STUDIES ON GASTRIC EMPTYING IN PONIES

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ABSTRACT

Disordered gastric emptying has been associated with several medical disorders in the horse, but little is known about the physiology of equine gastric emptying, largely due to the inefficiency of the methods available for its assessment. The ^{13}C-octanoic acid breath test (^{13}C-OBT) was recently developed as a non-invasive method for assessment of gastric emptying in man and the aim of this study was to investigate the feasibility of applying this test for assessment of gastric emptying in ponies.

The ^{13}C-OBT involves the ingestion of a ^{13}C-octanoic acid substrate which is absorbed rapidly in the duodenum, and is excreted in the breath as $^{13}CO_2$. Because gastric emptying is the rate-limiting step in the digestion and metabolism of octanoic acid, the rate of excretion of $^{13}CO_2$ is proportional to the rate of gastric emptying. An initial study established that the basal level of breath $^{13}CO_2$ excretion in three healthy ponies was low, and remained stable over 12 hours. There was no significant change in the pattern of $^{13}CO_2$ excretion following ingestion of an unlabelled test meal in three ponies. These findings confirmed the potential for enrichment of breath $^{13}CO_2$ excretion in the pony, by administration of a ^{13}C-labelled substrate.

The test protocol was then repeated on three ponies following ingestion of test meals enriched with 125mg (n=3), 250mg (n=6) and 500mg (n=3) ^{13}C-octanoic acid. All doses produced a significant increase in the levels of breath ^{13}C, and this increase was proportional to the dose administered. The test was reproducible within individuals and inter-subject variation was low. The duration and frequency of breath sampling in these studies was lengthy, and the possibility of simplifying the protocol was investigated by recalculating the breath test parameters while progressively omitting data points. This investigation showed that in the healthy pony the test duration could be shortened to 6 hours, but the sampling frequency could not be altered without affecting the accuracy of the test.

The ingestion of energy dense meals has been shown to cause delayed gastric emptying of solids in man, and in the horse. A second aim of this study was to investigate the effect of increasing test meal energy density on the parameters of the ^{13}C-OBT in healthy ponies. There was a significant difference in the breath test parameters following ingestion of high and intermediate energy density meals, when compared with a low energy density meal, but no difference between intermediate and high energy density meals. These findings indicated that the ingestion of an energy enriched test meal was associated with delayed gastric emptying, but this delay was not directly proportion to the calorific load ingested.

The results of these studies suggest that the ^{13}C-OBT is a non-invasive and reproducible method for the assessment of gastric emptying in the horse, and further validation is justified.

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Wyse, C. Murphy, D. Preston, T. Morisson, D and Love, S (1998) A preliminary study of the [13C]-octanoic acid breath test for assessment of gastric emptying in ponies. *British Equine Veterinary Association,* Annual Congress, Birmingham, 1998.

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CHAPTER ONE

INTRODUCTION

Delayed gastric emptying is a feature of numerous medical disorders in the horse, including gastroduodenal ulceration (Becht and Byars, 1986), grass sickness (Milne, Doxey and Gilmour, 1990) and gastric neoplasia (White, 1992). The physiology of gastric emptying in the horse is poorly understood because of the limitations of methods currently available for the investigation of gastric function in this species. The development of non-invasive tests that can assess gastric emptying in different physiological and pathological conditions will lead to further advances in the understanding of equine digestive disease. In this chapter a brief review of the functional anatomy of the stomach and the physiological control of gastric emptying will be presented. The techniques available for evaluating gastric emptying will then be described and their potential applications in equine medicine evaluated. Finally, a review of the clinical conditions that result in primary and secondary disruption of gastric emptying in the horse will be presented.

1.1 Gastric Anatomy of the Horse

The stomach of the horse is relatively small (8-15 litres), comprising 8.5% of the total gut capacity (Pfeiffer and MacPherson, 1990) and necessitating frequent ingestion of food to satisfy energy requirements. The stomach can be divided anatomically into five regions, the cardia, fundus, body, antrum and pylorus (Figure 1.1).

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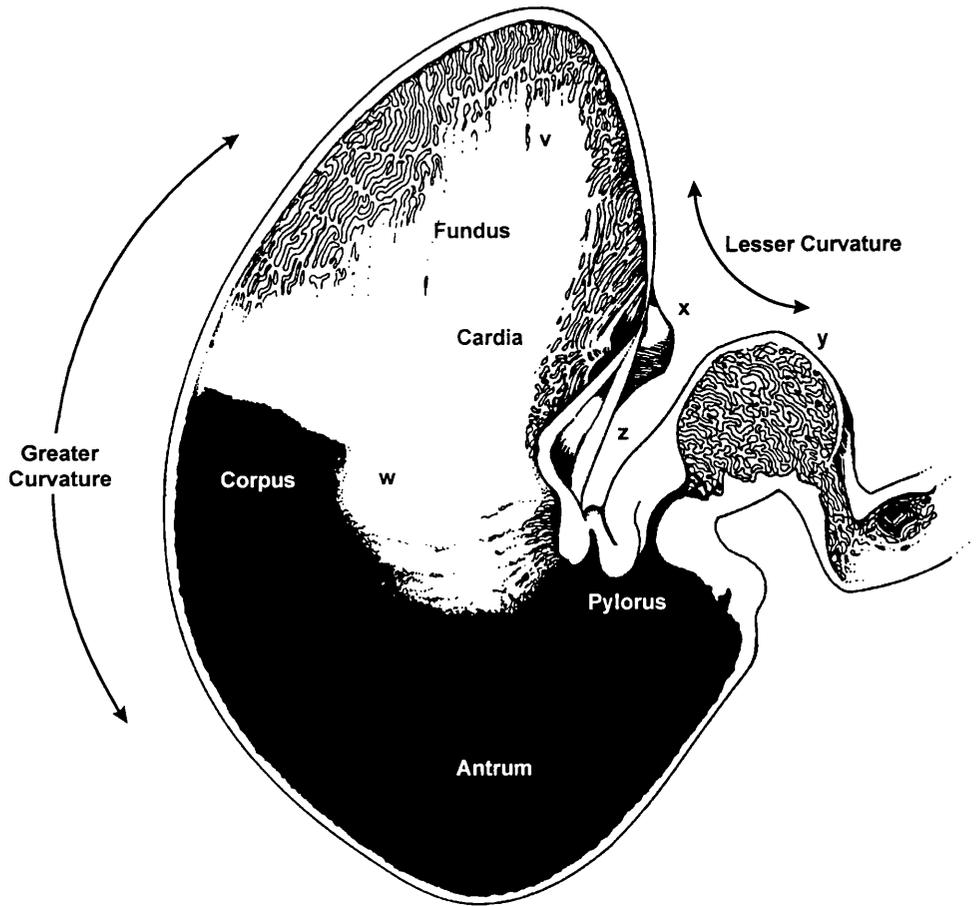
Figure 1.1: A diagrammatic illustration of the anatomical regions of a frontal section of the equine stomach and proximal duodenum (Redrawn from Sisson, 1976).

v	*saccus caecus ventriculi*
w	*margo plicatus*
x	oesophagus
y	duodenum
z	*incisura anglurais*

The stomach is located in the dorsal part of the abdomen, caudal to the diaphragm and to the left of the midline. It has two surfaces and two distinct radii of curvature. The *parietal* surface lies dorsally against the diaphragm and is bordered ventrally by the left lobe of the liver and laterally by the spleen (Nickel, Schummer and Seiferle, 1979).

The *visceral* surface faces caudo-ventrally and is related dorsally to the transverse colon and ventrally to the jejunum (De Boon, 1975). The *greater curvature* of the stomach ascends dorsally from the cardia and descends in a wide arc ending at the pylorus; the *lesser curvature* forms a smaller arc to describe an acute angle *(incisura anglurais)* between the termination of the oesophagus, and the pylorus (Figure 1.1). At the left extremity of the stomach there is a rounded fundic expansion called the *saccus caecus ventriculi* that is a feature unique to the equine stomach (Sisson, 1976). A raised irregular edge called the *margo plicatus* marks the division between the glandular and non-glandular regions of the stomach (Figure 1.1).

The gastrophrenic ligament attaches the apex of the *saccus caecus* to the diaphragm, and is continuous dorsally with the phrenicosplenic ligament and ventrally with the gastrosplenic ligament (De Boon, 1975). The greater omentum originates on the *saccus caecus* and is continuous with the gastrophrenic ligament; it follows the greater curvature of the stomach and runs over the large colon before rejoining the gastrophrenic ligament (Nickel, Schummer and Seiferle, 1979). The lesser omentum attaches the stomach to the liver via the hepatogastric ligament (Nickel, Schummer and Seiferle, 1979). The greater curvature normally rests on the dorsal colon, but the volume, position and form of the equine stomach is subject to variation depending on the gastric contents and rate of gastric emptying. When the stomach is empty the pylorus becomes contracted and loops of small intestine lie ventrally to its visceral surface. When the stomach becomes distended with food material, gas or fluid, the small intestine, spleen, small colon and the left dorsal part of the large colon are pushed caudally. The equine stomach is completely enclosed within the bony thorax, and for this reason, it not directly affected by the abdominal musculature and is inaccessible to clinical examination or manipulation from the exterior.

The stomach wall consists of four tissue layers, an outer serosal coat, a muscular layer, a sub-mucosal and an innermost mucosal layer. The serosa is derived from the peritoneum and covers the entire gastric surface except at the attachment of the greater and lesser omentum. The muscular layer is composed of an external layer of longitudinal fibres, a middle layer of circular fibres and an internal layer of oblique muscle fibres (Sisson, 1976). The longitudinal fibres are present only along the curvatures, and are replaced by elastic fibres from the middle of the greater curvature to the pyloric antrum (Sisson, 1976). The longitudinal fibres on the lesser curvature are continuous with those of the oesophagus. At the pyloric antrum, a layer of exceptionally well developed longitudinal fibres is formed separately to that of the curvatures, and this layer, together with a slight retraction of the circular fibres, demarcate the entrance into the pyloric canal. The circular fibres form a uniform layer throughout the glandular region of the stomach, but are not present in the fundic region (Sisson, 1976). The external and internal oblique layers cover the fundic region. The external oblique layer is a continuation of the longitudinal fibres of the oesophagus. The internal oblique layer is a continuation of the circular fibres of the stomach and oesophagus, and exchanges fibres with the external oblique layer (Sisson, 1976). At the lesser curvature, the edges of the layer of internal oblique fibres form a groove, *the sulcus ventriculi*, that is spanned crosswise in its floor by circular fibres (De Boon, 1975). This groove may function as a channel, enabling water to empty along the lesser curvature (Rooney, 1997).

The cardia of the horse is characterised by the proficiency of the cardiac sphincter, and this, along with the oblique angle of entrance of the oesophagus, accounts for the horse's inability to vomit (Dyce, Sack and Wensing, 1987). The cardiac sphincter of the horse

is not a typical sphincter, but consists of two staggered semi-lunar muscular folds; the proximal fold is formed by a loop of internal oblique fibres and the more distal fold is formed by a loop of circular fibres (De Boon, 1975). This arrangement describes a muscular occlusion that is more proficient than that of a typical sphincter. The pyloric sphincter is formed by a ring of circular muscle at the junction of the pylorus and duodenum; a more proximal ring of muscular constriction marks the entrance to the pyloric antrum (Nickel, Schummer and Seiferle, 1979).

In the human stomach, a mid-gastric transverse muscular band is thought to represent an anatomical separation of the functional units of the stomach and to play a role in regulating the emptying of the fundus into the antrum (Moore, Dubois, Christian, Elgin and Alazraki, 1986). This mid-gastric division is a physiological state requiring intra-gastric food, and is not always detectable in the empty stomach (Moore *et al.* 1986). In the horse, the *margo plicatus* is often the site of a similar muscular constriction in the moderately full stomach (Nickel, Schummer and Seiferle, 1979) and may represent a similar mid-gastric anatomical division.

The submucosa is a layer of loose connective tissue that connects the mucosal and muscular layers, and this is where vessels and nerves ramify before entry into the mucosal layer. The mucosa is divided into glandular and non-glandular regions (Figure 1.2). The non-glandular or pro-ventricular region (*pars proventricularis*) is located at the proximal stomach and is similar in structure to the mucosal layer of the oesophagus. This region is lined with stratified squamous epithelial cells, that are keratinised at the surface and appear rough, white and flaky under low magnification (Pfeiffer and MacPherson, 1990). The non-glandular region is non-secretory and is not structurally adapted for transport mechanisms because of its thick outer *stratum corneum*.
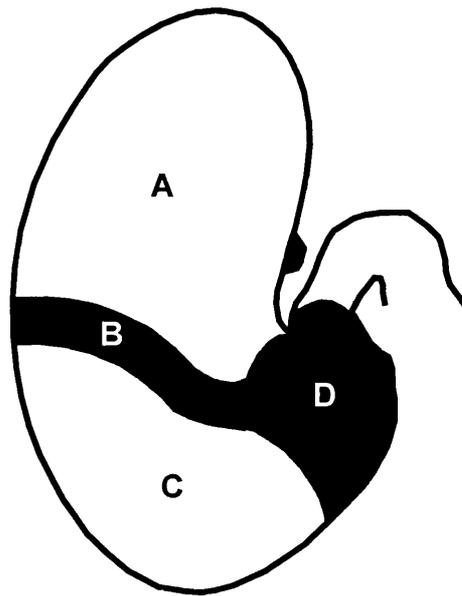
Figure 1.2 The glandular regions of the equine stomach. The non-glandular or proventricular region (A) is composed of non-secretory squamous epithelial cells. The cardiac region (B) secretes bicarbonate ions and mucus, the gastric zone (C) secretes hydrochloric acid and pepsinogen and the pyloric region (D) secretes mucus.

The *margo plicatus* marks the transition into the glandular region of the stomach (Figure 1.1). The glandular region can be roughly divided into zones according to the type of secretory gland present. The cardiac region is located adjacent to the *margo plicatus* and contains cardiac glands which secrete bicarbonate ions (HCO_3^-) (Argenzio, 1990), and mucus secreting cuboidal cells (Pfeiffer and MacPherson, 1990). The gastric zone is thick and vascular and contains gastric pits that are lined with chief, parietal and G-cells responsible for the secretion of hydrochloric acid (HCl), pepsinogen and gastrin (Figure 1.2) (Argenzio, 1990). The more distal pyloric region contains pyloric glands that secrete mucus. The stomach is supplied with parasympathetic input from the left and right vagus nerves, and sympathetic innervation is supplied from the coeliac plexus. The coeliac artery supplies blood and venous return is provided by the gastric veins and the portal vein (Sisson, 1976).

1.2   Gastric Motor Patterns

The basic component of gastrointestinal motility is the ability of the smooth muscle cell to generate cyclic changes in its resting membrane potential, that under certain conditions can give rise to a spontaneous wave of contraction. These periodic fluctuations in membrane potential are known as slow waves and represent the basic electrical rhythm of the gastric smooth muscle cell. Slow waves are generated in the longitudinal muscle layer and are not associated with muscular contraction. In the horse, gastric slow waves have been recorded at frequencies of 2 - 2.5 per minute (Merritt, Campbell-Thompson and Lowrey, 1989). Possible mechanisms for slow wave generation include oscillations of the electrogenic Na^+ pump, oscillations of intracellular Ca^{2+} release or through the contribution of specialised pacemaker cells (Mayer, 1994). Superimposed onto some slow waves are spikes or action potentials, which are associated with the influx of Ca^{2+} across the sacrolemma, and it is this electrical activity that is responsible for muscular contraction. Slow waves do not actually initiate contraction, but rather set the pace at which the superimposed action potentials generate muscular contraction (Rao and Schulze-Delrieu, 1993). A muscular contraction is generated when a slow wave spreads to the circular layer, and this occurs approximately every five minutes in the stomach of the horse (Phaneuf, Grivel and Ruckebusch, 1972; Phaneuf and Ruckebusch, 1983).

The equine gastrointestinal tract, similar to other mammals, exhibits three phases of motor activity: phase I of motor quiescence, Phase II of irregular spiking activity and Phase III of regular sustained contractions, where each slow wave gives rise to an action potential (Gerring, 1991). This phasic motor activity is called the migrating motor complex (MMC) and in the horse it passes through the gastrointestinal tract once every

50 minutes (Ruckebusch, 1981). The MMC is not interrupted by feeding in the horse as it is in man and other non-herbivores (Bueno and Fioramonti, 1983). The horse is a trickle feeder, and such continuous pattern feeding is generally associated with little alteration of the MMC (Ruckebusch, 1981). In the stomach of the horse, periods of regular spiking activity are prolonged, and these are interrupted by shorter periods of gastric quiescence (Baker, 1992). There is a perfect co-ordination between the occurrence of periods of gastric quiescence and phase III contractility in the proximal small intestine, and this correspondence appears to involve a more complex mechanism than simply distal migration of the gastric phase III motor activity (Baker, 1992). Simultaneous recording of gastric motility and pH in the horse, revealed that this co-ordination between gastric motor quiescence and the jejunal MMC, was associated with a rise in gastric pH caused by duodenal reflux (Gerring, 1991). Patterns of gastrointestinal motility are poorly understood in the horse, despite the proposed role of alterations in gut motility in the aetiology of gastrointestinal disease (Gerring, 1991), and this is an area that warrants further research.

1.3   Gastric Digestion and Emptying

Gastric emptying is a product of the tonic pressure maintained in the fundus, antral contraction and the inhibitory forces of pyloric and duodenal contraction (Parkman, Harris, Krevsky, Urbain, Maurer and Fisher, 1995). The function of gastric emptying is to deliver chyme to the duodenum at a rate and in a form that optimises the digestion and absorption of nutrients, and the conveyance of ingesta to the large intestine for further digestion. The main components of gastric function are listed below:

- reservoir for ingesta

- emptying of liquids

- triturition

- emptying of indigestible solids

- chemical digestion

1.3.1   Reservoir for Ingesta

The proximal stomach is composed of the fundus and the body, and in man, acts as an expansile and contractile reservoir for the storage of food (Read and Houghton, 1989). The equine stomach has limited potential as a reservoir for ingesta because of its small size. Ingestion of food, deglutition and gastric distension cause a reflex relaxation of the fundus, followed by a slow increase in fundic tone that allows material to pass to the antrum and prevents reflux into the proximal stomach during antral contractions (Read and Houghton, 1989). Two reflexes contribute to the reservoir function of the stomach. The first is known as 'receptive relaxation' and this reflex was proposed by Cannon and Lieb in 1910. They were able to demonstrate that relaxation of the proximal stomach occurred during sham feeding in the cat, and that this reflex was associated with deglutition (Cannon and Lieb, 1910). Receptive relaxation allows the stomach to expand to accommodate ingesta, without significantly affecting the intra-gastric pressure gradient that controls gastric emptying. Receptive relaxation is mediated by a non-cholinergic, non-adrenergic vagal reflex, and dopamine and enkephalin have roles as transmitters (Minami and McCallum, 1984). Vagal denervation of the proximal stomach in the dog caused increased intra-gastric pressure, due to failure of the receptive relaxation reflex (Wilbur and Kelly, 1973). Reflex relaxation of the stomach also occurs in response to gastric distension, and is known as "gastric accommodation" or "adaptive relaxation" (Mayer 1994). During reflex accommodation, the gastric smooth muscle lengthens to accommodate increases in gastric volume. This reflex can be initiated in the isolated stomach (Schulze-Delrieu, 1983), and a vaso-vagal reflex is

thought to have only a modulatory role (Mayer, 1994). It has been suggested that accommodation does not occur in the equine stomach, and that immediate gastric emptying of ingesta is necessary to avoid gastric impaction (Phaneuf and Ruckebusch, 1983). The fundus of the horse is relatively large, a typical characteristic of the herbivorous stomach, and the accommodative reflex has been demonstrated in the stomach of a similar monogastric herbivore, the guinea pig (Schulze-Delrieu, 1983). As many as 60% of cases of gastric rupture in the horse are thought to be idiopathic, with no predisposing cause identified (Kiper, Traub-Dargatz and Curtis, 1990). There appears to be a higher incidence of idiopathic gastric rupture in the horse relative to man (Dale, 1986). The inability of the horse to vomit undoubtedly contributes to the incidence of gastric rupture, but a poorly developed gastric accommodative reflex could also predispose the horse to this condition. There is a well-recognised correlation between the reservoir function of the stomach, and the tension-length characteristics of gastric smooth muscle strips *in vitro* (Paton and Vane, 1963; Schulze-Delrieu and Shirazi, 1987), and the ability of the equine stomach to accommodate ingesta could be investigated in this way.

1.3.2   Gastric Emptying of Liquids

Fundic contractions are responsible for the maintenance of the pressure gradient between stomach and duodenum that facilitates gastric emptying of liquids, and experimental denervation of the fundus caused accelerated gastric emptying of liquids in the dog (Wilbur and Kelly, 1973). The pattern of liquid emptying in the horse is exponential, similar to that in man (Baker and Gerring, 1994a). However, the *rate* of gastric emptying of liquids occurs more rapidly in the horse, than in man (Argenzio, Lowe, Pickard and Stevens, 1974a; Baker and Gerring, 1994a; Baker and Gerring, 1994b; Sosa León, Hodgson and Rose, 1997).

1.3.3   Triturition

Triturition refers to the mechanical breakdown and mixing of food to a semi-liquid chyme, and this is achieved via the repeated to-fro movement of ingesta in the antrum. Antral contraction is composed of circular rings of muscular contraction (peristalsis) that increase in amplitude and velocity as they travel distally towards the pylorus (Minami and McCallum, 1984). Solid food is milled to a semi-liquid chyme and allowed to mix with digestive juices, before being propelled towards the pylorus. As the wave of contraction approaches the distal antrum, the pylorus and proximal antrum close and particles too large to pass through the pylorus (>2mm) are propelled back into the body of the stomach. It is this action of contractile retro-pulsion that facilitates the stomach's role as a mill for the processing of solids into a form suitable for digestion in the small intestine. Because the antral contractions are responsible for this action, the distal stomach is thought to control the rate of emptying of solids from the stomach. In man, the triturition function of the distal stomach is represented by a period of decreased gastric emptying known as the lag phase. The lag phase follows ingestion of a meal and its rate and characteristic is dependant on the composition of the meal (Nusynowitz and Benedetto, 1994). The lag phase is followed by linear emptying of the stomach and together, these two patterns of gastric emptying depict a model of the gastric digestion of solids (Nusynowitz and Benedetto, 1994). A lag phase was evident in a scintigraphic study of gastric emptying of solids in the horse, and the solid marker exhibited an exponential pattern of gastric emptying (Ringger, Lester, Neuwirth, Merritt, Vetro and Harrison, 1996). Further research is necessary to elucidate the pattern and mechanisms controlling the gastric emptying of solids in the horse.

1.3.4   Gastric Emptying of Indigestible Solids

Food particles that cannot be broken down into a form that can be propelled into the duodenum by normal intra-gastric pressure, are emptied in man and in the dog, by the phase III contractile activity of the migrating myoelectric complex (Minami and McCallum, 1984). Indigestible solids were found to be retained in the stomach for prolonged periods of time in the horse (Argenzio *et al.*, 1974a). In a more recent study the gastric emptying of indigestible radio-opaque markers was highly variable in the horse, and feeding did not produce any clear effect on this variable pattern of emptying (Baker and Gerring, 1994c). These findings led to the conclusion that the gastric emptying of indigestible solids cannot be related to a single bowel motility cycle in the horse, and mechanisms other than just the phase III contractile activity of the MMC are responsible for ensuring gastric emptying of indigestible solids (Baker and Gerring, 1994c).

1.3.5   Chemical Digestion

Pepsinogen has been isolated from the equine stomach, and been shown to have some *in vitro* proteolytic activity (Khittoo, Vermette, Nappert and Lariviere, 1991), but it is unlikely that the gastric enzymatic digestion of protein is of any great physiological importance in the horse. Hydrochloric acid (HCl) is released into the stomach from the parietal cells in response to acetylcholine released from post-ganglionic cholinergic fibres, or histamine which reacts with specific type 2 histamine receptors on the parietal cell membrane (Argenzio, 1990). Acid secretion is also stimulated by the release of gastrin from the G-cells, in response to gastric distension, protein and increased luminal pH (Murray, 1997b).

1.3.6 Microbial Fermentation

There is thought to be considerable microbial fermentation in the stomach of the horse, leading to the generation of lactic acid and volatile fatty acids (VFA) (Argenzio, Southworth and Stevens, 1974b; Healy, Siciliano and Lawrence, 1995). Microbial digestion in the stomach is probably insignificant in providing an energy source, but may serve some physiological function in the production of vitamin B12 (Argenzio, 1990).

1.4 Physiological Control of Gastric Emptying

Several physiological conditions affect gastric motility, but perhaps the most significant determinant of the rate of gastric emptying, is the composition and form of the meal. The physiological control of gastric emptying is mediated by neural and hormonal influences.

1.4.1 Meal Composition and Form

1.4.1.1 Nutrient Density

Meal composition exerts a strong regulatory effect on gastric emptying. Increasing energy density causes decreased gastric emptying, an effect mediated by chemoreceptors in the duodenum (Hunt and Stubbs, 1975). In this way the rate of gastric emptying is sensitive to the nutritive density of the ingested meal, and chyme is delivered to the duodenum at a rate at which it can be digested and absorbed. In meals of high nutritive density, the rate of gastric emptying may not decrease sufficiently to prevent increased delivery of energy to the duodenum (Read and Houghton, 1989). It is the nutritive density (kcal/ml) of a meal that determines the rate at which it is emptied from the stomach, and this is independent of the ratio of fat to carbohydrate from which this energy density is derived (Hunt and Stubbs, 1975). The chemoreceptors in the

duodenum are polymodal, that is they are responsive to various stimuli including electrolytes, carbohydrates, amino acids and to the mechanical effects of stroking the epithelium (Wood, 1987). Pancreatic-biliary secretion in the duodenum, and pH of duodenal contents are not thought to have major roles in the intestinal regulation of gastric emptying (Miller, Malagelada, Taylor and Go, 1981). Increasing energy density was shown to delay the gastric emptying of solid meals in horses (Sojka and Cantwell, 1989), but studies have failed to identify any effect of increasing energy density on the gastric emptying of liquid meals in the horse (Baker and Gerring, 1994a; Sosa-León, Hodgson and Rose, 1997).

1.4.1.2   Meal Volume

In man, gastric volume influences the rate of gastric emptying and the gastric emptying of liquids of low energy density is directly proportional to the volume present in the stomach (Minami and McCallum, 1984). The effect of increased gastric distension on gastric emptying is mediated by mechanoreceptors in the gastric musculature (Minami and McCallum, 1984). The emptying of complex nutrient meals is controlled by feedback from chemoreceptors sensitive to increased nutrient concentration in the duodenum, and in these conditions the regulatory effect of the intestinal receptors is thought to override the effects of increased intra-gastric volume (Miller et al., 1981). No data is available on the effect of meal volume on gastric emptying in the horse.

1.4.1.3   Particle Size

Particle size is an important determinant of the rate of gastric emptying (Read and Houghton, 1989), large particles leave the stomach at a slower rate than smaller particles. This is partially because the greater inertia of large particles tends to divert them to the lateral sides of the flow of food being propelled towards the pylorus (Read

and Houghton, 1989). These slower moving particles are pushed back into the antrum by the strong retropulsive forces that accompany pyloric contraction. As the antral contraction moves distally, the pylorus partially closes, allowing only small particles to enter the duodenum and further decreasing the rate of gastric emptying of large particles.

1.4.2   Exercise

There are conflicting reports on the effect of exercise on the rate of gastric emptying in man, but in general it has been demonstrated that mild to moderate exercise increases the rate of gastric emptying, while maximal exercise decreases the rate of gastric emptying. The stimulatory effects of moderate exercise on gastric emptying are probably mediated by the release of neurotransmitters (Read and Houghton, 1989). Exercise at intensities above 70% of maximal oxygen consumption (VO_2 max) induced delayed gastric emptying due to pyloric closure and decreased antral motility (Brown, Ketelaar, Schulze-Delrieu, Abu-Yousef and Brown, 1994). These effects were thought to be mediated through the exercise-induced release of endogenous opioids, or through increased sympathetic tone, and catecholamine release (Brown *et al.,* 1994). Maximal exercise (>70% VO_2 max) did not have any effect on the gastric emptying of liquids in horses (Sosa-León, Hodgson and Rose, 1997), although there is some anecdotal evidence for delayed gastric emptying following acute exercise in the horse (Owen, 1975; Pocock, 1975; MacKinnon, 1975).

1.4.3   Stress

In human medicine, it has been known for some time that psychological or physiological stress can affect the rate of gastric emptying. In the classic experiments on "Tom" a chronic fistulated human patient, it was noticed that gastric motility

increased when the subject became angry but decreased when he became fearful (Wolf and Wolff, 1943). This observation illustrated that the type of stress is important in determining its effect on gastric emptying. In other studies, a consistent inhibitory effect of stress on gastric emptying has been reported (Musial and Enck, 1993), although a biphasic response has been demonstrated using some techniques, with an initial increase followed by a subsequent delay (Glavin and Mikail, 1977). For many years, it was believed that stressful life events were involved in the pathogenesis of gastroduodenal ulceration, but the evidence to support this association remains anecdotal, despite extensive research (Soll, 1997). However it is possible that alterations in gastric emptying induced by stress could affect duodenal clearance of biliary/pancreatic secretions, causing increased gastroduodenal reflux, and ulcer formation (Soll, 1997). Although alterations in gastric function induced by stress may interact with other factors to potentiate ulcerogenesis, it is no longer believed that stress has a primary role in the pathogenesis of gastroduodenal ulceration (Soll, 1997).

