INVESTIGATION OF THE PHYSIOLOGICAL MECHANISMS MODULATING AMINO ACID-INDUCED SATIETY IN HUMAN BEINGS

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To Matt
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Summary

The investigations carried out during the course of this research are concerned with the short-term physiological mechanisms modulating protein and amino acid-induced satiety in human beings, an area which has recently received little attention in the field of appetite regulation.

Research into the effect of the essential amino acid, phenylalanine, on satiety demonstrated that 5 g phenylalanine reduced food intake by 13 % (p<0.05). Phenylalanine (5 g) increased plasma cholecystokinin (CCK) concentrations (basal 1.6 pmol/L ± SEM 0.4; t+35 min 4.6 pmol/L ± SEM 1.1, p<0.05) whereas there was no significant increase following the cornflour control. Aspartame (L-aspartyl-L-phenylalanine methyl ester), previously shown to reduce food intake, was hypothesised to increase CCK secretion. This was not shown in the current research. Both aspartame and its constituent amino acids shortened gastric emptying times (by 8 and 27 % respectively, p<0.005) and suppressed glucagon-like peptide-1 (GLP-1) secretion (by 74 and 59 %, p<0.05) following a test meal. The amino acids also reduced subjective desire to eat following the test meal (p<0.05), and this was associated with a rapid increase in post-prandial plasma phenylalanine levels (p<0.005).

The hormonal and metabolic responses to different protein types and their effect on satiety was investigated using test meals containing casein or whey protein. Previous work had shown that food intake is reduced following a meal containing whey compared to casein. The current work demonstrated that whey test meals led to a 28 % increase in post-prandial plasma amino acid concentrations over 3 hours compared to casein (incremental AUC (iAUC) p<0.05). There was also a statistically significant increase in plasma CCK by 60 % (iAUC p<0.005), GLP-1 by 65 % (iAUC p<0.05) and glucose-dependent insulinotropic polypeptide (GIP) by 36 % (iAUC p<0.01) following the whey meal compared to casein. The two meals also resulted in different time courses for plasma paracetamol concentrations, an indicator of gastric emptying rate. Greater subjective satiety followed the whey test meal (p<0.05).

Electrical impedance epigastrography (EIE), used in the current research for the measurement of gastric emptying, was compared to other methods, firstly with the "gold standard" method scintigraphy, and secondly, with paracetamol absorption and
the $^{13}$C-octanoic acid breath test. EIE distinguished between low and high fat test meals adequately, but half-emptying times were short and correlated poorly with scintigraphy, the breath test, and also paracetamol absorption parameters.

The investigations into protein- and amino acid-induced satiety show that large doses of phenylalanine affect satiety and plasma CCK levels, but smaller doses of phenylalanine, while still enhancing satiety, do not involve these hormonal changes. Studies with whole proteins emphasise the importance of considering the impact of protein type on the appetitive responses to fat and carbohydrate.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2-DG</td>
<td>2-deoxy-D-glucose</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin, 5-hydroxytryptamine</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-isoxazole</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>Analysis of covariance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APE</td>
<td>Atom percent excess</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CHO</td>
<td>Carbohydrate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CR</td>
<td>Coefficient of repeatability</td>
</tr>
<tr>
<td>CSS</td>
<td>Charcoal stripped serum</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAGP</td>
<td>Donkey anti-guinea pig</td>
</tr>
<tr>
<td>DAR</td>
<td>Donkey anti-rabbit</td>
</tr>
<tr>
<td>DEBQ</td>
<td>Dutch eating behaviour questionnaire</td>
</tr>
<tr>
<td>DMN</td>
<td>Dorsal medial nucleus of the hypothalamus</td>
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<tr>
<td>DOPA</td>
<td>Dihydroxyphenylalanine</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3,4-dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>Dipeptidyl peptidase-IV</td>
</tr>
<tr>
<td>DTE</td>
<td>Desire to eat</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylenediaminepentaacetic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EIE</td>
<td>Electrical impedance epigastrography</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GET</td>
<td>Gastric emptying parameter</td>
</tr>
<tr>
<td>GIP</td>
<td>Glucose-dependent insulinotropic polypeptide</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>IAUC</td>
<td>Incremental area under the curve</td>
</tr>
<tr>
<td>KA</td>
<td>Kainic acid</td>
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<tr>
<td>KJ</td>
<td>Kilojoule</td>
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<tr>
<td>LH</td>
<td>Lateral hypothalamus</td>
</tr>
<tr>
<td>LNAA</td>
<td>Large neutral amino acids</td>
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<tr>
<td>NA</td>
<td>Noradrenaline</td>
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<tr>
<td>NGPS</td>
<td>Normal guinea pig serum</td>
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<td>NLE</td>
<td>Norleucine</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NSB</td>
<td>Non-specific binding</td>
</tr>
<tr>
<td>NTS</td>
<td>Nucleus of the solitary tract</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PITC</td>
<td>Phenylisothiocyanate</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>RSCH</td>
<td>Royal Surrey County Hospital</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>T50</td>
<td>Gastric half emptying time</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TFEQ</td>
<td>Three factor eating questionnaire</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VAS</td>
<td>Visual analogue scale</td>
</tr>
<tr>
<td>VMH</td>
<td>Ventromedial hypothalamus</td>
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Chapter One
Chapter 1

INTRODUCTION

The regulation of appetite is an area of research that has preoccupied scientists for decades. Recently it has become increasingly important to understand the regulation of appetite in the light of accelerating levels of overweight and obesity in the UK, and alarming predictions for the next few decades. It is also an area that has caught the attention of the public and media, with more and more newspaper space and broadcasting time devoted to the problem of overweight and obesity.

The two main concepts within the field of appetite research are “hunger” and “satiety”. “Hunger” and “satiety” are distinct terms and should not be regarded as simply the two extremes of a continuum. “Hunger” describes a biological drive to eat and in evolutionary terms is arguably the primary eating drive. Humans evolved in an environment where obtaining enough food was a primary force for natural selection: hunger was dominant and biological systems developed to protect from deprivation. In affluent societies, such systems are increasingly redundant, and may only be irregularly experienced by the well-nourished. For these individuals, the hunger drive may only become dominant in specific physiological states such as rapid growth, or recovery from starvation or illness. The initiation of an eating episode, or meal, does not wholly rely on hunger sensations, since the palatability and sensory properties of the food can stimulate food intake when the hunger drive is not necessarily present. This is often termed the desire to eat, and although it is definitively distinct from hunger, these two terms are frequently used interchangeably. Thus control of food intake in industrialised societies requires systems to minimise excess by inhibiting food intake. “Satiety” describes the inhibition of the hunger drive and desire to eat; this involves metabolic and systemic signals, a sensation of gastric distension (fullness) and the sensory and cognitive suppression of the pleasantness of food. These events, initiated and intensified during a meal, can be collectively termed as the satiation process.

Appetite regulation is a complex function of behavioural factors influencing eating behaviour, and physiological and metabolic factors regulating energy balance and body composition. Figure 1.1 illustrates the complexity behind the term “appetite regulation” and indicates the multitude of psychological and physiological processes involved.
Central nervous system
Neuropeptides - neurotensin, PYY, NPY, substance P, galanin, MCH, CRH, GLP-1, CART, aMSH
Neurotransmitters - dopamine, serotonin, GABA, glutamate

Eating behaviour
Food cues - mouthfeel, palatability
Environmental cues - cultural, social, economic, stress, time of day, smoking
The individual - attitudes to food, psychological factors (emotional, external restraint), eating disorders, obesity, gender

Satiety

Figure 1.1. Mechanisms of food intake regulation. "Appetite regulation" is a complex interaction between a multitude of psychological and physiological processes involved in the regulation of meal initiation through hunger, and meal termination through satiety. The powerful physiological processes that mediate hunger and satiety are modified by a wide range of non-physiological factors which determine eating behaviour. For instance, cultural-environmental influences, e.g. differences in body shape preference or religious taboos; external stimuli, such as the sight of food; and internal emotions may all influence food intake. Food itself can influence appetite through sensory properties and palatability. Sensory specific satiety, the decrease in perceived pleasantness of a food that occurs during the course of a meal, eventually inhibits eating. The perceived sensory qualities of food can increase desire to eat, and this is linked to the cephalic phase response (where the sight, smell or thought of food is known to trigger physiological responses in the body in preparation for a meal). Hunger or a desire to eat leads to meal initiation and ingestion of food, and involves signals that derive from tissue macronutrient stores monitoring. Food macronutrients enter the stomach and cause gastric distension, generating immediate satiety signals sent via neural pathways to the brain. Macronutrients (apart from alcohol) in the intestine produce short-term satiety signals via the enteric and sympathetic nervous system, and by inducing the secretion of gastrointestinal hormones and insulin. Absorbed nutrients are transported in the circulation to be taken up into the liver and other tissues for storage or metabolic consumption via oxidation. Hepatic metabolic consumption appears to be specifically important for monitoring fuel balance and signalling to the CNS to control food intake. Body pools of carbohydrate, fat and protein each appear to influence appetite. Depletion of glycogen may trigger an increase in food intake to replenish carbohydrate stores (Flatt, 1996). Adipose tissue secretes the hormone leptin, which is responsible for long-term regulation of body fat. Signalling by the concentration and pattern of circulating amino acids to the CNS may acutely control food intake. Changes in the net flux between circulating and tissue protein-bound amino acids especially skeletal muscle, as determined by overall nutritional state and stage of growth and development, may also signal longer-term food intake possibly via changes in the circulating free amino acid pool. Neuropeptides and neurotransmitters within the brain participate in the conversion of these peripheral neural and hormonal monitoring signals to feelings of hunger or satiety.
The following introduction will briefly summarise present knowledge of the control of appetite, particularly in regard to the role of protein/amino acids. Peripheral and central factors involved in protein/amino acid-induced appetite control such as gastric emptying, gut hormones and neuronal stimulation will be discussed. In addition to the physiological mechanisms, psychological aspects of satiety will also be briefly addressed – both areas that are pertinent to current research in this report.

1.1 Theories for the control of appetite

Metabolic control mechanisms have been previously discussed under four main headings. These relate to body stores of the three principal macronutrients (or their precursors) and to energy balance regulation. Some of the major hypotheses are outlined below.

1.1.1 Energostatic theory

The energostatic hypothesis is based on the theory that the energy content of a meal is the key factor that regulates food intake. The macronutrients – protein, carbohydrate, and fat – would each be expected to exert equal amounts of negative feedback to suppress further food intake if a positive energy balance was reached. Evidence from a few studies suggested that individual macronutrients were having no differential effect on satiety where the fat and carbohydrate intake ratio were manipulated in human beings (Van Stratum et al., 1978; Goldberg et al., 1998).

Many researchers dispute the idea of an energostatic control of food intake. It has been suggested that habituation to eating a constant weight or volume of food may lead to passive overconsumption if energy density is increased (Poppitt & Prentice, 1996). Much work has been done to show that each macronutrient has a different effect on food intake. A high fat, and therefore energy dense diet leads to passive overconsumption in animals and human subjects (De Castro, 1987; Lissner et al., 1987; Tremblay et al., 1989; Tremblay et al., 1991; Thomas et al., 1992; Rolls et al., 1994). Protein and carbohydrate seem to suppress subsequent energy intake to a greater extent than fat (Lissner et al., 1987; Tremblay et al., 1989; van Amelsvoort et al., 1990; Thomas et al., 1992; Blundell et al., 1993; Rolls et al., 1994; Stubbs et al., 1996). In the light of these findings, most investigations have been directed at the macronutrient composition of a diet rather than energy content when looking at the control of food intake.
1.1.2 Glucostatic Theory

The original glucostatic theory proposed by Jean Mayer (Mayer, 1955) was that an increase in peripheral glucose utilisation would suppress subsequent food intake, and a decrease in peripheral glucose utilisation would stimulate feeding. These changes would be detected by gluco-sensitive receptors in the brain and the liver, and were perhaps complementary to a longer-term lipostatic mechanism for the control of energy intake (Van Itallie, 1990). The glucostatic model was modified into a glycogenostatic model by Russek (Russek, 1963), who proposed that hepatic gluco-sensors are involved in the control of feeding. Flatt (Flatt, 1987; Flatt, 1996) developed a two-compartment model for a glycogenostatic mechanism using the idea that the respiratory quotient must match the food quotient for fuel mix in order to maintain energy balance. A high fat, low carbohydrate diet would be predicted to deplete glycogen stores, which would trigger an increase in food intake to replenish carbohydrate reserves. Thus it would be predicted that hunger would be driven by carbohydrate reserves.

There is much human and animal experimental evidence in support of the glucostatic theories. Peripheral administration of glucose in rats suppresses food intake (Booth & Jarman, 1976; Novin & Vanderweele, 1977; Tordoff et al., 1989) and central injections of glucose anti-metabolites like 2-deoxy-D-glucose (2-DG) stimulates glucoprivic feeding (Oomura, 1983). Experiments with 2-DG in the liver have also provided evidence for hepatic gluco-sensitive receptors in the control of feeding in animals (Laanghans, 1996). Transient declines in blood glucose in rats precede a meal, with a transient spike in insulin just before the glucose decline (Campfield & Smith, 1990). It was proposed that CNS glucose receptors are triggered by these transient declines, to initiate feeding, based on the observation that vagotomised rats are not as sensitive to transient declines in blood glucose in their feeding behaviour. Similar transient declines in blood glucose in human beings were then observed just before a meal request or a change in hunger (Campfield, 1997). 2-DG infusions in healthy men increased hunger and decreased sensations of fullness (Thompson & Campbell, 1977). In the 1950s, arteriovenous glucose differences were measured to represent the rate of tissue removal of glucose (Van Itallie, 1990). Good correlations between sustained elevations of peripheral arteriovenous differences and satiety, and between sustained negligible peripheral arteriovenous differences ("metabolic hypoglycaemia") and hunger were reported. However, later studies found
that these differences didn’t consistently correlate with hunger and satiety (Stunkard & Wolff, 1954; Bernstein & Grossman, 1956).

