 *In vitro* Determination of Enzymatic Starch Digestion

Due to the importance of the rate and extent of starch digestion in the small intestine and the relative expense and difficulty experienced when measuring these dietary parameters via *in vivo* methods such as the glycaemic and insulin responses, many attempts have been made to develop an *in vitro* assay that is able to rapidly and reliably predict small intestinal starch digestion. Brand *et al.* (1985) and Jenkins *et al.* (1987) incubated food samples in dialysis bags at biological temperatures, with fresh human saliva used as a source of α-amylase.

Glucose, maltose and 'oligosaccharide' concentrations were measured in the 'diffusate' to calculate the extent of starch digestion. In both experiments the glycaemic response was employed to determine *in vivo* starch digestion of the selected foodstuffs in humans and good positive correlations between *in vitro* and *in vivo* starch digestion estimates were observed.

In a further step to imitate and predict small intestinal starch digestion, Heaton *et al.* (1988) employed an *in vitro* assay using both α-amylase and amyloglucosidase (AMG) to carry the starch digestion process to completion, measuring the concentration of glucose to determine extent of starch digestion. Oat, wheat and corn grains, processed to varying particle sizes were incubated (temperature unknown) first in α-amylase then in AMG, with supernatant glucose concentration measured at the conclusion of both incubations. *In vivo* small intestinal starch digestion was also estimated in humans using the glycaemic and insulin response methods. Positive correlations between *in vitro* and *in vivo* estimates of starch digestion were evident, however, Heaton *et al.* (1988) observed some anomalies. For example, correlations existed between the *in vitro* assay and the glycaemic response, but no correlation was present between the *in vitro* assay and the insulin response. Large differences in apparent starch digestion between coarse and fine wheat flours *in vivo* were not evident *in vitro* and in the reverse there were large differences between whole and cracked maize *in vitro* that were not observed *in vivo.* A further inconsistency observed was that oats, which was just as, if not more extensively digested *in vitro* than wheat and corn, induced a glycaemic response which was lower than that initiated by the wheat and corn meals. The authors hypothesised that these inconsistencies were due to a lack of protein digestion in an assay containing only starch digesting enzymes; the fact that there is nothing to simulate chewing in the *in vitro* assay; and the effect of soluble and viscous fibres such as β-D-glucans found in the oat husk on small intestinal viscosity and *in vivo* small intestinal starch digestion, (Heaton *et al.*, 1988).

By simulating digestion in the gastric stomach and including protein digesting enzymes in the assay (60 min incubation with pepsin at pH 1.5 prior to incubation with α-amylase), Holm *et al.* (1989), were able to achieve a significant positive correlation between *in vitro* and *in vivo* (glycaemic response) estimations of small intestinal starch digestion in rats. If the pepsin digestion step was omitted from the *in vitro* assay, the correlation between the *in vitro* assay and the glycaemic response was no longer evident.

More recently *in vitro* assays have been developed to determine the safety and nutritional value of high starch feedstuffs in animal diets. Wiseman *et al.* (2000) developed a rapid method for determining the nutritional quality of wheat for broilers that involved incubating isolated wheat starch granules or wheat based poultry meals with pancreatic porcine α-amylase. Total soluble carbohydrate production was measured to determine the extent of starch digestion. *In vivo* starch digestion of the wheat meals was determined using a 'standard assay' with 12-day old broiler chicks. As previously observed in human subjects,

a significant, positive correlation existed between *in vitro* and *in vivo* estimations of small intestinal starch digestion.

Similarly Bird *et al.* (1999) developed an *in vitro* assay to determine small intestinal starch digestion in ruminants and monogastrics based on the total starch determination method of McCleary *et al.* (1997). The assay employs the two major starch digesting enzymes, α-amylase (from *Bacillus licheniformis*) and AMG (from *Aspergillus niger*) to digest starch, with the concentration of glucose in the supernatant being used as the measure for starch digestion. The grains to be digested are incubated in the presence of excess enzyme at pH 7 for one hour at 39^0C, thus simulating conditions that prevail in the ruminant or monogastric small intestine. The *in vitro* assay of Bird *et al.* (1999) was negatively related (R^2=0.74) to an *in vivo* measure (slaughter trial utilising ytterbium as a marker) of pre-caecal starch digestion in horses on various grain diets (Rowe *et al.*, 2001). During this study, the starch concentration at the ileo-caecal valve decreased as predicted *in vitro* starch digestion increased (Rowe *et al.*, 2001). Thus it is possible that an *in vitro* assay may be used to provide accurate and reliable estimations of small intestinal starch digestion in equines.