1.5   Methods for Assessment of Gastric Emptying

Methods for assessing gastric emptying in the horse should be simple, reliable and non-invasive. The test procedure must not in itself affect the gastric emptying rate, and sedation of the animal should not be required during the test. The methods that are currently available are in most cases unsuitable for use in practical situations, due to unreliable results or the necessity for complex and expensive test protocols. There are several methods available for assessing gastric emptying in man that could potentially be adapted for investigation of equine gastric emptying. Each method will now be briefly reviewed, and its potential application in equine gastroenterology evaluated.

1.5.1   Diagnostic Imaging

1.5.1.1   Radiography

The assessment of gastric motility using radiography was first described by Cannon in 1898, and radiography is a widely used method of providing qualitative and limited quantitative data on human gastric motility (Corazziani and Torsoli, 1993). Following administration of a radio-opaque fluid, radiography can be used in human medicine to diagnose mucosal defects, anatomical deformities, gastric obstruction, gastric neoplasia and the presence of foreign bodies (Parkman *et al.,* 1995). Gastric radiography can provide an indication of gastric shape and size, and while it is not a good method for quantitative measurement of motility, radiography does allow visualisation of antropyloric co-ordination (Corazziani and Torsoli, 1993). Fluoroscopy and cinefluoroscopy can be used for radiographic evaluation of gastric motility, and still or slow motion pictures of wall contractions and luminal distension can be scrutinised (Corazziani and Torsoli, 1993).

While radiography is a valuable method for evaluating oesophageal and intestinal motility, there are several disadvantages associated with its use for the evaluation of gastric emptying. The motor activity of the proximal stomach is difficult to image radiographically, as the slow tonic variation causes little detectable change in the gastric profile. The radiographic examination of the stomach can only reveal qualitative information about gastric emptying, and recognition of subtle changes in motility cannot be identified using this method. In addition, the information that radiography can provide on gastric emptying may be of limited physiological significance, due to the necessity for the use of contrast medium that is not chemically or physiologically similar to food (Corazziani and Torsoli, 1993). Furthermore, the poor physiological and

quantitative measures of gastric motility obtained using radiography, do not justify the risk of radiation exposure of the patient and medical personnel presented by this technique (Parkman *et al.,* 1995). Despite these limitations, radiography continues to be used as a simple and widely available investigative tool in human and veterinary gastroenterology.

The technique for radiographic examination of the equine stomach has been described, although its usefulness is limited by the necessity for powerful equipment (500-1000mA, 150-200kV) to penetrate the abdomen (Dik and Kalsbeek, 1985). Many horses require sedation for radiography and in one study the administration of the alpha-2-adrenoreceptor agonist, detomidine, was shown to significantly affect the radiographic assessment of oesophageal motility (Watson and Sullivan, 1991). Nevertheless, radiography has been successfully applied to demonstrate delayed gastric emptying in horses with pyloric occlusion (McGill and Bolton, 1984; Church, Baker and May, 1986), and has also identified the presence of a gastric squamous cell carcinoma (Aronoff, Reed and Aronson, 1997). Radiography was used experimentally to monitor the gastric emptying of metal weights in ponies, but due to poor resolution, it was not thought that this technique could be applied to horses (Baker and Gerring, 1994c). Because rectal palpation is often impossible, radiography is a valuable aid in the diagnosis of gastrointestinal disease in the foal, and it is possible to obtain standing lateral abdominal radiographs of good resolution. (Fischer, Kerr and O' Brien, 1987). A barium oesophogram can be used to demonstrate reduced oesophageal motility in cases of equine grass sickness (Greet and Whitwell, 1986), but because of the poor resolution of radiographs of the equine abdomen, it is unlikely that this technique could be of real use in the diagnosis of reduced gastric motility, even in small ponies.

1.5.1.2   Endoscopy

Fibreoptic endoscopes of 2-3m in length have recently become available to the veterinary clinician, allowing endoscopy of the equine stomach to be carried out (Brown, Slocombe and Derksen, 1985). The procedure of gastric endoscopy is relatively simple, the endoscope is passed over the cricopharyngeal sphincter to the oesophagus via the dorsal pharynx, and an oblique cleft marks the entrance to the stomach at the cardia (Murray, 1997a). The horse is usually starved for 12 hours prior to gastroscopy to minimise gastric contents (Proudman and Baker, 1994). Gastric secretion is a continuous process in the horse, and secretion in the distal stomach may prevent endoscopic visualisation of the glandular region, despite a period of starvation prior to examination (Hanson, Bartz, Stone, Darien and Markel, 1993). Clinically, gastroscopy has been used for the diagnosis of gastric ulceration (Murray, Grodinsky, Anderson, Radue and Schmidt, 1992), and gastric squamous cell carcinoma (Keirn, White, King and Tennant, 1982). Gastroscopy is a useful method for assessing gross aberrations of gastric emptying associated with conditions such as gastric neoplasia or pyloric stenosis; however it is not possible to detect subtle changes in the rate of gastric emptying using this method.

1.5.1.3   Scintigraphy

In human medicine, the evaluation of gastric motility by scintigraphic imaging following ingestion of a radionuclide-labelled marker, was first described in 1966 (Griffith, Owen and Kirkman, 1966) and is now considered to be the gold standard method for investigation of gastric motility (Parkman et al., 1995). ^{99}Technetium sulphur-colloid (^{99}Tc-SC) and ^{111}Indium-diethylene triamine pentacetic acid (^{111}In-DPTA) are the two radiopharmaceuticals most widely employed in the study of gastric emptying. These radionuclides have short half lives of 6 hours and 2.2 days

respectively (Harding and Notghi, 1993), and they are not absorbed through the gastric mucosa. Because ^{99}Tc and ^{111}In, emit gamma radiation at different energies they can be used to preferentially label the solid and liquid phases of gastric emptying. Specific analysis of the gastric emptying of solids and liquids using one radio-labelled meal is called the "dual isotope" method, and allows the mechanisms and rates of each emptying phase to be monitored simultaneously (Parkman *et al.,* 1995).

Precise test protocol and reference ranges for normal and abnormal rates of gastric emptying are established by each individual test centre. Many centres have adopted a test meal of scrambled egg with incorporated ^{99}Tc-SC, or chicken liver surface labelled using *in vitro* techniques (Parkman *et al.,* 1995). The test meal containing the radiolabel is standardised for composition, content and form, as these are factors known to affect gastric emptying (Hunt and Stubbs, 1975). The test meal in ingested following a 12-24 hour fast, and the patient is then required to remain in front of the gamma camera while the gastric emptying of the test meal is monitored.

There are some inherent sources of error associated with the measurement of gastric emptying using scintigraphy, and correction factors have been derived to compensate for inaccuracies associated with the technique. Correction factors may be necessary to allow for radioactive decay of the isotope over the duration of the test. Furthermore, anterior movement of the marker in the stomach may cause self-attenuation of the radionuclide, and the lag phase of gastric emptying may be overestimated because the marker has moved anteriorly to lie closer to the camera (Christian, Datz, Sorenson and Taylor, 1983). Indeed some authors believe that the lag phase of gastric emptying may be caused by an artefact produced during the imaging process (Moore, Christian, Taylor and Alazraki, 1985). However, most authors are in agreement that the lag phase is

representative of a period of gastric digestion that occurs prior to emptying of food into the duodenum, rather than an artefact of scintigraphy (Parkman *et al.* 1988; Siegal, Urbain, Adler, Charkes, Maurer, Krevesky, Knight, Fisher and Malmud, 1988; Nusynowitz and Bendetto, 1994). Other errors that may occur during the measurement of gastric emptying by scintigraphy are self-attenuation caused by the spreading of food in the stomach leading to initial counts greater than 100%, and overlap of activity in the small intestine over the area of interest in the stomach. Scintigraphy was the method used to describe the lag phase and in the derivation of mathematical definitions for the process of gastric emptying (Siegal *et al.,* 1988; Nusynowitz and Bendetto, 1994). Therefore, intrinsic errors associated with this technique could potentially affect understanding of the physiology and function of gastric emptying.

Despite the problems associated with scintigraphy, it remains the most useful method for the investigation of disorders of gastric motility in human medicine. The principal advantage of scintigraphy in clinical diagnosis is that it is a method for evaluation of the gastric emptying of solids; abnormal gastric emptying of solids is not always detectable in the liquid phase (Parkman *et al.,* 1995). The test meal closely resembles food and the imaging process is non-invasive, so the test is carried out under relatively normal physiological conditions. Preferential measurement of solid and liquid emptying is possible. The major disadvantage of scintigraphy for evaluation of gastric emptying is the risk presented to medical personnel and patients by the necessity for a radionuclide label. This risk precludes the use of the test in children and in pregnant woman, and limits the opportunities for serial tests in one individual (Parkman *et al.,* 1995). Other problems associated with the use of scintigraphy in the measurement of gastric emptying, are that it is an expensive and prolonged test that requires access to specialist equipment and experienced personnel.

Scintigraphy was recently applied to the measurement of gastric emptying in the horse, and was reported to be a well tolerated and accurate method for measuring the rate of gastric emptying of solids and liquids (Sojika and Cantwell, 1989; Levy and Sojika, 1991; Neuwirth, 1994; Ringger *et al.,* 1996). The use of scintigraphy as a method for measuring the rate of gastric emptying in the horse will facilitate further investigation of the role of gastric emptying in equine gastric disorders and the potential for pharmacological modulation of gastric motility.

1.5.1.4   Magnetic Resonance Imaging

The measurement of the gastric emptying of liquids in man, using magnetic resonance imaging (MRI) was recently described. This technique provides three dimensional images of the stomach and allows gastric emptying and gastric motility to be assessed simultaneously and non-invasively (Schwizer, Fraser, Borovicka, Crelier, Boesiger and Fried, 1994). Magnetic resonance imaging is the only method that allows gastric morphology to be visualised at the same time that gastric motility is evaluated (Maughan and Leiper, 1996). This facility could facilitate investigation of gastric structural disorders and their effects on gastric motility. Magnetic resonance imaging has not yet been applied for the measurement of the rate of gastric emptying of solids, and the use of this method in clinical diagnostics awaits the description of a solid marker. Magnetic resonance imaging is a relative time-consuming method and requires expensive equipment that is not yet widely available (Parkman *et al.,* 1995). Technical difficulties combined with the high costs of MRI, currently preclude its use as a method for evaluation of gastric emptying in the horse.

1.5.1.5   Ultrasonography

The development of techniques of high resolution, real-time ultrasonography has facilitated the application of this method for the assessment of gastrointestinal motility. The application of ultrasonography for the investigation of gastrointestinal motility initially focused on the measurement of the rate of gastric emptying, and in 1985 a close correlation between the rate of gastric emptying measured by ultrasonography and by scintigraphy was demonstrated (Bolondi, Bortolotti, Santi, Salleti, Gaiani and Labo, 1985). In these early studies, gastric volume was measured by dividing the stomach into serial elliptical cross sections, and the practical application of this technique was limited by these cumbersome and time-consuming measurements (Ahluwalia and Thompson, 1993). However, ultrasonography has evolved into a valuable investigative tool, and using various techniques it is now possible to measure gastric and antral emptying, antral rhythmicity, assess antro-duodenal co-ordination and gastroduodenal flow (Ahluwalia and Thompson, 1993). Ultrasonography allows clear visualisation of antral contraction, and peristaltic contraction can be calculated by using the plotting facility to measure the area enclosed by the relaxed state and the peristaltic contraction. Integration of real-time ultrasound with a video interface allows recordings to be replayed and the onset of contraction identified (Ahluwalia and Thompson, 1993). Furthermore, the development of coloured Doppler ultrasonography has allowed detailed analysis of the antro-duodenal contraction and luminal flow (Hauskin, Odegaard, Matre and Berstad, 1992). Using this method, retropulsive gastric flow can be visualised in a different colour to propulsive flow, allowing the to-and-fro movement of the luminal contents and the superimposed peristaltic-related propulsive flow patterns to be observed. This is a very useful method for identifying abnormal retropulsive flow, or reflux of material from duodenum to antrum (Ahluwalia and Thompson, 1993). Ultrasonography is a safe technique that does not involve exposure of patient or

personnel to ionising radiation, and it is completely non-invasive (Parkman *et al.,* 1995). Ultrasonography does not require a lengthy test protocol and can be used to evaluate gastric motility in children and pregnant women.

The rate of gastric emptying measured using ultrasound is based on the observed change in antral volume, and this may not represent a true measurement of gastric emptying, particularly when antral contents are retropelled into the fundus (Ahluwalia and Thompson, 1993). Ultrasonography is generally used to measure the rate of emptying of liquids and is of less use in the assessment of the gastric emptying of solids (Parkman *et al.,* 1995). The technique requires an experienced operator for imaging and interpretation, and access to specialised equipment. Ultrasonography is a subjective method for measuring gastric emptying and the variability of the results is reliant to some degree on the skill and expertise of the operator, although studies have shown that this variance between operators is not significantly large (Irvine, Tougas, Lappalainen and Bathurst, 1993). The use of ultrasonography in clinical diagnosis is currently limited by a lack of knowledge of the application of this technique in different pathological conditions, and it is likely that continuing research will result in greater validation of this method as an investigative tool in gastroenterology (Ahluwalia and Thompson, 1993).

Ultrasonography is a useful method for the diagnosis of gross pathology of the equine stomach such as gastric squamous cell carcinoma (Rantanen, 1986). The use of this technique as a method for evaluation of gastric emptying is limited by the density of the abdominal wall and the poor resolution of the images. The stomach of the horse cannot be scanned trans-rectally and gas in the bowel and lungs prevent effective trans-abdominal imaging (Rantanen, 1986). The use of ultrasonography for the assessment of

gastric emptying in the horse was attempted, but the gastric antrum could not be visualised, even using a 3MHz transducer to maximise tissue penetration. Visualisation of more dorsal areas of the stomach using the spleen and liver as acoustic windows, was prevented by gas in the bowel and stomach (Baker, 1992).

1.5.2   Electrical Resistance

1.5.2.1   Impedance Epigastrography

The rate of gastric emptying can be measured using the technique of impedance epigastrography to monitor changes in the electrical resistance across the abdomen (Maughan and Leiper, 1996). The ingestion of a meal causes increased electrical resistance across the stomach and this is followed by an exponential decrease in impedance that is an indirect representative of gastric emptying. The impedance of current through the stomach is monitored using a pair of standard electrocardiogram electrodes to pass an alternating current at a frequency of 100kHz across the abdomen. A second pair of electrodes is used to monitor fluctuations in potential difference across the region (Spyrou and Castillo, 1993). The technique of impedance epigastrography was first applied to the measurement of the rate of gastric emptying in 1985 (Sutton, Thompson and Sobnack, 1985) and further studies demonstrated a good correlation with gastric emptying rates using scintigraphy (Mangnall, Barnish, Brown, Barber, Johnson, and Read, 1988). Initial studies of gastric emptying using impedance epigastrography required the use of test meals of low conductivity, such as orange cordial, that did not stimulate gastric secretion (Sutton *et al.*, 1985). Pharmacological inhibition of gastric secretion allowed liquids of high conductivity to be used and these produce a greater increase in electrical resistance and a more sensitive measure of gastric emptying (Mangnall *et al.*, 1988). Impedance epigastrography has been confined to the measurement of the gastric emptying of liquids and a solid marker for use with this

method has not yet been described. The necessity for pharmacological inhibition of gastric secretion and test meals that do not resemble food mean that the measurement of gastric emptying using impedance epigastrography can never be carried out under true physiological conditions. The technique is very sensitive to artefact induced by even slight body movement during the test (Spyrou and Castillo, 1993). The advantages of impedance epigastrography as a method for measuring gastric emptying are that it is non-invasive, does not involve exposure to ionising radiation and yields results in real time.

Impedance epigastrography was recently used to measure the rate of gastric emptying in pony foals (Baker and Gerring, 1994b). The use of canvas slings to restrain the animals during the test allowed body movement to be reduced sufficiently to permit the acquisition of adequate data. In this instance, gastric emptying was successfully measured in the horse using impedance epigastrography. However the restraint in canvas slings used in this study would be difficult to reproduce in a clinical setting and impedance epigastrography in the horse is probably confined to use in the research laboratory.

1.5.2.2   *Applied Potential Tomography*

Applied potential tomography (APT) is based on the same principle as epigastric impedance but a multi-electrode array is used to enclose the upper abdomen. An alternating current is passed through the abdomen and the potential difference between all combinations of the other 14 electrodes is measured. Successive neighbouring electrodes are used to input the current until all possible configurations have been used. The resulting array of data is used to construct grey-scale plots that represent the conductivity distribution of a cross-sectional image of the stomach (Spyrou and Castillo,

1993). For measuring the rate of gastric emptying, the data is collected at a rate of one frame per second, and this is known as low-speed tomography. Studies where APT was used to calculate the rate of gastric phasic contractions, used the higher rate of data collection (24 frame/sec) called high speed tomography. Applied potential tomography is a more accurate method for measuring the rate of gastric emptying than impedance epigastrography. Applied potential tomography can also be used to monitor the gastric emptying of a solid meal, provided that the meal is of either higher or lower conductivity than the body tissue. Disadvantages associated with this method are that the positioning of the electrodes can be difficult, and as with epigastric impedance, pharmacological inhibition of gastric secretion is necessary.

1.5.3   Plasma Tracer Studies

Plasma tracer studies rely on the detection of a marker substance in the blood following ingestion of a labelled substrate that travels through the stomach and is quickly released into the circulation upon reaching the duodenum (Spiller, 1993).

1.5.3.1   Paracetamol and Acetominophen Absorption Test

The paracetamol absorption test has been described as a method for measuring the rate of gastric emptying of the liquid phase in human patients (Heading, Nimmo, Prescott and Tothill, 1973). Plasma drug concentrations are measured in serial blood samples following ingestion of 1.5g paracetamol, and the rate of gastric emptying is related to the appearance of paracetamol in the blood (Heading *et al.,* 1973). The acetaminophen absorption test is based on the same principle as the paracetamol absorption test: acetaminophen is poorly absorbed in the stomach, rapidly absorbed from the duodenum, and serum acetaminophen can be correlated with the rate of gastric emptying (Clements, Heading, Nimmo and Prescott, 1978). The acetaminophen absorption test was recently

used to measure the rate of gastric emptying in the horse, and to demonstrate prevention of endotoxin-induced gastric ileus by cisapride and phenylbutazone (Doherty, Andrews, Provenza and Frazier, 1998; Valk, Doherty, Blackford, Abraha and Frazier, 1998). When used to determine the effects of pharmacological agents on the rate of gastric emptying in ponies, the acetominophen absorption test was able to detect the gastric prokinetic effect of metoclopramide, and inhibitory effect of atropine (Doherty *et al.,* 1998). Serum acetaminophen and paracetamol can be measured easily by chromatography (Spiller, 1993), and the absorption test protocol is relatively simple. However, this method of measuring gastric emptying is invasive and unsuitable for measuring the gastric emptying of the solid phase. Both drugs may be subject to limited absorption in the stomach, that could result in substantial variation in the results (Heading *et al.,* 1973).

1.5.3.2   *Oral Glucose/Xylose Tolerance Test*

The oral glucose or xylose tolerance test is an established method for assessing gastrointestinal function in the horse (Roberts and Hill, 1973). A glucose solution (1g/kg body weight) is administered by naso-gastric tube following a 12 hour fast, and serum glucose levels are monitored over the following six hours (Roberts and Hill, 1973). Glucose is actively absorbed in the small intestine (Roberts and Hill, 1973) and the rate of increase in blood glucose concentration following ingestion of a xylose or glucose solution is determined, to a limited extent by the rate of gastric emptying. The glucose tolerance test is not widely used to assess gastric emptying in human medicine as it is an insensitive test that is influenced by diet, age, absorptive capacity and intestinal disorders and can only be used as an indicator of liquid emptying (Thompson, Wingate, Thomas and Harrisson, 1982). However, gross delays in gastric emptying will result in delayed delivery of glucose to the small intestine, and a consequent delay in the

appearance of increased blood glucose concentration. Although, the OGTT in the horse is also subject to variation with diet and age (Murphy, 1997), this test has been applied for the assessment of gastric emptying in horses, as no other suitable method was available. The glucose tolerance test was used in the investigation of cases of pyloric occlusion with secondary delayed gastric emptying (McGill and Bolton, 1984; Church, Baker and May, 1986; Laing and Hutchins, 1992; Murphy, Howie and Love, 1994). In two cases the results of the test were indicative of delayed gastric emptying, but in two cases glucose absorption was within reference ranges, despite radiographic evidence of grossly delayed gastric emptying and subsequent diagnosis of pyloric occlusion (McGill and Bolton, 1984; Laing and Hutchins, 1992). It was postulated that the dose of glucose generally administered in the OGTT is sufficient to produce a normal plasma glucose curve despite delayed gastric emptying (McGill and Bolton, 1984). These results suggest that the OGTT is an insensitive and unsuitable method for the detection of delayed gastric emptying in the horse.

1.5.4   Gastric Aspiration

Gastric tracer studies involve the serial aspiration of samples of stomach contents (through a naso-gastric tube or a gastric cannula) following ingestion of a known concentration of a non-absorbable marker substance. The rate of gastric emptying is proportional to the decrease in concentration of the marker.

1.5.4.1   Saline Load Test

The saline load test is a simple method for demonstrating gross delays in the gastric emptying of liquids. Saline (750mls) is administered by naso-gastric tube, and total gastric contents are aspirated after thirty minutes. The aspiration of more than 300-400mls is indicative of delayed gastric emptying (Goldstein and Boyle, 1965).

1.5.4.2   Indicator Dilution Techniques

Dilution techniques involve the oral administration of a known concentration of a non-absorbable indicator substance (such as polyethylene glycol, phenol red or chromium oxalate) and assume that a homogenous suspension is formed with the gastric secretion. Serial samples of gastric fluid are taken through a naso-gastric tube or gastric cannula and the changes in concentration of the marker are used to calculate the rate of gastric emptying. These methods are seldom used in human medicine as they are invasive, unreliable and confined to the assessment of liquid emptying (Parkman *et al.,* 1995). A phenol red indicator dilution technique was used to measure the rate of gastric emptying in the horse, and although the results were promising, the method was laborious (Baker and Gerring, 1994a). This method was successfully used to investigate the gastric emptying of oral rehydration solutions following exercise in horses (Sosa-León, Hodgson and Rose, 1997). The dye dilution methods for measuring gastric emptying are useful research tools for investigation of liquid emptying in the horse, but the complex protocol associated with these methods preclude their use in clinical investigations.

1.5.4.3   Indigestible Solids

Indigestible solids, such as lengths of plastic tubing or beads, can be administered by naso-gastric intubation or in food, and their subsequent passage through the gastrointestinal tract monitored at necropsy. This method can provide only very crude information on gastric emptying. However, for many years, data obtained using this method was the only information available to describe equine gastric emptying. In a classic investigation of equine gastrointestinal physiology, Argenzio *et al.,* (1974a) observed the gastrointestinal transit of indigestible solid and liquid markers, and demonstrated the rapid exit of liquids and retention of particulate solids in the equine

stomach. This method was also more recently used to investigate the effect of neostigmine methylsulphate on gastric emptying in horses Adams and MacHarg, 1985).

1.5.5 Breath Tracer Studies

Analysis of exhaled breath is increasingly utilised in human medicine. Typically, the tests involve detection of a gas and/or isotope either produced in response to the ingestion of a meal, or administration of a labelled substrate. The gastric emptying breath test is based on the principle that the main parameter determining the enrichment of the label in the breath following ingestion of a labelled substrate, is the rate of delivery of the substrate to the duodenum (Ghoos, Maes, Geypens, Mys, Hiele, Rutgeerts and Vantrappen, 1993). Substrates for use in gastric emptying breath tests must be completely and rapidly absorbed in the duodenum to allow rapid entry of the label into the bicarbonate pool and subsequent enrichment of the breath with $^{13/14}CO_2$. The level of enrichment of labelled carbon ($^{13/14}C$) is determined by assessing the ratio of naturally occurring $^{13}C:^{12}C$, in the expired CO_2 by isotope ratio mass spectrometry in the case of ^{13}C, or by β–scintillation counting for ^{14}C. Breath samples are collected into evacuated tubes at frequent intervals for several hours following ingestion of a carbon-labelled test meal (Ghoos *et al.,* 1993), and samples can be stored for up to 60 days before analysis (Schoeller, Schneider, Solomons, Watkins and Klein, 1977).

The first stable isotope breath tests to be described for measuring the rate of gastric emptying were the ^{13}C-acetate breath test (Mossi, Meyer-Wyss, Beglinger, Schwizer, Fried, Ajami, Brignoli, 1991; Braden, Adams, Duan, Orth, Maul, Lembcke, Hör and Caspary, 1995) and the ^{13}C-bicarbonate breath test (Bjorkman, Moore, Klein and Graham, 1991). Despite promising initial results, the ^{13}C-bicarbonate gastric emptying breath test could not be correlated with a simultaneous scintigraphic study, and ^{13}C-

bicarbonate is not a suitable substrate for use in gastric emptying breath tests (Bjorkman *et al.*, 1991). The ^{13}C-acetate breath test remains a useful marker for the liquid phase of gastric emptying, although this is of little significance in clinical diagnosis, as delayed gastric emptying rarely manifests in the liquid phase (Parkman *et al.*, 1995).

The ^{13}C-octanoic acid breath test (^{13}C-OBT) was developed as a marker of the solid phase of gastric emptying, and results of this test were correlated with scintigraphy (Ghoos *et al.*, 1993; Ziegler, Schadewaldt, Pour Mirza, Piolot, Schommartz, Reinhardt, Vasberg, Brösicke and Gries, 1996; Perri, Clemente, Festa, Quitadamo, Niro and Andriulli, 1998). The ^{13}C-OBT is based on the detection of ^{13}C enrichment in breath, following the rapid absorption of the ^{13}C-labelled substrate, octanoic acid from the duodenum. Because gastric emptying is the rate-limiting step in the process of absorption and metabolism of the labelled substrate, the appearance of the label in the exhaled breath is a direct reflection of the rate and pattern of gastric emptying. Octanoic acid has been shown in human and animal studies to be absorbed completely and rapidly from the small intestine, following gastric emptying (Schwabe, Bennet and Bowman, 1964). Octanoic acid is easily hydrolysed in the small intestine by pancreatic lipase and other esterases (Jandacek, Whiteside, Holcombe, Volpenhein and Taulbee, 1987). Following absorption octanoic acid is carried by the portal vein to the liver where it undergoes oxidation to produce acetyl-CoA, most of which enters the Krebs cycle to be oxidised to $^{13}CO_2$ (Figure 1.3). Octanoic acid is preferentially oxidised to CO_2 in the hepatocyte (Maes, 1994), and the $^{13}CO_2$ enters the bicarbonate (HCO_3^-) pool and is excreted in the breath as $^{13}CO_2$. Following entry of the $^{13}CO_2$ into the HCO_3^- pool, there is a slight delay before the produced $^{13}CO_2$ is represented by an increased ^{13}C-enrichment in the exhaled breath (Pallikarakis, Sphiris and Lefebvre, 1991). This delay precludes direct comparison of the gastric emptying coefficients obtained using

the ^{13}C-octanoic acid breath test with quantitative methods such as scintigraphy, unless a correction factor is used. The kinetics of ^{13}CO$_2$ exhalation from the HCO$_3^-$ pool are not dose dependent (Meineke *et al.,* 1993) and in human studies, a time correction factor of 66 minutes is currently used to allow for the inevitable delay between gastric emptying of the substrate and detection of the ^{13}C signal in breath (Maes, 1994).