Insulin may be important in the control of food intake. Its reduction of satiety in rats (Woods & Porte Jr., 1983), and the relationship between fasting insulin and adiposity (Jen et al., 1985), provoked a specific insulinostatic theory of feeding behaviour. Woods and Porte (1983) proposed that increased circulating insulin following a meal entered the brain via the cerebrospinal fluid, modulated by the amount of adipose tissue. The discovery of insulin-responsive neurons in the paraventricular nucleus (PVN) of the medial hypothalamus supported this hypothesis (Oomura, 1983). It was later suggested that insulin may be transported into the brain by insulin receptors present in the endothelial cells (Woods et al., 1990). However, as regards the idea of insulin regulating appetite via a role as an adiposity signal, as suggested by Woods and Porte (Woods & Porte Jr., 1983), there seems to be little evidence to support this. A new adipose tissue-linked factor has now been discovered— the \( \text{ob} \) gene and its protein product leptin — and this has recently been the subject of many insights in the field of appetite regulation (Campfield et al., 1995; Halaas et al., 1995, Pellymounter et al., 1995).

1.1.3 Lipostatic theory

When reviewing the evidence for a glucostatic regulation of food intake (particularly the investigations that addressed the relative satiating properties of the macronutrients) there is little to support a lipostatic theory, since fat is a poor regulator of energy balance. The original hypothesis (Kennedy, 1953) proposed that there might be a centre in the brain that monitored plasma levels of fat metabolites, which were directly proportional to fat stores. However, Welch and colleagues (Welch et al., 1985) found that intravenous administration of lipid in human subjects failed to produce any satiety effects.

The most convincing evidence for a body fat-derived signalling pathway for the control of appetite, in accordance with Kennedy’s original hypothesis, comes from the study of hormones. Insulin was regarded as an important signalling of adiposity, since it is secreted in proportion to body fat and insulin receptors are expressed in various regions of the brain (Jen et al., 1985; Woods & Seeley, 2000). In support of this, insulin has been shown to reduce food intake under some
circumstances (Woods & Porte Jr., 1983; Woods & Seeley, 2000). Amylin is another suggested candidate, as this pancreatic β-cell polypeptide is also secreted in proportion to body fat and is transported into the brain (Woods & Seeley, 2000). Amylin strongly inhibits gastric emptying (Young et al., 1996), and peripheral and central administration decreases food intake in rats (Young, 1997). The discovery of leptin, a hormone that is actually secreted by adipose tissue, is transported into the brain, and decreases food intake when centrally administered, heralded the strongest candidate for a circulating adiposity signal yet (Campfield et al., 1995; Halaas et al., 1995, Pellymounter et al., 1995). Leptin is also distinct in that it is a chronic regulator of body weight, rather than signalling short-term satiety signals as insulin and amylin are postulated to do.

### 1.1.4 Aminostatic Theory

There is abundant animal and human evidence that varying dietary concentrations of either protein or amino acids can determine short and long-term appetite regulation.

#### 1.1.4.1 The original theory

The animal literature on food selection and intake regulation is extensive in relation to both amino acid imbalance (e.g. Harper et al., 1970; Harper & Peters, 1989; Gietzen, 1993), and variation in protein concentration (reviewed in Millward, 1995). Early studies revealed that feeding behaviour was suppressed by dietary amino acid imbalance (Leung et al., 1968a; Leung et al., 1968b; Leung et al., 1968c), and very high protein diets (Harper et al., 1970; Rogers & Leung, 1973; Gurr et al., 1980) with self-selection of an optimum protein intake over lower or high protein concentrations (Peng et al., 1975).

It was Mellinkoff who originally suggested that there might be an aminostatic mechanism controlling appetite and food intake. In a series of experiments on human volunteers (Mellinkoff et al., 1956), feelings of hunger increased after ingestion of a protein-rich breakfast as serum amino acid levels declined. Intravenous infusion of amino acids and glucose produced similar relationships, and although appetite decreased with increasing blood glucose the correlation was weaker than that for amino acids. Administration of casein hydrolysate intravenously or by mouth also led
to diminished feelings of hunger as plasma amino acid concentrations rose, and even as blood glucose levels decreased.

Several subsequent studies, although not all, have confirmed these initial findings. Thus with protein, a reduced short-term food intake and/or increased subjective satiety ratings, has been shown following a protein preload or during a high protein meal compared to fat or carbohydrate, (Booth et al., 1970; De Castro, 1987; Barkeling et al., 1990; Hill & Blundell, 1990; Johnstone et al., 1996; Vandewater & Vickers, 1996; Stubbs et al., 1996; Porrini et al., 1997; Latner & Schwartz, 1999). The minority of reports that do not show such effects, (Sunkin & Garrow, 1982; Geliebter, 1979; De Graaf et al., 1992), are characterised by differing experimental designs and type, size and timing of preload in relation to test meal. Individual amino acids also suppress food intake in the case of tryptophan (Hrboticky et al., 1985; Hill & Blundell, 1988; Muurahainen et al., 1988) and phenylalanine (Muurahainen et al., 1988; Ballinger & Clark, 1994). Subjects also reduced their food intake half an hour after an oral load of mixed amino acids (phenylalanine, valine, methionine, and tryptophan) compared to the placebo (magnesium trisilicate) in a study by Butler and colleagues (Butler et al., 1981).

1.1.4.2 Why is the regulation of protein and amino acid intake important?

An aminostatic-mediated inhibition of food intake by dietary protein is to be expected as part of amino acid and protein homeostasis. The accumulation of certain free amino acids has to be avoided as the aromatic, branched chain and sulphur-containing amino acids are toxic at high concentrations (Millward & Rivers, 1988). The capacity for disposal of protein through storage is limited with a fixed upper limit of the skeletal muscle mass as a function of height (Millward, 1995). While disposal of excess dietary protein as fat can occur biochemically, the lipogenic pathway is energetically expensive so that energy storage of dietary protein as fat is inefficient (Millward et al., 1974). Furthermore, simple use of dietary protein as a metabolic fuel is disadvantageous compared with glucose and fat since amino acid oxidation includes a number of oxidative steps not linked to ATP production. Thus complete oxidation of amino acids is inherently less efficient than glucose or fat and would be associated with greater levels of thermogenesis. Even without the need for amino acid disposal to avoid toxicity, the energy cost of the ureagenesis, gluconeogenesis and lipogenesis required to dispose of surplus amino acids, or the increased
thermogenesis associated with their complete oxidation, is such that the evolution of a regulatory system to limit protein intake rather than glucose and especially fat, would be expected.

With bodily function dependent on adequate cellular protein, a hunger drive in growing animals which varies with the rate of protein deposition in lean tissue might also be expected, and is indeed found (Webster, 1993). This has been reviewed previously (Millward, 1995), where it was stated that the nutrient requirements of a growing animal are determined principally by its impetus for lean tissue growth, with appetite and meal size dependent upon the dietary protein level, when this is below the optimal requirement level. It is thought that a similar process occurs in human beings during recovery from malnutrition, thereby allowing catch-up growth (Ashworth & Millward, 1985). However, these increases in hunger drive associated with net protein accretion relate to nutritional circumstances that may never be experienced in the life of an adult human being.

1.1.4.3 Recent modifications to the aminostatic theory

Many of the original theories of appetite, including Mellinkoff’s (Mellinkoff et al., 1956), had the concept of central monitoring of circulating substrate levels as their central premise. There is now growing evidence for appetite signalling pathways originating from all macronutrients at the level of terminal fuel oxidation (Friedman, 1998), i.e. by hepatic ATP availability. It has been shown that metabolic blockers used with glucose and fatty acids in animal models lead to an increase in hunger and food intake (Novin & Vanderweele, 1977; Friedman et al., 1990). Some evidence for a satiety effect of hepatic fatty acid oxidation exists in human beings, with medium chain triglycerides, which are more readily oxidised, suppressing food intake in non-dieting humans (Stubbs & Harbron, 1996), and the stimulation of eating in men (especially those that have a high baseline level of fatty acid oxidation) by the use of a fatty acid oxidation inhibitor (Kahler et al., 1999). Stubbs developed this approach (Stubbs, 1995; Stubbs, 1998), so that the extent to which the three macronutrients are able to act as precursors for oxidative metabolism, as opposed to being stored, determines their satiating power through some cellular energy balance-sensing mechanisms. Regulation of food intake (usually inhibition) in relation to dietary macronutrient composition is described in terms of a protein>carbohydrate>fat hierarchy of macronutrient satiating efficiency reflecting the extent to which their
metabolic pools are more tightly autoregulated by oxidation, i.e. oxidation increases with intake. Fat, the least satiating nutrient, is primarily stored when ingested with other macronutrients, and an increase in fat oxidation does not necessarily follow a fat-rich eating episode (Friedman, 1990; Himaya et al., 1997; Stubbs, 1998) unless the fat is easily oxidised as with dietary medium chain triglycerides. Thus protein, the macronutrient with the least flexible autoregulation in the body, is also the most satiating macronutrient in this “hierarchical oxidation hypothesis”.

There are a number of problems with this theory in terms of protein-related food intake regulation. This model is focused on fuel oxidation as the key regulatory metabolic process, but the supporting experimental studies concentrate entirely on glucose and fat oxidation (Ritter & Calingasan, 1994; Friedman, 1998). It has not been demonstrated experimentally that the inhibition of amino acid oxidation increases food intake. The physiological relevance of inhibiting fuel oxidation is questionable in any case, since oxidation rates are linked to fuel availability, i.e. a decrease in food intake would lead to reduced fuel oxidation, not a complete cessation of oxidation. Increased food intake, associated with a greater rate of fuel oxidation, would be expected to increase satiety, but it is not yet clear whether increased levels of fuel oxidation are directly responsible for satiety. In fact, it can be shown that an increase in amino acid oxidation does not always cause a decrease in food intake. A study in malnourished children showed that when two levels of protein were fed during catch-up growth, similar intakes of energy intake occurred with quite different levels of oxidation (Badaloo et al., 1999). The relative ease with which amino acids can be disposed of either as protein deposition or oxidation may be the important factor in aminostatic appetite regulation. In this way, the key metabolic responses sensed by the body relate to the changes in free amino acid pool sizes subsequent to feeding, similar to Mellinkoff’s original theory.

1.1.4.4 Adaptive changes in protein metabolism and protein-related satiety

There is a small amount of evidence that variation in long-term protein intake in healthy adults influences food intake. Protein:energy ratio was weakly negatively correlated with total energy intake \((r = -0.45)\) in free-living post-menopausal women; in contrast, the carbohydrate:energy ratio was unrelated \((r = 0.00)\) while the fat:energy ratio was very weakly positively correlated with energy intake \((r = 0.18)\) (Bingham et al., 1994). Protein intakes vary within society (mainly a function of
levels of meat intake) and the weak relationship between protein:energy ratio and energy intake in this sub-group is surprising in view of the fact that protein is the most satiating macronutrient. The adaptive changes in amino acid metabolism that occur in response to changes in habitual protein intake ought to be taken into consideration when attempting to unravel aminostatic mechanisms.

One adaptive response to a habitually high dietary protein intake is an increasing capacity to dispose of dietary amino acids (Price et al., 1994; Millward, 1998). This includes a greater capacity for amino acid oxidation as a result of adaptive changes in key enzymes in catabolic pathways for amino acids occurs (Millward & Rivers, 1988), not only after feeding (as would be expected from subjects in overall nitrogen balance) but also increasing postabsorptive oxidation rates. This in turn results in an increasing capacity for postprandial protein gain. Thus the amplitude of the diurnal changes in body protein (Garlick et al., 1973; Millward et al., 1974; Clugston & Garlick, 1982; Rennie et al., 1982; Hoffer et al., 1985) increases with protein intake (Price et al., 1994). Therefore, with greater dietary protein intakes, the increasing ease of its disposal through transient deposition and oxidation via adaptation may equalise the relative satiating power of a wide range of different dietary protein concentrations. Given this adaptive variation in amino acid disposal, the satiating effect of a fixed protein intake is therefore likely to vary inversely with habitual protein intake. Protein-related appetite mechanisms in the longer term are thus more likely to be observed when habitual protein intakes change. There is evidence to support this prediction from measurements of the effect of habitual dietary protein intake on subjective ratings of appetite (Long et al., 2000a). As shown in figure 1.2, a high protein meal was more satiating to subjects habituated to a low compared with a high protein diet especially after their dietary intake was manipulated to further increase the differences in protein intakes.
Adaptive changes in amino acid oxidation and diurnal protein gains and losses are not the only possible explanation of the findings of Long *et al.* (2000a). The protein-induced delay in gastric emptying was attenuated over time in rats following a habitual high-protein diet (Shi *et al.*, 1997). Thus subjects who habitually consumed a high protein diet could exhibit faster gastric emptying rates for the protein meal with less gastric distension.

1.2 Peripheral Mechanisms

1.2.1 Pre-absorptive mechanisms for protein and amino acid induced satiety

Of the many potential mechanisms by which satiety signals may be generated in response to protein ingestion and amino acid absorption, it is useful to separate those that may originate pre- or post-absorptively. There is much to indicate that protein-induced satiety includes a gut-mediated component, through an intestinal neuronal response, hormonal/neuropeptide responses (specifically cholecystokinin
(CCK)), and via gastric emptying which is in turn controlled by both neural and hormonal/neuropeptide pathways. This arrangement of pre-absorptive signalling pathways from the gastrointestinal tract has been conceptualised by Smith (Smith, 2000) into a model for the direct control of meal size (the effect of nutrients on vagal mechano- and chemo-receptors and various hormones/neuropeptides/neurotransmitters that are triggered by nutrients contacting with mucosal receptors in the gastrointestinal tract), requiring only the hindbrain; this is modulated by indirect controls, e.g. metabolic, cognitive.