2.5.3 Non-Invasive parameters for Determining Extent of Hindgut Starch Fermentation

Volatile Fatty Acid and Lactic Acid Concentrations

On a roughage diet, increased activity of *Ruminococcus albus* causes a higher production of acetate in the rumen of cattle, whereas on a concentrate diet increased activities of *Streptococcus bovis* and *Bacteroides amylophilus* cause the raised production of propionate and succinate (Hungate, 1966). The expected VFA ratios for a ruminant maintained on a roughage or a concentrate diet are presented earlier in Table 2.5.

These changing VFA ratios with alterations in diet have been reported by Orskov *et al.* (1969) who observed that as the percentage of starch entering the abomasum of sheep increased, the molar percentage of acetate in the caecum decreased. Likewise, sheep consuming barley have been observed to have significantly lower molar concentrations of acetate and significantly higher concentrations of propionate and butyrate in the caecum in comparison to sheep fed dried grass only. The ratio of acetate to propionate to butyrate in the caecum of sheep fed dried grass was 70: 16: 6 while in sheep fed barley, the ratio was changed to 56: 20: 13 (Orskov, 1970).

With similar population distributions and numbers of micro-organisms in the equine hindgut as found in the rumen (Kern *et al.*, 1973), closely related patterns of VFA production can be expected in the hindgut. Horses fed a concentrate diet have been observed to have significantly lower molar percentages of acetate and significantly higher molar percentages of propionate in the caecum in comparison to horses consuming hay only (de Fombelle *et al.*, 1999; Willard *et al.*, 1977). Zeyner *et al.* (1992) also report that as the concentrate: roughage ratio in the diet increases, the acetate: propionate ratio in faecal matter is decreased. Thus the ratio of acetate to propionate to butyrate in the hindgut or

faeces may be able to be used to determine if starch substrate is fermenting in the hindgut of equines.

Because high starch cereal grains are fermented at a faster rate than cellulose, there is also a more rapid production of fermentation end products, namely the volatile fatty acids acetate, propionate and butyrate (Russell *et al.*, 1995). Thus total concentrations of VFAs tend to increase when the micro-organisms in the hindgut have access to starch as a fermentation substrate. Siciliano-Jones *et al.* (1989) observed that steers fed a high grain diet had total caecal VFA concentrations that were 80% higher than animals maintained on a low grain diet and that the acetate to propionate ratio was significantly decreased from 4.80 on a high roughage diet to 3.38 on a high grain diet. Similarly DeGregorio *et al.* (1982) observed that total VFA concentrations in the hindgut of sheep increased as starch intake rose. The caecal acetate to propionate ratio was also significantly lower in animals consuming the high starch diet.

The ratio of acetate to propionate to butyrate and total VFA production cannot however be looked upon as 100% reliable indicators of starch fermentation in the hindgut. Lee *et al.* (1977) found that sheep maintained on an *ad libitum* whole-wheat diet had significantly lower concentrations of acetate, propionate and butyrate in the caecum in comparison to sheep consuming *ad libitum* lucerne hay. However the caecal pH of sheep on the wheat diet was significantly lower than that observed in the caecum of sheep on the lucerne hay diet (4.91 vs. 6.96). Thus it is possible that the low pH of 4.91 is inhibiting production of VFA by all bacteria in the rumen and that a possible large accumulation of lactic acid in the caecum of sheep on the wheat diet is contributing to the low pH. This indicates that hindgut pH and lactic acid concentrations should be measured in conjunction with VFA concentrations.