The ^{13}C-OBT was sensitive enough to detect subtle changes in the rate of gastric emptying induced pharmacologically by propantheline and erythromycin, in humans (Maes, Hiele, Geypens, Rutgeerts, Ghoos and Vantrappen, 1994) and recent reports have described the use of the ^{13}C-octanoic acid breath test to evaluate gastric motility in neonates (Veereman-Wauters, Ghoos, Vander Schoor, Maes, Hebbalkan, Devlieger and Eggermonts, 1996), children (Maes, Ghoos, Geypens, Hiele and Rutgeerts, 1995) and pregnant women (Maes, Hiele, Spitz, Rutgeerts, and Van Trappen, 1993). Clinically the ^{13}C-OBT has been used successfully in human medicine to assess the rate of gastric emptying in diabetic patients (Ziegler *et al.,* 1996) and in patients with non-ulcer dyspepsia (Maes, Ghoos, Hiele and Rutgeerts, 1992). In veterinary medicine the ^{13}C-OBT was recently used to detect reticular groove contraction in cattle, and hence determine the route taken of orally administered therapeutic agents or nutrients (McLeay, Carruthers and Neil, 1997). While a breath test (hydrogen breath test) has been validated for the measurement of oro-caecal transit time in ponies (Murphy, Reid and Love, 1999), no such test has yet been validated for the measurement of gastric emptying in this species. The ^{13}C-OBT is a very attractive method for measuring the rate of gastric emptying in equine medicine because of the non-invasive nature of the test, and because it would be possible to perform the test in field conditions.
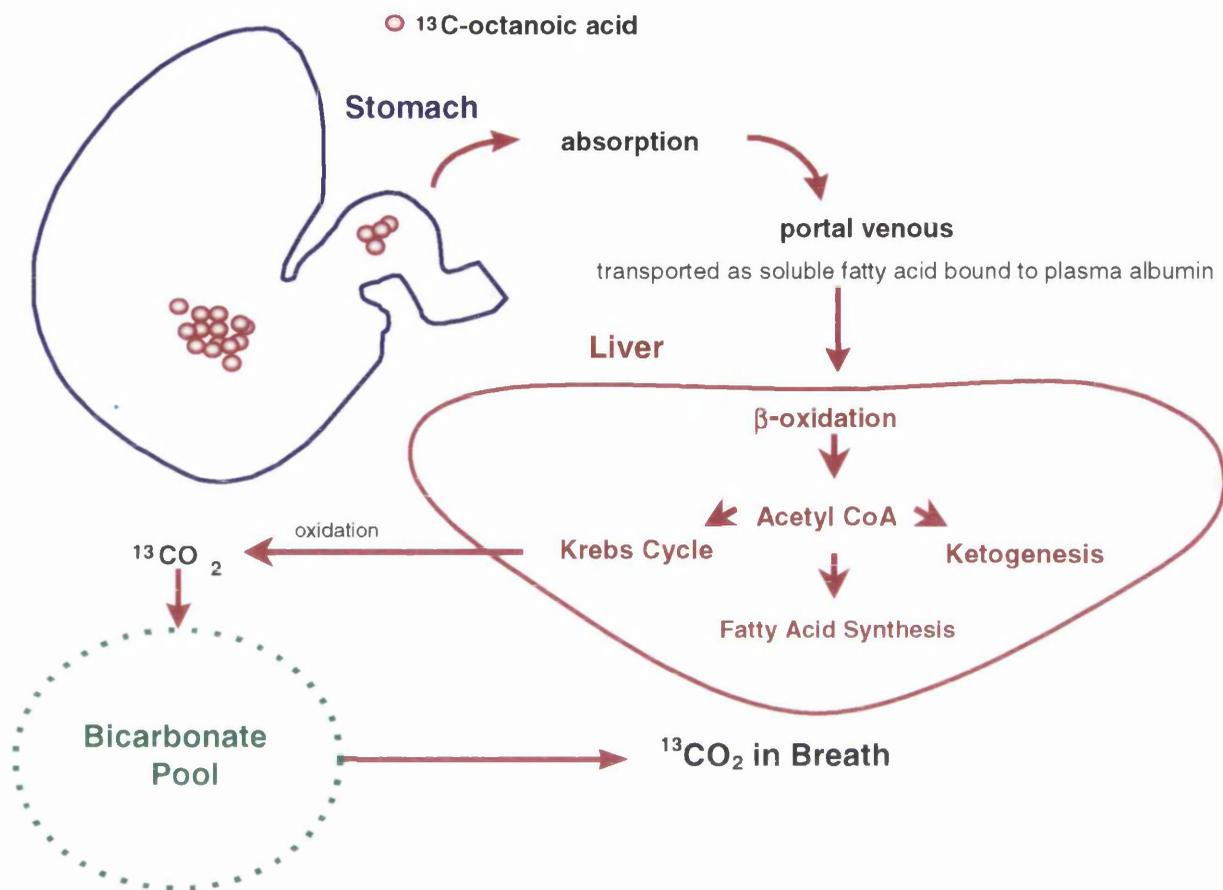
Figure 1.3   A schematic drawing illustrating the rationale of the ^{13}C-OBT. The ^{13}C labelled substrate, ^{13}C-octanoic acid is absorbed in the duodeneum and carried to the liver for β-oxidation, producing acetyl CoA, most of which enters the Krebs cycle for further oxidation, to produce ^{13}CO$_2$ which enters the bicarbonate pool, producing a detectable increase in breath ^{13}CO$_2$. A small proportion of the labelled substrate is lost to other metabolic pathways, such as ketogenesis or the *de novo* synthesis of fatty acids.

1.6 Delayed Gastric Emptying in the Horse

Delayed gastric emptying may result from pyloric or gastric obstruction or from a primary or secondary disruption of gastric motility, but this condition has rarely been quantified in the horse, possibly because a simple method for assessing gastric emptying in horses is not available.

1.6.1 Grass Sickness

Grass sickness is a pan-dysautonomia of the horse that is characterised pathologically by lesions of the autonomic and enteric ganglia, with restricted central nervous system lesions (Obel, 1955; Griffiths, Smith, Kyriakides and Barrie, 1994), and clinically, by a gastrointestinal propulsive deficit (Scholes, Valliant, Peacock, Edwards and Kelly, 1993a). Grass sickness affects grazing horses and ponies, typically in cool dry weather between April and July, and occurring with greatest incidence in Scotland and Northern England (Milne, Woodman and Doxey, 1994). Grass sickness appears to bear some epidemiological, pathological and clinical resemblance to other primary dysautonomiae of animals, such as the *Mal Seco* condition of horses reported in Argentina (Uzal and Robles, 1993), dysautonomia of the hare (Whitwell, 1991; Griffiths and Whitwell, 1993), and the Key-Gaskell Syndrome of the cat (Key and Gaskell, 1982). Like grass sickness, the incidence of Key-Gaskell syndrome in cats, and dysautonomia of the hare is confined almost exclusively to the UK (Pollin and Griffith, 1992). Clinical signs of grass sickness are associated with autonomic dysfunction and the three forms of the disease, acute, sub-acute and chronic, represent degrees of an identical pathological condition (Edwards, 1987). Acute grass sickness causes clinical signs of colic, dysphagia, naso-gastric reflux, impaction of the colon, patchy sweating and tachycardia (Milne, Doxey and Gilmour, 1990). Sub-acute cases show similar, though less severe signs, and the prognosis is hopeless in both acute and sub-acute grass sickness (Milne,

Woodman and Doxey, 1994). Chronic grass sickness is associated with a more insidious development of clinical signs including weight loss, patchy sweating, dysphagia, rhinitis and muscle tremour (Milne, Woodman and Doxey, 1994). The prognosis in cases of chronic grass sickness is poor, although some cases have been reported to recover (Milne, Woodman and Doxey, 1994). Reduced gastrointestinal motility is a clinical feature of all three forms of grass sickness, and investigation of the rate of gastric emptying could be a useful aid in the diagnosis and prognosis of this condition. Delayed gastric emptying is a feature of the autonomic dysfunction associated with human type I diabetes, and the magnitude of delay reflected in the coefficients of gastric emptying correlated significantly with the degree of cardiovascular autonomic dysfunction (Ziegler *et al.,* 1996; Merio, Festa, Bergmann, Eder, Eibl, Stacher-Janotta, Weber, Budka, Heckenberg, Bauer, Francesconi, Schernthaner and Stacher, 1997).

Despite extensive research, the aetiology of grass sickness remains poorly understood. A major advancement in the understanding of this condition was made in 1973, when intra-peritoneal injection of sera from horses with acute grass sickness into experimental ponies, was shown to produce similar autonomic ganglion lesions to the natural disease (Gilmour, 1973). Surprisingly, these experimental lesions were not associated with clinical signs, and although the sera were shown to contain a neurotoxic compound, (Gilmour, 1973), this has not yet been identified (Pemberton, Hodgson, Gilmour and Doxey, 1990). A further study demonstrated that typical ganglional lesions could be induced by injection of sera from cases of acute grass sickness into the parotid gland of healthy horses (Griffiths, Smith, Doxey, Whitwell and Love, 1994). This finding led to the conclusion that the putative neurotoxin is either ingested or produced within the intestine before gaining direct access to the nerve terminals of the gut wall. The toxin is

then thought to be transported retrogradely up the axon terminal, giving rise to the autonomic dysfunction that typifies equine grass sickness (Griffiths *et al.,* 1994). Marked changes in the gut regulatory peptide system have been demonstrated in cases of grass sickness (Hodson, Edwards, Barnett, Bishop, Cole, Probert, Bloom and Polak, 1982), suggesting that the gut peptides may play a contributory role in the pathophysiology of this condition, and may also have potential as diagnostic indicators.

Clinical signs (Milne, Woodman and Doxey, 1994), peritoneal fluid analysis (Milne, Doxey and Gilmour, 1990) and barium contrast radiography (Greet and Whitwell, 1986) are useful diagnostic indicators of grass sickness, but cannot confirm the presence of the condition. A definitive *in vivo* diagnosis of grass sickness can only be reached following histological identification of typical morphological changes in the enteric neurones of a sample of ileal tissue, taken during laparotomy (Scholes, Valliant, Peacock, Edwards and Kelly, 1993b). Because cases of grass sickness may present with clinical signs similar to other conditions, the reliability of a diagnosis based on clinical signs is questionable (Edwards, 1987). However, the justification for the use of an invasive technique (laparotomy) in order to reach a definitive diagnosis of a condition for which there is no effective treatment, is equally questionable. A reliable and non-invasive method for the diagnosis of grass sickness in the living animal would greatly benefit research into this perplexing condition.

1.6.2   Gastroduodenal Ulceration

Equine gastro-duodenal ulceration was first recorded as an incidental finding at *post mortem* in foals that died due to other causes (Rooney, 1964). The advent of veterinary gastroscopy in the 1980s facilitated further investigation of the prevalence of this condition and gastric ulceration is now recognised as an important cause of foal

morbidity and mortality (Becht and Byars, 1986; Murray, Murray, Sweeny, Weld, Wingfield-Digby and Stoneham, 1990). In 1986, routine *post mortem* examination of 195 Thoroughbred racehorses revealed that 66% had evidence of gastric ulceration, despite the absence of clinical signs (Hammond, Mason and Watkins, 1986), illustrating that gastric ulceration is also a common condition in the adult horse.

Gastric ulceration in horses is usually confined to the non-glandular (fundic region), particularly the region immediately adjacent to the *margo plicatus*; ulceration of the glandular region of the stomach does occur, but is less common. Gastric ulceration in the adult horse is associated with poor performance, decreased appetite and poor body condition (Murray, 1992b). However, many horses with endoscopic evidence of gastric ulceration are asymptomatic (Murray, Grodinsky, Anderson, Radue and Schmidt, 1992), and the true clinical significance of gastric ulceration in the adult horse remains to be determined.

Ulceration of the glandular region accounts for 9% of all gastric ulcers in foals, and is usually associated with the stress of concurrent illness or hospitalisation (Furr, Murray and Ferguson, 1992). Gastric ulceration in foals is often accompanied by more acute clinical signs that may include colic, bruxism, salivation and in some cases gastric or duodenal perforation, peritonitis and death (Becht and Byars, 1986). However, an endoscopic survey revealed that up to 50% of normal foals had gastric ulceration that was not associated with any clinical signs (Murray and Murray, 1990). Desquamation of the gastric epithelia in the first few days of life is a normal physiological process in the foal, and this combined with the aggressive properties of HCl and pepsin, may account for the foal's susceptibility to gastric ulceration (Murray and Mahaffey, 1993)

Little is known about the prevalence of duodenal ulceration in the horse, due to the difficulty in passing an endoscope through the pyloric sphincter (Becht and Byars, 1986). Duodenal ulceration does occur in foals, often secondary to gastric ulceration (Becht and Byars, 1986), but is rarely thought to affect the adult horse (McGladdery, 1997).

The pathophysiology of gastroduodenal ulceration is complex, with environmental, physiological and possibly psychological factors interacting to produce gastric mucosal damage (Soll, 1997). Gastric ulceration arises from an imbalance between the protective and aggressive factors of the gastric mucosa. The protective factors include, bicarbonate and mucus secretion, prostaglandin-E_2 (PGE_2) and mucosal blood flow, while the aggressive factors are gastric dysmotility and hypersecretion (Baker, 1992). The pathophysiology of the disruption of this balance in the horse, and consequent ulceration of the gastric mucosa, is poorly understood, but may involve parasitism and gastric foreign bodies (Rooney, 1964), stress (Lloyd, 1993), delayed gastric emptying, rotavirus infection (Becht and Byars, 1986) and treatment with non-steroidal anti-inflammatory drugs (Snow, Bogan, Douglas, Thompson, 1979).

In 1936, Seyle implicated stress in the pathophysiology of gastric ulceration, which was considered to be one of the signs of the "general adaptation syndrome" evoked in response to chronic physiological stress. "Stress" is a term that is open to several interpretations, but in the context of equine gastric ulceration, refers to a *physiological* failure to cope adequately with environmental conditions (Broom and Johnson, 1994). It is important to distinguish this type of stress from the psychological stress that has been implicated in the aetiology of human gastric ulceration (Lloyd, 1993), but was not thought to affect the incidence of gastric ulceration in the horse (McGladdery, 1997).

The stress of severe illness is a recognised cause of gastric ulceration in human patients (Gillespie and Thompson, 1983), and the glandular ulcer described in foals (Becht and Byars, 1986) is likely to have a similar aetiology (Murray et al., 1990; Furr, Murray and Ferguson, 1992).

The stress of race-training has also been implicated in the aetiology of gastric ulceration in the adult horse, based on the high prevalence of gastric ulceration in Thoroughbreds in training (Hammond, Mason and Watkins, 1986) and on the greater incidence of gastric ulceration in horses that have recently raced (Murray, Schusser, Pipers and Gross, 1996). This increased prevalence of gastric ulceration in the racing Thoroughbred is probably associated with the physiological stress induced by inappropriate exercise and feeding regimens. Racehorses are maintained on a bolus-fed, energy dense diet, and they are generally confined in stables during the training period, with only relatively short periods of high intensity daily exercise. Bolus feeding of energy dense feed is known to cause increased release of gastrin and subsequent increased gastric secretion (Smyth, Young and Hammond, 1989). Furthermore, energy dense feeds cause the rate of gastric emptying to be delayed in the horse (Sojka and Cantwell, 1989), thus compounding the destructive effect of increased acidic secretion on the gastric mucosa. The horse continually secretes gastric acid, even when not eating (Murray, 1997a) and intermittent feed deprivation, associated with stable confinement quickly induces gastric ulceration (Murray and Eichorn, 1996). Exercise causes gastrin release, increased gastric secretion and decreased gastric pH in the horse (Furr, 1991; Furr, Taylor and Kronfield, 1994), while acute exercise (greater than 70% of VO_2 max) causes delayed gastric emptying in man (Brown et al., 1994). Thus it may be hypothesised that the combined effect of decreased gastric motility and increased gastric secretion associated with the acute exercise of racing, bolus feeding of energy density

feeds, and periods of food deprivation associated with stable confinement is responsible for the high prevalence of gastric ulceration in the Thoroughbred racehorse.

Gastric ulceration associated with non steroidal anti-inflammatory drug (NSAID) toxicosis, has been recorded in horses, and usually involves ulceration of the glandular mucosa (Traub, Gallina, Grand, Reed, Gainn and Paulson, 1983; Trillo, Soto and Gunson, 1984). In one retrospective study, non-steroidal anti-inflammatory drugs were not associated with the presence or severity of gastroduodenal ulceration (McGladdery, 1997). Furthermore, gastric ulceration was widespread amongst a population of horses with no history of administration of these drugs (Hammond, Mason and Watkins, 1986). Therefore, NSAIDs are unlikely to play an important role in the pathogenesis of gastric ulceration in the horse.

Gastric infection with *Helicobacter pylori* was recently discovered to play a major role in the pathogenesis of gastric ulceration in man (George, 1990), but attempts to isolate this organism from gastric biopsies of horses with gastric ulceration have been unsuccessful (Sprouse, Jones and Barthel, 1991). One study reported an outbreak of gastric ulceration in a group of foals with rotaviral infection (Rebhun, Dill and Power, 1982), but gastric ulceration may have occurred secondary to the stress of disease in these cases, rather than as a consequence of the rotaviral infection. *Candidia spp.* have been isolated as opportunist agents, causing infection secondary to gastric ulceration in foals (Gross and Mayhew, 1983). To date, there is no evidence supporting the implication of an infectious agent in the aetiology of equine gastric ulceration.

The pathophysiology of ulceration of the proximal stomach (Type I, gastric ulceration) in man is associated with delayed gastric emptying of solids but normal gastric

emptying of liquids (Miller, Malagelada, Longstreth and Go, 1980). The delayed emptying of solids in this condition can be reversed by metoclopramide suggesting that antral dysmotility is a factor in the aetiology of Type I lesions (Miller *et al.,* 1980). Indeed, delayed gastric emptying is thought by some workers to be an essential pathogenic factor in ulcer formation (Dragstedt and Woodward, 1970). Delayed gastric emptying has been reported in foals with gastric ulceration (Becht and Byars, 1986), and the role of delayed gastric emptying as a primary factor in the aetiology of gastric ulceration in the adult horse is worthy of further investigation.

Treatment of gastric ulceration is aimed at suppressing gastric acid secretion to allow healing to take place (Murray, 1997a). It is possible to suppress secretion of gastric acid, and increase gastric pH using agents, such as ranitidine, cimetidine and omeprazole. The clinical use of these drugs in the horse is justified by a reduction in gastric lesions following treatment, in horses with clinical signs of gastric ulceration (Furr and Murray, 1989). However, in many cases, the lesions may have reduced despite treatment, especially if the training regimen of the horses with clinical signs was reduced in intensity (Murray, 1992a). In one controlled study, ranitidine was not significantly effective in accelerating healing of gastric ulceration in horses (MacAllister and Sangiah, 1993). Sucralfate is a sulphated polysaccharide that protects gastric lesions from acid-digestion, by adhering to ulcerated mucosa, and stimulating mucus secretion. The efficacy of sucralfate in the prophylaxis of the gastric squamous epithelial ulcer, has been questioned, as defective mucus secretion is not an important factor in the aetiology of this condition (McGladdery, 1997). Prokinetic drugs such as metoclopramide and bethanechol can be used in the treatment of equine gastric ulceration (Murray, 1997a). There are no data available on the success of these drugs in

the treatment of equine gastric ulceration, and further investigation has been precluded by the side effects that these drugs produce in horses.

1.6.3   Post Operative Ileus

Ileus can be defined as an obstruction of the intestine caused by decreased gut motility (Adams, 1988). In the horse, ileus may be associated with colic, drug administration, peritonitis or enteritis, but occurs most commonly following intestinal surgery (Adams, 1988). Post operative ileus (POI) is thought to be the largest cause of death in the 2-3 days following intestinal or colic surgery in the horse (Gerring, 1991). Post operative ileus was responsible for 42.9 % of all post operative fatality in 259 cases of surgical colic, and a mortality rate of 85.7 % was associated with this condition (Hunt, Edwards and Clarke, 1986). The clinical signs of POI are the result of obstruction to the passage of ingesta and compromise of the absorptive functions of the gut (Adams, 1988). This leads to accumulation of fluid and gas into the gut lumen, initiating a vicious circle of intestinal distension, ischaemic damage and bowel necrosis. Fluid loss into the intestine causes haemoconcentration, hyperkalaemia and hypochloraemia, and shock (Hunt, Edwards and Clarke 1986). The pathophysiology of POI is poorly understood and several mechanisms are thought to have contributory roles. Early investigations of POI revealed hypokalaemia and hypochloraemia and led to the implication of electrolyte imbalance in the aetiology of this condition. However, studies have failed to establish a relationship between changes in electrolyte status and gut activity in POI (Gerring and Hunt, 1986; Hunt, Edwards and Clarke, 1986). An experimental model of POI was developed in ponies to produce reproducible and reversible changes in gut activity that were typical of POI (Gerring and Hunt, 1986). The most significant derangement of motility in this model was in gastro-duodenal co-ordination, and this derangement was reversed following administration of the dopamine antagonist metoclopramide (Gerring

and Hunt, 1986). Furthermore, intravenous infusion of dopamine has been shown to induce an initial inhibition of gastrointestinal motility in the horse (King and Gerring, 1988). These findings have led to the conclusion that the pathophysiology of non-endotoxic POI involves a disruption in gastro-duodenal co-ordination that is mediated by dopaminergic hyperactivity (Gerring and Hunt, 1986). The release of inhibitory gut peptides has been implicated in the pathophysiology of POI (Adams, 1988). Increased release of the inhibitory peptides, vasoactive inhibitory peptide, somatostatin or decreased release of excitatory peptides such as cholecystokinin, motilin or substance-P, may be responsible for the induction of ileus in some cases (Adams, 1988). Endotoxaemia is a common sequel to intestinal surgery in the horse, and has also been associated with the development of POI (Hunt, Edwards and Clarke, 1986). Intravenous infusion of an *E. coli* endotoxin at a low physiological dose produced a decrease in intestinal motility (Clark and Moore, 1992). Non-steroidal anti-inflammatory drugs such as phenylbutazone and flunixin were effective in ameliorating the gastrointestinal effects of endotoxic ileus (King and Gerring, 1989) suggesting that endotoxic ileus is mediated at least in part by the products of the cyclooxygenase pathway. Various gastrointestinal prokinetic drugs have been used to treat or prevent post-operative ileus. Neostigmine, an anticholinesterase drug, has been used to treat POI as it increases propulsive motility of the colon (Adams, 1988). However, neostigmine caused delayed gastric emptying of indigestible solid markers in ponies, an effect which could potentiate the effects of POI (Adams and MacHarg, 1985). The acetylcholine agonist, cisapride was shown in a clinical trial to be effective in preventing idiopathic POI but not endotoxic ileus (Gerring, King, Edwards, Walmsley, and Greet, 1991). However, in a more recent study using the acetaminophen absorption test, cisapride attenuated the delay in gastric emptying induced experimentally by intravenous endotoxin (Valk *et al.,* 1998).

1.6.4 Gastric Neoplasia

Gastric neoplasia can cause disruption of gastric motility by obstructing the outflow of ingesta from the stomach, and delayed gastric emptying has been reported in cases of gastric neoplasia in the horse (White, 1992). Gastric squamous cell carcinoma is the most common primary neoplasia of the equine stomach (Cotchin, 1977). Other documented incidents of gastric neoplasia in the horse are limited to a single case of gastric leiomyoma, three cases of gastric adenocarcinoma (Cotchin, 1977), and one case of lymphosarcomatous infiltration of the gastric mucosa (Van der Hoven and Franken, 1983). Gastric squamous cell carcinoma most often affects middle aged horses, and there is not thought to be any breed predilection (Tennant, Keirn, White, Bentinck-Smith and King 1982; Olsen, 1992). One study reported an increased incidence of squamous cell carcinoma among male horses (Moore and Kintner, 1976) but no sex predisposition was found in two subsequent studies (Tennant *et al.* 1982; Olsen, 1992). There are insufficient reports in the literature to permit detailed analysis of the epidemiology of this condition. The aetiology of gastric squamous cell carcinoma is unknown. Dietary or parasitic conditions causing chronic irritation of the gastric mucosa may have some association with the incidence of the disease (Olsen, 1992). In cattle, a direct relationship has been shown in Western Scotland between the prevalence of viral papilloma of the upper gastrointestinal tract, bracken fern infestation of pasture and the incidence of squamous cell carcinoma of the upper gastrointestinal tract (Jarrett, 1980). Geographical location is known to be important in the distribution of cancer in man (Doll, 1969). It is likely that the pathogenesis of equine gastric squamous cell carcinoma is multifactorial, and that environmental and geographical factors are important considerations in the aetiology of this disease. Squamous cell carcinoma occurs in the non-glandular region of the stomach and the tumour projects cauliflower-like into the gastric lumen (Proudman and Baker, 1994), with occasional metastases into

the thoracic and abdominal cavities (Meagher, Wheat, Tennnant and Osburn, 1974).

Clinical signs are varied but the condition is usually associated with progressive weight

loss, inappetance and slight anaemia (Meagher *et al.* 1974). A low grade recurrent colic

and poor performance are often the initial presenting signs (Proudman and Baker,

1994), and oesophageal obstruction, dysphagia, ventral oedema and periods of pyrexia

are other possible manifestations of this condition (Tennant *et al.,* 1982). In some cases

abdominal masses or adhesions can be palpated *per rectum* (Tennant *et al.,* 1982) and

laboratory findings often reveal hypoproteinaemia and anaemia (Olsen, 1992). A

definitive diagnosis of squamous cell carcinoma can be made by histological

examination of biopsy specimens taken during endoscopy, necropsy or exploratory

laporotomy, or by the identification of exfoliated neoplastic cells in peritoneal or gastric

fluid (McKenzie, Mills and Bolton, 1997). The prognosis is hopeless and euthanasia is

indicated as soon as a diagnosis of gastric squamous cell carcinoma is confirmed.

1.6.5   Primary Gastric Dilation

Gastric dilation occurs when fluid or gas accumulates in the stomach to produce acute

gastric distension (Owen, 1983). Secondary gastric dilation may occur as a sequel to

gastrointestinal obstruction, post-operative ileus, adynamic ileus, grass sickness or

anterior enteritis (Edwards, 1993). Primary gastric dilation refers to the distension

produced by the intra-gastric fermentation of ingesta or accumulation of fluid or gas due

to reduced gastric motility (Müller, Donandt, Pingen and Zeitelhack, 1995). The

principle clinical signs of gastric dilation are acute colic, tachycardia, depression, and

dyspnoea due to the pressure exerted by the stomach on the diaphragm (Edwards,

1993). There have been few documented cases of primary gastric dilation in the

literature. Müller *et al.* (1995) described one case of chronic gastric dilation, but no

aetiological factor was identified. A case of acute gastric dilation was reported in a

standardbred mare, and this condition was thought to be related to the ingestion of hay while the stomach was hypomotile following a race (Owen, 1975).

The aetiology of primary gastric dilation is not well documented but it is likely that reduced gastric motility is an important factor. Primary gastric dilation can result from the ingestion of feedstuffs that can be fermented by gastric microflora such as sugar beet, fresh grass or grain. This intra-gastric fermentation produces gas that causes gastric distension, and also generates considerable amounts of volatile fatty acids (Argenzio *et al.,* 1974b). The effect of these volatile fatty acids is to delay gastric emptying, thus promoting further fermentation and exacerbating gastric dilation (Edwards, 1983). Primary gastric dilation may also be induced by ingestion of cold water after exercise, causing pyloric spasm and consequent transient gastric dilation (Edwards, 1983). The ingestion of air during crib-biting and wind-sucking has been proposed as a cause of gastric dilation (Campbell-Thompson and Merritt, 1999), but this is unlikely, as the sequence of events during cribbing and wind-sucking is not related to deglutition and air is not swallowed to the stomach (McGreevy, Richardson, Nicol, and Lane, 1995). Other factors that may be involved in the aetiology of primary gastric dilation in the horse are reduced gastrointestinal motility, loss of gastric innervation, and weakened intramural gastric connective tissue (Müller *et al.* 1995). Gastric decompression usually effects a cure in cases of primary gastric dilation, although excessive gastric distension can cause neuromuscular damage to the stomach wall leading to gastric atony, and possibly chronic gastric impaction (Edwards, 1993).

1.6.6 Primary Gastric Impaction

Gastric impaction is generally thought to refer to the abnormal accumulation of dry, solid, poorly-fermentable ingesta in the stomach (Foerner, Barclay and Philips, 1983).

In most cases primary gastric impaction is caused by ingestion of indigestible fibrous material such as straw, persimmon seeds or coarse grass (Owen 1983; Honnas and Schumacher, 1985; Owen, Jagger and Jagger, 1987). Other factors that have been implicated in the aetiology of this condition are gastric atony, defective gastric secretion and dental disease (Honnas and Schumacher, 1985). The clinical signs of gastric impaction are chronic (as opposed to the acute signs seen in gastric dilation), and include anorexia, dysphagia, mild colic and weight loss (Owen, Jagger and Jagger, 1987). Four cases of primary gastric impaction were successfully treated by injecting saline into the stomach during exploratory laporotomy (Barclay, Foerner, Phillips and MacHarg, 1982). However, gastric impaction may have been induced in these cases, by repeated administration of the adrenergic agonist drug, xyalzine to treat colic (Owen, 1983), especially as no predisposing factor could be identified (Barclay *et al.* 1982). Two cases of gastric impaction caused by retention of fibrous material were successfully treated by gastrotomy (Owen, Jagger and Jagger, 1987).