1.2.1.1 Neuronal responses

Nutrients entering the gastrointestinal tract trigger a sequence of neural pathways that signal elsewhere in the gut or to the brain. Starting in the stomach, there are vagal afferent nerve endings in the circular and longitudinal muscle, the myenteric plexus, the submucosa and mucosa, indicating that these neurons send messages to the nucleus of the solitary tract (NTS) in response to gastric distension and contraction, enteric neural transmissions (from elsewhere in the gastrointestinal tract) and direct nutrient-chemical stimulation from the lumen. There is a dense distribution of vagal afferent nerve fibres around the pylorus and upper duodenum, with the density gradient lessening in the distal duodenum. As in the stomach, these fibres innervate several regions including the muscle layers, the myenteric plexus and submucosal plexus, mucosal crypts, the submucosa, the lamina propria - even as far as the basal lamina of the luminal epithelial cells (Schwartz, 2000). These vagal afferent terminations also innervate the intestine down to the jejunum and ileum. Duodenal afferent responses can be mechanical and chemical, with nutrient-specific chemoreceptors on vagal afferent fibres to intestinal infusions of carbohydrates (Mei, 1978), amino acids (Jeanningros, 1982) and lipids (Randich et al., 2000). It was also shown that there are low affinity CCK-A receptors (see section 1.2.1.2.1) on vagal afferent terminations situated near to CCK-secreting cells, an important pathway whereby nutrient- and distension-induced satiety is enhanced by endogenous production of paracrine CCK in response to a meal (Moran et al., 1992; Weatherford et al., 1993; Schwartz, 2000).

With regard to aminostatic mechanisms of appetite control, phenylalanine and tryptophan appear to be particularly potent in the suppression of food intake in either sham or naturally feeding rats when the intestine is infused with amino acids (Meyer
et al., 1998). There is evidence that this is a preabsorptive effect. Intestinal infusion of amino acid mixtures or individual amino acids in anaesthetised cats increases the firing rate of many vagal neurons as a very early response (Jeanningros, 1982), with 37% of lateral hypothalamus neurons responding with a short latency (Jeanningros, 1983). In comparison, neurons of the ventromedial hypothalamus (VMH) took much longer to respond and far fewer responded, suggesting this to be a postabsorptive effect of the intestinal infusion. This experiment provides good evidence that amino acids can be monitored in the intestine before absorption, and that the exact mechanism involves vagal afferent neurons that relay signals to the lateral hypothalamus.

1.2.1.2 Hormonal responses

The regulation of food intake by circulating physiological factors can be roughly grouped into gastrointestinal hormones, neuropeptides, neurotransmitters and other circulatory central and peripheral factors (see figure 1.1). None of these categories are exclusive, and many of these factors are interdependent. Two important satiety gut peptides will be described here.

1.2.1.2.1 Cholecystokinin (CCK)

CCK is a hormone and neuropeptide released following a mixed meal, and it is considered to be a major satiety factor. Many different forms of CCK have been identified, with CCK-8 being the most potent, and CCK-33 and -58 the most prominent in human plasma (Liddle, 1997). Apart from the satiety action of CCK, this peptide also stimulates gallbladder contraction (Liddle et al., 1989), the secretion of pancreatic enzymes (Schmidt et al., 1991), delays gastric emptying (Jin et al., 1994) and inhibits gastric acid secretion (Konturek et al., 1993).

CCK is produced by endocrine cells in the duodenum and proximal jejunum, with a graded increase in concentration towards the top of the small intestine, and also by some pituitary and adrenal medullary cells. CCK is one of a group of peptides termed "brain-gut peptides" and is distributed throughout the brain (Moran & Schwartz, 1994). CCK is also distributed throughout the peripheral autonomic nervous system, specifically the vagus nerve, coeliac plexus, submucous plexus, myenteric plexus, and neurons around the islets of Langerhans in the pancreas (which
may reflect the ability of CCK to stimulate insulin and glucose release) and in the circular muscle of the distal small intestine and colon.

CCK release is stimulated by the entry of fat and protein into the duodenum. Perfusion of the duodenum with amino acids increased CCK secretion, especially following phenylalanine, methionine and valine (Go et al., 1970). The potent effect of intraduodenal phenylalanine on CCK release in human beings was later confirmed (Owyang et al., 1986), and was associated with a reduction in food intake (Ballinger & Clark, 1994). A recent study has provided evidence that phenylalanine stimulates CCK secretion via a calcium-dependent mechanism in the CCK-secreting cells (Mangel et al., 1995). Intraduodenal perfusion of trypsin has been shown to suppress the secretion of CCK in human subjects (Kanarek et al., 1995). It was proposed that there is a trypsin-sensitive releasing factor for CCK, secreted into the lumen of the small intestine by the pancreas – this would be degraded by trypsin but protected by the entry of protein into the gut (Calam et al., 1987). Since CCK stimulates pancreatic secretion and trypsin would block the release of CCK, this proposal provides a possible mechanism for the negative feedback regulation of CCK.

Evidence in animals for a satiety effect of CCK is strong (Gibbs et al., 1973) but the importance of CCK as an endogenous mediator of hunger and satiety in human beings is less clear. It was shown that peripheral CCK administration reduced appetite in some human studies (Stacher et al., 1979; Kissileff et al., 1981; Pi-Sunyer et al., 1982), but other researchers challenged their findings. Exogenous CCK administration may have produced a pharmacological response rather than a physical one in these experiments (Schick et al., 1991), and effects on food intake or appetite ratings may have been mostly due to the aversive and sedative effects produced. Evidence that CCK administration produced conditioned taste aversions was presented, but subsequent studies failed to replicate this effect with doses that could suppress food intake by up to 50% (Kulkosky, 1985).

The stimulation of endogenous CCK secretion by intraduodenal administration of phenylalanine strongly reduced food intake and increased subjective sensations of fullness (Ballinger & Clark, 1994); and peripheral administration of physiological levels of CCK also decreased food intake (Ballinger et al., 1995) and increased satiety sensations in human subjects (Lieverse et al., 1994; Lieverse et al.,
Any further doubts over the importance of endogenous CCK in mediating satiety were dismissed using a different methodological tool – CCK antagonists.

There are two types of CCK receptor: CCK-A receptors (peripheral receptors responsive to the sulphated tyrosine moiety of CCK-8 and CCK-33, found in the pancreas and on vagal afferent and enteric neurons in the small intestine and stomach, and also in some brain sites) and CCK-B receptors (predominantly central receptors, binding gastrin, desulphated CCK and CCK fragments, found in brain, afferent vagus nerve and within stomach). Studies with loxiglumide in human subjects, an antagonist that inhibits CCK-A receptors, found that it accelerated gastric emptying (GE) (Meyer et al., 1989), and increased food intake (Lieverse et al., 1994). However, a similar effect on food-stimulated GE and satiety was not observed in all studies (Corazziari et al., 1990; Konturek et al., 1990; Drewe et al., 1992; Lieverse et al., 1995). CCK-A receptors exist in low and high affinity forms and antagonist studies that differentiate between these types have shed light on the inconsistencies previously found for type A receptors. The antagonist effects of CCK-JMV-180 to endogenous CCK (an antagonist at the low affinity CCK-A receptors and an agonist at high affinity CCK-A receptors) implied that endogenous CCK acts on low affinity CCK-A receptors. Since the physiological concentrations of CCK normally present in blood plasma are only sufficient to stimulate high affinity CCK-A receptors, it is thought that endogenous CCK acts to decrease food intake via paracrine and neurocrine pathways rather than endocrine pathways (Moran, 2000).

Overall, there is good experimental evidence for a CCK-mediated satiety mechanism, but presenting a clear picture of how CCK can exert its effects is problematical. CCK acts peripherally on satiety, gastric emptying and gastric secretion via CCK-A receptors on afferent vagal fibres and circular muscle cells in the pylorus (French et al., 1993; Schwartz & Moran, 1994; Moran, 2000). Total vagotomy has been shown to block the satiety effect of CCK in the rat (Lorenz & Goldman, 1982), although if vagal transection was limited to the gastric branches the CCK action was still blocked effectively (Smith et al., 1981). The vagal afferent satiety response to CCK is further potentiated by gastric loads, which became more effective in increasing activity in vagal afferent fibres when different doses of CCK were administered locally (Moran & Schwartz, 1994). Moran and colleagues found that pylorectomised rats still reduced their food intake at low doses of CCK but the
dose response effect was curtailed at a higher CCK dose (Moran et al., 1988). In contrast, rats with afferent vagotomies reduced the potency of low and high doses of CCK, with low doses leaving food intake unaffected (Moran et al., 1997). This led Moran to postulate that low doses of CCK activate CCK-A receptors on vagal afferent fibres to reduce food intake, and that higher doses of CCK only reduce food intake indirectly via pyloric CCK-A receptors, and the consequent inhibition of gastric emptying (Moran, 2000). In this way, it is hypothesised that CCK can influence appetite directly and via inhibition of gastric emptying.

The role of central CCK systems in satiety has been the subject of some attention, especially as the brain contains sites where CCK-A receptors have been detected (nucleus accumbens, the dorsal medial nucleus of the hypothalamus (DMN), and NTS) (Hill et al., 1992). However, although studies on farm and experimental animals indicate central CCK-mediated satiety (Crawley & Corwin, 1994; Baldwin et al., 1998), a study by Corp and colleagues found no evidence for this in rats using CCK-A and CCK-B receptor antagonists (Corp et al., 1997).

In conclusion, the importance of CCK in the regulation of food intake cannot be disputed, and it is increasingly apparent that there is a large amount of interaction with other hormones and neuromodulators, a huge area of research that is still rapidly expanding.

1.2.1.2.2 Glucagon-like peptide-1 (GLP-1)

GLP-1 (7-36 amide) is a relatively recently discovered gastrointestinal hormone, now agreed to be the major incretin factor in human beings, i.e. it potentiates glucose-induced insulin secretion (Nauck et al., 1993; Wang et al., 1995; Scrocchi et al., 1996). It is released from L-cells in the distal small intestine and colon into the circulation in response to mixed meals, especially if high in fat content, or oral carbohydrate loads (Orskov et al., 1991; Elliott et al., 1993). The mechanism of GLP-1 release from ileal L cells is under debate since the peptide is rapidly secreted following ingestion of a meal, long before the digesta has reached the distal small intestine. Evidence for similar GLP-1 levels in ileostomy patients and normal subjects further diminishes the likelihood of direct nutrient stimulation of the L cell being a primary mechanism for GLP-1 release (D'Alessio et al., 1993). GLP-1 may be released in response to stimulation by GIP (Damholt et al., 1998), especially as
immunocytochemical research (Damholt et al., 1999) has shown that the GIP K cells and GLP-1 L cells overlap in the jejunum (30% of the L cells in the middle intestine are adjacent to K cells), suggesting the existence of a paracrine action. Researchers have recently hypothesised that a neuroendocrine loop stimulates the L cells when nutrients enter the duodenum, involving the stimulation of GIP secretion, which acts on vagal afferents to send a signal to the brain in rats (Rocca & Brubaker, 1999). A vagal efferent signal is then postulated to act via gastrin-releasing peptide (GRP) neurons in the myenteric plexus to stimulate the L cell in the distal small intestine (Rocca & Brubaker, 1999).

GLP-1 exerts a number of other effects in addition to stimulation of insulin secretion, including inhibition of gastric acid secretion (Wettergren et al., 1993; Layer et al., 1995; Fung et al., 1998), inhibition of food intake (Schick et al., 1992), and delaying of gastric emptying (Wettergren et al., 1993; Schirra et al., 1996; Young et al., 1996; Imeryuz et al., 1997). Specifically, GLP-1 delays gastric emptying by inhibiting antro-duodenal contractility, which reduces emptying rate, and stimulating pyloric contractions to reduce the volume emptied (Schirra et al., 2000).

Plasma concentrations of intact GLP-1 are very low due to rapid metabolism by dipeptidyl peptidase-IV (DPP-IV), which cleaves off the two N-terminal amino acids (Mentlein et al., 1993). It is thought that this rapid degradation of active GLP-1 occurs within 1 to 1.5 min of entering the circulation, so that the majority of GLP-1-induced effects on gastrointestinal motility and secretion occur before the molecule has left the gut, i.e. by interacting with sensory nerve endings in the lamina propria before entering the capillaries (Holst, 2000). In support of this, vagal afferent denervation in rats abolished the gastric inhibitory effects of GLP-1 (Imeryuz et al., 1997). The GLP-1 receptor is expressed in areas of the brain that theoretically could be accessed by peripheral GLP-1 through leaks in the blood brain barrier (the subfornical organ and area postrema) (Orskov et al., 1996), and also areas of the brain that can only be stimulated by centrally-secreted GLP-1, including the hypothalamus (Kanse et al., 1988; Kreymann et al., 1989; Larsen et al., 1997) and the PVN and the antral nucleus of the amygdala (Turton et al., 1996). The importance of central GLP-1 is further supported by a study on rats where central GLP-1 reduced food intake but intraperitoneal GLP-1 did not have any effect on feeding (Oomura, 1983). When exendin was used to block the central inhibitory effect of GLP-1, food intake was
more than doubled in satiated rats, although there was little effect in fasted rats. This, and other studies on central GLP-1 in rats, suggests that GLP-1 may be an important satiety factor (Gunn et al., 1996; Tang-Christensen et al., 1996).