Due to the nature of structural carbohydrate fermentation and the equilibrium achieved between lactic acid producers and utilisers, lactic acid rarely accumulates in the hindgut or rumen of animals when no starch substrate is available for fermentation (Leek, 1993; Phillipson, 1952). However if starch substrate becomes available, lactic acid production accelerates and lactate utilisers are unable to maintain the equilibrium between production and utilisation of lactic acid and thus lactic acid commonly accumulates when starch is being fermented in the hindgut. Willard *et al.* (1977) and de Fombelle *et al.* (1999) observed higher hindgut lactic acid concentrations in horses consuming concentrate diets in comparison to horses consuming roughage. Likewise Godfrey *et al.* (1992) observed that sheep receiving no introduction to a high grain diet had significantly higher L-lactate concentrations in the hindgut than sheep receiving a gradual introduction to grain. Similarly DeGregorio *et al.* (1982) found that lambs consuming an 80% corn diet had higher caecal lactate concentrations than lambs on a roughage diet. The trend is also present in cattle, with lactic acid concentrations in the caecum escalating with increasing concentrate to roughage ratios (Siciliano-Jones *et al.*, 1989).

A definite relationship between lactic acid concentration in the hindgut or faeces and quantity of starch fermenting in the hindgut has not been reported for horses. This suggests that hindgut lactic acid concentration may be used only as an indicator of starch fermentation in the hindgut, with the presence of lactic acid in hindgut or faecal matter indicating the fermentation of starch.

Caecal and Faecal pH

Due to the rapid synthesis of VFAs and production of lactic acid by amylolytic bacteria, it may be expected that hindgut pH will be lower when the resident bacteria have access to starch substrate. It has been observed in pigs that as concentrations of resistant starch are increased in the diet, the pH in the large intestine declines (McDonald *et al.*, 1998; Pluske *et al.*, 1998; Pluske *et al.*, 1997). Similarly in beef cattle that were maintained on high starch diets, faecal starch content was negatively correlated to colon pH (R^2=0.94), and faecal pH (R^2=0.92), indicating that as the quantity of starch reaching the hindgut increased, colon and faecal pH decreased (Wheeler *et al.*, 1977). In sheep, animals allowed *ad libitum* access to wheat had significantly lower caecal and colonic pH readings than animals maintained on an *ad libitum* lucerne hay diet (Lee, 1977).

Similarly, horses maintained on a grain concentrate diet tend to display mean caecal pH values that are substantially lower than for horses consuming a hay diet, suggesting that part of the soluble carbohydrate portion of the concentrate diet reaches the caecum (Johnson *et al.*, 1998; Willard *et al.*, 1977). Radicke *et al.* (1991) noted that corn (pre-caecal starch digestibility 71%) depressed caecal pH further than oats (pre-caecal starch digestibility 98%). In agreement with these observations, it has been shown that the caecal pH of horses on concentrate diets is negatively related (R^2=0.91) to the quantity of starch entering the caecum (Rowe *et al.*, 2001).

Therefore caecal pH may be a good indicator of the amount of starch escaping enzyme degradation in the small intestine and fermenting in the hindgut. For caecal pH to be measured the animal must be fitted with a caecal cannula or slaughtered to allow access to caecal contents. Alternatively, it is possible that pH in the caecum may be estimated by measuring the pH of faecal samples. A strong positive relationship (R^2=0.99) between faecal pH and caecal pH in sheep has been observed, with caecal pH being an average of 0.78 pH units lower than faecal pH (Clayton *et al.*, 1999).

Several studies have also been carried out in which caecal pH and distal colon pH has been recorded in pigs. A strong positive relationship exists between caecal pH and distal colon pH in pigs (R^2=0.61). As was observed in sheep, the pH of digesta increased as it moved along the digestive tract with the pH in the porcine distal colon being an average of 0.46 pH units higher than the pH of digesta in the caecum (Pluske *et al.*, 1998; Pluske *et al.*, 1997; Pluske *et al.*, 1996). A strong positive relationship between caecal pH and faecal pH also exist in cattle (R^2=0.97) (Wheeler *et al.*, 1977) and in horses (R^2=0.74) (Rowe *et al.*, 2001). Unlike sheep and pigs, differences between caecal pH and faecal pH in cattle and horses

were minimal amounting to just 0.05 and 0.12 pH units respectively. Thus faecal pH, a non-invasive and simple measure, may be an accurate tool for estimating caecal pH and thus the extent of hindgut starch fermentation occurring in horses.