1.6.7   Pyloric Occlusion

Pyloric occlusion refers to a functional resistance to gastric emptying at the pylorus, that is congenital, or is acquired secondary to ulceration, carcinoma or hypertrophic tissue. Congenital pyloric stenosis with associated gastric retention has been described in foals (Crowhurst, Simpson, McEnery and Greenwood, 1975; Barth, Barber and McKenzie, 1980) and congenital muscular hypertrophy of the pylorus with gastric impaction was described in a yearling (Munroe, 1984). Acquired pyloric occlusion has been reported secondary to the growth of fibrous tissue (McGill and Bolton, 1984; Laing and Hutchins, 1992) and exuberant granulation tissue (Mackay, Iverson and Merritt, 1981; Church, Baker and May, 1986). Clinical signs associated with pyloric obstruction may include progressive weight loss, inappatence, ptyalism and bruxism (Church, Baker and

May, 1986) poor performance, post-prandial pain and the spontaneous reflux of gastric fluid following passage of a naso-gastric tube (Laing and Hutchins, 1992). Barium contrast radiography of the gastrointestinal tract has been used to demonstrate delayed gastric emptying in cases of acquired pyloric occlusion (McGill and Bolton, 1984; Church, Baker and May, 1986), but in other cases no abnormalities of gastric emptying or structure could be identified radiographically (Laing and Hutchin, 1992; Murphy, Howie and Love, 1994). The oral glucose tolerance test was used to assess gastric function in cases of pyloric occlusion, and was indicative of delayed gastric emptying (Church, Baker and May, 1986; Murphy, Howie and Love, 1994). However, in other cases the results of the oral glucose tolerance test were within reference ranges despite radiographic evidence of delayed gastric emptying and subsequent diagnosis of pyloric occlusion (McGill and Bolton, 1984; Laing and Hutchins, 1992). The breath hydrogen test was used to investigate gastric function in a case of pyloric occlusion, and an early rapid increase in breath hydrogen following administration of a xylose or lactulose substrate, was attributed to gastric fermentation associated with the retention of the substrate in the stomach (Murphy, Howie and Love; 1994).

1.6.8   Pyrrolizidine Alkaloid Toxicosis

Ingestion of *Senecio jacobea* (Ragwort) in pasture or hay, is a common cause of pyrrolizidine alkaloid toxicosis in Britain (Milne, Pogson and Doxey, 1990). The principle clinical signs associated with this condition are the result of hepatic failure, but three cases of gastric impaction secondary to ragwort poisoning have been reported (Milne, Pogson and Doxey, 1990). Pyrrolizidine alkaloid toxicosis was also listed as a cause of secondary gastric impaction by Owen, Jagger and Jagger, (1987). Liver failure is associated with an impairment of neural function in the horse, which could affect gastric motility, causing secondary gastric impaction (Milne, Pogson and Doxey, 1990).

1.6.9   Gastric Parasites

Gastric infection with the bot fly larva, *Gasterophilus spp.* has been implicated in the aetiology of gastric ulceration in horses (Rooney, 1964), but ulceration was widespread in a population of horses completely free of *Gasterophilus* (Hammond, Mason and Watkins, 1986). Furthermore, no association could be found between the incidence of gastric ulceration and *Gasterophilus spp.* infection in a survey of carcasses at an abattoir (Sweeney, 1990) and this organism is unlikely to play a major role in the aetiology of gastric ulceration. There have been isolated reports of gastric perforation associated with *Gasterophilus spp.* infestation (Dart, Hutchins and Begg, 1987), and in one instance, disrupted oesophageal motility, identified during endoscopy, was attributed to *Gasterophilus spp.* infection in the proximal stomach (Edens and Murray 1992). However, the pathogenicity of *Gasterophilus spp.* infection remains to be determined (Proudman and Baker, 1992).

1.7   Project Aims

The ^{13}C-octanoic acid breath test has been developed as a non-radioactive, non-invasive and accurate method for the assessment of gastric emptying of solids in human medicine (Ghoos *et al.,* 1993). The overall aim of this study was to investigate the feasibility of applying this test for the assessment of gastric emptying in ponies. The specific aims were as follows:

1.   to establish natural fluctuations in $^{13}CO_2$ excretion in ponies, at rest, following a 14-16 hour fast, and following a small test meal

2.   to develop a suitable test meal, experimental protocol, and substrate dosage (^{13}C-octanoic acid) to facilitate the use of the ^{13}C-octanoic acid breath test for assessment of gastric emptying in ponies

3.   to apply a mathematical model to describe ^{13}C excretion, and use this model to calculate the coefficients of gastric emptying

4.   to evaluate the effect of energy density of test meal on the coefficients of gastric emptying as determined using the ^{13}C-octanoic acid breath test

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1   Animals

Four adult British native-breed ponies were used in these studies. They were aged between 6 and 15 years, and the group consisted of two mares and two stallions. One of this group (Pony No 101) was reared indoors and had been stabled since birth, one had been kept stabled at night (Pony No. 104), and the remaining two ponies (Pony Nos. 102 and 103) had been maintained at grass. All individuals in this study had been stabled for at least one month prior to inclusion in any investigations. The ponies were maintained on a hay diet, and bedded on straw. They were regularly turned out to exercise in a riding arena. Anthelminthics were administered regularly to all ponies during the study. Food was provided twice daily at 08:00 and 16:00 hours and access to water was *ad libitum.*

2.2   Breath Collection

Breath collection was carried out using a previously validated method for breath sampling in the pony (Murphy, 1997). Breath samples were collected using a plastic tube (nasal tube) (40cm long, internal diameter 12mm) with a valve attached at one end, two centimetres from which was situated a side exit port (8FG Dog Catheter, Rocket of London) (Figure 2.1). The nasal tube was inserted into the ventral nasal meatus of the pony, and this procedure was well tolerated by all the animals in this study (Figure 2.2). The animal was allowed to breathe through the tube on a number of occasions and the valve at the distal end of the tube served as an indicator of the period of expiration. End expiratory breath samples were taken in 20ml syringes (Plastipak, Becton Dickinson) by aspiration through the side port (Figure 2.3). The syringes were sealed with a three-way

tap (Rocket of London), and samples were immediately transferred into collection tubes (Exetainer, Labco Systems), by attaching the syringe to a 19 gauge 2 inch needle (Millpledge), piercing the lid of the tube, and then opening the three-way tap to allow the sample to enter the tube under pressure of the vacuum. In the study detailed in Chapter 4, evacuated tubes were not used; the breath sample was simply deposited into the tube, via a 19 gauge, 2 inch needle (the validation of this method is detailed in Appendix 4).

Figure 2.1: The nasal tube used for breath collection in this study. Samples were taken via the side port, and the period of expiration was easily identified by the rubber valve at the distal end of the tube.

Figure 2.2: The tube was inserted into the ventral nasal meatus, and the pony was allowed to breathe normally through the tube for a few moments.

Figure 2.3: Expiratory breath samples were taken in 20ml syringes by aspiration through the side port.

2.3   Test Meal

The test meal consisted of 75g oats and 50g bran; water and soya oil were added, depending on the particular study. Bran, oats and soya oil were chosen as components of the test meal as they were not likely to be natural sources of ^{13}C-enrichment that could interfere with the signal provided by metabolism of the labelled substrate. The isotopic composition of carbohydrate depends on the photosynthetic process by which carbon was fixated (Stellaard and Geypens, 1998). Plants that fix carbon into a three carbon intermediate (C_3) via the Calvin-Benson reaction, produce carbohydrates with higher percent ^{13}C, than plants that fix carbon into a four carbon (C_4) intermediate, via the Hatch-Slack pathway (Schoeller, Klein, Watkins, Heim and MacLean, 1980). Thus, food produced from C_3 plants such as sugar beet, cane sugar or wheat flour, could provide a significant source of natural ^{13}C-enrichment and were not suitable test meal components in this instance.

Octanoic acid is an 8-carbon, medium chain fatty acid found in esterified form in dietary fats such as coconut oil and butter. ^{13}C-octanoic acid is an ideal tracer for gastric emptying, as it is rapidly metabolised to $^{13}CO_2$ and remains firmly retained in the solid phase of gastric emptying (Maes, 1994). The octanoic acid substrate was added to an egg yolk, which was then baked in a microwave oven, before being mixed into the test meal. Egg yolk is an ideal solvent for the highly lipophilic octanoic acid, and baking increases the binding of the substrate to the solid phase, without affecting the molecular structure of the octanoic acid (Maes, 1994). All test meals were consumed voluntarily by the ponies.

2.4 Experimental Protocol

Following an overnight fast (12-14 hours), the pattern of $^{13}CO_2$ excretion in the breath was determined after the ingestion of a test meal, the specific details of which are outlined in the relevant sections. Baseline breath samples were collected in duplicate, one hour and again immediately before ingestion of the test meal. All meals were consumed in less than five minutes and breath samples were taken at regular intervals over the following 12 hours (every 15 minutes for four hours, every 30 minutes for four hours and every hour for four hours).

2.5 Measurement of Labelled CO_2

All samples were analysed within 3 weeks of collection by continuous flow isotope ratio mass spectrometry (IRMS) (ABCA, Europa Scientific, Crewe England) (Figure 2.4). The ratio of $^{13}CO_2$:$^{12}CO_2$ in the sample was calculated as the relative difference between the isotope ratios of the sample and a CO_2/N_2 standard gas. The delta notation (δ) was derived to express this relative difference (McKinney, McCrea, Epstein, Allen and Urey, 1950). By international convention, $\delta^{13}C$ is expressed relative to the PDB standard, a limestone fossil of *Belemnitella americana* from the Cretaceous Pee Dee formation in South Carolina. The PDB standard has a $\delta^{13}C$ value of 0% and an absolute ^{13}C:^{12}C of 0.0112372 and results are expressed as $\delta^{13}C_{PDB}$. The sign of the $\delta^{13}C$ value for any sample of gas indicates whether it has a higher or lower ratio of ^{13}C:^{12}C than the PBD standard; a negative sign indicates a lower ratio of ^{13}C:^{12}C.

Figure 2.4: A continuous flow gas-chromatography isotope ratio mass spectrometer with attached Gilson autosampler, and computer.

The breath sample, along with the CO_2/N_2 standard gas (itself calibrated against the Pee Dee Belemnite (PDB) international standard), was introduced into the IRMS. Carbon dioxide was separated by gas chromatography, and then ionised to positive ions by exposure to an electron beam generated by a heated tungsten filament. This ion beam travelled down an evacuated tube to a curved tube situated in a magnetic field. The radius of the path of deflection followed by each ion in this magnetic field was determined by the individual mass/charge ratio (m/e), as the strength of the magnetic field (H) and the accelerating potential (V) remained constant. The deflected ions were collected by a detector system consisting of three faraday collectors for mass 44, 45 and 46 (Figure 2.5). In this way the major species of CO_2 are separated and each arrives at separate faraday collectors. It is necessary to measure mass 46 to correct for the [17]O portion in mass 45 (Perri, Clemente, Festa, Quitadamo, Niro and Andriulli, 1998a).

Ions of any particular m/e ratio can be drawn across the collecting slit by varying the accelerating potential or the strength of the magnetic field. The mass of the ions was derived from the formula:

$$m/e = \frac{R^2H^2}{2V}$$

m/e   mass/charge ratio
R   radius of curvature of ion trajectory
H   accelerating potential
V   accelerating voltage
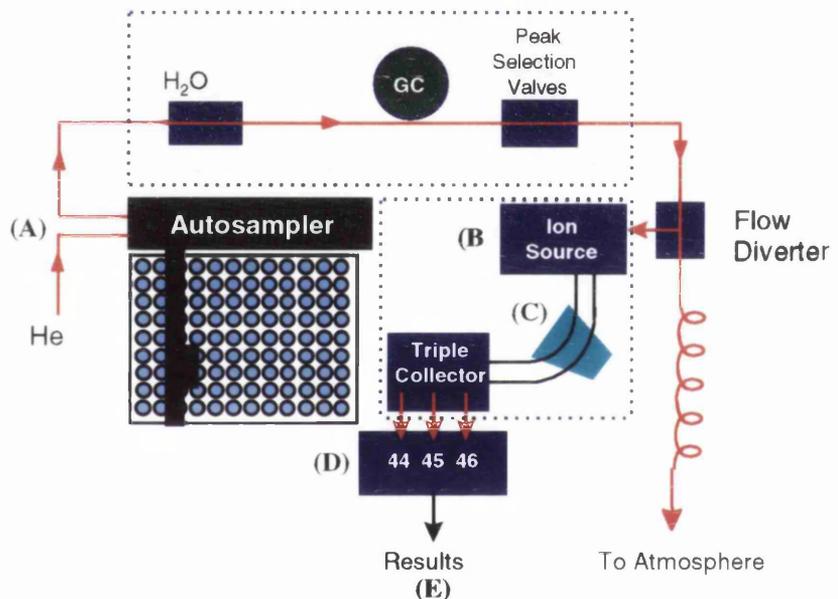
Figure 2.5: A schematic diagram of the continuous flow gas chromatography (GC) isotope ratio mass spectrometer.

A.   Sample or standard is introduced into the IRMS
B.   Ion source, where gases are ionised
C.   Curved flight tube takes gases through magnetic field. The radius of the path of deflection followed by each ion in this magnetic field is determined by the individual mass/charge ratio.
D.   Faraday cup collectors for collecting and amplifying ion beams
E.   Computer system processes data and controls instruments

2.6 Calculation of Results

The $\delta^{13}C$-values given by the IRMS were converted to percentage ^{13}C recovery per hour, of the initial dose administered (Maes, 1994). This was expressed according to the formula:

% dose/h = $\qquad$ **$\dfrac{\text{mmol excess }^{13}Ct\text{ in breath}}{\text{mmol excess }^{13}C\text{ administered}} \times 100$** $\qquad\qquad$ **(1)**
$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad$ **(2)**

(1) *mmol excess ^{13}Ct in breath* $\quad = \quad \dfrac{\%^{13}Ct - \%^{13}Cto \times CO_2 \text{ production}}{100}$

$\%^{13}Ct$	=	relative concentration, expressed as percentage of ^{13}C at time t after ingestion of substrate
$\%^{13}Cto$	=	relative concentration, expressed as percentage of ^{13}C at time zero
$\%^{13}Ct - \%^{13}Cto$	=	atomic percent excess (APE)
	=	$\dfrac{\delta t /(1000 + 1) \times 0.0112372}{[(\delta t/(1000 + 1) \times 0.0112372] + 1}$

δt	=	δ–value at time t
0.0112372	=	$^{13}C{:}^{12}C$ standard

CO_2 Production $\qquad$ 1744.8mmols/hr[*] x Body Surface Area (BSA)/h[†]
$\qquad\qquad\qquad\qquad\qquad$ BSA = 10.5 x Body Weight (g)/10,000[‡]

(2) *concentration of ^{13}C provided by the labelled substrate minus the baseline production of ^{13}C*

$\qquad\qquad = \qquad \dfrac{(\%^{13}Cs - \%^{13}Cto)}{100} \times \dfrac{m}{M} \times n$

%Cs	=	relative concentration of ^{13}C in administered substrate (i.e. 99% for 1-^{13}C-octanoic acid)
%Cto	=	relative concentration of ^{13}C before ingestion of substrate (t = 0)
m	=	amount of substrate administered (mg)
n	=	number of ^{13}C-labelled atoms in the substrate (n=1, as only one carboxyl group is labelled in 1-^{13}C-octanoic acid)
M	=	molar mass of the substrate

The results were expressed as either the percentage of the administered dose recovered per hour (% dose/hr) or parts per million (ppm) ^{13}C enrichment, and were plotted against time (mins).

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[*] Mauderly, 1974
[†] CO_2 production is presumed to be constant
[‡] Orr, Bisgard, Forster, Rawlings, Buss and Will, 1975

2.7   Mathematical Modelling

The formula described by Maes, (1994) was used to describe the $^{13}CO_2$ excretion curve in this study.

$$y = at^b e^{-ct}$$

where y   =   % $*CO_2$ excreted in breath
     t   =   time (hours)
     a, b and c   =   regression constants

Non-linear regression analysis was performed by the least squares method, using the solver function on an Excel 7.0 computer program. The predicted curves derived using this model were plotted on X-Y graphs (Figs. 3.4 and 3.5). The mathematical model allowed the calculation of three parameters that describe the rate and pattern of $^{13}CO_2$ excretion and gastric emptying.

The gastric emptying coefficient (GEC) is a purely mathematical parameter, which is directly related to the ascending and descending parts of the gastric emptying curve, and is therefore defined as a global index of the rate of gastric emptying (Maes, 1994). It is defined mathematically as the natural log of a (ln a).

The gastric half emptying time (t½) is the area under the fitted culminative $*CO_2$ excretion curve when half of the administered dose of ^{13}C has been excreted. The gastric half emptying time is defined numerically as :

$$t(\tfrac{1}{2}) = \int_0^{0.5} f(t)\, dt$$

t½ was calculated using the Excel function Gammainv (0.5; b + 1; 1/c).

The time taken to reach maximal gastric emptying was defined as t(max):

$$t(\text{max}) = \int_0^{\text{max}} f(t)\, dt$$

t(max) was represented by the time at which the function is at its maximum, b/c [tmax (b)]

2.8   Data Organisation and Statistical Analysis

Data organisation and presentation were facilitated using several computer software packages, including Microsoft Word for Windows 95 version 7.0, Microsoft Excel for Windows 95 version 7.0 and Microsoft Powerpoint for Windows 95 version 7.0 (Microsoft Corporation). Statistical analysis and data modelling were performed using Microsoft Excel for Windows 95 version 7.0.

CHAPTER THREE

THE ^{13}C OCTANOIC ACID BREATH TEST IN PONIES - A PRELIMINARY VALIDATION

3.1 Background

Breath test technology is a volatile and rapidly developing facet of clinical diagnosis in human medicine, and carbon isotope breath tests are currently available for assessing gastric emptying (Ghoos et al., 1993), pancreatic insufficiency (Vantrappen, Rutgeerts, Ghoos and Hiele, 1989), liver function (Irving, Schoeller, Nakamura, Baker and Klein, 1982) and *Helicobacter pylori* infection (Graham, Klein, Evans, Evans, Alpert, Opekun and Boutton, 1987). The non-invasive nature of the breath test, and the feasibility of carrying out the test away from the analytical centre, makes breath testing an attractive method for research and clinical diagnosis in the horse.

The ^{13}C-octanoic acid breath test (^{13}C-OBT) was recently developed as a non-invasive, non-radioactive technique for the measurement of the rate of gastric emptying of solids in human medicine, and good correlation with the reference method of scintigraphy has been demonstrated (Ghoos et al., 1993). An earlier study described a ^{13}C-bicarbonate breath test as a measure of gastric emptying of liquids, but due to poor correlation with a simultaneous scintigraphy study, this test was dismissed as an unreliable indicator of gastric emptying (Bjorkman et al., 1991). A ^{13}C-acetate breath test has been validated for the measurement of the rate of gastric emptying of liquids (Mossi et al., 1994), and a combination breath test of ^{13}C-glycine and ^{14}C-octanoic acid substrates (dual isotope method), has facilitated the simultaneous measurement of the rate of gastric emptying of solids and liquids (Maes, Ghoos, Geypens, Mys, Hiele, Rutgeerts and Vantrappen, 1994).

The sensitivity of the ^{13}C-OBT was demonstrated by performing the test before and after administration of drugs known to affect the rate of gastric emptying. Significantly increased rates of gastric emptying were detected using the ^{13}C-OBT, following administration of erythromycin (Maes *et al.,* 1994) and cisapride (Duan, Braden, Caspary, Lembke, 1995). Decreased rates of gastric emptying induced by the administration of the anti-cholinergic drug, propantheline (Maes *et al.,* 1994) and in another study, octreotide (Maes, 1994) were also detected using the ^{13}C-OBT. The results of these studies confirm the sensitivity of the ^{13}C-OBT, and the potential for application of this test in pharmacological studies.

Because the ^{13}C-OBT is completely non-invasive, and presents no risk of radiation exposure, it simplifies investigation of the incidence of delayed gastric emptying both in specific disease, and in different physiological conditions. For example, gastric emptying in groups of patients with diabetes and non-ulcer dyspepsia has already been investigated using the ^{13}C-OBT (Maes *et al.,* 1997; Ziegler *et al.,* 1996). The ^{13}C-OBT is particularly useful for assessment of gastric emptying in pregnant women and children, as the radiation risk presented by scintigraphy prohibits its application in these groups (Maes *et al.,* 1995; Veereman-Wauters *et al.,* 1996). Unlike other methods of assessing gastric emptying, the breath test can be easily carried out in the field, and this distinction allowed the investigation of the rate of gastric emptying in soldiers exercising in intense heat (Mudambo, Leese and Rennie, 1997), and also presents much potential for epidemiological studies.

3.1.1 Applications of the ^{13}C-OBT in Veterinary Medicine

The ^{13}C-OBT has many potential applications in diagnostic and experimental veterinary medicine. The simplicity of this method would be of particular advantage in equine medicine, allowing the test to be carried out away from the analytical centre, and with no requirement for specialised equipment or complex protocol. The ^{13}CO$_2$ remains stable in the sample for up to 60 days, enabling samples to be delivered to the nearest laboratory by post (Schoeller *et al.*, 1977). The ^{13}C-OBT is completely non-invasive, and could be used to assess gastric emptying under experimental conditions while causing minimal disturbance to the animal. The ^{13}C-OBT could potentially be made available to the general practitioner for routine diagnosis of disrupted gastric emptying, and to the epidemiologist for the study of the role of delayed gastric emptying in equine gastrointestinal disease.

3.2 Specific Objective

The purpose of this study was to assess the patterns of ^{13}C excretion in the breath of adult ponies following voluntary consumption of a test meal and, in doing so, evaluate the potential of the ^{13}C-OBT as a diagnostic/research tool for the investigation of pathological and/or physiological alterations in equine gastrointestinal function.

3.3 Materials and Methods

3.3.1 Animals

Four British native-breed ponies were used in this study (Pony Nos. 101-104). Full details of the maintenance of the ponies is given in Chapter 2, and a detailed description of each animal is presented in Appendix 2.

3.3.2 Experimental Procedure

Following an overnight fast (12-14 hours) the ratio of $^{13}CO_2{:}^{12}CO_2$ excretion in breath was determined over a twelve hour sampling period, as described in Chapter Two. Three ponies ingested either no test meal (Test I), a test meal with 250mg ^{12}C-octanoic acid (Test II), or a test meal with 125mg (Test III), 250mg (Test IV) or 500mg (Test V) ^{13}C-octanoic acid (Table 3.1). Test III (three ponies) was repeated in each individual on three occasions, and Test IV (four ponies) was repeated in each individual on six occasions. Each test was started between 5-6am and continued for thirteen hours. Breath samples were collected in duplicate at one hour (t = -60mins) and immediately before (t = 0mins) ingestion of the test meal, and then every fifteen minutes for four hours, every thirty minutes for another four hours and every sixty minutes for a further four hours. All samples were analysed within four weeks of collection, and at least one week was allowed between tests on each individual pony. Animals had access to water at all times during the test, but access to food was denied until the end of the sampling period.

Table 3.1 :   Study Design

Test No.	Meal	Substrate	Animals
I	None	None	101, 102 & 103
II	75g oats 50g bran 1 egg	250mg ^{12}C-octanoic acid[1]	101, 102 & 103
III	75g oats 50g bran 1 egg	125mg ^{13}C-octanoic acid[2]	101, 102 & 103 (Repeated 3 occasions)
IV	75g oats 50g bran 1 egg	250mg ^{13}C-octanoic acid	101, 102, 103 & 104 (Repeated 6 occasions)
V	75g oats 50g bran 1 egg	500mg ^{13}C-octanoic acid	101, 102 & 103

3.3.3   Measuring Techniques

The ratio of ^{13}C:^{12}C in the breath samples was measured by isotope ratio mass spectrometry, as described in Chapter Two.

3.3.4   Data Analysis

Analysis of test results utilised the mean of duplicate measurements taken at each time point. Samples with CO_2 concentrations that were less than 10% of the CO_2/N_2 standard gas (0.3%) were rejected, as they were not considered to be adequate samples of expiratory breath, and in these cases a single replicate measurement was used. Results were expressed as % dose administered recovered per hour (PDR), and breath $^{13}CO_2$ excretion curves were analysed individually. The $^{13}CO_2$ excretion curve (% dose

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[1]Caprylic acid (n-octanoic acid) Sigma Chemicals®
[2]Octanoic acid-1-^{13}C (minimum 99% atom %^{13}C), CK Gas Products Ltd.

recovered per hour against time, PDR), was plotted using the formula described by Maes (1994), and full details of the method of calculation are given in Chapter Two. For each test, three variables were calculated, the gastric emptying coefficient (GEC), the time of maximal gastric emptying (t(max)), and the gastric half emptying time (t½). A repeated measures analysis of variance (ANOVA) was used to perform statistical analyses for significance and to estimate the reproducibility of the test. A further measure of reproducibility was provided by the calculation of intra- subject coefficients of variation (standard deviation/mean X 100%). The models derived using the 250mg (Test IV) and 500mg doses (Test V) (n = 15) were used to examine the effect of reducing the duration and frequency of sampling on the gastric emptying indices, by repeating mathematical curve fitting using longer sampling intervals and shorter sampling durations. The re-calculated gastric emptying indices were compared with the corresponding values using a Students t-test for paired samples.

3.4   Results

Data that contributed to the results presented below are detailed in Appendices 4-6, and an explanation of the methods used to analyse the data is given in Chapter 2. The pattern of $^{13}CO_2$ excretion remained constant over the twelve hour sampling period following both the fasted test (Test I) and the ingestion of the unlabelled test meal (Test II). Mean ^{13}C excretion over the twelve hour sampling period was 20.8 ± 5.03 ppm (mean ± sd) in the fasted study (Test I) (Figure 3.1), and -11.44 ± 5.45 (mean ± sd) following ingestion of the test meal and unlabelled octanoic acid (Test II) (Figure 3.2). There was a negative shift in baseline associated with the ingestion of the unlabelled test meal (Test II).
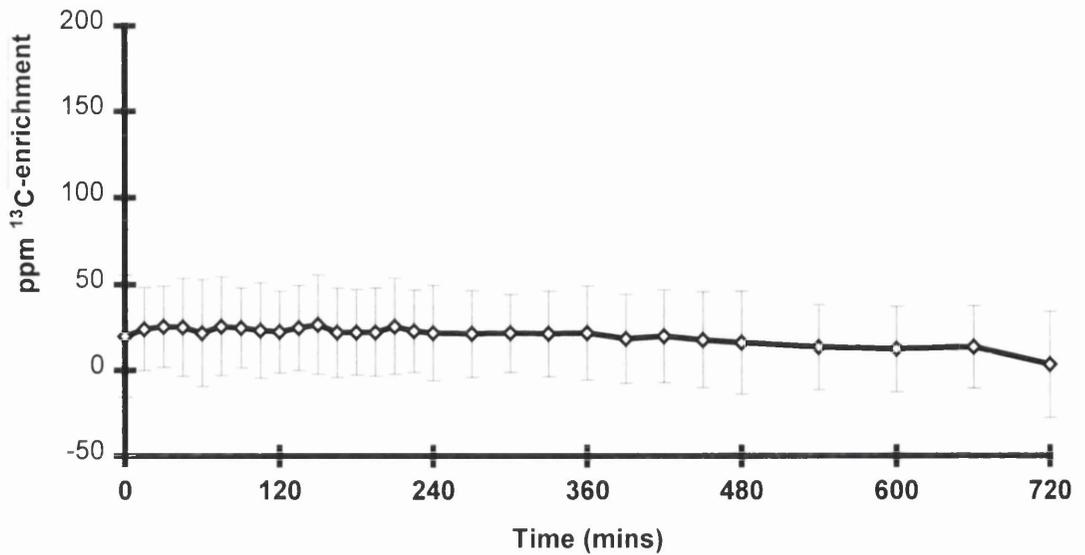
Figure 3.1: Mean excretion of ^{13}C ($\pm$ standard deviation) for all animals (n=3) over the twelve hour test period. The ponies fasted for 12-14 hours prior to commencement of the study, and no test meal was ingested (Test I).
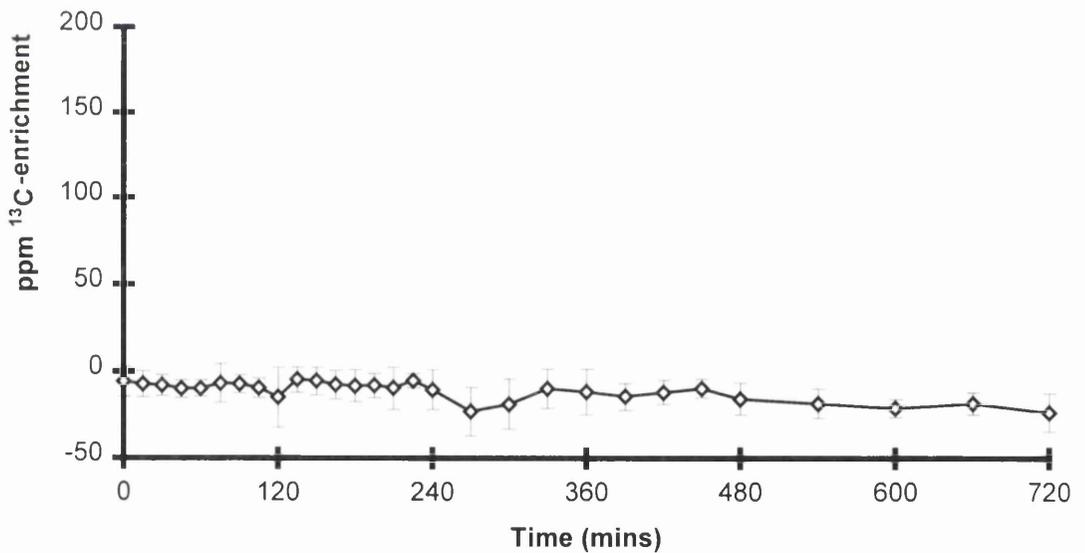
Figure 3.2: Mean excretion of ^{13}C ($\pm$ standard deviation) for all animals (n=3) over the twelve hour test period, following ingestion of a test meal and unlabelled substrate (250mg ^{12}C-octanoic acid)(Test II).