However, Thiele and colleagues (Thiele et al., 1997) showed that intraventricular GLP-1 but not leptin produced a conditioned taste aversion in rats. This means that it is possible that the reduced food intake observed in these animals after the central infusion of exogenous GLP-1 may reflect aversive effects rather than a biological satiety response. It can also be said that the demonstration of a conditioned taste aversion at high doses of central GLP-1 does not rule out the possibility that GLP-1 can independently induce satiety at lower doses, and that central and peripheral mechanisms for a GLP-1-induced suppression of food intake may co-exist. Other researchers have shown a reduction in food intake and subjective appetite ratings in human beings following peripheral administration of GLP-1 (Flint et al., 1998; Naslund et al., 1998; Gutzwiller et al., 1999). It is impossible to say from the literature available whether the satiety effect is independent of gastric inhibitory effects.

1.2.1.3 Gastric emptying and gastric distension

1.2.1.3.1 The stomach

The stomach is a J-shaped, sac-like organ, and is located between the oesophagus and small intestine in the upper abdomen. Its functions are to act as a reservoir for ingested food, to break down the food into small particles, and to deliver to the small intestine in a form of a solution called chyme. The stomach can be divided into anatomical parts (see figure 1.3).
The surface of the gastric mucosa is covered by columnar epithelial cells, and the epithelial layer turns in on itself into the mucosa to form tubular glands, or gastric pits. Mucous, alkaline fluid, hydrochloric acid, intrinsic factor, pepsinogen and gastrin are all secreted from the stomach. The stomach is innervated by sympathetic and parasympathetic nerve fibres which relay long reflexes via the CNS, and the enteric nervous system (consisting of the myenteric and submucosal plexuses) which relay short reflexes located wholly within the gastrointestinal tract wall. Afferent nerves carry signals to the brain from chemo-receptors and mechano-receptors in the stomach wall and efferent nerves lead to motor, endocrine, secretory and vascular responses in the stomach.

The peristaltic waves that originate in the body of the stomach produce powerful contractions in the antral area, which mixes the stomach contents and closes the pyloric sphincter. This forces the liquid chyme to be expelled into the duodenum and also forces the larger particles of digesta back into the body of the stomach for further dissolution. The basic electrical rhythm generated by the pacemaker cells in the longitudinal smooth muscle layer determines gastric peristaltic wave frequency (three peristaltic waves per minute). Contraction strength depends on the frequency of action potentials triggered by the slow waves, which are influenced by excitatory

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**Figure 1.3.** The anatomy of the stomach showing the three gastric regions: fundus, body and antrum. Reproduced from Vander, A.J. (1994), Human physiology: the mechanisms of body function, 6th ed. New York, London: McGraw-Hill.
neurotransmitters and hormones acting on the antral area. Distension of the stomach also increases the force of antral contractions through long and short reflexes, i.e. a larger volume of food will increase gastric emptying rate. However, this is counteracted by the presence of nutrients, acid and sheer volume in the duodenum, triggering mechano-, osmo-, pH- and chemoreceptors to activate long vagal and short local reflexes to inhibit gastric emptying (McHugh et al., 1982; Greenberg et al., 1990; Schwartz & Moran, 1998). The sieving action of the pylorus means that the antrum will only pass digestible particles less than 1mm in size into the duodenum (Meyer et al., 1981).

1.2.1.3.2 Why measure gastric emptying?

Methods that have been developed to measure gastric emptying have many clinical and research applications. Disorders of the gastrointestinal tract, e.g. following surgery for duodenal ulcer, leading to symptoms such as gastric stasis, dumping or diarrhoea, require clinical evaluation of emptying. From a research point of view, the rate or duration of gastric emptying can be measured to evaluate the effects of different nutrients, whole foods (i.e. their physical properties or biochemical consequences) or drugs on absorption, postprandial parameters and satiety.

1.2.1.3.3 Methods of measuring gastric emptying

Gastric emptying rates have been measured since 1833 (Beaumont, 1833, quoted in Pickworth, 1984), and a range of methods have been developed in order to do this. Intubation is a direct way of assessing how much of a meal is left in the stomach, by aspirating the stomach contents through a naso-gastric tube. The dye-dilution method is similar but involves the labelling of the test meal with phenol-red to monitor changes in concentration of the dye. Another method is to measure the appearance of iodine in saliva after the consumption of potassium iodide in a barium meal, which reflects the absorption of the meal from the duodenum, from which gastric emptying rate is extrapolated. The passage of fluids, granules, pellets or meals containing barium sulphate through the gut can be measured radiographically. Absorption of paracetamol from the duodenum can also be taken to reflect gastric emptying by measuring concentrations in blood samples at sequential time points. Another tracer method is the $^{13}$C-octanoic acid breath test, where the cumulative
percentage of dose recovered in the breath is used to calculate gastric half emptying times (Ghoos et al., 1993). Ultrasound scanning, applied potential tomography, magnetic resonance imaging and scintigraphy are all imaging techniques used for the measurement of gastric emptying. Electrical impedance epigastrography (EIE) uses the change in impedance of an electrical current after a liquid meal to calculate gastric emptying parameters such as half-emptying time. Similar techniques include applied potential tomography and impedance gastrography. See appendix I for a summary.

1.2.1.3.4 The physical state of a meal

The emptying of non-nutrient liquids, nutrient-containing liquids, solid meals, and solid-liquid meals all follow different patterns. A non-nutrient liquid meal will empty mono-exponentially from the stomach if the solution is pH-neutral and iso-osmolar, such as physiological saline (Urbain & Charkes, 1995). The greater the volume of the liquid, then the faster the GE rate. A liquid that contains nutrients will empty linearly (Urbain & Charkes, 1995). Emptying of a solid meal, however, shows an initial lag phase, a long phase with a constant rate of emptying, followed by a slower phase of emptying, which produces a sigmoidal gastric emptying curve (Urbain & Charkes, 1995).

Many researchers have looked at gastric emptying of solid-liquid meals using the scintigraphic dual isotope method. The solid phase is usually labelled with ⁹⁹ᵐ⁻Technetium-sulphur colloid incorporated into chicken liver and the liquid phase ¹¹³ᵐ⁻In-DTPA (diethylenetriaminepentaacetic acid). These studies have found that the liquid phase is rapidly emptied, but at a rate that is directly correlated with the energy density of the solution (usually 10.5 – 13.0 kJ/min) (Macdonald, 1996). In another study, the liquid component emptied exponentially (Notivol et al., 1984) depending on the nutrient content, and the solid component emptied linearly (Notivol et al., 1984) depending on particle size, viscosity etc. If the liquid phase contained 25% dextrose the liquid and solid emptying was delayed compared to saline due to a prolonged lag period and increased retention in the proximal stomach (Collins et al., 1991). The pylorus exhibits a sieving action of the liquid phase, the emptying of which is mainly controlled by the distal stomach, whereas the proximal stomach retains the solid portion until it is in a liquid enough form to enter the antrum. Solid particles that are too large are propelled by antral contractions back into the proximal stomach until adequately “liquefied” (Urbain & Charkes, 1995). Emptying solid
meals made of large particles is slower than meals of small particles due to this liquefying process, a difference that is absent in patients who have undergone gastric surgery (Holt et al., 1982). The fibre content of a meal can have a strong effect on gastric emptying, whether due to viscosity, bulk, or digestibility of the stomach contents (Benini et al., 1995).

### 1.2.1.3.5 Viscosity, osmolality, pH and temperature

As the viscosity of a meal increases the rate of gastric emptying is slowed and stronger antral contractions are observed (Wilmshurst & Crawley, 1980; Marciani et al., 2000). Osmolality of a meal is an important determinant of gastric emptying rate – physiological saline empties faster than lower or higher concentration salt solutions, and isotonic sugar solutions empty faster than water (Thomas, 1957). The pH of a meal can affect gastric emptying, with acids prolonging gastric emptying, especially weak acids of high molecular weight (Hunt & Knox, 1972), weak alkalis increasing the rate and strong alkalis delaying gastric emptying (Thomas, 1957). However, the range of acids or alkalis likely to be present in food are insignificant for normal gastric emptying. Initial gastric emptying is faster for cooler liquid loads compared to liquids at body temperature, although the majority of the subsequent gastric emptying half-time was unaffected (Bateman, 1982).

### 1.2.1.3.6 Volume versus energy content

The duration of gastric emptying is related to the volume of the liquid meal (Cohnheim & Best, 1910; Bateman, 1982), or the weight of a solid meal. Moore and colleagues (Moore et al., 1981) found that the rate of solids transferred into the duodenum stayed constant for larger meals (1692g and 900g) but was slower for the 300g meal. The rate of energy transferred to the duodenum increased with greater meal volume and with greater energy density, with a doubling of volume increasing gastric emptying rate slightly more than a doubling of energy density (+3.01 kJ/min and +2.59 kJ/min respectively) in a study by Hunt et al (Hunt et al., 1985). Wisen et al. (Wisen et al., 1993) also found that the higher the energy density of the meal, the slower the gastric emptying, but that this did not fully compensate for the increasing energy density in terms of energy delivered to the duodenum. Moore et al (Moore et al., 1984) found that a 300% increase in meal weight led to a much greater increase in gastric emptying rate in terms of grams of solid emptied per minute compared to a
300% increase in energy content (388% increase compared to a 43% increase, respectively). On the whole it is believed that the gastric emptying of liquids is controlled so that around 837 kJ per hour are delivered to the duodenum, and that the greater the energy density of a meal the less is the volume emptied per minute (Hunt, 1983; Carbonnel et al., 1994).

1.2.1.3.7 Carbohydrate

The glucose content of a meal can also alter the rate of gastric emptying. Carbohydrate empties more rapidly than fat or protein (Cannon, 1911). Intravenous glucose administration leading to hyperglycaemia slowed gastric emptying of fat and protein meals, but emptying of non-nutrient saline meals were unaffected (Macgregor et al., 1976). However, the emptying of the saline meal is significantly slowed if glucose is infused into the duodenum, indicating the role of nutrient receptors in the duodenum in feedback control of gastric emptying (Brener et al., 1983). There is also some evidence that habituation to glucose in the diet can occur, with dietary glucose supplementation resulting in faster gastric emptying of a glucose test meal while emptying of a protein test meal was unaffected (Cunningham et al., 1991b).

1.2.1.3.8 Fat

Gastric emptying is slower when fat is added to a test meal (Cunningham & Read, 1989; Houghton et al., 1990). Lipid meals (instilled into the stomach) of 2.5% or greater concentration increased relaxation of the proximal stomach (McLaughlin et al., 1998), a determinant of gastric emptying, independently of energy content or osmolality. There is evidence for desensitisation of gastric emptying mechanisms for a high fat diet and a high fat test meal (as seen for glucose supplementation and high glucose meals) (Cunningham et al., 1991a), and this is thought to be linked to a reduced potency of CCK's inhibitory effects (Covasa & Ritter, 2000). Evidence has been reported that pancreatic lipase is needed for fat-mediated inhibition of gastric emptying in dogs (Meyer et al., 1994), and chylomicron formation is required for delayed gastric emptying (Raybould et al., 1998). Long chain fatty acids are less effective than short or medium chain fatty acids at delaying gastric emptying, and fatty acids are more effective than di-or triglycerides (Hunt & Knox, 1968; Cortot et al., 1982). Fat in the intestine leads to CCK secretion from endocrine cells in the
intestinal mucosa, which seems to almost entirely account for the delay in gastric emptying after a fatty meal (Moran et al., 1993).

1.2.1.3.9 Protein and amino acids

Protein and amino acid meals also liberate CCK and therefore delay gastric emptying. Phenylalanine and tryptophan are the most potent amino acids in slowing gastric emptying (Stephens et al., 1975; Byrne et al., 1977; Fisher & Hunt, 1977; Mangel & Koegel, 1984). Egg albumin had little effect on gastric emptying unless it was boiled (and therefore denatured) which led to a slowing of gastric emptying (Hunt, 1983). This suggests that protein has to be readily digestible in order to delay gastric emptying. Feeding rats with a high protein diet for three weeks led to faster gastric emptying rates following a protein containing meal, indicating an adaptive response (Shi et al., 1997), similar to that found for high fat and high glucose diets.

It was noticed that boiled milk, which produces small, soft curds in the stomach, empties faster than raw milk, which produces larger, tougher curds (Hawk et al., 1926). The casein in the milk is responsible for curd formation in the low pH environment of the stomach, and so the milk is emptied in a liquid and solid phase. Casein is also a potent secretagogue for CCK, which would delay gastric emptying still further (Lewis et al., 1990, cited in Maes et al., 1995). Casein was emptied most slowly in human subjects compared to glucose, partially hydrolysed gelatin, and intact or denatured egg albumin (Low, 1990).

1.2.1.3.10 Other influences on gastric emptying

Other potential influences on gastric emptying include gender (Notivol et al., 1984; Knight et al., 1997), body size (Lavigne et al., 1978; Brogna et al., 1998), and age (Moore et al., 1983; Maes et al., 1995).

1.2.1.3.11 Gastric emptying and distension in relation to appetite

An empty stomach would be expected to cause a greater feeling of hunger compared to a full stomach, with degrees of appetite correlating with stomach content in between the two extremes. However, the available evidence has not always proven the relationship between appetite and gastric emptying. Fructose emptied more rapidly than glucose or xylose in rhesus monkeys, but this had no effect on the
amount of food ingested subsequently (Moran & McHugh, 1981). Although relationships were demonstrated between plasma CCK response and subjective appetite scores, and between plasma CCK response and gastric emptying, there were no significant correlations between gastric emptying rate or half-emptying times and subjective appetite scores (French et al., 1993).