Faecal Starch

It appears that as the quantity of starch escaping digestion pre-caecally increases, the quantity of starch appearing in the faeces will also be elevated and thus may be used as an indicator for the quantity of starch entering the hindgut. Ørskov *et al.* (1970) observed that as infusions of starch into the caecum of sheep increased, the quantity of starch excreted in the faeces increased. In this situation it appeared that the hindgut of sheep had a limited capacity for hindgut starch fermentation as any starch infused above a rate of 138 g/day generally appeared in the faeces

In data presented by Karr *et al.* (1966) it is evident that the quantity of starch present in the posterior ileum of cannulated steers is well correlated to the quantity of starch found in the faeces (R^2=0.97) with starch recovery in the faeces increasing as starch escaping digestion in the rumen and small intestine increases. There was an average of 79% less starch in the faeces in comparison to that present in the posterior ileum. Similarly in horses, the quantity of starch in the caecum has been observed to be related (R^2=0.70) to the concentration of starch in the distal colon. On average there was 76% less starch present in the distal colon (Ben Barwick, University of New England, *pers. comm*). Thus measurement of faecal starch may allow an estimation of the quantity of starch escaping pre-caecal digestion.

Faecal Nitrogen

Bacteria found in the rumen of cattle are approximately 65% protein and constitute an important source of protein for ruminant animals as they are digested in the small intestine (Hungate, 1966). It could be expected that bacteria in the hindgut of equines are also 65% protein, however, unlike ruminants where the bacteria are digested in the small intestine for the benefit of the host, bacteria from the hindgut of the horse are excreted in the faeces. Given optimum supplies of energy and protein, ammonia or urea, micro-organisms in the rumen and hindgut are able to multiply at the highest possible rate. Cellulolytic bacteria given optimum conditions are only able to double their population every 18 hours, whereas amylolytic bacteria are capable of doubling their population numbers in as little as 15 minutes (Leek, 1993). Thus logic may lead us to believe that the quantity of high protein bacteria in the hindgut of the horse will be higher when the hindgut micro-organisms have access to starch substrate and thus faecal nitrogen will increase with increasing starch fermentation. Such trends have been observed in sheep and ponies.

Orskov *et al.* (1970) observed that increasing starch infusions into the caecum of two sheep from 20 g/day to 300 g/day caused faecal nitrogen excretion to increase by 82% and 52% for sheep A and B respectively. Similarly small colon and rectal crude protein concentrations in ponies were increased from 12.6% of dry matter in ponies on an all

roughage diet to 16.7% of dry matter in ponies on a 1: 4 roughage: grain diet (Hintz *et al.*, 1971). Thus an increase in faecal nitrogen may indicate that hindgut starch fermentation is occurring, however further data detailing the effect of hindgut starch fermentation on faecal nitrogen needs to be collected before the parameter may be used as a predictive tool for estimating quantities of starch entering and fermenting in the hindgut.

Faecal Dry Matter

It has been repeatedly observed that animals experiencing the fermentation of starch in their hindgut often have lower faecal dry matter contents and thus faecal dry matter may be used as and indicator of starch fermentation in the hindgut. Lee (1977) observed that wethers on an *ad libitum* whole-wheat diet had a significantly lower digesta dry matter content in the colon than wethers maintained on an *ad libitum* lucerne hay diet. Similarly Orskov *et al.* (1970) found that increasing infusions of starch into the caecum of sheep above 138 g/day caused faeces of the sheep to become soft and unpelleted.

Likewise, Godfrey *et al.* (1992) observed that sheep with a low hindgut pH and high concentration of L-lactic acid had significantly more water (by weight) in the hindgut in comparison to sheep with more neutral pH values and lower concentrations of lactic acid. In contrast however Hintz *et al.* (1971) observed that horses on a grain/roughage diet had significantly higher colon-rectal dry matter percentages than horses fed an all roughage diet. Thus dry matter may not be a reliable predictor of hindgut starch fermentation across all species.

The following chapter describes a survey conducted to investigate grain-feeding practices in the Australian Thoroughbred Industry. The incidence of hindgut starch fermentation and acid accumulation and thus the potential risks involved with grain feeding to thoroughbred horses, were identified using the *in vivo* faecal analysis methods described here. *In vitro* methods were used to analyse various grain samples, and data was collected regarding the feeding practices of a sample of thoroughbred horse trainers. Details of the methods, materials, findings and their implications form the following Chapter (3).