In tests III, IV and V, ingestion of the labelled substrate (^{13}C-octanoic acid), was associated in all cases with significant increases above baseline levels of $^{13}CO_2$ excretion, and these increases were proportional to the dose administered (Figure 3.3).
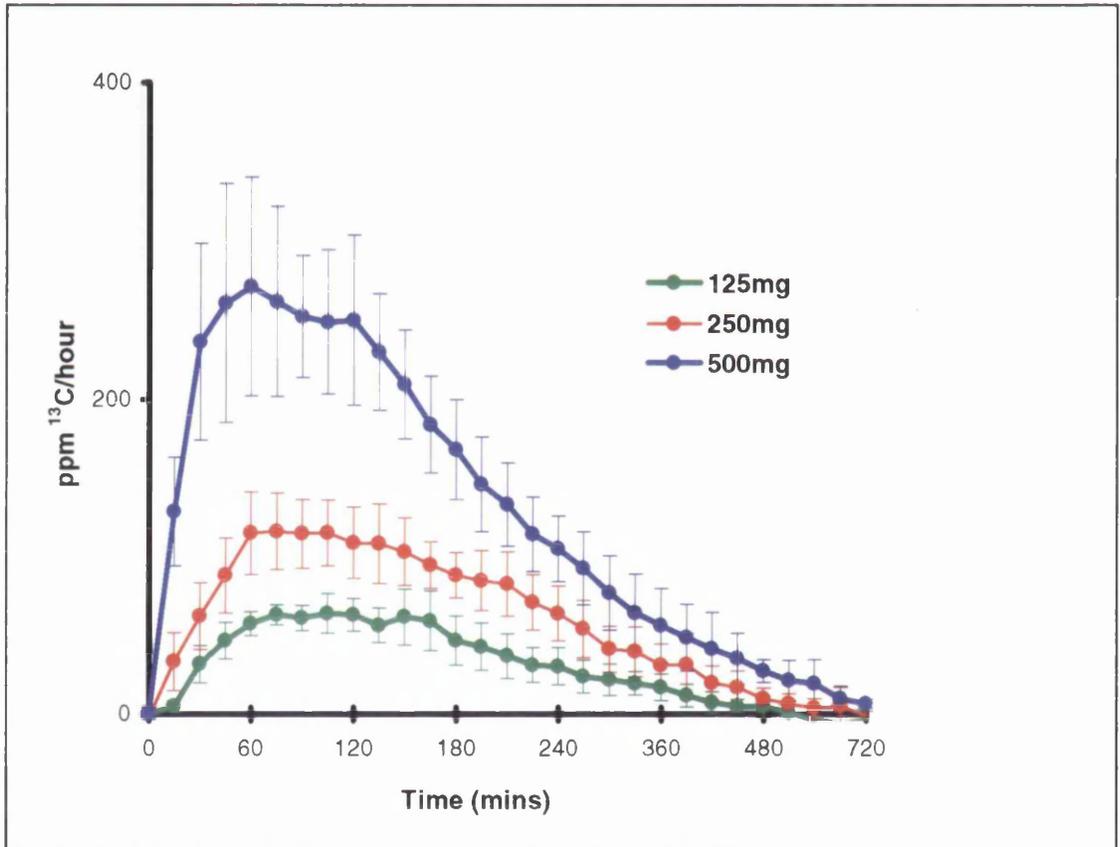
Figure 3.3: Mean excretion of ^{13}C (± standard deviation) in three ponies (Pony Nos. 101, 102 and 103) over a twelve hour test period, following ingestion of a test meal and 125mg (Test III, n=3), 250mg (Test IV, n=3) and 500mg (Test V, n=1) labelled substrate (^{13}C-octanoic acid).

The $^{13}CO_2$ excretion curve plotted after ingestion of the labelled substrate showed the typical pattern of a CO_2 excretion curve, characterised by an initial ascending slope, followed by a point of peak excretion, containing a point of inflexion, and a descending exponential slope containing a second inflexion point (Figure 3.4).
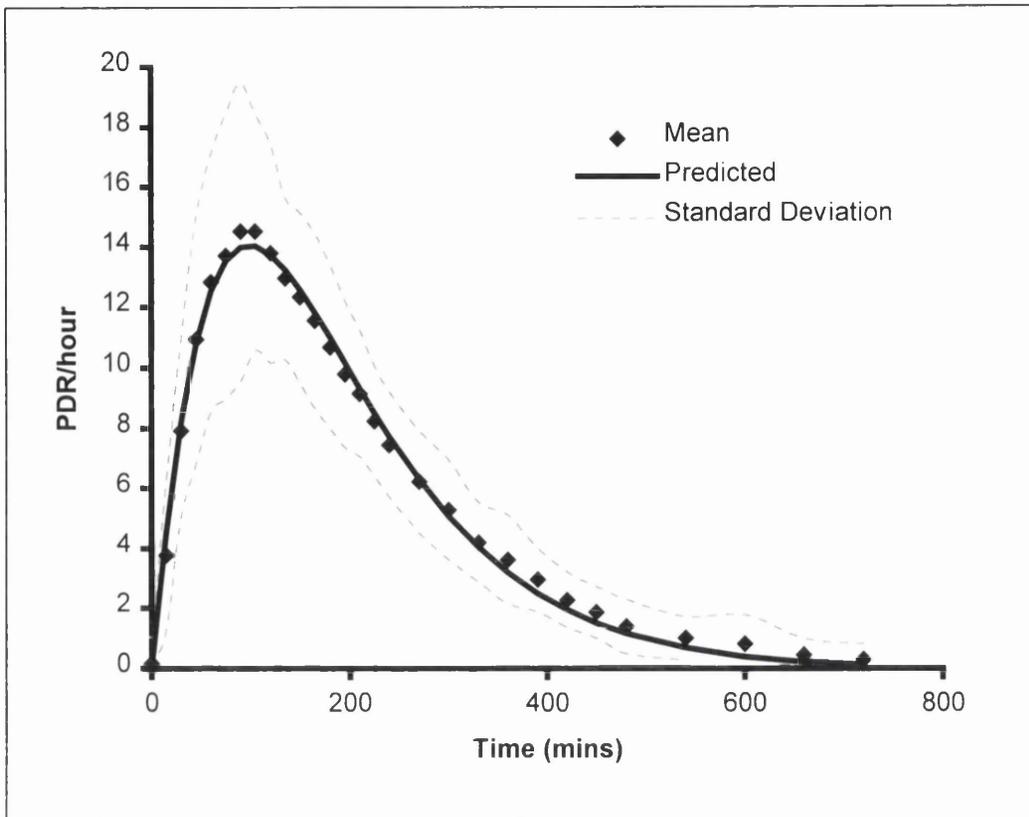
Figure 3.4: Mean excretion of ^{13}C over a twelve hour test period, following ingestion of a test meal labelled with 250mg ^{13}C-octanoic acid (Test IV), on six separate occasions in each individual. Each data point represents the mean PDR/hour (percentage dose recovered per hour) of 24 individual tests at each time point; the standard deviation of the mean is shown by a dotted line.

The mean calculated values for the GEC, t½ and t(max) following ingestion of 125mg (Test III), 250mg (Test IV) and 500mg (Test V) are shown in Table 3.2. Reference ranges for the gastric emptying breath test parameters in the ponies in this study were calculated using data obtained when 250mg ^{13}C-octanoic acid was ingested on six separate occasions by four individuals (Table 3.3). In eight of the twenty four (33%) gastric emptying curves analysed, there was evidence of a dual-phase pattern of ^{13}C excretion (Figure 3.5).

Table 3.2: Gastric emptying parameters (mean ± standard deviation) for individual ponies following ingestion of 125mg (Test III), 250mg (Test IV) or 500mg (Test V) ^{13}C-octanoic acid. Data are represented as the mean ± standard deviation.

Dose	Variable	Pony 101	Pony 102	Pony 103	Pony 104	Mean
125mg (n=3)	GEC	3.15 ± 0.49	NM	3.05 ± 0.55	2.72 ± 0.3	2.97 ± 0.44
	t½(hr)	2.85 ± 1.1	NM	1.59 ± 1.33	3.79 ± 1.0	2.74 ± 1.14
	t(max)(hr)	1.76 ± 0.45	NM	1.84 ± 0.36	1.98 ± 0.16	1.86 ± 0.32
250mg (n=6)	GEC	3.11 ± 0.26	3.47 ± 0.7	2.91 ± 0.25	3.02 ± 0.05	3.13 ± 0.31
	t½(hr)	2.34 ± 0.38	2.36 ± 0.69	2.79 ± 0.78	2.88 ± 0.19	2.59 ± 0.51
	t(max)(hr)	1.40 ± 0.13	1.62 ± 0.67	1.96 ± 0.28	1.69 ± 0.14	1.61 ± 0.30
500mg (n=1)	GEC	3.22	NM	3.19	3.25	3.22 ± 0.03
	t½(hr)	2.16	NM	2.37	2.4	2.31 ± 0.13
	t(max)(hr)	1.1	NM	1.32	1.14	1.18 ± 0.11

NM   not measured
GEC   gastric emptying coefficient
t½   gastric half emptying time : hours
t(max)   time of maximal rate of gastric emptying : hours

Table 3.3 Reference ranges of the gastric emptying breath test parameters in the ponies in this study following ingestion of 250mg ^{13}C-octanoic acid on six occasions by four individuals. GEC, the gastric emptying coefficient, $t_{½}$ the gastric half emptying time and t(max), the time of maximal gastric emptying.

	GEC	$t_{½}$ (hours)	T(max) (hours)
Mean	3.21	2.60	1.64
Standard Deviation	0.40	0.42	0.36
Range	2.28 - 4.17	1.76 - 3.28	0.99 - 2.4
Percentile 25	3.01	2.30	1.39
Percentile 75	3.30	2.94	1.91
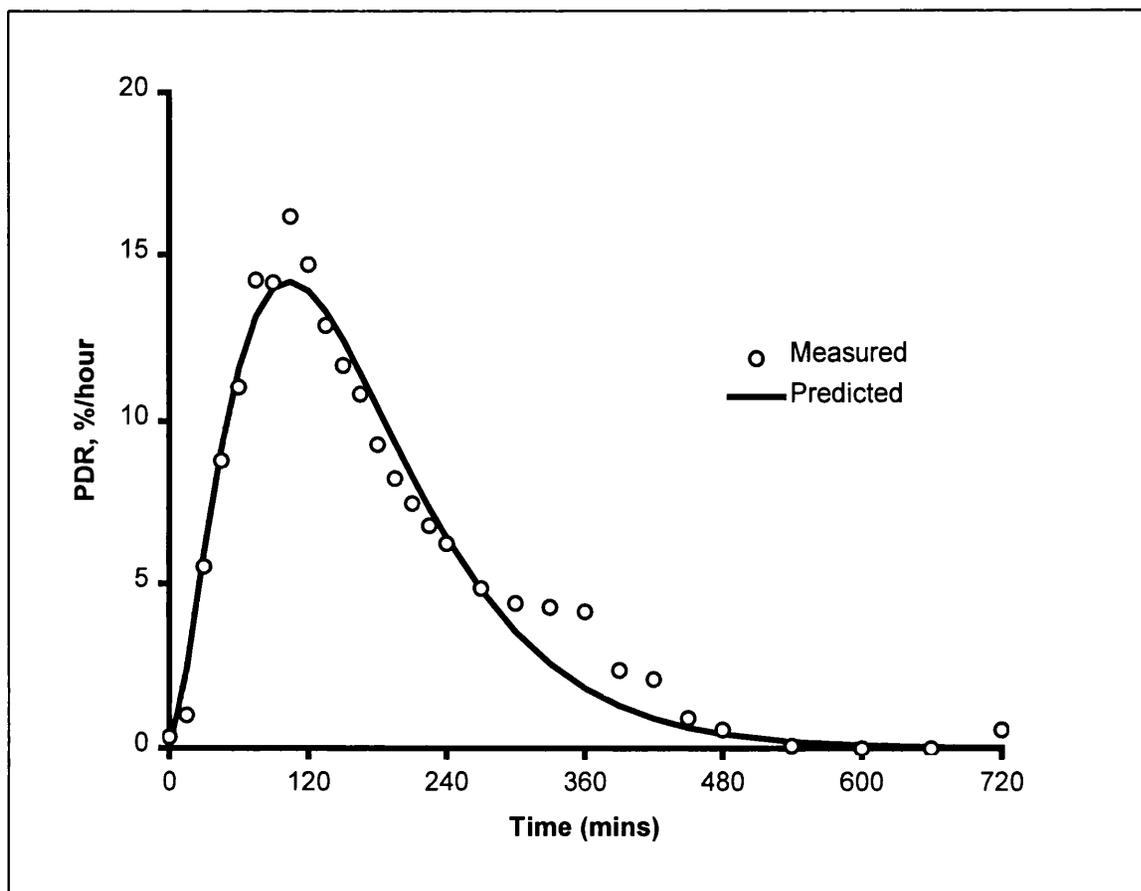
Figure 3.5: Sample ^{13}C-excretion curve illustrating typical dual phase pattern seen in eight of twenty four gastric emptying curves analysed. (Test III, Pony No. 101, Replicate 1). The percentage of the ^{13}C dose recovered (percentage dose recovered, PDR) is plotted over the 12 hour test duration.

The intra-subject or day-to-day variation was assessed by comparing the parameters calculated in six separate tests on each individual animal. There was no significant difference between the replicate tests in each animal, p=0.65, 0.76 and 0.50 for GEC, t(max) and t½, respectively. The inter-subject variation or variation between animals was not significant, p=0.46, 0.48 and 0.36 for GEC, t(max) and t½ respectively. Inter-subject variation was also described using the coefficient of variation and the mean variation in the three gastric emptying parameters for all animals is shown in Table 3.4.

Table 3.4 Coefficients of variation (standard deviation/mean X 100) of the gastric emptying breath test parameters in healthy ponies - GEC, the gastric emptying coefficient, $t_{1/2}$ the gastric half emptying time and t(max), the time of maximal gastric emptying (250mg ^{13}C-octanoic acid ingested by 4 ponies on 6 occasions).

	GEC	$T_{1/2}$	T(max)
Pony No. 101	19.6	12.4	20.6
Pony No. 102	13.9	25.3	33.8
Pony No. 103	5.1	6.6	8.5
Pony No. 104	8.3	10.3	20.8
Mean	11.7	13.6	20.9

The possibility of simplifying the test protocol by reducing the sampling frequency was examined by comparing the gastric emptying indices derived using progressively increasing intervals between samples in the first four hours of the test. Reducing sampling frequency from every fifteen minutes to every thirty or sixty minutes resulted in significant aberration of the test results (probability values are shown in Table 3.5 below). The possibility for reducing the test duration from 12 hours, was also examined in the same way, by re-calculating the gastric emptying parameters while progressively shortening the test duration. Reducing the test duration from 12 to 6 hours did not have a significant effect on the parameters of gastric emptying (Table 3.6).

Table 3.5   Comparison of gastric emptying parameters calculated using different sampling intervals over the first four hours.   The mean difference between the two sampling intervals is shown and the standard deviation (sd) and p-value for each test.

Sampling Interval	GEC			t½			t(max)		
	mean difference	sd	p	mean difference	sd	p	mean difference	sd	p
15 versus 30mins	0.62	0.85	0.0001	0.39	0.49	0.16	0.93	0.82	0.01
15 versus 60mins	1.91	0.27	0.0001	1.21	0.48	0.0001	2.23	0.78	0.001

sd	standard deviation
GEC	gastric emptying coefficient
t½	gastric half emptying time : hours
t(max)	time of maximal rate of gastric emptying : hours
p	probability

Table 3.6   Comparison of gastric emptying parameters calculated using different test durations.   The mean difference between the two test durations is shown and the standard deviation (sd) and p-value for each test.

test duration	GEC			t½			t(max)		
	mean difference	sd	p	mean difference	sd	p	mean difference	sd	p
12 versus 10hr	0.02	0.08	0.86	0	0	1.0	0	0	0.99
12 versus 8hr	0.01	0.01	0.96	0	0	1.0	0.02	0.02	0.92
12 versus 6hr	0.09	0.06	0.47	0.01	0.02	0.94	0.13	0.10	0.39
12 versus 4hr	0.36	0.17	0.01	0.06	0.08	0.64	0.38	0.23	0.01

sd	standard deviation
GEC	gastric emptying coefficient
t½	gastric half emptying time : hours
t(max)	time of maximal rate of gastric emptying : hours
p	probability
hr	hour

3.5　Discussion

The results of this study indicated that the ^{13}C-OBT was a non-invasive, simple and reproducible technique for the assessment of gastric emptying in the pony.

Breath Collection

The breath collection technique (nasal tube) used in this study was previously validated as a method of breath collection for measurement of breath Hydrogen in ponies (Murphy, 1997). The good correlation (Appendix 3), that existed between replicate samples in this study, established that the nasal tube was equally applicable for collection of breath samples for measurement of expiratory $^{13}CO_2$.

Natural Enrichment of $^{13}CO_2$ in Ponies following 12-14 hour Fast, and Test Meal

$^{13}CO_2$ breath tests are carried out against a natural isotopic abundance of approximately 1.1% ^{13}C in exhaled CO_2, and the natural variation in this abundance is a reflection of the source of endogenous fuel, and to a lesser extent metabolic activity (Schoeller *et al.*, 1980). The ponies in this study were fasted and kept at rest during the test protocol in order to minimise the natural variation in the isotopic abundance of ^{13}C in breath. When $^{13}CO_2$ was monitored over 12 hours in ponies kept under these conditions, there was no significant fluctuation in the natural fasting isotope ratio of breath CO_2. This finding confirmed that the effect of background variation in $^{13}CO_2:^{12}CO_2$ on the ^{13}C-OBT in ponies, is negligible.

The test meal functioned essentially as a carrier for administration of the ^{13}C-octanoic acid substrate and the meals were ingested quickly and voluntarily by all the ponies. It was important that the test meal did not in itself provide a source of ^{13}C, that could

interfere with the signal produced by the metabolism of the ^{13}C-octanoic acid tracer. The metabolism of a test meal produces a shift in background ^{13}C-abundance, that is a reflection of the enrichment above or below natural atomic abundance of the carbon contained in the meal (Schoeller *et al.*, 1980). There was no significant change in ^{13}CO$_2$ excretion in the twelve hours following ingestion of an unlabelled test meal in any of the ponies in this study. Although there was a slight negative shift in baseline breath ^{13}CO$_2$ associated with ingestion of the test meal (indicating enrichment below natural atomic abundance), this change was unlikely to significantly affect the detection of the test signal. These findings confirmed that the test meal was an appropriate vehicle for administration of the labelled substrate. It was concluded that neither the ingestion of the test meal, nor the 12-14 hour fasting period induced a change in the rate or pattern of ^{13}CO$_2$ excretion that could have deleterious effects on the detection of the signal provided by the labelled substrate.

Enrichment in Exhaled ^{13}C:^{12}CO$_2$ after Ingestion of ^{13}C-Octanoic Acid

All animals exhibited significant increases above baseline levels of ^{13}C:^{12}CO$_2$ in exhaled breath following ingestion of a test meal incorporating either 125mg, 250mg or 500mg of the labelled substrate, ^{13}C-octanoic acid. The pattern of excretion was similar to that reported in human subjects, and the mathematical model derived by Maes, (1994) to describe the human gastric emptying curve was able to fit the pony data obtained in this present study. The concentration of ^{13}C-enrichment above baseline in this study, was proportional to the concentration of labelled substrate administered, a further indication of the complete and rapid absorption and oxidation of octanoic acid in the pony. Of the three dose rates examined in this study 250mg was judged to produce an appropriate

enrichment in breath ^{13}C, and this dosage was used in all further investigations described in this thesis.

The pattern of gastric emptying in ponies in many cases (33%) described a dual-phase pattern with the movement of the substrate out of the stomach represented by two or more peaks (Figure 3.5). This apparent dual-phase pattern could have been produced by intra-gastric separation of the meal components (oats and bran), possibly due to their respective particle size or caloric density. The dual-phase pattern could have resulted from absorption of octanoic acid in the stomach, although this was unlikely, or absorption in the distal small intestine. The gastric pH of the foal has been shown to undergo periods of "spontaneous alkalisation", thought to result from reflux of ingesta and bile from the duodenum into the stomach (Baker, 1992). Experimental evidence to support this theory was provided by the demonstration that peaks of duodenal activity coincided with gastric motor quiescence and an increased gastric pH (Gerring, 1991). Reflux of the labelled octanoic acid marker from the duodenum to the stomach could explain the dual-phase pattern of gastric emptying observed in this study.

Test Reproducibility

Mathematical analysis of the ^{13}C-excretion curves allowed the calculation of parameters that describe the rate and pattern of gastric emptying (GEC, t½ and t(max)) (Maes, 1994), and these were used to assess the reproducibility of the test. No data is available on the reproducibility of solid-phase gastric emptying in ponies, but the mean gastric emptying coefficient recorded for the ponies in this study was within the reference range reported for human subjects, (2.7-3.98) (Ghoos *et al.*, 1994), while the overall rate of gastric emptying as determined by the t½ values was slower in ponies than in man.

However, differences in the caloric density and volume of test meal administered limit direct comparisons between test protocols. The calculation of the gastric emptying parameters, t½, t(max) and GEC permitted an analysis of the repeatability of the ^{13}C-OBT among the ponies in this study. There was no significant difference in the gastric emptying parameters within individuals, and the inter-subject coefficient of variation was low, 11.7% for the GEC and 13.6% for t½. Similar inter-subject coefficients of variation for the same indices have been obtained using the ^{13}C-OBT in human subjects (n = 16), 9.65% for the GEC and 20.39% for t½ (Ghoos et al., 1993). Significant inter-subject variation in gastric emptying parameters have been reported when gastric emptying was assessed in man, using the ^{13}C-octanoic acid breath test (Choi et al. 1998a; Choi et al., 1998b), and using radioscintigraphy (Brophy, Moore, Christian, Egger and Taylor, 1986). The higher level of reproducibility of this test between the individual ponies in this study may be a reflection of the uniform diet and exercise regimens under which the animals were maintained, conditions that would be difficult to reproduce in a human trial.

Test Validation

Initial experiments during the validation of the ^{13}C-octanoic acid breath test in man demonstrated that the breath test parameters correlated well with those obtained using the reference method of radioscintigraphy (Ghoos et al., 1994), and a correction factor (arbitrarily given the value of 66 minutes) was derived to permit direct comparison between the two methods (Maes, 1994). However, a further investigation failed to elucidate any significant correlation between the ^{13}C-octanoic acid breath test and radioscintigraphy, and questioned the reliability of this breath test as a method for assessing gastric emptying (Choi, Camilleri, Burton, Zinsmeister, Forstrom,

Sreekumaran Nair, 1997). The poor correlation between the ^{13}C-OBT and scintigraphy was attributed to inter-subject differences in the absorption, metabolism and excretion of the labelled octanoic acid (Choi *et al*, 1997), processes thought to remain constant in earlier studies (Ghoos *et al.*, 1993). The study carried out by Choi *et al.*, (1997) was based on data obtained from 15 subjects, and other studies using larger sample groups have demonstrated a good correlation between scintigraphy and the ^{13}C-OBT (Ghoos *et al.*, 1993; Ziegler *et al.*, 1997; Perri *et al.*, 1998b; Delbende, Perri, Couturier, Leodolter, Mauger, Bridgi, Bizais, Bruley des Varannes, Andriulli and Galmiche, 1998). Furthermore, when the protocol was repeated in a larger sample group (n=30) by the same authors (Choi *et al.*,1998), significant correlation between t½ measured using scintigraphy and the ^{13}C-OBT was demonstrated. The accuracy and sensitivity of the ^{13}C-OBT is currently the subject of a European multicentric study and an initial report described a excellent correlation between the ^{13}C-OBT and scintigraphy (Delbende *et al.*, 1998).

There was no significant inter-subject variation in the breath test parameters of the ponies in this study, suggesting that the processes of absorption and metabolism of the labelled substrate was relatively constant between individuals. However, the ^{13}C-OBT should be correlated against radioscintigraphy in the horse, in order to confirm that gastric emptying is the rate limiting step in the absorption and metabolism of octanoic acid, and to validate the test as an accurate method for the assessment of gastric emptying in the horse.

Modification of Protocol

One disadvantage of the use of the ^{13}C-OBT was that the initial protocol followed in this study was very time-consuming, and would be a major impediment to the use of the test

as a clinical diagnostic tool. An investigation of the feasibility of reducing the sampling frequency and length of the test revealed that in the four healthy ponies in this study the test period could be reduced from 12 to 6 hours without significantly affecting the gastric emptying parameters. In human test protocols, the initial four hour period of fifteen minute sampling cannot be reduced without affecting the accuracy of the test (Maes, 1994; Choi *et al.*, 1998b), and the same was true for this pony study. The results of the ^{13}C-OBT are a reflection of the dynamic process of gastric emptying, a function that could not be accurately described by short test protocols. In this present study, the least time consuming and most accurate protocol was found to consist of four hours of fifteen-minute sampling, followed by two hours of thirty-minute sampling. Reducing the sampling frequency and test length in this way makes the test less labour intensive, as well as substantially reducing the costs associated with sample analysis. While the six hour protocol was adequate for assessment of gastric emptying in the healthy ponies in this study, further evaluation of the test protocol will be necessary when the test is performed on animals with delayed gastric emptying.

The experiments described in this chapter have confirmed the potential for enrichment of breath $^{13}CO_2$ in ponies, and demonstrated the reproducibility of the ^{13}C-OBT between and within individual ponies. Administration of the substrate and collection of breath samples was completely non-invasive, and the test protocol was simple and well-tolerated by all animals. Repetition of the ^{13}C-OBT test in a larger group of healthy horses, and in animals with delayed gastric emptying will be necessary in order to establish reference ranges. These studies provided preliminary evidence of the potential value of the ^{13}C-OBT for assessment of gastric emptying in the horse, and further validation, including correlation with scintigraphy, is now justified.

CHAPTER FOUR

THE EFFECT OF TEST MEAL ENERGY DENSITY ON SOLID PHASE GASTRIC EMPTYING
IN PONIES

4.1   Introduction

Gastric emptying is inhibited by meals enriched with nutrients, acids and osmotically
active substances (Hunt and Stubbs, 1975; Meeroff, Go and Phillips, 1975). However,
the regulation of the rate of gastric emptying is mainly a function of the nutrient density
of the meal; osmolarity becomes of physiological significance only at high tonicity
levels (Calbet and MacClean, 1997). All three nutrients, carbohydrate, fat and protein,
exert similar inhibitory effects on gastric emptying (Hunt and Stubbs, 1975). Although
regulatory mechanisms controlling gastric emptying are also sensitive to increasing
intra-gastric volume (Hunt and Stubbs, 1975), intestinal nutrient receptors can override
those sensitive to gastric distension, ensuring that nutrients are delivered to the
duodenum at a rate matched to the absorptive capacity of the gut (Miller *et al.,* 1981).
Intestinal regulation of gastric motility is a very sensitive process, and at any point, the
rate of gastric emptying bears a direct relationship to the energy density of the previous
delivery of kilocalories to the duodenum (Brener, Hendrix and McHugh, 1983). The
chemoreceptors which regulate the rate of gastric emptying in response to nutrient
density are located in the small intestine, and the degree of inhibition is related to the
length of small intestine exposed to the nutrient, rather than simply the nutrient
concentration (Lin, Doty, Reedy and Meyer, 1989). In this way, the recruitment of
increasing numbers of receptors results in a greater inhibition of gastric emptying
(Stanghellini, Borovicka and Reed, 1994). Further credence was added to this theory by
the observation that the more distal the presence of glucose in the small intestine, the
more potent the inhibition of gastric emptying (Lin, Kim, Elashoff, Doty, Gu and

Meyer, 1992). The mechanisms by which the intestinal chemoreceptors respond to changes in the nutrient density of the post-prandial chyme may involve sensitivity to osmotic effects (Barker, Cochrane, and Corbett, 1974) or the binding of calcium on the luminal border of the enterocyte, by the products of digestion of triglycerides (Hunt, 1983). Cholecystokinin is thought to be involved in the mediation of the response of the rate of gastric emptying to the post-prandial chemoreceptor (Stanghellini, Borovicka and Read, 1994). The gastric motor response to intestinal chemoreceptors may be mediated by the central nervous system, since the responses of the gut to the presence of food can be inhibited by truncal vagotomy (Sarna, 1985). Furthermore, ablation of vagal primary sensory afferents by capsaicin abolished the inhibition of gastric emptying induced by fat, in the rat (Holzer, Tukelson, Solomon, Raybould, 1991). Excitation of the intestinal chemoreceptors inhibits the pumping action of the antrum and increases the motor activity of the proximal part of the duodenum, so increasing resistance to gastric emptying (Weisbrodt, Wiley, Overholt and Bass, 1969).