Some researchers have been successful in demonstrating a link between gastric emptying and appetite. It has been shown that with the use of guar gum to increase viscosity and therefore gastric emptying time, there was a relationship between gastric transit time and time of self-rated maximum hunger in obese subjects (Wilmshurst & Crawley, 1980). Postprandial fullness was related to the retention of dextrose in the antrum in a study by Hveem et al. (Hveem et al., 1996). Hunger was decreased and gastric emptying time increased in a decubitus position following an oil and aqueous meal, compared to a sitting position, and hunger was significantly related to the retention of oil in the stomach in the decubitus position (Horowitz et al., 1993). Bergmann et al. (Bergmann et al., 1992) showed that the use of psyllium (a soluble dietary fibre) could delay gastric emptying in the later stages of emptying, and that subjective sensations of hunger and satiety were significantly correlated with gastric emptying time. Rigaud et al. (1998), however, went on to show that if psyllium was ingested fifteen minutes before the test meal, although hunger and subsequent food intake was still reduced there was no delay in gastric emptying compared to the placebo. This finding shows that a significant correlation such as increased gastric emptying time with reduced hunger does not necessarily mean that hunger is directly affected by gastric emptying.

Gastric distension is a state that is related to gastric emptying (since the latter is a function of intra-gastric pressure), and is also associated with pre-absorptive appetite control, especially with regard to feelings of fullness. Vagal afferent nerves terminate in the circular and longitudinal muscle layers of the stomach, sensitive to contraction or stretch in the muscle tissue. In rats, the discharge rate of vagal afferent fibres increases with gastric loads (Schwartz et al., 1991), and gastric loads volume-dependently reduce food intake independently of nutrient content when the pylorus was occluded (Phillips & Powley, 1996). This suggests that gastric distension-related signals are of primary importance in the satiation process. However, this relationship between gastric distension and gastric-related fullness is not straightforward, as
vagotomy does not block the satiating effect of food in the stomach of rats (Kraly & Gibbs, 1980). During the course of a meal, nutrients are emptied from the stomach and enter the intestine, where they also trigger satiety signals from mechanoreceptors, chemoreceptors and hormone/neuropeptide secretion, thereby modulating gastric distension sensations. It has been shown that CCK augments the sensations of gastric distension (subjective ratings of fullness) (Melton et al., 1992), and also stimulates gastric and duodenal vagal mechanosensitive afferents, sensitising and amplifying the afferent response to gastric loads (Davison & Clarke, 1988; Schwartz et al., 1991; Schwartz & Moran, 1994). Gastric distension-type feelings of fullness probably initiate the satiety process, especially following meals of large volume or bulk. In fact, it has been suggested that gastric distension dominates the fullness sensations experienced after a meal (Cecil et al., 1998). Distension-induced satiety signals are then enhanced and prolonged while the food is emptying from the stomach by intestinal nutrient-related signals, both direct (e.g. direct stimulation of mucosal vagal afferent termination fibres) and indirect (e.g. via stimulation of gastrointestinal hormone secretion).

1.2.1.4 Summary

In summary, hormonal, neurological and gastric emptying studies point to pre-absorptive signals arising from amino acids and protein in the gastrointestinal tract as important regulators of short-term food intake. These neuroendocrine signals appear to be key to the satiation process during the course of a meal.

1.2.2 Post-absorptive mechanisms for protein- and amino acid-induced satiety

Ritter & Calingasan (Ritter & Calingasan, 1994) interpreted studies based on metabolic drugs and brain lesions as indicating that lipoprivic signals (i.e. those generated through an inhibition of fat oxidation) are monitored peripherally and reach the brain via vagal sensory neurons, while glucoprivic signals (generated through an inhibition of glycolysis) act both peripherally and centrally, i.e. there are no vagal afferents arising from the periphery that are exclusively essential for the glucoprivic feeding responses. On the basis of experiments with glucose and fatty acid oxidation, Friedman (Friedman, 1998), postulated that metabolic signals may originate at the most basic cellular level – changes in intracellular ATP are sensed by the liver and signalled vagally to the brain. With amino acid oxidation involving a variety of
pathways in gastrointestinal mucosal cells, liver, skeletal muscle and kidney, there are many potential candidate metabolic pathways and organs but as yet no identifiable experimental evidence.

1.2.2.1 Signals from hepatic amino acid sensors

Russek (Russek, 1971) proposed that hepatic glycogen and protein reserves were the main factors that influenced the regulation of food intake. Following feeding, these reserves would be replenished and an absence of "hunger signals" would follow. As these hepatic glycogen and protein reserves decreased, receptors in the liver would start signalling to the CNS to increase feelings of hunger. Russek's ideas prompted a great deal of research and it is widely believed that the liver is a likely candidate for fuel utilisation monitoring and signalling to hunger and satiety centres in the CNS.

In fact, because the liver plays a direct regulatory role in postprandial amino acid homeostasis, a hepatic source for an amino acid-derived satiety signal is a logical theory. Following a meal the inhibition of hepatic proteolysis (Garlick et al., 1973) allows the accumulation of hepatic protein (Millward et al., 1974) which buffers the large increase in blood amino acids and maintains a steady supply of amino acids to peripheral tissues for up to eight hours (Wahren et al., 1976). Furthermore inhibition of proteolysis appears to increase progressively with increasing protein intake (Pacy et al., 1994) and is mediated in part by amino acid supply with a specific role for alanine as a coregulator of proteolysis (Pösö & Mortimore, 1984) probably acting through a receptor-mediated mechanism (Miotto et al., 1992). With alanine the major end product of both skeletal muscle and intestinal amino acid oxidation, alanine uptake and disposal in the liver is an obvious signalling source that might theoretically regulate food protein intake.

Hepatic amino acid supply can initiate a neural output with sensors in the rat hepato-portal system sensitive to alanine and other amino acids (Tanaka et al., 1990). Intraportal infusion of an amino acid mixture decreases the firing rate of hepatic vagal afferent neurons responsive to amino acids, in a dose-dependent way (Niijima & Meguid, 1994), and individual amino acids act either to excite (arginine, alanine, histidine, leucine, lysine, serine, tryptophan and valine) or inhibit (cysteine, glycine, isoleucine, methionine, phenylalanine, proline and threonine) the discharge rate
(Niijima & Meguid, 1995). Although, it is not understood why some amino acids are excitatory while others are inhibitory, it provides a theoretical mechanism by which amino acid supply in the periphery may be monitored centrally thereby acting as a sensitive regulatory system for the control of food intake.

1.2.2.2 Amino acids flux into skeletal muscle as a hypothetical locus for appetite regulation

There is as yet no identifiable evidence of amino-acid sensitive signalling mechanisms in muscle comparable to those identified in the liver. However control of skeletal muscle growth has been proposed to link the regulation of growth and body composition to an aminostatic mechanism of appetite control (Millward, 1995). Muscle protein synthesis, which is part of the postprandial responses to dietary amino acids observed in the whole body (Pacy et al., 1994; Gibson et al., 1996; Millward et al., 1996), is sensitive to amino acid supply in the leg (Gelfand et al., 1988; Bennet et al., 1989; Bennet et al., 1990), and in insulin-clamped pig skeletal muscle (Watt et al., 1992). Thus post-feeding disposal of amino acids occurs in muscle as both net protein deposition and amino acid oxidation (mostly branched chain amino acids, comprising some 25% of dietary protein). Given the size of muscle relative to body size, this may be a major proportion of total body net protein deposition and amino acid oxidation. Most importantly, within the protein-stat hypothesis proposed by Millward (1995), muscle is identified at a higher level than other organs within a hierarchy of growth regulation: i.e. the canalised linear growth of long bones mediates the rate of and target size for muscle growth, which in turn determines energy expenditure, energy requirements and food intake, the latter regulating splanchnic organ mass. In this sense, muscle mass determines food intake while food intake determines splanchnic organ mass. When major changes in food intake are required, e.g. during early postnatal life or during nutritional rehabilitation, much of the increased protein and energy requirements are for skeletal muscle growth and it is postulated within the protein-stat hypothesis that the signalling mechanisms for the required changes in intake originate from muscle.

However, the identification of a metabolic locus in the muscle for aminostatic signal transduction is problematic. There are no neural pathways apart from those involved in pain or muscle length detection. It may be that simply through its role as the largest single component of the lean body mass, muscle regulates food intake
through its ability to remove or release amino acids from the circulating pool, with sensors elsewhere monitoring such changes and mediating aminostatic signal transduction. It may also be the case that such a mechanism is only operative during early postnatal life, growth spurts during adolescence, catch-up growth, and recovery from illness where muscle wasting has occurred. In the well nourished adult (with muscle fibres replete, minimal net fluxes of amino acids between muscle and the circulation, and with modest protein requirements limited to the replacement of postabsorptive losses), the role of muscle in appetite regulation may be of minor importance. In this case, with protein-related hunger mechanisms unnecessary, aminostatic components of appetite regulation may be managed elsewhere by gut mediated, hepatic, or central mechanisms.

1.3 Central mechanisms

1.3.1 Evidence that circulating and central nervous system amino acid concentrations influence food intake.

Previously, through work on animals with hypothalamic lesions or neural transactions, it was established that the hypothalamus was the main brain region responsible for the control of food intake, especially the VMH, DMN, PVN, and LH. Extensive subsequent research has uncovered the extent of the complexity of the hypothalamic appetite circuitry and its connections to the periphery and other regions of the brain.

1.3.1.1 Animal studies

Infusion of amino acids into the circulation depresses food intake in rats (Walls & Koopmans, 1992), suggesting that the gastrointestinal tract is not the sole mediator of the satiety response. Circulating amino acid levels may be regulated by a central receptor, since rats with a reduced food intake, due to an amino acid-imbalanced diet, normalise their feeding when the limiting amino acid is infused into the carotid artery but not the jugular vein (Rogers & Leung, 1973). One site may be the hypothalamus, since feeding is inhibited by a balanced amino acid solution injected into this area (Panksepp & Booth, 1971). However, food intake remains responsive to dietary amino acid manipulations in rats with hypothalamic lesions (Rogers & Leung, 1973), so the hypothalamus is unlikely to be the only receptive region of the brain. Lesions in the prepyriform cortex and the medial amygdala
prevented the reduction in food intake observed when rats were fed amino acid-imbalanced diets but not amino acid deficient or high protein diets. Lesions in the septal area did not appear to be important in the regulation of amino acid intake, and lesions in the hippocampus did not affect the feeding response to diets containing amino acids in excess (Leung & Rogers, 1979). Concentrations of a limiting amino acid were not decreased in rats fed an imbalanced amino acid diet in the three hypothalamic regions studied (VMH, LH and PVN), but noradrenaline concentrations were shown to be increased in the VMH. (Gietzen et al., 1989). Since noradrenaline concentrations were also decreased in the prepyriform cortex this implicated the noradrenergic system in the regulation of intake of imbalanced amino acid diets (Leung & Rogers, 1971).

Another approach to locate the receptive areas of the brain is to examine actual neuronal activity of the various likely brain centres in response to variation in amino acid concentrations. Examination of the discharge frequency response of specific neurons in the cerebral hemisphere and LH to the electrophoretic application of individual essential amino acids revealed that none of the cells in the cerebral cortex responded to any of the amino acids, but neurons in the thalamus, zona incerta and LH were responsive, especially to threonine, tryptophan, methionine and valine (Wayner et al., 1975). More recently perfusion of cerebrospinal fluid (CSF) containing normal physiological concentrations of all amino acids increased the excitability of neurons in the VMH, which was then shown to be mainly due to glutamine (Nishimura et al., 1995). Although the importance of excitatory amino acid receptors in appetite regulation could not be clarified (see section 1.3.4), a direct link between the excitatory amino acid, glutamate (whose precursor is glutamine), in the LH and the initiation of a strong immediate eating response in satiated rats was shown (Stanley et al., 1993). Another study of single neuron activity found that specific set of LH neurons were more active in response to dietary lysine in lysine-deficient rats whereas the neurons of normal lysine-status rats responded non-differentially, a phenomenon that was reflected in their preference for lysine (Torii et al., 1998). The authors suggested that neurons in the LH are particularly important in recognising nutrient deficiencies and that neural plasticity in response to deficiency or learned preferences may play an important role in amino acid intake regulation.
A lack of temporal associations between plasma and brain amino acid levels and feeding has led some investigators to question a postabsorptive aminostatic theory. Ng and Anderson (Ng & Anderson, 1992) did observe reductions in food intake following intragastric or intraperitoneal administration of tryptophan and tyrosine but they were unable to link this with changes in concentrations of these amino acids in the plasma or brain at the time of feeding. In subsequent rat studies, Anderson and colleagues (Anderson et al., 1994) point to a lack of temporal association between protein-induced satiety and plasma amino acid responses. Oral intake of albumin, as protein or its constituent amino acids, did reduce food intake during the first hour of feeding, but plasma amino acids did not change until the end of that hour. They also reported a lack of consistent change in brain amino acid concentrations in the study, further reinforcing their arguments.

1.3.1.2 Human studies

As far as human evidence is concerned we are still very reliant on Mellinkoff’s original work that changing concentrations of plasma or brain amino acids influence feeding behaviour (Mellinkoff et al., 1956). One study, not specifically designed to examine aminostatic mechanisms, did show that in healthy males, voluntary oral intakes of a covert liquid diet were decreased to a greater extent by intravenous infusions of a combination of amino acids, glucose, and fat than with glucose or fat alone (Gil et al., 1991). However, the design does not allow resolution of the influence of the amino acids per se as opposed to a combined satiety effect of all three macronutrients. Even more straightforward studies are not entirely unambiguous. Gielkens et al. (Gielkens et al., 1999) reported that food intake was unaffected by intravenous infusion of two concentrations of amino acids but that subjective feelings of satiety were increased dose-dependently, with a small, short-lived (first half hour) increase in plasma CCK concentrations during the high dose infusion.