In a similar manner to other nutrients, oil inhibits gastric emptying in man (Hunt and Stubbs, 1975), and in the dog (Meyer, Mayer, Jehn, Gu, Fink, and Fried, 1986). Fats are known to empty from the stomach at a similar rate to equi-calorific solid meals (Edelbroek, Horowitz, Maddox and Bellen, 1992), and fats generally empty along with the solid phase (Meyer *et al.*, 1986). However, the mechanisms by which the stomach emulsifies fat and controls concurrent emptying of fats with the solid phase of gastric emptying, remain unclear.

There have been very few studies addressing the effects of meal energy density on gastric emptying in the horse. Two studies of gastric emptying of fluids in the horse

have failed to demonstrate any delay in gastric emptying with increasing energy density (Baker and Gerring, 1994a; Sosa-Leon, Hogson and Rose, 1997), although it is possible that the energy densities administered were insufficient to cause a delay in gastric emptying. Mare's milk was shown to empty from the foal's stomach at a slower rate than the same volume of saline, or test meal with identical lipid concentration (Intralipid 1.25%) (Baker and Gerring, 1994a). This suggests that factors other than the calorific density of the mare's milk were responsible for the induced delay in gastric emptying, and this was attributed to intrinsic elements present in the milk (Baker and Gerring, 1994a). Interestingly, a study of the gastric emptying of milk in children, using the ^{13}C-OBT, reported delayed gastric emptying mediated by factors other than energy density (Maes *et al.*, 1995).

The gastric emptying of solids does appear to be delayed with increasing meal energy density in the horse. Significant delays in gastric emptying was demonstrated using scintigraphy in three horses when meal energy density was increased by the addition of dextrose (one litre 25% solution in one pound grain), although there was considerable variation between animals (Sojka and Cantwell, 1989). Blood glucose and insulin levels were significantly lower in horses fed a grain test meal supplemented with soya oil, and this effect was attributed to differences in the rate of gastric emptying when fat was included in the diet (Pagan, Rotmensen and Jackson, 1996).

The ^{13}C-OBT has already been applied for the investigation of the effect of test meal energy density on the rate of gastric emptying in man (Maes *et al.*, 1995; Maes,1994), and in a recent study gastric emptying of an oil enriched test meal was examined successfully using this method (Mathers, 1999). A previous *in vitro* study has

established that the octanoic acid substrate is well retained in oil incubated in gastric juice, and is a suitable marker for oil-enriched meals (Maes, 1994). These studies suggest that the ^{13}C-OBT could be used to examine the effect of an oil-enriched meal on the parameters of the ^{13}C-OBT in the horse.

4.2 Specific Objective

The specific objective of this study was to assess the effect of test meals of different calorific content on the rate of gastric emptying in healthy ponies, using the ^{13}C-OBT.

4.3 Materials and Methods

4.3.1 Animals

Four British native-breed ponies were used in this study (Pony Nos. 101-104). Full details of the maintenance of the ponies are given in Chapter 2, and a detailed description of each animal is presented in Appendix 2.

4.3.2 Test Meal

Three test meals of low (Meal 1), intermediate (Meal 2) and high (Meal 3) energy density were used in this experiment. The energy density was increased by adding either 35mls (Meal 2) or 70mls (Meal 3) soya oil (Dodson and Horrell Ltd, UK, 8.54Mkcal/kg) to the meal. An appropriate volume of water was added to each meal to ensure that meal volume remained constant. To ensure that the test meal did not provide a significant source of ^{13}C, an unlabelled test meal was fed to two ponies, and breath ^{13}C excretion monitored over 12 hours. All meals were ingested voluntarily by all ponies in less than five minutes. Details of each test meal are given in Table 4.1, below.

Table 4.1 : Details of the test meals ingested during study of effect of density on $^{13}CO_2$ excretion in ponies

Meal	Energy Density	Meal Components
1	Low (kcal)	75g oats +50g bran + 170ml H_2O 250mg ^{13}C-octanoic acid + 1 egg
2	Intermediate (kcal)	75g oats +50g bran + 135ml H_2O + 35ml soya oil 250mg ^{13}C-octanoic acid + 1 egg
3	High (kcal)	75g oats +50g bran + 100ml H_2O + 70mls soya oil 250mg ^{13}C-octanoic acid + 1 egg

4.3.3   Experimental Procedure

Following an overnight fast (12-14 hours) the ratio of $^{13}CO_2$:$^{12}CO_2$ excretion in breath was determined over a twelve hour sampling period, as described in Chapter Two. The ponies ingested Meal 1, 2 and 3 on three separate occasions (i.e. 9 separate tests in each individual), and in a randomly allocated order (details of the order of ingestion of test meals by each animal, are given in Appendix 7). The test meal was labelled with 250mg ^{13}C-octanoic acid, as described in Chapter Two. Each test was started between 5-6am and breath samples were collected in duplicate at one hour (t = -60mins) and immediately before (t=0mins) ingestion of the test meal, and at regular intervals over the next twelve hours. All samples were analysed within four weeks of collection. Animals had access to water at all times during the test, but access to food was denied until the end of the sampling period.

4.3.4   Measuring Technique

The ratio of ^{13}C:^{12}C in the breath samples was measured by isotope ratio mass spectrometry, as described in Chapter Two.

4.3.5   Data Analysis

Analysis of test results utilised the mean of duplicate measurement taken at each time point (Appendix 5). Samples with CO_2 concentrations less than 10% (0.3%) of the standard gas used in these studies were rejected, as they were not considered to be adequate samples of expiratory air, and in these cases a single replicate measurement was used.   Results were expressed as % dose administered recovered per hour (PDR/hour), and breath $^{13}CO_2$ excretion curves were analysed individually.   For each test, three variables were calculated, the gastric emptying coefficient (GEC), the time of maximal gastric emptying (t(max)), and the gastric half emptying time (t½). Statistical analysis for significance was performed using a two way ANOVA with repeated measures with factors being replicate, animal and test meal. A Student's paired t-test was used to further investigate the relationship between energy density and gastric emptying. Statistical significance was accepted at $p < 0.05$.

4.5   Results

The basal excretion of breath ^{13}C was low and remained stable over a 12 hour sampling period following ingestion of a unlabelled high energy density test meal. A summary of the gastric emptying parameters recorded following each test meal is presented in Table 4.2, below.   The gastric emptying coefficient (GEC) was significantly reduced following ingestion of the high energy meal compared to the low energy density meal (p = 0.0001). A significant difference between the rate of gastric emptying of the high and low energy density meals was also reflected in the gastric half emptying time (t½) (p = 0.0002) and the maximal rate of gastric emptying (t(max)) (p = 0.003).   The gastric emptying of the intermediate energy density meal was also significantly different from

the low energy density meal, as determined by the GEC (p = 0.0016), t½ (p = 0.0049)

and t(max) (p = 0.0156).

Table 4.2 :   Effect of test meal energy density on gastric emptying in four healthy ponies. Data are presented as mean ± standard deviation of the three gastric emptying breath test parameters - GEC, the gastric emptying coefficent, $t_{\frac{1}{2}}$ the gastric half emptying time and t(max), the time of maximal gastric emptying. Each individual pony ingested each meal on three separate occasions in random order (n = 36).

Energy Density	GEC	$t_{\frac{1}{2}}$	t(max)
Low	3.35 ± 0.28	2.62 ± 0.41	1.67 ± 0.33
Intermediate	2.80 ± 0.35*	3.35 ± 0.79**	2.03 ± 0.53*
High	2.61 ± 0.32***	3.87 ± 0.78***	2.22 ± 0.41**

*   p < 0.05   significantly different from the results of the low energy density test meal
**   p <0.001
***   p < 0.0001

There was no significant difference between the gastric emptying parameters derived

following the high and intermediate energy density meals, GEC (p = 0.185), t½ (p =

0.238) and t(max) (p = 0.425). The effects of increasing energy density on the GEC, t½

and t(max), are illustrated in Figure 4.1.
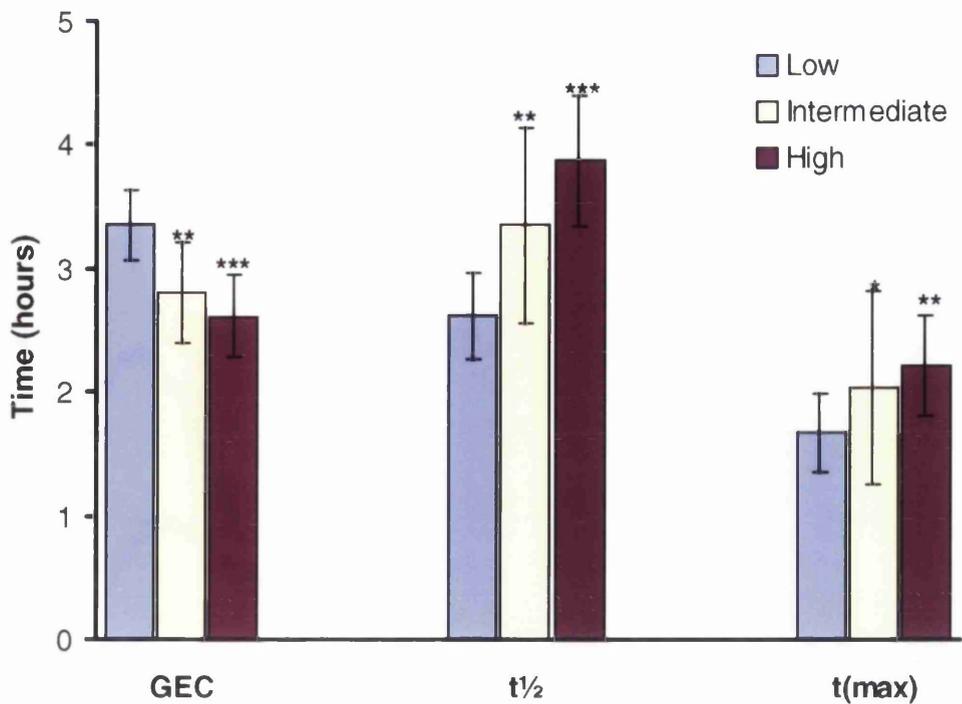
Figure 4.1: Gastric emptying parameters for three test meals of increasing energy density. Data are presented as the mean ± the standard deviation; each test meal was administered to each pony on three occasions, resulting in 36 individual experiments. Results that differ significantly from the results of the low energy density meal test are indicated *p<0.05, **p<0.001 and ***p<.0001.

Intra-individual variation was not statistically significant for the GEC, t½ or t(max) within the energy density groups. There was some variation between animals reflected in the t½ (p = 0.001), and the GEC (p=0.03). There was no significant variation between animals following the low energy density meal for the GEC (p=0.44) t½ (p=0.27) or t(max) (p=0.43).

The mean recovery of ^{13}C for the three individual test meals is illustrated in Figure 4.2, below. The addition of oil to the test meal had a considerable effect on the shape of the gastric emptying curve, as well as the rate of gastric emptying. The slope of the ascending and descending part of the curve decreased and the time of peak excretion of

$^{13}CO_2$ was later for the intermediate and high energy density meals when compared to the low energy density meal.
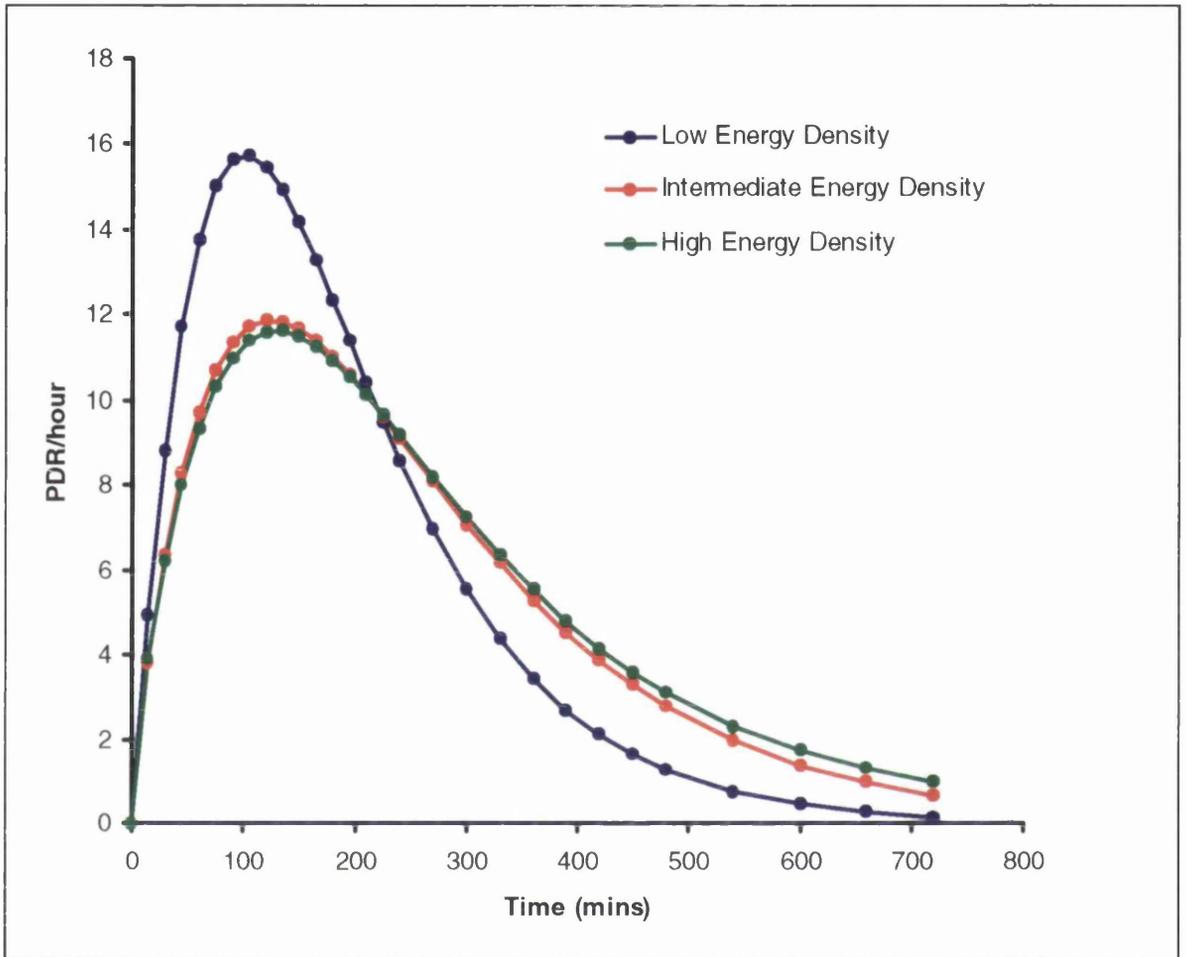
Figure 4.2: Gastric emptying curves derived using the ^{13}C-octanoic acid breath test following administration of test meals of increasing energy density to four healthy ponies. Data shown are mean predicted values for percentage dose recovered (PDR) at each time point. Each meal was ingested by four ponies on three occasions (n=36).

4.4   Discussion

In this investigation the addition of soya oil to a test meal labelled with ^{13}C-octanoic acid, caused a significant delay in the recovery of ^{13}CO$_2$ in the breath of healthy adult ponies, and this was interpreted as evidence of delayed gastric emptying. Lipids are known to exert an inhibitory effect on the rate of gastric emptying, but the mechanisms controlling the gastric emptying of oil have not been studied in the horse, and are poorly understood in man. The delayed gastric emptying of lipids is known to involve small intestinal receptors which are sensitive to the digestion products of fats, and co-ordinate motor mechanisms responsible for retrograde movement of ingesta from distal to proximal stomach (Edelbroek *et al.,* 1992). This retrograde movement may be mediated by the transverse mid-gastric band (Houghton and Mangnall, 1990) or by the lower specific gravity of the oil (Cortot *et al.,* 1981). A similar mid-gastric band has been described in the horse (Nickel, Schummer and Seiferle, 1979), and could play a similar physiological role.

The rate of gastric emptying can be influenced by test meal volume (Moore *et al.,* 1984), osmolarity (Hunt and Stubbs, 1975), and viscosity (Cortot *et al.,* 1981), as well as energy density (Hunt and Stubbs, 1975). The inhibitory effect of increasing meal energy density is not always sufficient to offset the enhancing effects of increasing meal volume on the rate of gastric emptying (Moore *et al.,* 1984). The energy dense nature of soya oil allowed the calorific load administered in this study to be manipulated without necessitating significant alteration in test meal volume. Meal osmolarity is not thought to affect gastric emptying under physiological conditions (Calbert and MacClean, 1997), and is unlikely to have a significant effect on the gastric emptying of the test meals used in this study. Test meal viscosity is thought to affect the rate of gastric

emptying (Edelbroek *et al.*, 1992). The higher viscosity and lower density of the oil added to the test meal in this study could have resulted in intragastric "layering", (Cortot, Phillips and Malagelada, 1981; Jian, Vigneron, Najean and Bernier, 1982) or separation of separate solid and oil phases of gastric emptying. In a human study using phase-specific non-absorbable markers, Mayer *et al.*, (1986) demonstrated that the extracellular fat portion of a solid-liquid meal emptied on average with the solid phase, although an oil phase was identified. In a scintigraphic study of the gastric emptying of a solid-oil meal, there was no significant difference in total stomach emptying of the solid and oil components, although the oil tended to be retained in the proximal stomach, possibly facilitated by the mid-gastric band (Edelbroek *et al.*, 1992). The ^{13}C-octanoic acid breath test was used to monitor the gastric emptying of the oil phase of gastric emptying in man, and there was no significant difference between the gastric emptying of the oil and solid phases (Maes, 1994). Although it is possible that separation of an oil-phase may have occurred in this present study, it is unlikely that the significant changes in the rate of gastric emptying were mediated by factors other the increased calorific content of the test meal.

Numerous studies in humans have demonstrated a correspondence between the rate of gastric emptying and the energy density of the ingested meal (Hunt and Stubbs 1975; Moore, Christian, Brown, Brophy, Datz, Taylor, and Alazraki, 1984; Hunt, Smith and Jiang, 1985), and this effect is independent of the specific nutrient from which the energy is derived (Hunt and Stubbs, 1975). The present study suggested that the rate of gastric emptying of solids in the horse was significantly affected by the addition of soya oil to a test meal, and this is in agreement with a previous study of the gastric emptying of solids in the horse, which demonstrated using scintigraphy, that the rate of gastric

emptying was decreased following ingestion of an energy-dense meal (Sojka and Cantwell, 1989). Soya oil was fed experimentally to Thoroughbred horses in an investigation of the effect of dietary oil on the glycaemic response to a grain meal. The addition of oil was associated with a delayed increase in blood glucose and insulin following the grain meal, and it was proposed that this effect could have been caused by a decreased rate of gastric emptying in response to the oil-enriched meal (Pagan, Rotmensen and Jackson, 1996). The present investigation has demonstrated that the ingestion of soya oil was associated with delayed gastric emptying in the horse, as assessed using the ^{13}C-OBT. Delayed gastric emptying could account for the previously reported delayed blood glucose and insulin response to feeding in the horse following ingestion of an oil-enriched meal (Pagan, Rotmensen and Jackson, 1996).

The rate of gastric emptying is proportional to the energy density of the ingested meal in man (Hunt and Stubbs, 1975) and in the dog (Lin *et al.,* 1989) and ingestion of meals of increasing energy density produces a corresponding effect on gastric emptying in man (Moore *et al.,* 1984). There was no significant difference between the rate of gastric emptying of the intermediate and high energy density meals in the ponies in this present study and a progressive delay in the rate of gastric emptying of test meals of increasing energy density could not be demonstrated. Previous studies in horses have failed to demonstrate any effect of test meal energy density on the rate of gastric emptying of liquid meals (Baker and Gerring 1994a; Sosa-Leon, Hogson and Rose, 1997). Although the rate of gastric emptying of solids was affected by test meal energy density in this study and a previous report (Sojka and Cantwell, 1989), this effect did not appear to be directly proportional to test meal energy density. In man, intestinal control of the rate of gastric emptying is directed towards regulation of the reservoir function of the stomach

(Miller *et al.,* 1981), and allows nutrients to be delivered to the duodenum at a rate that matches absorptive capacity (Brener, Hendrix and McHugh, 1983). The relatively small stomach of the horse limits its capacity for storage of food, and necessitates frequent ingestion of food to satisfy energy needs. The horse may have a reduced capacity for intestinal regulation of gastric emptying, as its small stomach and fibrous diet make it dependent on a constant rate of *intake* of nutrients, rather than a constant rate of nutrient delivery from the stomach.

A dual phase pattern of gastric emptying was apparent in the ponies in this present study. Possible mechanisms responsible for the dual phase pattern have been discussed in Chapter Three. Separation of oil and solid phases of gastric emptying cannot account for the dual phase pattern, as this pattern of gastric emptying was also evident following test meals that did not contain oil, in this present study, and in the experiments described in the previous chapter. Further investigation is necessary to elucidate whether the dual-phase pattern of gastric emptying reported in these studies, is an artefact of the test method, or a true physiological event associated with gastric emptying in the horse.

Variation in the breath test parameters of the individual ponies following the three test meals (intra-subject variation) was low, but there was some inter-subject variation within the test meal groups. This variation was likely to reflect differences in the absorption and digestion of fat between the ponies, especially considering the low intra-subject variation within the test meal groups. Furthermore, no significant variation was apparent between individuals when the gastric emptying of a low energy density meal was assessed in ponies using the ^{13}C-OBT in this study, and in the previous studies

(Chapter Three). The results of this study have shown that the ingestion of an oil-enriched test meal is associated with delayed gastric emptying in ponies, as determined using the ^{13}C-octanoic acid breath test. The ^{13}C-octanoic acid breath test was sensitive enough to detect this delay, and this is a further confirmation of the potential usefulness of the test as a tool for the investigation of gastric emptying in horses.

GENERAL DISCUSSION AND CONCLUSIONS

5.1   Gastric Emptying in the Horse

This discourse began with a review of gastric anatomy and physiology of the horse, with particular emphasis on gastric motility, and factors affecting the rate of gastric emptying. The anatomical and physiological attributes of the equine stomach are a reflection of the role of the horse as a mono-gastric herbivore, requiring a constant intake of nutrients, and superbly adapted for the ingestion and metabolism of fibrous material. As a consequence of this role, the stomach of the horse is small, with limited functional capacity to act as a reservoir, or to accommodate large volumes of ingesta. The migrating motor complex is not interrupted by feeding in the horse, as it is in man, and this continuous pattern of gastric motility is common among herbivores (Bueno and Fioramonti, 1983). The rate of gastric emptying in the horse is also likely to be a continuous process, with the horse relying on the constant passage of low-energy-density nutrients through the gut, rather than the storage and slow release of high-energy density food from the stomach. Most methods of assessing gastric emptying were developed in species with interrupted feeding patterns, such as cats, dogs and humans, and the gastric emptying of a single meal following a period of fasting is a reasonable test of normal gastric function in these animals. The assessment of the gastric emptying of a single "bolus" meal is a questionable reflection of the true physiological nature of gastric emptying in a "trickle feeder" such as the horse. While such a test may be an adequate indicator of alterations in equine gastric emptying produced by disease or pharmacological agents, investigation of the true physiological function of the equine stomach may require tests involving more lengthy protocols, and perhaps continuous

ingestion of fibrous test meals in order to satisfy the horse's natural requirement for a constant nutrient intake.

The results of this study suggest that solid-phase gastric emptying in the horse is reproducible within individuals and variation between animals maintained under the same conditions was low. The addition of soya oil to the test meal caused the rate of gastric emptying to be delayed, suggesting that the rate of solid-phase gastric emptying in the horse is dependent on the energy density of the ingested meal. However a direct linear relationship between meal energy density and gastric emptying could not be demonstrated in the ponies in this study, although such a relationship has been previously demonstrated in man (Hunt and Stubbs, 1975) and in the dog (Brener, Hendrix and McHugh, 1997). The horse is dependent on continuous ingestion of low energy food to satisfy energy requirements, rather than the controlled release of nutrients from the stomach. Therefore, it is conceivable that the stomach of the horse may be less equipped for controlling the rate of gastric emptying of nutrients, than the stomach of man or the dog.

A dual-phase pattern of gastric emptying was frequently evident in the ponies in this study. A previous study demonstrated concurrency between antral contraction, duodenal relaxation and spontaneous gastric alkalisation, which was concluded to represent periodic duodeno-gastric reflux, a feature associated with normal gastric motility in the horse (Gerring, 1991; Baker and Gerring, 1993). Reflux of octanoic acid from the duodenum into the stomach could have been responsible for dual-phase recovery of ^{13}C in the breath. The dual-phase pattern observed in these studies may have been caused by intra-gastric separation of the test meal components, or by

absorption of octanoic acid in the stomach, or in the distal duodenum. Further research is necessary to elucidate the physiological events associated with solid-phase gastric emptying in the horse, and to confirm that octanoic acid is absorbed quickly and completely in the duodenum of the horse.

5.2 Methods for Assessing Gastric Emptying in the Horse

Disordered gastric emptying has already been associated with several equine medical disorders, despite the fact that there is no simple method available for the investigation of gastric emptying in the horse. The location of the equine stomach deep within the abdomen makes assessment of gastric emptying using conventional methods such as radiography or ultrasonography, impossible. The development of a test that would allow simple and accurate assessment of gastric emptying in the horse would facilitate research into the aetiology, diagnosis and treatment of equine gastrointestinal disease.

Gastric aspiration (Baker and Gerring, 1994a), acetaminophen absorption (Doherty *et al.*, 1998) and impedance epigastrography (Baker and Gerring, 1994b) have all been successfully used to assess the gastric emptying of liquids in the horse. All of these methods involve invasive or complex protocol, and are confined to the assessment of liquid emptying which is not always a good indicator of clinically disordered gastric emptying. Radiography, ultrasonography and radioscintigraphy are methods that have been applied for the assessment of solid-phase gastric emptying in the horse. Radioscintigraphy was the only method to provide accurate results, and despite the radiation risk and expense, this is the gold standard method for the assessment of solid-phase gastric emptying in man, and in the horse.

5.3　The ^{13}C-Octanoic Acid Breath Test

The aim of this study was to investigate the feasibility of applying the ^{13}C-octanoic acid breath test for the assessment of solid-phase gastric emptying in the horse. Preliminary investigations demonstrated that natural ^{13}C enrichment in the breath of three fasting ponies was low, and remained constant over a twelve-hour sampling period. The excretion of ^{13}C remained consistently low when this protocol was repeated following ingestion of a test meal. These findings confirmed the potential for enrichment of breath ^{13}C in the horse, for the purposes of the ^{13}C-octanoic acid breath test, but also for other breath tests that utilise the ^{13}C label. The protocol was then repeated using the ^{13}C-octanoic acid substrate, and in all cases there followed significant enrichment of breath ^{13}CO$_2$ that was proportional to the dose of ^{13}C-octanoic acid administered. Non-linear regression analysis of these results allowed the gastric emptying curve to be plotted, and parameters to describe the rate of gastric emptying to be calculated. The reproducibility of the test between individuals was good, and intra-subject variation was low.

This study confirmed that the ^{13}C-octanoic acid breath test is a feasible investigative method for application in the horse. Palatable test meals can be developed so that the substrate is ingested voluntarily, and breath samples are collected easily using a non-invasive method. Samples remain stable in sealed tubes for 60 days (Schoeller *et al.*, 1977) and can be posted to the laboratory for analysis. Because there is no radiation burden or toxic effect associated with the test substrate or protocol, serial tests can be performed in one individual, to monitor progress during a disease, or the effects of a drug. Interpretation of the results of the ^{13}C-octanoic acid breath test can be completely automated, unlike radioscintigraphy, where the necessity for operator interpretation confers an inevitable degree of subjectivity on the test.

The major disadvantage of the ^{13}C-octanoic acid breath test is that the results provided are not real-time physiological measurements, as the recovery of $^{13}CO_2$ is subject to delays imposed by the absorption and metabolism of ^{13}C-substrate. The information provided is therefore a qualitative assessment of the rate and pattern of gastric emptying, and complex correction methods must be applied to enable comparison with quantitative methods, such as radioscintigraphy. The breath sampling protocol required for the ^{13}C-octanoic acid breath test is time-consuming, requiring regular sampling over at least six hours. Attempts to reduce the frequency and duration of breath sampling resulted in significant aberrations in the accuracy of the test in this study and in previous human studies (Maes, 1994; Choi et al., 1998). This finding is a recognition of the function of breath $^{13}CO_2$ as a representative of the dynamic process of gastric emptying that is by definition, best described by interval assessment over a long test period. Because ^{13}C occurs naturally as about 1.1% of the total carbon, all ^{13}C-breath tests are carried out against a natural background level of ^{13}C (Schoeller et al., 1980). In order to minimise fluctuations in the ratio of ^{13}C:^{12}C during the test, fasting is necessary and ^{13}C-rich food must be avoided for several days before the test. The subject must usually remain at rest during the test in order to prevent alterations in the source of endogenous fuel, or CO_2 output.