1.3.2 Phenylalanine, tyrosine and dopamine

This section will review the individual amino acid phenylalanine, its conversion to tyrosine, and the importance of the availability of this precursor to dopamine formation and the appetite effects of dopamine.
1.3.2.1 Phenylalanine

The upper requirement for total aromatic amino acids was set at 14mg/kg body weight per day for adults in the 1985 FAO/WHO/UNU report. However, this has not been agreed upon, and subsequent studies have also disagreed on the level of phenylalanine intake required for phenylalanine balance (Waterlow, 1996). The main reason for the uncertainty surrounding the correct requirement for balance is lack of agreement on the proportion of phenylalanine that undergoes hydroxylation to tyrosine. A more recent study suggested that a mean intake of 39mg/kg/d is required in healthy adults (Basile-Filho et al., 1998). In practice, daily consumption of the average mixed diet in adults greatly exceeds this value (Harper, 1984).

Phenylalanine is widely ingested as the sweetener, aspartame; a high intensity, non-nutritive sweetener, and a methyl ester of the dipeptide L-aspartyl-L-phenylalanine. One 330ml (12 oz) can of “diet” carbonated beverage contains 200mg of aspartame (112mg of phenylalanine) (Federal Register, Vol. 46, No142, July (1981), p.38290 (US), in Pardridge, 1986). In a child weighing 30 kg, a can of this drink would provide 3.7 mg/kg body weight of phenylalanine in the child and 1.6 mg/kg body weight in an adult. These seem insignificant amounts compared to the projected 99th percentile of aspartame ingestion (34 mg/kg/day) set by the Food and Drug Administration in the USA (Pardridge, 1986). Stegink et al (Stegink et al., 1988; Stegink et al., 1989; Marcou et al., 1987) found that repeated ingestion of an aspartame-sweetened beverage throughout the day does not lead to an accumulation of plasma phenylalanine and other aspartame metabolites beyond normal postprandial levels. Plasma phenylalanine is cleared relatively quickly, with a half time of approximately 60-90 minutes (Møller, 1991). Therefore it would be surprising if there were an accumulation of phenylalanine in the plasma even at the highly unlikely levels of consumption administered in the experiments carried out by Stegink and colleagues.

Phenylalanine may influence satiety via peripheral mechanisms, e.g. increasing CCK secretion, or centrally by its conversion to tyrosine. Møller (1991) investigated the plasma phenylalanine and tyrosine response in human beings to ingestion of phenylalanine in the form of aspartame and found that it is quickly absorbed, reaching a peak at 15 minutes, and that it increased plasma tyrosine levels. Aspartame increased plasma phenylalanine/large neutral amino acid (LNAA) ratio at
15 minutes, and phenylalanine/LNAA as a % of basal was significantly increased for 2½ hours. Tyrosine/LNAA ratios were also significantly increased by aspartame from 30 minutes to 4 hours. These elevated phenylalanine/LNAA and tyrosine/LNAA ratios represent an increased availability of these amino acids to the brain after aspartame. This, potentially, could mean that there is greater synthesis of catecholamines such as dopamine and noradrenaline (see section 1.3.2.3).

Other studies had also shown that aspartame loading results in some of the phenylalanine being converted to tyrosine (Stegink et al., 1977; Stegink et al., 1987; Stegink et al., 1988; Mangel et al., 1995; Maffei et al., 1996). In a recent 24 hour stable isotope tracer study (Basile-Filho et al., 1998), the rate of phenylalanine hydroxylation and tyrosine oxidation varied with the level of phenylalanine in the diet. The high phenylalanine diet led to a different pattern of whole-body phenylalanine and tyrosine metabolism during the fed and fast phases compared to the intermediate and low phenylalanine diet, with phenylalanine concentrations increasing for the former and staying the same or decreasing for the latter.

Tyrosine is a precursor for the formation of the catecholamines dopamine (DA, dihydroxyphenylethyl amine), noradrenaline (NA) and adrenaline. Catecholamines are “organic compounds that contain a catechol nucleus (a benzene ring with 2 adjacent hydroxyl substituents) and an amine group” (Cooper et al., 1996). Tyrosine is converted to 3,4-dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase. Then, in the brain or the peripheral neuron (in the case of NA) a number of enzymatic steps take place to form one or other of the catecholamines. Tyrosine transport into the brain involves the same large neutral amino acid carrier as tryptophan; and a tyrosine/LNAA supply-limited mechanism of DA synthesis is an obvious candidate for appetite regulation, similar to the purported tryptophan-serotonin link (see section 1.3.3).

1.3.2.2 Dopamine influences eating behaviour

Dopamine (DA) is strongly believed to be involved in the regulation of food intake but the evidence does not clearly indicate the direction of this effect. Depletion of ventrolateral striatal DA in rats (Salamone et al., 1993) and genetic DA-deficiency in mice (Szczypka et al., 1999) both reduce food intake. However, DA concentrations in the cerebrospinal fluid are depressed during food deprivation and normalised by
ingestion of food in rats (Bednar et al., 1991), while DA agonists (for D₁ and D₂ receptors) inhibit feeding in rats (Bednar et al., 1995). DA also acts to inhibit food intake via CCK-mediated mechanisms (Bednar et al. 1991).

1.3.2.3 Plasma tyrosine and dopamine synthesis

Rat studies have shown changes in brain concentrations of DA metabolites in response to normal feeding or dietary manipulation of tyrosine and phenylalanine intake (Biggio et al., 1977; Thurmond et al., 1980; Glanville & Anderson, 1986; Hernandez & Hoebel, 1988; Lehnert & Wurtman, 1993; Yeomans et al., 1997; Greenberg et al., 1990). The plasma tyrosine to phenylalanine ratio correlated with long term energy intake in weanling rats (Anderson & Ashley, 1977), and some authors have shown that an increase in brain tyrosine can enhance NA synthesis (Gibson & Wurtman, 1978; Brady et al., 1980), but not DA synthesis (Spring et al., 1987). However the inference that this represents a tyrosine-mediated control of food intake can be questioned by the failure to show an increase in NA levels after phenylalanine supplementation, despite increased concentrations of phenylalanine and tyrosine in the brain (Thurmond et al., 1980). Moreover, directly increasing brain tyrosine produced no increase in the rate of catecholamine synthesis (Sved, 1983). The low Km for tyrosine hydroxylase leads to its saturation with tyrosine under normal conditions, and therefore it is no longer thought to act as a rate-limiting step for catecholamine synthesis (Cooper et al., 1996). The product of tyrosine hydroxylase, DOPA (dihydroxyphenylalanine), is more likely to rate-limit catecholamine synthesis. This is present in the brain at very low concentrations, due to its rapid conversion to DA by aromatic amino acid decarboxylase.

It may be that tyrosine does influence neurotransmitter synthesis when noradrenergic or dopaminergic pathways are physiologically stimulated, and peripheral sympathetic or central noradrenergic or dopaminergic neurons are rapidly firing (Spring et al., 1987). When this occurs in the relevant neurons, tyrosine hydroxylase becomes less inhibited by the end product due to a greater affinity for its cofactor (tetrahydrobiopterin) as a result of phosphorylation (Lehnert & Wurtman, 1993). This relationship can be demonstrated in DA neurons in the retina, which fire actively in the light but are inactive in the dark. Tyrosine hydroxylase activity increases in retinal DA neurons in the light, a process that is enhanced when tyrosine is injected (Fernstrom & Fernstrom, 1994). It was also shown that there is a
competitive LNAA transport into the retina, and therefore the plasma tyrosine to LNAA ratio should influence tyrosine hydroxylation rates in the retina. Another example is cold stressed animals, which show increased synthesis and release of NA in response to increased brain tyrosine concentrations, an effect which is also achieved by administration of probenecid (Spring et al., 1987). However, DA release was not affected.

1.3.2.4 The effect of meals on brain tyrosine and dopamine synthesis

Feeding has been reported to increase extracellular concentrations of DA in the nucleus accumbens, a part of the brain that is a major target of the mesolimbic dopaminergic system (Radhakishun et al., 1988; Yoshida et al., 1992; Young, 1998). Rats fed a protein meal had increased retinal tyrosine levels and tyrosine hydroxylation rates, and effect that was not observed following a protein-free meal (Fernstrom & Fernstrom, 1994). This showed that tyrosine hydroxylation could be increased by feeding as well as by direct tyrosine injection. However, this physiological effect has not been proven in any brain DA neurons, which would need to be actively firing ones. Tyrosine hydroxylation rates were shown to be higher in the hypothalamus of rats ingesting greater amounts of protein for two weeks, but it was not known if this was occurring in DA or NA neurons (Fernstrom & Fernstrom, 1994). In other rat studies, the palatability of food influenced the magnitude of the increase in DA and its metabolites in the nucleus accumbens (Martel & Fantino, 1996), suggesting either that mesolimbic dopaminergic system activity is related to the rewarding aspects of feeding, or that the higher ingested energy density with the highly palatable food was responsible. Plasma DOPA has been observed to fall in human subjects after a mixed meal (Eldrup et al., 1997), possibly reflecting increased DA synthesis, since there were also large increases in plasma total DA, not all of which could be accounted for by the meal DA content. However, the responses could also reflect increased uptake of DOPA into muscle following postprandial increases of insulin, especially since there was no relationship between plasma DOPA and plasma NA. Plasma tyrosine and phenylalanine increased after the meals but concentrations did not correlate with plasma DOPA, DOPAC (3,4-dihydroxyphenylacetic acid) or DA-sulphate concentrations. Clearly meal-feeding studies of sympathetic nervous activity, in which DA is provided in food and could account for some of the responses (e.g., the observed increase in plasma DOPAC
concentrations), create difficulties in their interpretation. These results neither support nor rule out the possibility that increased tyrosine uptake in the brain after the meals may have contributed towards increased DA synthesis and release.

1.3.2.5 Conclusions

DA is involved in food intake regulation, although it appears that this relationship is not straightforward. The plasma tyrosine:LNAA ratio can be increased by high protein meals which enhances tyrosine entry into the CNS. However, the rate-limiting step of tyrosine hydroxylation is only effective in rapidly firing DA neurons, otherwise the enzyme is saturated under normal conditions. The areas of the brain that contain rapidly firing DA neurons are poorly understood, and whether these project to appetite regulation centres remains to be discovered. There is little evidence in animals or human beings that meals of differing composition can modulate DA synthesis and release, or that this influences eating behaviour.

1.3.3 Tryptophan and serotonin

There is considerable literature on the relationship between tryptophan, serotonergic activity, and eating behaviour (Wurtman et al., 1981; Hrboticky et al., 1985; Hill & Blundell, 1988). Serotonergic, or 5-HT neurons, (serotonin is 5-hydroxytryptamine, 5-HT), certainly play an important part in the regulation of appetite with serotonergic agents (5-HT releasers, re-uptake blockers and direct agonists) inducing a prompt anorexia; as a result they are used to control obesity and eating disorders (Blundell & Hill, 1992). This has, not surprisingly, raised the possibility that aminostatic regulation of appetite may be simply explained by amino acids acting centrally through their role as neurotransmitter precursors to influence eating behaviour. This popular hypothesis has in fact proved difficult to demonstrate.

1.3.3.1 Effects of diet or tryptophan on serotonin synthesis

Tryptophan is a precursor for 5-HT, with tryptophan uptake into the brain thought to regulate 5-HT synthesis, and with tryptophan competing with other large neutral amino acids (LNAA) (phenylalanine, tyrosine, isoleucine, leucine and valine) at the blood-brain barrier transporter (Wurtman et al., 1981). Tryptophan concentrations are low in most dietary proteins, and the plasma tryptophan to LNAA ratio (tryptophan/LNAA) after a meal depends on the net dynamic effect of insulin secretion and subsequent tissue uptake of amino acids, and the postprandial rise in
circulatory amino acids. In a series of rat experiments, Fernstrom & Wurtman (Fernstrom & Wurtman, 1972) established that brain tryptophan and 5-HT concentrations could be increased by injections of tryptophan or insulin, or consumption of high carbohydrate diets, and that this response was abolished with protein consumption. It has been assumed that a high carbohydrate meal, which increases insulin secretion, will lower plasma LNAA concentrations through promotion of their uptake into tissue protein. In particular, concentrations of plasma branched-chain amino acids would be reduced and the tryptophan/LNAA ratio would increase (Wurtman et al., 1981).

It is also argued that the insulin-mediated inhibition of release of non-esterified fatty acids into the plasma frees up albumin binding sites for tryptophan, although the importance of this has been disputed with the suggestion that most plasma tryptophan (70-80%) may be “free” in the brain capillaries and available for transport into the brain (Leathwood, 1989). Since tryptophan hydroxylase is not normally saturated by tryptophan, tryptophan uptake, a function of the plasma tryptophan:LNAA ratio becomes the rate-limiting step (Cooper et al., 1996). A raised brain tryptophan level will therefore increase conversion to 5-hydroxytryptophan (5-HTP) and then 5-HT by hydroxylation. The problem with this hypothesis is that the addition of even a small amount of protein to a high-carbohydrate meal is enough to prevent this tryptophan-mediated 5-HT increase (Teff et al., 1989), and large excursions in brain tryptophan concentrations are needed to produce observable increases in 5-HT (Ashley et al., 1985). Indeed, the majority of available evidence does not support the idea that protein or carbohydrate meals alter plasma amino acids sufficiently to cause significant changes in brain 5-HT synthesis (Ashley et al., 1982; Leathwood, 1989; Teff et al., 1989). In addition, the functional 5-HT pool does not necessarily relate to total 5-HT concentrations in the brain. Functional 5-HT may be inhibited in order to regulate neurotransmission rates, either by intracellular metabolism before synaptic release, or the adaptive slowing of raphe neuron firing rates if there is a large increase in 5-HT synthesis (Leathwood, 1987).

1.3.3.2 Effects of diet or tryptophan on food intake

The reduction in energy intake in human subjects following oral tryptophan, albeit as a large dose (2 g encapsulated supplement), initially provided partial support for this theory (Hrboticky et al., 1985). However, the hypothesis that serotonergic
effects on appetite are related to a feedback loop for specific macronutrients (Fernstrom et al., 1979; Woodger et al., 1979) was not supported since the proportion of carbohydrate or protein eaten was not affected. Furthermore, a tryptophan and carbohydrate lunch, (expected to enhance 5-HT synthesis), did not inhibit food intake, while high protein and high protein plus tryptophan meals did (Blundell & Hill, 1987), i.e. tryptophan appeared to exert its effects through its combination with protein rather than carbohydrate, therefore not through its capacity to increase 5-HT activity in the brain. Studies manipulating carbohydrate and protein intake that have shown associated changes in plasma tryptophan/LNAA ratios and differences in macronutrient selection, do not rule out other intermediary mechanisms (Pijl et al., 1993). The elimination of this purported feedback regulation of macronutrient intake by treatment with fluoxetine, which suppressed food intake, suggests that this short-term effect was not necessarily 5-HT-mediated since it was not enhanced by a 5-HT re-uptake blocker.

Studies of postprandial satiety and plasma amino acids in male subjects after the ingestion of beef, chicken and fish indicated that the greater satiety after a fish meal was correlated with differences observed in the postprandial tryptophan to large neutral amino acid ratio (Uhe et al., 1992). However, this merely hints at supporting the increasingly flimsy premise that dietary amino acids in normal meals can alter 5-HT release to affect appetite – it does not offer firm evidence that these changes in plasma LNAA profile were sufficient to alter 5-HT synthesis in amounts that could influence satiety and that some alternative neurochemical pathway was not involved. A non-serotonergic mechanism of tryptophan action was certainly indicated by studies of infused tryptophan metabolism and kinetics (Huether et al., 1992). 1, 3 and 5 g doses of tryptophan failed to alter whole blood 5-HT concentrations but did change concentrations of many other neuroactive tryptophan metabolites, none of which were saturated by tryptophan, as well as increasing amino acid utilisation for protein synthesis, to the detriment of brain tyrosine availability for catecholamine synthesis.

Thus the carbohydrate-tryptophan-serotonin satiety pathway suggested by animal studies appears difficult to demonstrate in humans, with tryptophan exerting its effects on appetite through its combination with protein rather than carbohydrate, and little evidence that serotonin is involved in this dietary paradigm. Although there
is no doubt that serotonin is an important regulator for appetite, the original carbohydrate-tryptophan-serotonin theory of behaviour and appetite is oversimplistic, with solutions more likely to involve other pathways of brain neurochemistry.

1.3.3.3 Other pathways for serotonergic effects on food intake

It seems that although serotonergic mechanisms can regulate food intake as shown by the agonist responses, whether food intake influences serotonergic activity is by no means clear. 5-HT has a large number of receptors, with many subtypes identified. Sumatriptan, a 5-HT1A receptor agonist, has been shown to inhibit motilin action and suppress motor activity in the stomach (Tack et al., 1998), as well as inhibiting gastric emptying rates (Coulie et al., 1997) and causing a post-prandial dilatation of the distal and proximal stomach (Vingerhagen et al., 2000). Since 5-HT has been identified in the myenteric plexus, this suggests that it may be acting on receptors in the enteric nervous system. Serotonin acting on specific receptors (5-HTIB/2C) in the hypothalamus may be inhibiting food intake by interacting with neuropeptide Y (NPY) and CCK (Cooper et al., 1992; Poeschla et al., 1993; Dryden et al., 1996; Voigt et al., 1998). These studies show the large degree of interdependence of neurotransmitters and neuropeptides in triggering satiety following food intake, pointing to a need for more information about the exact neural and systemic pathways that mediate these interactions.

1.3.3.4 Conclusions

There is no doubt that serotonergic neurotransmission can regulate food intake. It is also recognised that in certain specialised experimental conditions ingestion of tryptophan or pure carbohydrate can increase 5-HT synthesis in the brain. Whether this influences serotonergic neurotransmission is debatable. The evidence, however, that the tryptophan-carbohydrate-serotonin pathway is important in appetite regulation in free-living humans beings is unconvincing.

1.3.4 γ-aminobutyric acid (GABA) and glutamate

Amino acids not only act as precursors for neurotransmitters — some, or related metabolites such as GABA, are themselves neurotransmitters in the CNS. GABA is classed as an "inhibitory amino acid" (Cooper et al., 1996). Only trace amounts occur in peripheral nerve tissue but GABA is found throughout the brain and
spinal cord; high concentrations are found in the nigrostriatal system of the brain, with lower levels in the cerebral hemispheres, pons and medulla (Rang & Dale, 1991; Cooper et al., 1996). Glutamate is the precursor for GABA, itself an "excitatory amino acid" neurotransmitter. There is a mounting body of animal evidence to suggest that GABA neurons are involved in the regulation of feeding behaviour.

Low doses of inhibitors of GABA transaminase (raising brain GABA levels), led to the inhibition of feeding behaviour in rats (Nobrega et al., 1988); confirming earlier similar results in rats after ICV injections of GABA and GABA-transaminase inhibitor (Olgiati et al., 1980). The benzodiazepine system (benzodiazepine drugs, e.g. diazepam, potentiate the effects of GABA) has been investigated in relation to an appetite drive and taste palatability (Berridge & Pecina, 1995). The ability of benzodiazepine drugs to stimulate feeding is independent of their powerful sedative effects, and it is argued that these agonists increase feeding by enhancing pleasure and hedonic reactions to food (Cooper, 1980; Berridge, 1995).

Recent evidence has come to light to suggest that glutamate has a role in the control of food intake. Glutamate is an "excitatory amino acid" in the CNS (Cooper et al., 1996). Glutamate is present, like GABA, at high concentrations throughout the CNS, as well as throughout the body as part of the free amino acid pool (Rang & Dale, 1991). Many hypothalamic nerve terminals contain glutamate in their synaptic vesicles, and glutamate and its receptor agonists kainic acid (KA), α-amino-3-hydroxy-5-methyl-isoxazole (AMPA) and N-methyl-D-aspartate (NMDA) have particularly pervasive excitatory effects in the hypothalamus (van den Pol et al., 1990). Lateral hypothalamic injections of glutamate and glutamate agonists elicited strong, immediate feeding responses in satiated rats (Stanley et al., 1993). Strangely, an NMDA receptor antagonist enhanced chow intake in food-deprived rats, and increased liquid sucrose intake in both food-deprived and satiated rats (Burns & Ritter, 1997). NMDA receptor blockade thus appears to diminish satiety signals, although one might expect an NMDA antagonist to inhibit feeding. The limited evidence suggests that glutamate is indeed involved in the regulation of feeding behaviour, but that glutamate receptors cannot be regarded as simple on/off switches for satiety. Further research needs to be done into the exact behavioural effects of the glutamate neural system, bearing in mind the complex food reward behavioural
patterns observed in investigations into benzodiazepine-GABAergic and opioid neural systems.

1.4 Psychological and Social Aspects of Appetite Control

1.4.1 Introduction

Appetite regulation in human beings is a complex combination of events. A theoretical satiety or hunger signal from the brain does not follow a direct pathway to a physical consummatory or terminating action – there are many social and psychological ramifications along the way. The following section is a brief summary of these facets of human eating behaviour.

1.4.2 The Food

Eating behaviour can be recorded and classified in terms of the microstructure of eating, a term which refers to the pattern of biting, chewing, swallowing and pausing, as well as the duration of a bout of eating or meal. These parameters of eating behaviour may reflect attitudes to food or psychological profiles. For example, it has been suggested that obese people may have a different pattern of eating behaviour within a meal (a faster rate of chewing and swallowing, less pauses etc.), increasing the amount of food consumed before the physiological satiety signals take effect (Mela & Rogers, 1998). The microstructure of eating is determined by the palatability of the meal. Palatability can mean many things but it is generally taken to reflect an individual’s liking for a food based on its sensory (taste, flavour and texture) properties (Mela and Rogers, 1998). Palatability is distinct from food preference as the latter term can reflect other factors (economic, social, and psychological) in addition to palatability. Palatability alters eating behaviour. A more palatable meal enhances food intake in human subjects, and increases and prolongs subjective hunger ratings with the initiation of an eating episode (Yeomans et al., 1997).

Sensory-specific satiety is an expression used to describe the decrease in palatability that occurs during the course of a meal, which eventually inhibits eating, especially where only one type of food is provided as is the case in many eating behaviour studies. The decline in subjective ratings of pleasantness and attractiveness for a food may persist beyond termination of the meal (Rogers & Blundell, 1990). The sensory properties of a food are believed to be of great importance in short-term
satiety (Hetherington et al., 1989). Furthermore, the regulation of food intake may be more responsive to sensory-specific satiety than sensations of gastric fullness, and it was suggested that satiety may follow two phases, satiety to a specific food, and then general satiety for the meal (Hetherington, 1996). The sensory properties of a food have an effect on psychological and physiological appetitive processes. This phenomenon is illustrated particularly well when considering the process called the cephalic phase response. The sight and smell of food is known to trigger responses in the body in preparation for a meal, e.g. production of more saliva (Pavlov, 1927, cited in Mela and Rogers, 1998), gastric acid secretion and the release of insulin (Sjostrom et al., 1980). Gastrointestinal hormones are also released (Powley & Berthoud, 1985). There are a number of possible functions for these cephalic phase physiological responses: buffering the body against excess nutrient loads; providing an internal signal for hunger; or perhaps preparing the body for the ingestion, digestion and absorption of nutrients. These physiological reflex responses may be heightened by the increased palatability of the food (Louis-Sylvestre & Le Magnen, 1980), although this depends on the form of sensory stimuli in human beings (Bellisle et al., 1985; Teff et al., 1995; Teff & Engelman, 1996).

Food can affect eating behaviour via learned food preferences and aversions, demonstrated in laboratory animals and human subjects (Holman, 1968; Booth et al., 1982; Weingarten & Kulikovsky, 1989; Warwick & Weingarten, 1994; Sclafani, 1997). Food can also influence emotional state, cognitive function and fatigue through the macronutrient content of a meal (Lloyd et al., 1996), tryptophan intake (Spring et al., 1987), or its pharmacological constituents such as caffeine and alcohol (Rogers, 1995).

1.4.3 External influences

Eating behaviour and food intake is strongly influenced by external influences on a number of levels - religious, cultural, geographical, and social. Different cultures attach values to specific foods, e.g. people talk about “addiction” to chocolate. Foods have different uses (beyond that of nutrition) in certain contexts and are appropriate for different moods, e.g. comfort foods, food as a treat or reward, or food as a gift.
The time of day affects meal composition, size, frequency and duration. Time constraints due to work or family life are important in determining human feeding patterns but there is some evidence that these factors are secondary to preprandial factors such as time interval since last eating episode in terms of energy intake (De Castro et al., 1986). Social setting is also an important determinant of eating behaviour (Kim & Kissileff, 1996).

1.4.4 Psychological influences

Psychological factors influencing eating behaviour are complex. There is a great risk that many experimental designs, aiming to untangle the physiological regulators of appetite, are inherently flawed either because they fail to take account of the psychological profile of an individual or because the sensitivity of design is not great enough to pick up physiological patterns, above the "noise" of psychological influences. It is common practice now for researchers into eating behaviour to employ questionnaires, e.g. the Dutch Eating Behaviour Questionnaire (DEBQ) (Van Strien et al., 1986), the Three Factor Eating Questionnaire (TFEQ) (Stunkard & Messick, 1985), to measure a person's psychological profile and eating attitudes before a decision is made on whether to include them as a subject, or to target specific patterns of behaviour. These screening tools are based upon three main theories of eating behaviour – Externality theory, Psychosomatic (emotional) theory, and Restraint theory. The psychosomatic view stated that obesity is a result of overeating in response to emotional feelings (Rogers, 1993), and that excessive eating can be used as a way of coping with depression or stress. Another theory, the externality theory (Schachter, 1968) states that a person may disregard their internal state of satiety in response to external stimuli, especially food-related stimuli, a trait that was postulated to be a feature of obesity. The Restrained eating theory brings together both these theories of eating in response to emotions or external stimuli and disregarding internal signals. Pressure to diet causes unmaintainable self-restriction of food intake, triggering feelings of depression. A process called disinhibition, where self-imposed restrictions break down, frequently ensues leading to excessive eating and a perpetuation of the cycle (Herman & Polivy, 1984). The Dutch Eating Behaviour Questionnaire (DEBQ) was developed from a factor analysis of large groups of normal and overweight people chosen to reflect eating behaviours of interest (Van Strien et al., 1986). It measures three areas of eating behaviour theory – restrained
eating, emotional eating and external eating, with a choice of five responses: 'never', 'seldom', 'sometimes', 'often', 'very often', and where appropriate 'not relevant'. See appendix II.