5.4 Staple Isotope Breath Tests

Stable isotope breath tests are rapidly gaining popularity as methods of clinical diagnosis and research in human medicine, and a European Concerted Action Plan (BIOMED PL 93-2139) has been initiated specifically to exploit the use of stable isotopes for the investigation of functional and metabolic disorders (Stellaard and Geypens, 1998). The primary advantage of the stable isotope breath test is that it is

completely non-invasive, and carries no radiation risk, allowing the test to be carried out safely in situations where conventional radioisotope tests cannot be used, such as in children and pregnant women. Consequently, the application of stable isotope technology to date, has focused on the assessment of nutritional status and gastrointestinal function in babies and children. Stable isotope technology offers physiological and ethical advantages for use in nutritional and gastrointestinal research, as the labelled test meal is chemically identical to real food, and no radiation risk is presented to the patient. There is much scope for the development of further tests of organ function and metabolic status using stable isotopes such as ^{13}C, ^{18}O, ^{2}H, ^{15}N and ^{34}S, and because stable isotope breath tests can be carried out away from the laboratory, there is great potential for application of these methods in epidemiological studies.

This study has identified the potential application of stable isotope breath tests as non-invasive investigative tools in equine medicine and research. Stable isotope breath tests have been used in human medicine for investigation of various gastrointestinal and hepatological and metabolic functions, and many of these tests could have useful applications in equine medicine. For example, the ^{13}C-aminopyrine breath test has been used to investigate liver function in human medicine, and results have correlated well with histological lesions in chronic hepatitis (Mion, Queneau, Rousseau, Brazier, Paliard and Minaire, 1995). A ^{13}C-galactose breath test was developed for investigation of liver function and test parameters were altered early in the course of chronic hepatitis, and were significantly correlated with the degree of liver fibrosis (Mion, Rousseau, Paliard and Minaire, 1998). Sub-clinical pancreatic dysfunction can be detected in man using a ^{13}C mixed triglyceride breath test (Vantrappen et al., 1989) and carbohydrate metabolism can be assessed using substrates labelled with stable isotopes (Hiele, Ghoos,

Rutgeerts, Vantrappen and de Buyser, 1990). A ^{13}C-glycosyl-ureide breath test was developed for assessment of oro-caecal transit time (Heine, Barthold and Klein, 1995), and when a ^{13}C-lactose-ureide substrate was administered concurrently with ^{13}C-octanoic acid, the resulting ^{13}CO$_2$ excretion curve represented a study of the gastrointestinal transit of a single meal (Morrisson, 1998). The non-invasive nature of the breath test makes it an attractive tool for clinical diagnosis and research in the horse, and the many potential applications of these tests in veterinary medicine warrants future research.

5.5   Conclusions

The results of this preliminary study have indicated that the ^{13}C-octanoic acid breath test may prove to be a useful tool for the investigation of gastric emptying, but further validation is necessary before the test can be applied clinically or as a research tool. Although the results of the ^{13}C-octanoic acid breath test have correlated well with reference methods in human studies, comparison of radioscintigraphy and the ^{13}C-octanoic acid breath test should be carried out in the horse, to establish that ^{13}C-octanoic acid is a true marker of the solid phase of gastric emptying. The number of animals used in this study was small (n = 4) and repetition of the test in a larger group of animals, and in animals with delayed gastric emptying, will be necessary to establish reference ranges before clinical application is possible. The sensitivity of the ^{13}C-octanoic acid breath test was demonstrated in this study, when the test was able to detect delays in the rate of gastric emptying induced by alterations in the energy density of the test meal.   The use of motility-modifying drugs, such as erythromycin or metoclopramide would provide a further indication of the sensitivity of the test. This preliminary study produced very promising data, that suggest that the ^{13}C-octanoic acid

breath test is an accurate and non-invasive method for the assessment of gastric

emptying in the horse, and further validation is justified.

APPENDICES

APPENDIX ONE

SUPPLIERS OF REAGENTS AND EQUIPMENT

Becton Dickinson,

Becton Dickinson UK Limited, Between Towns Road, Oxford, England

Dodson and Horrell,

Kettering, Northhampton, England.

Europa Scientific,

Crewe, England.

CK Gas Products Ltd,

1 Marino Way, Finchampstead, Wokingham, Berkshire, England

Labco Ltd.,

Unit 2 Halifax House, Halifax Road, Cressex Business Park, High Wycombe, Buckinghamshire, England

Microsoft Corporation,

One Microsoft Way, Redmond, WA 98052-6399, USA

Millpledge,

Whinleys Estate, Clarborough, Retford, Nottingham, England.

Rocket of London,

Watford, England

Sigma,

P.O. Box 14508, St Louis, Missouri, US.

APPENDIX TWO

DETAILS OF ANIMALS

Pony Number	Sex	Age	Breed
101	Stallion	7yr	Native
102	Stallion	10yr	Fell
103	Mare	15yr	Native
104	Mare	12yr	Native

APPENDIX THREE

ASSESSMENT OF BREATH COLLECTION TECHNIQUE

The repeatability of the breath collection/storage technique was determined by analysing the duplicate breath $^{13}CO_2$ concentrations detected for all tests performed, in all ponies (i.e. 68 separate tests), following ingestion of a test meal with or without addition of the substrate, ^{13}C-octanoic acid, as described in Chapters Three and Four. For all duplicate measurements, the mean of each pair was taken as 100%, and the percentage deviation from the mean value was determined. The mean deviation from the mean value for all pairs analysed (n=1160), was calculated to be 27%, (median = 6%). When the analysis was repeated on duplicate measurements with initial readings of 2ppm ^{13}C-enrichement or greater (n = 1072) the average deviation decreased to 12%.

These results compare favourably with the validation of the breath hydrogen test in ponies, using the same method for breath collection, where a mean variation of 15% was reported for duplicate breath samples (Murphy, 1997). Based on the analysis presented above, the reproducibility of the breath collection method was regarded as being satisfactory and all data analyses performed in Chapters Three and Four, utilised the mean of duplicate measurements at each time point.

Figure 3A: Analysis of breath ^{13}C-enrichment of replicate breath samples (n=1160). The mean of each replicate pair was taken as 100% and each point represents the deviation of each measurement from the mean value.

VALIDATION OF METHOD FOR TRANSFER OF BREATH SAMPLE INTO SAMPLE TUBE

This experiment was designed to investigate the reliability of a method for transferring the breath sample from the syringe to the sample tube, which did not necessitate the use of an evacuated tube. This method would allow the breath sample tubes to be re-used, thus considerably reducing the cost of running a ^{13}C-octanoic acid breath test, particularly under experimental conditions, such as the present case, where large numbers of samples are taken. Two methods for transferring the breath sample into the sample tube were compared. Method A, was the method used in the studies described in Chapter Two, where the breath sample was allowed to flow into an evacuated tube, through a 19G needle. Method B, used screw top un-evacuated sample tubes (Exetainer®, Labco) and a 20ml breath sample was inserted at the bottom of the tube via a 19G 2" needle (Millpledge). The breath sample caused the air already in the 10ml tube to be displaced, and the screw top was quickly fastened to seal the sample.

Breath ^{13}C excretion was monitored in one pony, following ingestion of ^{13}C-octanoic acid, over 3 hours. Breath was collected (as described in Chapter Two) into a 30ml syringe, and 10mls was allowed to enter the evacuated tube (Tube A), and the remaining 20mls was used to flush and fill the used tube (Tube B). In this way, both Tubes A and B were filled with samples taken during a single exhalation.

The results were expressed as percentage dose recovered as described in Chapter Two, and a Students unpaired t-test was used to compare the data obtained using both methods. The ^{13}C concentration recorded at each time point, and using each method,

were plotted on a graph (Figure 1, below). The results revealed that the concentration of $^{13}CO_2$ collected using Method A (Tubes A), was not significantly different from the results obtained using Method B, and there was a high correlation between the two methods (rho = 0.975). This study indicates that Method B is a reliable method for transferring breath samples into used sample tubes, and based on these results, used sample tubes were used in the experiments described in Chapter Four.
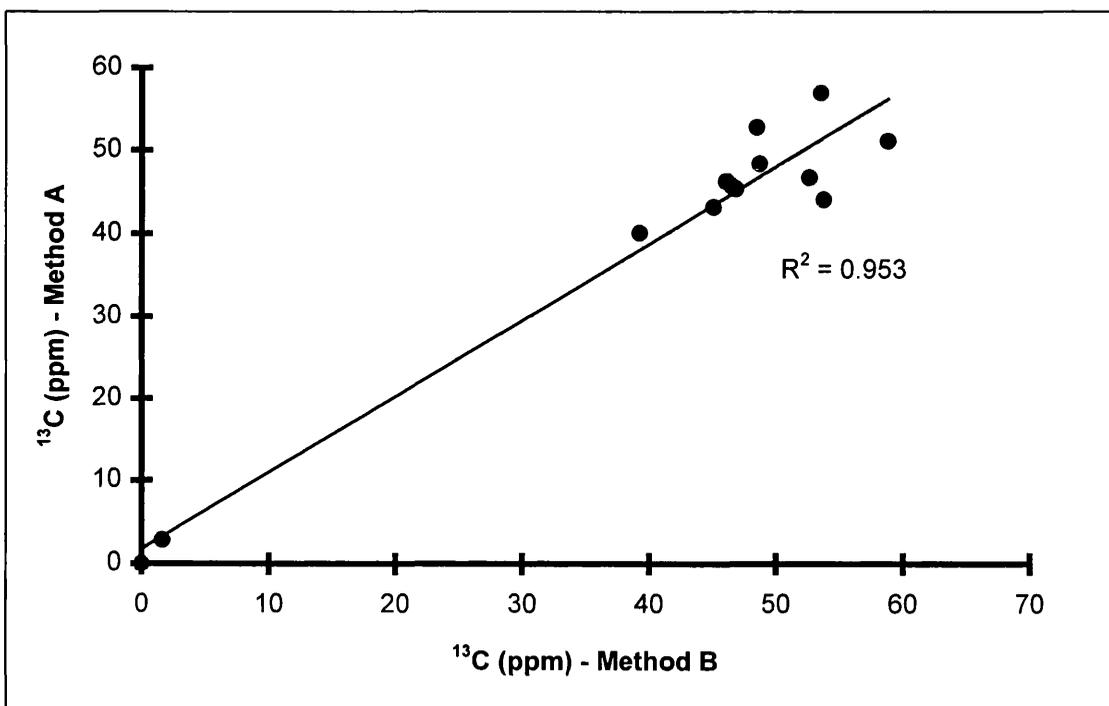

Figure 4A: Scatter graph comparing the concentration ^{13}C (ppm) collected at 14 time points over a three hour sampling period, using two different methods of transferring the breath sample to the sample tube (Methods A and B). The fitted regression line shows the good correlation between the two methods

APPENDIX FIVE

BREATH $^{13}CO_2$ ENRICHMENT FOLLOWING INGESTION OF LABELLED TEST MEAL

Breath test parameters in four ponies following ingestion of test meals labelled with 125mg (Test III), 250mg (Test IV) and 500mg (Test V) ^{13}C-octanoic acid. Test II and Test III were repeated in each individual on 3 occasions and Test IV on 6 occasions.

GEC	Gastric emptying coefficient
t½	Gastric half emptying time
t(max)	Time of maximal rate of gastric emptying
RMS	Square root of the mean of the residuals squared - measure of fit of the curve
PDR	Total percentage of the administered dose of ^{13}C label recovered

Pony No.	Test No.	Replicate	GEC	t½ (hr)	t(max) (hr)	RMS	PDR (%)
101		1	3.27	2.07	1.25	0.47	39
		2	3.30	2.29	1.33	0.81	52
		3	2.67	3.42	1.65	1.36	59
103	III	1	3.04	2.95	1.82	1.34	63
		2	2.68	3.53	1.97	0.83	62
		3	2.44	4.58	2.15	3.16	71
104		1	3.55	2.17	1.57	0.77	40
		2	3.15	2.53	1.71	1.08	46
		3	2.33	3.46	2.69	2.33	45
101		1	3.27	2.07	1.25	0.47	39
		2	2.93	2.61	1.51	0.93	46
		3	3.28	2.34	1.46	0.87	48
		4	4.17	2.08	1.43	1.78	76
		5	3.13	2.81	2.15	1.21	53
		6	3.56	2.29	1.38	0.84	69
102		1	2.64	3.16	2.40	0.99	46
		2	3.95	1.95	1.23	1.76	63
		3	3.84	1.97	1.24	1.10	59
		4	3.25	2.36	1.50	0.61	48
		5	3.30	3.06	1.91	1.57	89
	IV	6	3.30	1.76	0.99	0.98	55
		1	3.08	2.67	1.53	0.78	56
		2	2.97	3.03	1.73	0.56	64
103		3	3.02	2.95	1.82	0.98	62
		4	3.31	2.92	1.78	0.93	83
		5	3.20	2.74	1.96	0.84	55
		6	3.22	2.93	2.06	1.31	77
		1	3.01	2.71	1.92	0.49	45
		2	2.62	3.28	2.27	1.04	51
104		3	3.11	2.62	1.70	0.96	50
		4	3.28	2.87	1.81	1.37	76
		5	3.39	2.60	1.56	0.95	73
		6	2.37	4.00	2.18	1.10	57
101		1	3.22	2.16	1.10	1.01	49
103	V	2	3.25	2.40	1.14	0.95	62
104		3	3.19	2.37	1.32	0.53	51
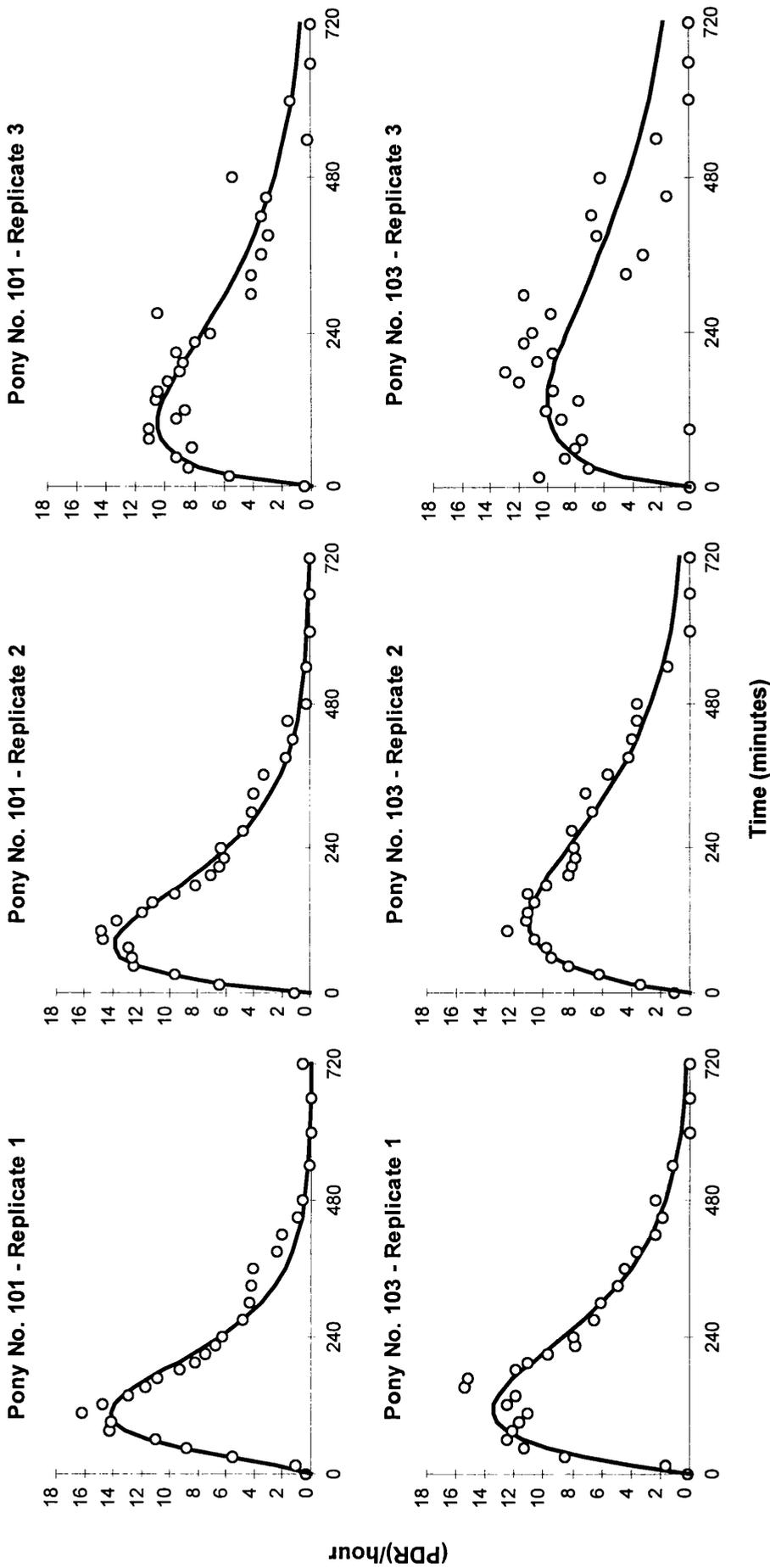
Figure 5A(i): Enrichment of breach $^{13}CO_2$ (mean of duplicate measurements) detected in exhaled breath of three ponies (Pony Nos. 101, 103 and 104), following ingestion of a test meal labelled with 125mg ^{13}C-octanoic acid (Test III). The test was repeated in each individual on three occasions (Replicates 1-3).

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Figure 5A(ii): Enrichment of breath $^{13}CO_2$ (mean of duplicate measurements) detected in exhaled breath of three ponies (Pony Nos. 101, 103 and 104), following ingestion of a test meal labelled with 125mg ^{13}C-octanoic acid (Test III). The test was repeated in each individual on three occasions (Replicates 1-3).

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Figure 5B(i): Enrichment of breath $^{13}CO_2$ (mean of duplicate measurements) detected in exhaled breath of four ponies (Pony Nos. 101 - 104), following ingestion of a test meal labelled with 250mg ^{13}C-octanoic acid (Test IV). The test was repeated in each individual on six occasions (Replicates 1-3). (Replicates 4-6 are illustrated in Figures 6A, 6B and 6C, Low Energy Replicates 1-3).
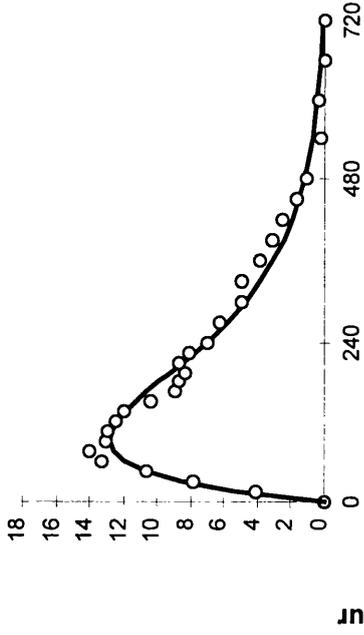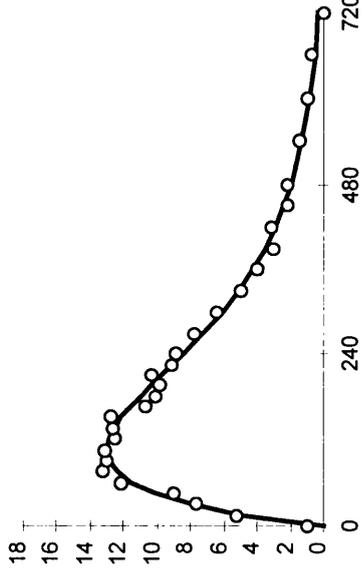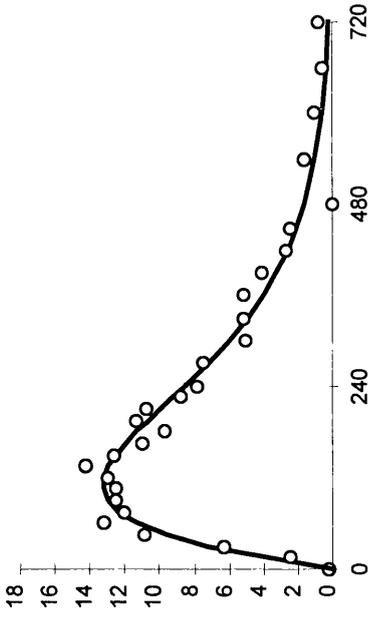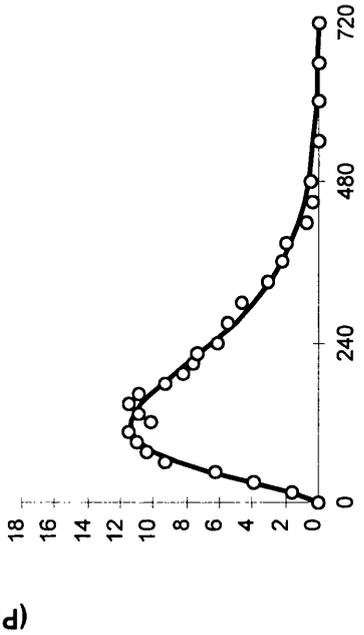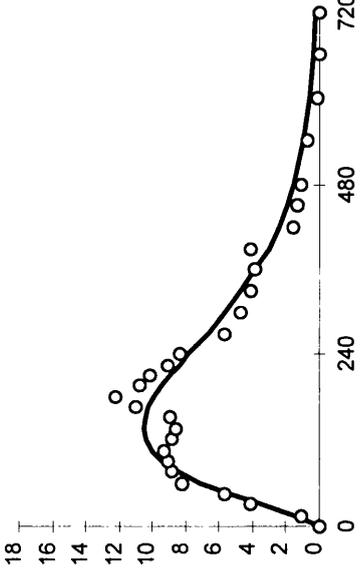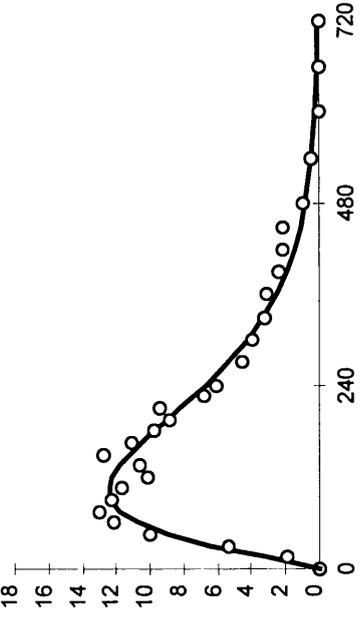
Figure 5B(ii): Enrichment of breath $^{13}CO_2$ (mean of duplicate measurements) detected in exhaled breath of four ponies (Pony Nos. 101 - 104), following ingestion of a test meal labelled with 250mg ^{13}C-octanoic acid (Test IV). The test was repeated in each individual on three occasions (Replicates 1-3).). (Replicates 4-6 are illustrated in Figures 6A, 6B and 6C, Low Energy Replicates 1-3).

113
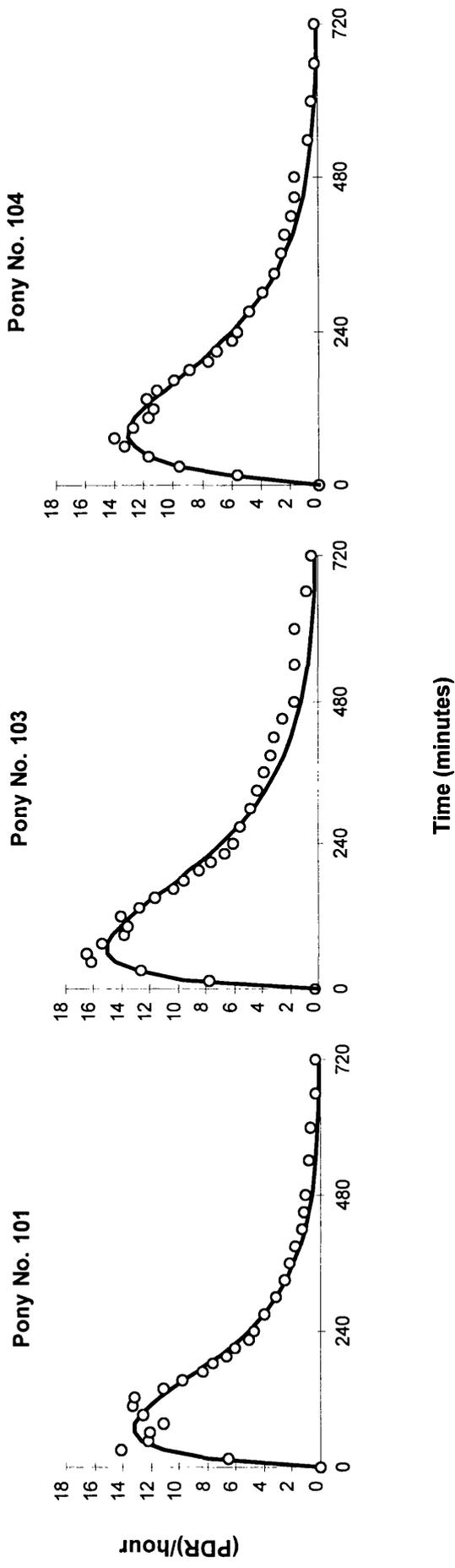
**Figure 5C(i):** Enrichment of breath $^{13}CO_2$ (mean of duplicate measurements) detected in exhaled breath of three ponies (Pony Nos. 101, 103 & 104), following ingestion of a test meal labelled with 500mg ^{13}C-octanoic acid (Test V).

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EFFECT OF TEST MEAL ENERGY DENSITY ON GASTRIC EMPTYING PARAMETERS

Gastric emptying breath test parameters in four ponies (Pony Nos. 101-104) following ingestion of test meals labelled with 250mg ^{13}C-octanoic acid, and enriched with 0mls (Low Energy), 35mls (Intermediate Energy) and 70mls (High Energy) soya oil. Each test meal was ingested by each animal on three separate occasions (Replicates 1 - 3), and in random order.

GEC	Gastric emptying coefficient
t½	Gastric half emptying time
t(max)	Time of maximal rate of gastric emptying
RMS	Square root of the mean of the residuals squared - measure of fit of the curve
PDR (T)	Total percentage of the administered dose of ^{13}C label recovered

Pony No.	Meal	Replicate	GEC	t½ (hr)	t(max) (hr)	RMS	PDR (T)
101		1	4.17	2.08	1.43	1.78	76%
		2	3.13	2.81	2.15	1.21	53%
		3	3.56	2.29	1.38	0.84	69%
102		1	3.25	2.36	1.50	0.61	48%
	Low	2	3.30	3.06	1.91	1.57	89%
	Energy	3	3.30	1.76	0.99	0.98	55%
103		1	3.31	2.92	1.78	0.93	83%
		2	3.20	2.74	1.96	0.84	55%
		3	3.22	2.93	2.06	1.31	77%
104		1	3.28	2.87	1.81	1.37	76%
		2	3.39	2.60	1.56	0.95	73%
		3	2.37	4.00	2.18	1.10	57%
101		1	2.77	3.60	2.57	1.21	77%
		2	3.19	3.32	1.70	0.85	94%
		3	3.13	3.05	1.98	1.93	76%
102		1	2.81	2.24	1.69	0.83	49%
	Intermediate	2	2.74	2.85	1.34	1.43	52%
	Energy	3	2.79	2.30	1.36	1.06	32%
103		1	2.62	4.17	2.64	0.82	84%
		2	2.95	3.49	2.02	1.30	77%
		3	2.58	3.93	1.70	1.30	63%
104		1	2.19	4.10	2.79	1.12	55%
		2	2.31	3.92	2.29	0.65	52%
		3	2.91	3.75	2.35	1.10	89%
101		1	2.71	3.32	2.55	1.42	57%
		2	2.94	2.92	1.52	1.90	77%
		3	2.88	3.60	2.20	0.95	82%
102		1	2.69	3.64	2.44	1.22	58%
		2	1.96	3.90	3.05	0.99	48%
	High	3	2.52	4.34	2.25	1.64	75%
	Energy	1	2.97	3.41	2.21	1.26	76%
103		2	2.13	3.55	2.32	0.98	42%
		3	2.60	4.30	2.00	1.18	77%
104		1	2.77	3.83	1.61	1.78	74%
		2	2.86	3.89	2.00	1.25	88%
		3	2.31	5.53	2.63	1.10	78%

Pony No. 101
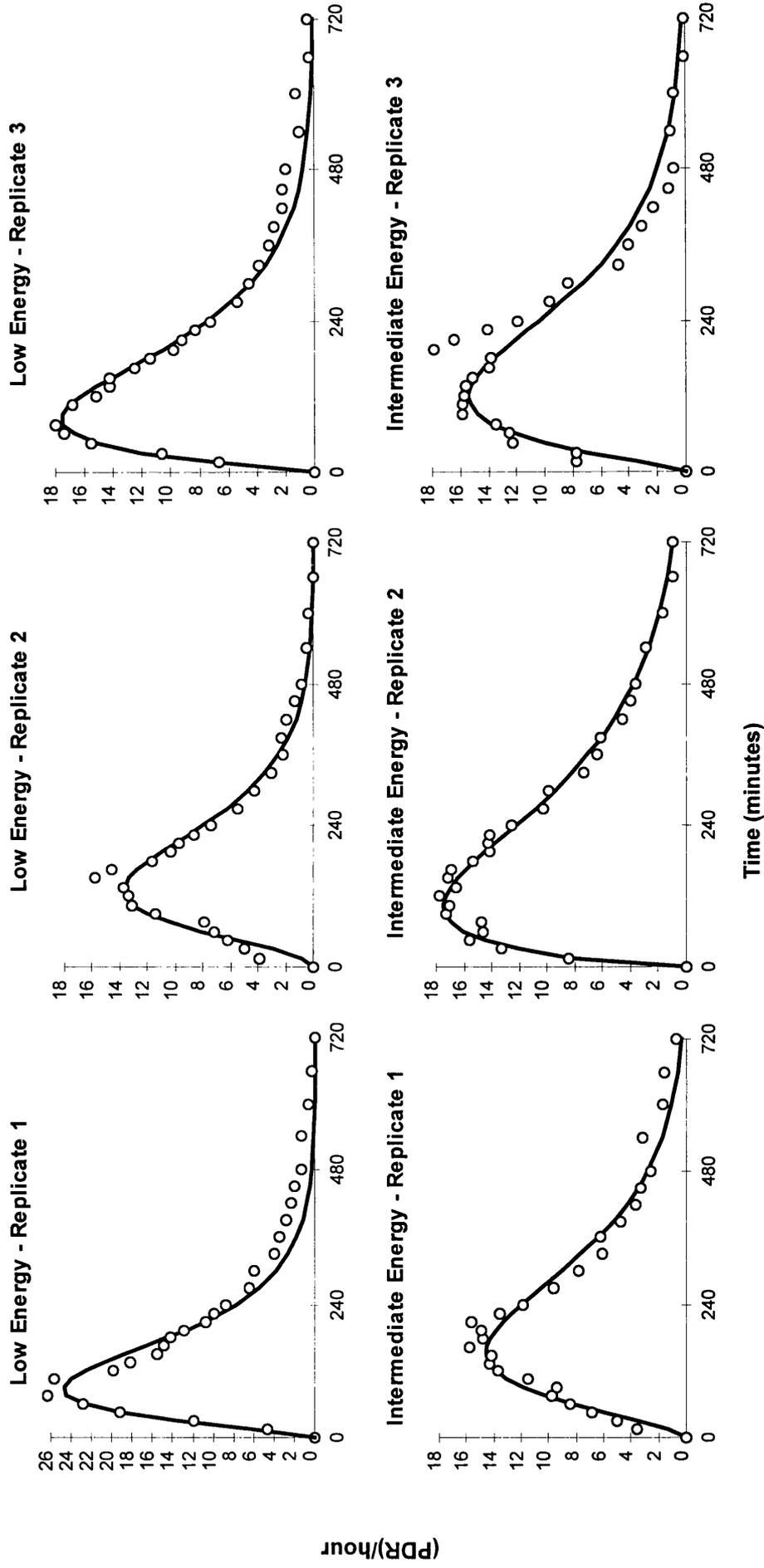
Figure 6A(i): Enrichment of breath $^{13}CO_2$ (mean of duplicate measurements) detected in exhaled breath of Pony No. 101, following ingestion of a test meal labelled with 250mg ^{13}C-octanoic acid, and enriched with 0mls (low energy) and 35mls (intermediate energy) soya oil. Each meal was ingested on three separate occasions (Replicates 1- 3).

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Pony No. 101

High Energy - Replicate 1

High Energy - Replicate 2

High Energy - Replicate 3

(PDR)/hour

Time (minutes)
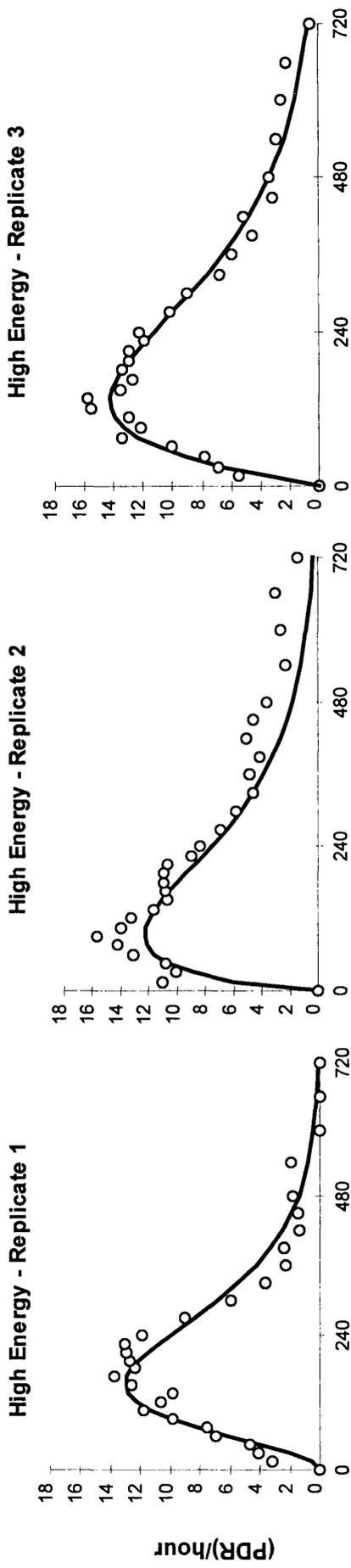
Figure 6A(ii): Enrichment of breath $^{13}CO_2$ (mean of duplicate measurements) detected in exhaled breath of Pony No. 101, following ingestion of a test meal labelled with 250mg ^{13}C-octanoic acid, and enriched with 70mls (high energy) soya oil. The meal was ingested on three separate occasions (Replicates 1- 3).

117

Pony No. 102

Low Energy - Replicate 1
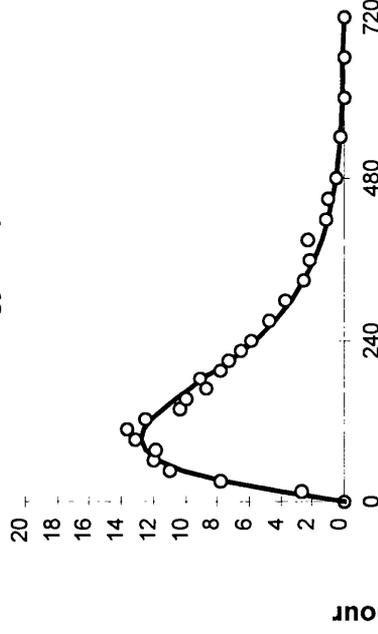
Low Energy - Replicate 2
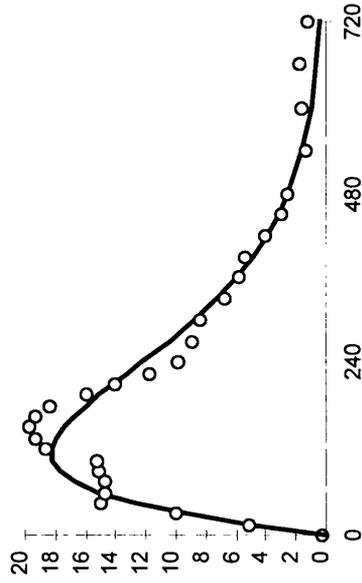
Low Energy - Replicate 3
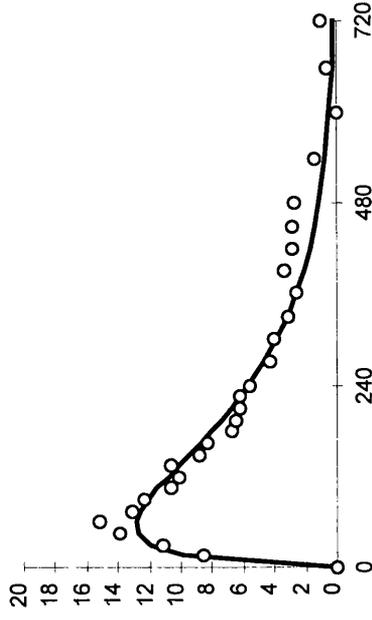
Intermediate Energy - Replicate 1
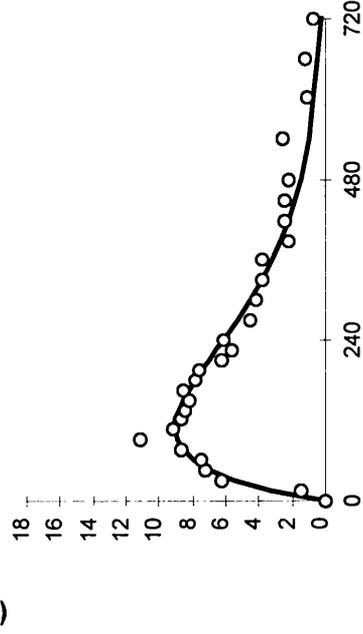
Intermediate Energy - Replicate 2

Intermediate Energy - Replicate 3

(PDR)/hour
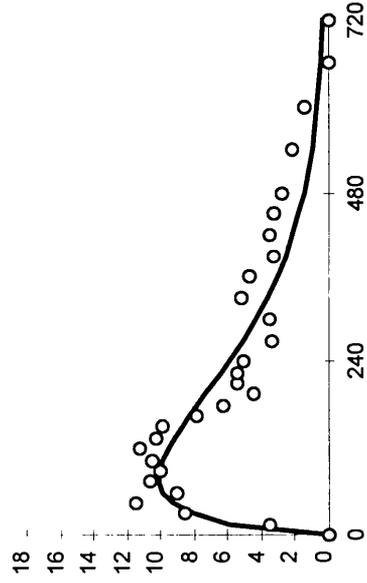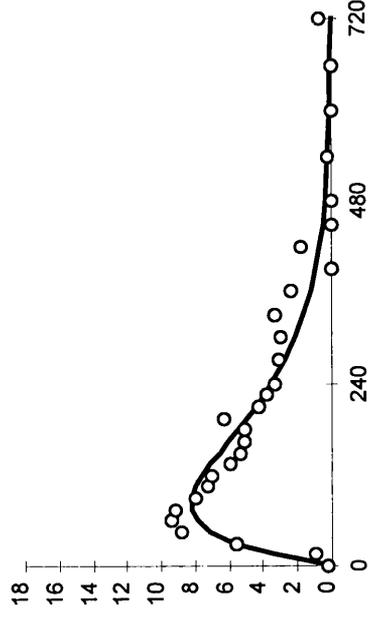
Time (minutes)

Figure 6B(i): Enrichment of breath $^{13}CO_2$ (mean of duplicate measurements) detected in exhaled breath of Pony No. 102, following ingestion of a test meal labelled with 250mg ^{13}C-octanoic acid, and enriched with 0mls (low energy) and 35mls (intermediate energy) soya oil. Each meal was ingested on three separate occasions (Replicates 1- 3).

118
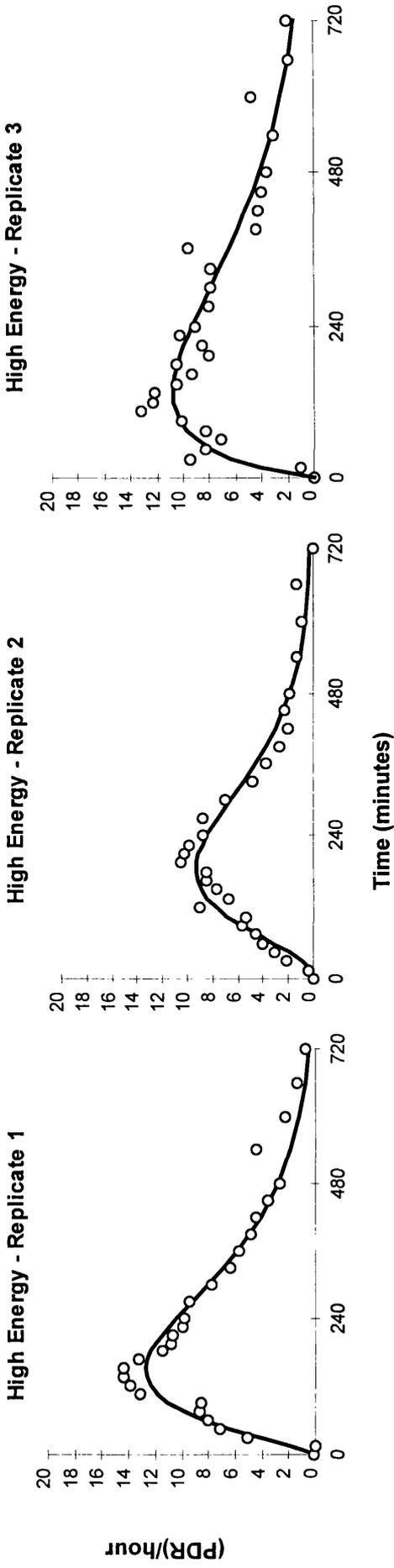
Pony No. 102

Figure 6B(ii): Enrichment of breath $^{13}CO_2$ (mean of duplicate measurements) detected in exhaled breath of Pony No. 102, following ingestion of a test meal labelled with 250mg ^{13}C-octanoic acid, and enriched with 70mls (high energy) soya oil. The meal was ingested on three separate occasions (Replicates 1- 3).

119
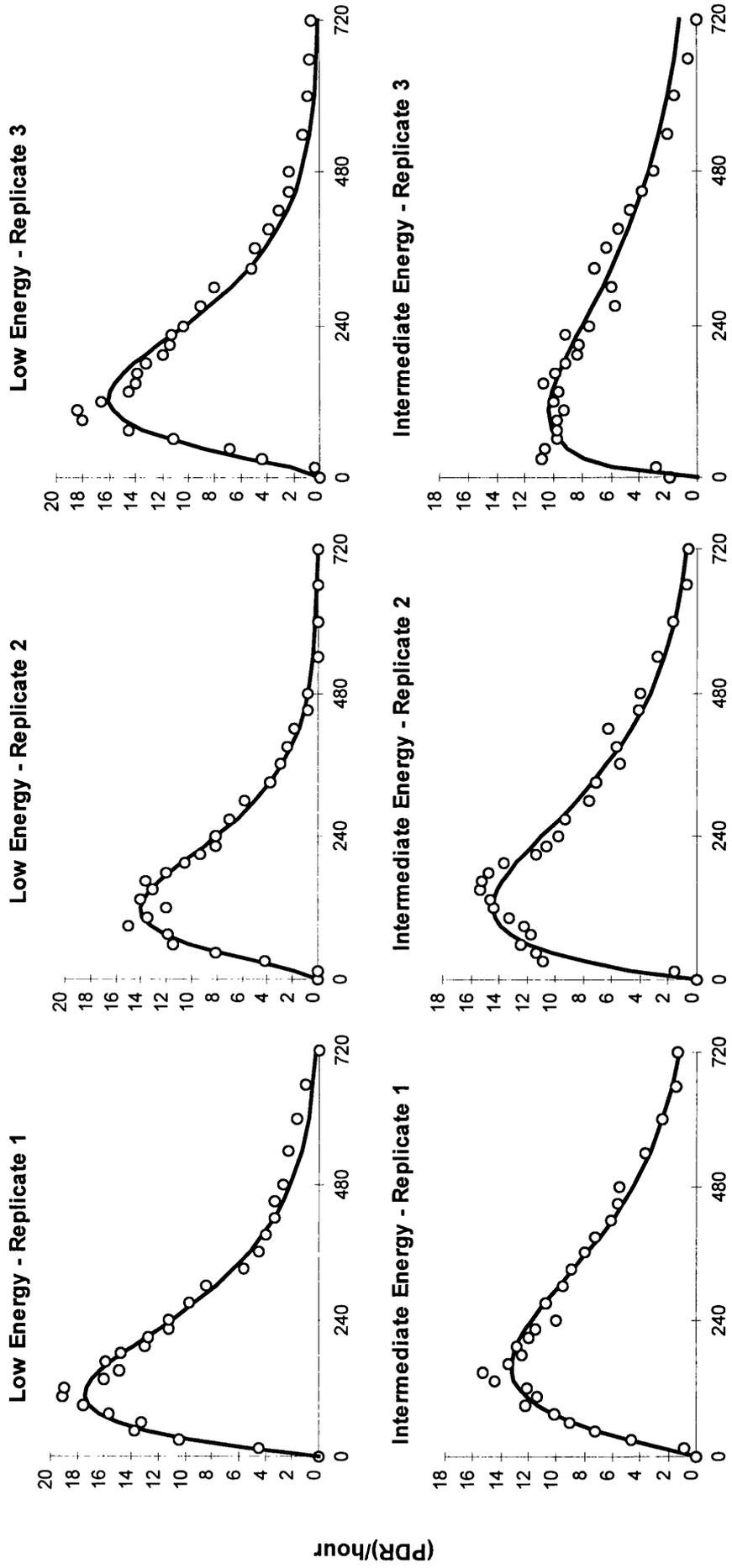
Figure 6C(i): Enrichment of breath $^{13}CO_2$ (mean of duplicate measurements) detected in exhaled breath of Pony No. 103, following ingestion of a test meal labelled with 250mg ^{13}C-octanoic acid, and enriched with 0mls (low energy) and 35mls (intermediate energy) soya oil. Each meal was ingested on three separate occasions (Replicates 1- 3).

120
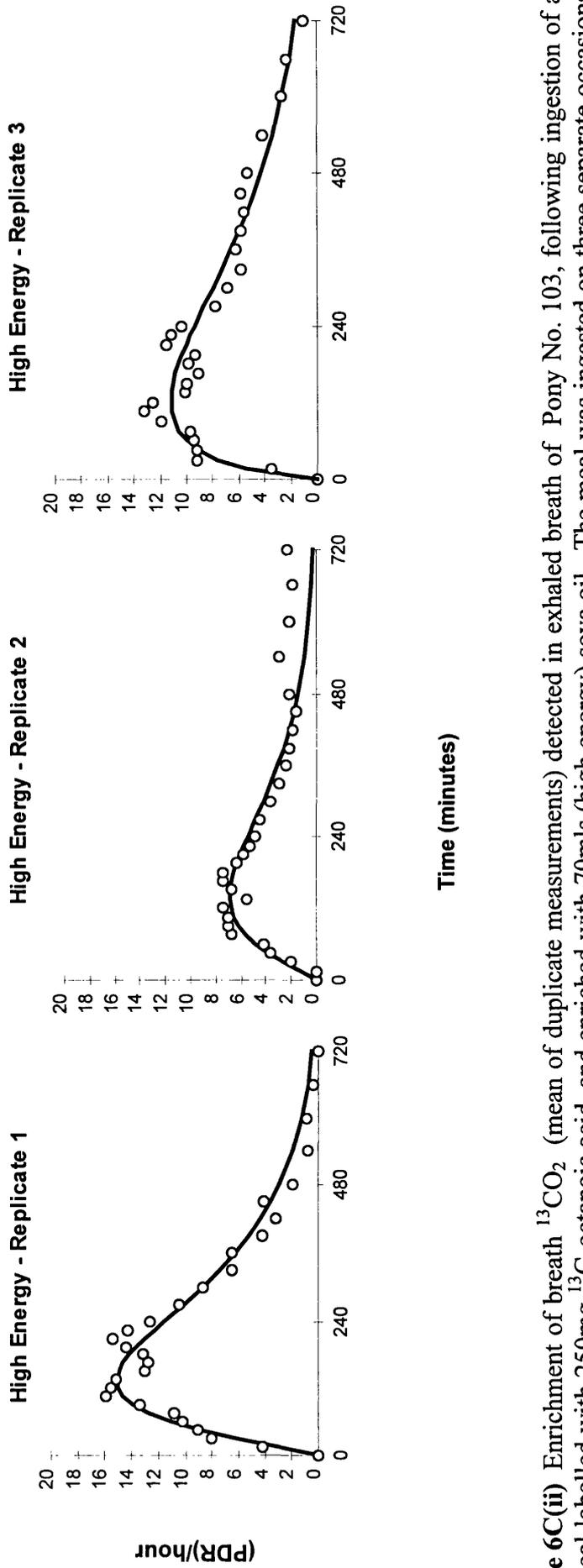
Pony No. 103

Figure 6C(ii) Enrichment of breath $^{13}CO_2$ (mean of duplicate measurements) detected in exhaled breath of Pony No. 103, following ingestion of a test meal labelled with 250mg ^{13}C-octanoic acid, and enriched with 70mls (high energy) soya oil. The meal was ingested on three separate occasions (Replicates 1- 3).

121
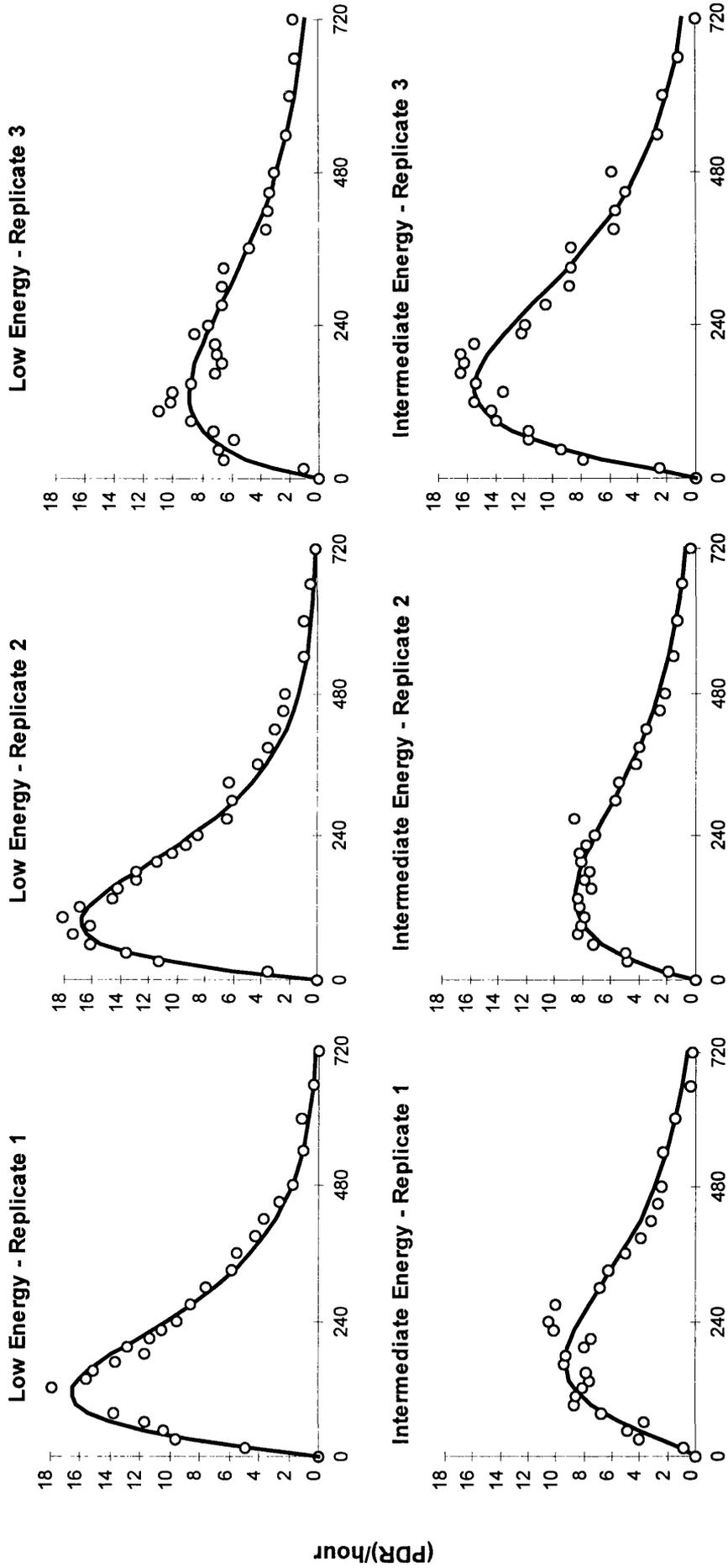
Pony No. 104

Figure 6D(i): Enrichment of breath $^{13}CO_2$ (mean of duplicate measurements) detected in exhaled breath of Pony No. 104, following ingestion of a test meal labelled with 250mg ^{13}C-octanoic acid, and enriched with 0mls (low energy) and 35mls (intermediate energy) soya oil. Each meal was ingested on three separate occasions (Replicates 1- 3).

122
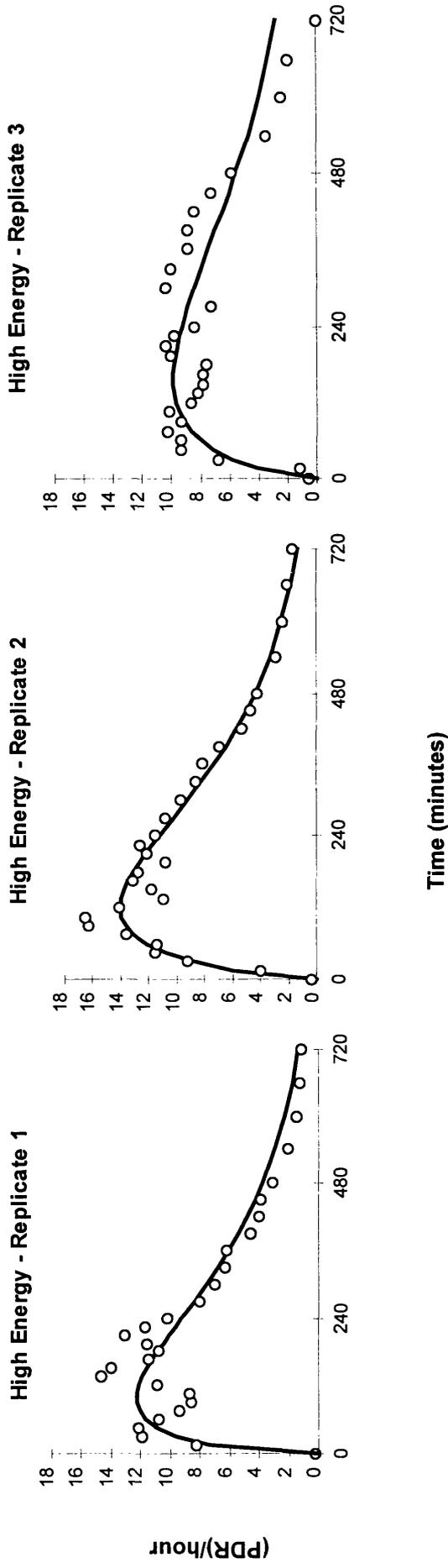
**Figure 6D(ii):** Enrichment of breath $^{13}CO_2$ (mean of duplicate measurements) detected in exhaled breath of Pony No. 104, following ingestion of a test meal labelled with 250mg ^{13}C-octanoic acid, and enriched with 70mls (high energy) soya oil. The meal was ingested on three separate occasion

123

TEST MEAL ALLOCATION

Test Number	Pony Number			
	101	102	103	104
1	Meal 2	Meal 3	Meal 3	Meal 1
2	Meal 3	Meal 1	Meal 2	Meal 1
3	Meal 1	Meal 1	Meal 1	Meal 3
4	Meal 1	Meal 3	Meal 2	Meal 3
5	Meal 2	Meal 2	Meal 3	Meal 2
6	Meal 2	Meal 2	Meal 1	Meal 3
7	Meal 3	Meal 1	Meal 1	Meal 2
8	Meal 0	Meal 3	Meal 3	Meal 2
9	Meal 3	Meal 2	Meal 2	Meal 1

Meal 1   High Energy Density   75g oats + 50g bran + 100mls water + 70mls soya oil
Meal 2   Intermediate Energy Density   75g oats + 50g bran + 135mls water + 35mls soya oil
Meal 3   Low Energy Density   75g oats + 50g bran + 170mls water

APT	Applied potential tomography
BSA	Body surface area
bwt	Body weight
^{13}C-OABT	^{13}C-octanoic acid breath test
*C	Carbon 12 or Carbon 13 or Carbon 14
CV	Coefficient of variation
GDU	Gastro-duodenal ulceration
GEC	Gastric emptying coefficient
hr	Hour
^{111}In DPTA	^{111}Indium diethylene triamine pentacetic acid
IRMS	Isotope ratio mass spectrometry
min	Minute
MMC	Migrating motor complex
MRI	Magnetic resonance imaging
n	Number of observations
NA	Not applicable
NSAID	Non-steriodal anti-inflammatory drug
OGTT	Oral glucose tolerance test
p	Probability
PDR	Percentage dose recovered
POI	Post operative ileus
ppm	Parts per million
SCC	Squamous cell carcinoma
sd	Standard deviation
spp.	Species
^{99}Tc-SC	^{99}Technetium sulphur colloid
t ½	Gastric half emptying time
t(max)	Time of maximal gastric emptying

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