1.5 Conclusions

In the light of the evidence discussed here, it appears that the notion of a single aminostatic model to explain the regulation of appetite is over-simplistic and that the physiological explanation for protein- and amino acid-induced satiety may embrace several different mechanisms. The phenomenology of protein/amino acids and human appetite is itself complex as shown in figure 1.4.
The mechanisms whereby protein and amino acids may influence hunger and satiety are shown in relation to body organs and compartments represented by the outer circle, the circulation, middle circle, and the central nervous system, the inner circle. Macronutrients in the stomach pass into the small intestine with proteins hydrolysed to polypeptides, peptides and amino acids. There are two possible routes whereby amino acids in the intestine can influence satiety. Amino acids can act directly to stimulate specific vagal afferent receptors in the upper intestinal mucosa, which may stimulate satiety centres in the brain, activating vagal efferent neurons that delay gastric emptying or increase secretion of CCK, or activating fibres of the enteric nervous system to stimulate CCK secretion. Another pre-absorptive route involves intestinal amino acids stimulating the CCK-secreting I cells bordering the small intestine lumenal wall. Protein may stimulate CCK by another route. Trypsin, secreted by the pancreas into the small intestine, may suppress CCK secretion by degrading a pancreatic CCK-releasing peptide (monitor peptide) and a luminal CCK-releasing peptide (Liddle, 1997). However, the entry of protein into the small intestine protects these CCK-releasing factors by binding trypsin (Calam et al., 1987). CCK acts peripherally to delay gastric emptying and amplify gastric distension via vagal afferent nerves originating from the stomach wall and intestine (Schwartz & Moran, 1994; French et al., 1993). CCK also acts centrally via CCK-A (peripheral) and CCK-B (central) receptors to increase satiety. Amino acids are eventually absorbed and enter the circulation. Absorbed amino acids may influence satiety by acting as neurotransmitters (GABA, glutamate) or precursors of neurotransmitters (DA, 5-HT) in the CNS. The intestinal carbohydrate stimulates insulin secretion from the pancreas (via secretagogue effects on insulinotropic factors), which increases uptake of circulating amino acids into the body protein mass. Hepatic amino acid uptake, protein accretion and amino acid oxidation are important in regulating circulating amino acid concentrations. Hepatic vagal afferents sensitive to individual amino acids may also be a pathway for an amino acid-derived satiety signal. Similar peripheral aminostatic signals monitored by the CNS have yet to be identified in skeletal muscle but might be expected given the large potential for modifying circulation amino acid concentrations during periods of rapid growth when substantial protein accretion occurs.
When metabolic demands for protein are low, as in the well nourished adult, hunger sensations are slight and protein acts as the most highly satiating macronutrient, although there can be minor adaptive changes in the degree of its satiating power with variation in habitual intake. In contrast the high metabolic demands for protein of rapid growth appear to result in a specific hunger drive for dietary protein and loss of its satiating power over a wide range of intakes. It is not known whether the changes in hunger and satiety involve separate mechanisms peripherally and centrally, or whether there is a simple inverse relationship between protein-related hunger and satiety as a function of the relationship between metabolic demand and dietary supply. However, an aminostatic mechanism for appetite regulation a) provides for biological requirements by monitoring the balance between dietary intake and metabolic demands for amino acids, especially in terms of net uptake into the body protein mass, which is then relayed to hunger centres, and b) protects against toxicity through strong satiety and hormonal/neuronal responses to specific amino acids or high protein loads involving both pre-absorptive and post-absorptive elements. The postprandial rise in circulating amino acids may also initiate signals to satiety centres in the CNS in the short term (i.e. in the hours following a meal) either directly or by hepatic mechanisms. In addition, the very short-term regulation of food intake, (i.e. during and immediately following a meal) is highly dependent on pre-absorptive satiety signals arising from protein/amino acids in the gastrointestinal tract. Protein- and amino acid-induced satiety mechanisms (as shown in figure 1.4) should be considered against a background of other regulatory systems that influence food intake, in particular the physiological and metabolic processes that are linked to glucostatic or lipostatic regulation. The available evidence supporting aminostatic control is meagre and this area of research is deserving of much more interest than it has received hitherto. There are some exciting leads in neurological science that may clarify the nature of amino acid-linked appetite signals.

1.6 Aims and objectives of the current research

The research projects described in this thesis originated from the desire for a greater understanding of the physiological mechanisms behind satiety, specifically with regard to appetite mechanisms arising from the ingestion of protein or individual amino acids. Scientific publications covering the role of protein or amino acids in appetite have shown that protein is the most satiating macronutrient, kilojoule for kilojoule, but few papers have been published on the biological importance and
physiological mechanisms behind these observations. The current research focuses on the importance of putative short-term satiety signalling pathways in relation to protein or amino acid ingestion, such as the release of two satiety gut hormones, CCK and GLP-1, and effects on gastric emptying.

The main objectives of the research were as follows:

a) **To further investigate the effectiveness and mechanisms of an individual amino acid, phenylalanine, in reducing appetite.** This idea was conceived within the context of using one single amino acid as a possible appetite suppressor that may prove useful in the treatment of obesity. Phenylalanine was a promising candidate for this due to existing research that had shown aspartame (of which phenylalanine is a major component) can suppress food intake in human volunteers (Rogers *et al.*, 1990). The effectiveness of phenylalanine in reducing appetite and the physiological mechanisms by which this occurred were thus investigated.

b) **To explore the physiological mechanisms underlying the differences in satiating properties of different protein types.** Satiety and hormone and metabolite responses following liquid meals, containing predominantly whey or casein protein, were measured with the purpose of understanding the mechanisms behind the appetite response to high-protein meals.

c) **To assess the repeatability of the methodology used to measure food intake and subjective appetite.** Repeated identical preloads and *ad libitum* buffet-style test meals were used on three study days to determine the reproducibility of methods frequently used in the current work, and by other researchers, to measure hunger and satiety.

d) **To compare different available methods of measuring gastric emptying rate.** Gastric emptying was measured by electrical impedance epigastrography (EIE) by colleagues in the Department of Physics as part of the current work. Initial studies showed that gastric emptying times obtained by this method did not correspond to those reported in the literature, and so further studies were planned to compare EIE gastric emptying times with those obtained by scintigraphy and other methods.
Chapter Two
Chapter 2

MATERIALS AND METHODS

The following chapter will describe the practical materials and methods used in carrying out the series of investigations. Individual protocols for each study will be described at the beginning of each chapter, together with any alterations to the methods described here.

2.1 Materials

2.1.1 Equipment

Butterfly needles, 0.8 x 22.2 mm (venisystems, Sligo, Ireland)
Cannulae, 1.1 x 28 mm (Y-Can, Simcare, Lancing)
Capsules, opaque, gelatine, size 0 (gift from Dr P. Rogers, University of Bristol, Bristol)
Chromatography columns, 35 micron filter (Evergreen Scientific, Los Angeles, USA)
Cobas Mira automated analyser (Roche Products Ltd, Welwyn Garden City)
Cuvettes (Roche Products Ltd, Welwyn Garden City)
Deltatrac (Datex, Helsinki, Finland)
Fluoride oxalate tubes, 1 ml (L.I.P. Ltd, Shipley)
Gamma counter (Wallac Wizard 1470 with Multicalc Level 4.M software)
HPLC Waters Pico-Tag System with Maxima 820 software, Millipore (1987) (Waters, Massachusetts, USA)
Lignocaine Hydrochloride Injection B.P. 2% w/v 5ml (Antigen Pharmaceuticals Ltd, Roscrea, Ireland)
Lithium heparin tubes, 5 ml/10 ml (L.I.P. Ltd, Shipley)
Needles, 0.5 x 16 mm, 0.8 x 40 mm (Terumo Europe N.V., Leuven, Belgium)
Potassium EDTA tubes, 1 ml/5 ml (L.I.P. Ltd, Shipley)
Test tubes, polystyrene, LP3 and LP4 (L.I.P. Ltd, Shipley)
Vacutainers, K3 EDTA 4.5 ml & PST Gel and lithium heparin 6 ml, (Becton Dickinson Vacutainer Systems Europe, Meylan, France)
2.1.2 Reagents

125I CCK-8 sulphated, labelled with Bolton and Hunter reagent (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, BUCKS)
125I-sodium iodide (3.7 GBq/ml) (ICN Biomedicals Inc., Thame, OXON)
125I-insulin (from Dr S.M. Hampton, University of Surrey)
5-Sulfosalicylic acid (Sigma Chemical Company Ltd, Poole)
Agarose – type III-A (Sigma Chemical Company Ltd, Poole)
Albumin, Bovine Serum (BSA) Fraction V powder (Sigma Chemical Company Ltd, Poole)
Albumin, Human Serum (HSA) Fraction V powder (Sigma Chemical Company Ltd, Poole)
Aprotinin (Sigma Chemical Company Ltd, Poole)
Charcoal, activated, Norit PN.5 (BDH Ltd, Poole)
Chloramine T, (Sigma Chemical Company Ltd, Poole)
Ethanol (Hayman Ltd, Witham Essex)
Glucose assay kit (Unimate 5 GLUC HK, Roche Diagnostics Systems, Welwyn Garden City)
Hydrochloric acid (HCl) (BDH Laboratory Supplies Ltd, Poole)
Methanol, HPLC grade (Fisher Scientific UK Ltd, Loughborough)
Paracetamol assay kit (Cambridge Life Sciences plc, Ely)
Phenylisothiocyanate (PITC) (Sigma Chemical Company Ltd, Poole)
Polyethylene glycol (PEG) (BDH Chemicals Ltd, Poole)
Sephadex, G-15 for gel filtration (Sigma Chemical Company Ltd, Poole)
Sodium metabisulphate (Sigma Chemical Company Ltd, Poole)
Triethylamine (TEA) (Sigma Chemical Company Ltd, Poole)

2.1.3 Peptides and amino acids

Amino acids for standard solution (Sigma Chemical Company Ltd, Poole)
CCK-8 sulphated (26-33) (Bachem UK Ltd, Saffron Waldon, Essex)
GIP, synthetic human (Sigma Chemical Company Ltd, Poole)
GLP-1 fragment 7-36 amide, synthetic human (Sigma Chemical Company Ltd, Poole)
Insulin standard (NIBSC, South Mimms, Potters Bar, Hereford)
2.1.4 Antisera

Donkey anti-guinea pig (DAGP) (Guildhay Antisera Ltd, Guildford)
Donkey anti-rabbit (DAR) (from Dr S.M. Hampton, University of Surrey)
Donkey anti-rabbit Sac-Cel (IDS Ltd, Boldon, Tyne & Wear)
Guinea pig anti-insulin antisera (from Dr S.M. Hampton, University of Surrey)
Rabbit anti-CCK antisera (from Professor J. Calam, Royal Postgraduate Medical School, Hammersmith Hospital)
Rabbit anti-GIP antisera, RIC 34 iii J (from Dr L.M. Morgan, University of Surrey)
Rabbit anti-GLP-1 antisera, G2-30/6/89 (from Dr L.M. Morgan, University of Surrey)

2.1.5 Sera

Normal Guinea Pig serum (from Dr S.M. Hampton, University of Surrey)
Normal Rabbit serum (Sigma Chemical Company Ltd, Poole)

2.1.6 Buffers

Reverse osmosis (RO) water was used for preparing all buffers unless otherwise stated.

**CCK radioimmunoassay buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaH2PO4-H2O (sodium dihydrogen orthophosphate 1-hydrate)</td>
<td>0.05M</td>
</tr>
<tr>
<td>EDTA (ethylenediaminetetraacetic acid)</td>
<td>0.01M</td>
</tr>
<tr>
<td>Gelatine powder</td>
<td>0.25%</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.02%</td>
</tr>
</tbody>
</table>

1 litre of water is added, warmed to dissolve gelatine, and pH is adjusted to 7.4 with NaOH. Stored at 4°C.

**GIP radioimmunoassay stock buffer**

(0.4M phosphate buffer, pH 6.5)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH2PO4 (potassium dihydrogen orthophosphate)</td>
<td>A 0.4M</td>
</tr>
<tr>
<td>Na2HPO4 (di-sodium hydrogen orthophosphate anhydrous)</td>
<td>B 0.4M</td>
</tr>
</tbody>
</table>

1 litre of each solution was prepared and solution A was added drop-wise to solution B to give required pH. Stored at 4°C.
GLP-1 radioimmunoassay stock buffer
(0.4M phosphate buffer, pH 6.5)
as above

Insulin radioimmunoassay stock buffer
(0.04M phosphate buffer, pH 7.4)
23 g Na₂HPO₄ (di-sodium hydrogen orthophosphate anhydrous)
5.97 g NaH₂PO₄·2H₂O (sodium dihydrogen orthophosphate)
Made up to 5 litres and stored at 4°C.

Bicarbonate buffer for column affinity purification of GIP and GLP-1
(0.1M Na₂CO₃/NaHCO₃ buffer, pH 9.8)
10.6 g Na₂CO₃ (sodium carbonate)
8.5 g NaHCO₃ (sodium hydrogen carbonate)
Dissolved in 1 litre water and stored at 4°C.

Buffers for iodination of GIP and GLP-1
0.4M phosphate buffer, pH 7.4
(see insulin RIA stock buffer)

0.1M sodium acetate, pH 5.0
13.61 g C₂H₃O₂Na·3H₂O (sodium acetate trihydrate)
in 1 litre water. Titrated to pH 5.0 with 1M glacial acetic acid. Stored at 4°C.

Sodium acetate buffer added to blood collection tubes for CCK analysis
1.02 g NaCl (sodium chloride)
3 ml C₂H₄O₂ (glacial acetic acid)
6.3 ml 1M NaOH (sodium hydroxide)
Made up to 100 ml with water and adjust to pH 3.6 with HCl. Stored at 4°C.

Eluent 1 for Waters Pico-Tag HPLC analysis of amino acids in plasma
70mM sodium acetate trihydrate, pH 6.45 + 2.5% acetonitrile
38.1 g C₂H₃O₂Na·3H₂O (sodium acetate trihydrate)
Dissolved in 4 litres Milli-Q water and adjust pH to 6.45 with 10% acetic acid. Filtered under vacuum (0.45 micron filter). Added 100 ml of acetonitrile to 3900 ml buffer. Stored at 4°C.

**Eluent 2 for Waters Pico-Tag HPLC analysis of amino acids in plasma**

450 ml C$_2$H$_3$N (MeCN) (acetonitrile HPLC grade)
400 ml H$_2$O (Milli-Q)
150 ml CH$_3$OH (MeOH) (methanol HPLC grade)

2.1.7 Miscellaneous

Aspartame, NutraSweet powder (donated by Forum Products Ltd, Redhill)
Casein protein powder, strawberry flavour (Tropicana World Ltd, Birmingham)
Cornflour (Sainsbury's)
Double cream (Tescos)
L-alanine for consumption (Scientific Hospital Supplies, Liverpool)
L-aspartic acid for consumption (Scientific Hospital Supplies, Liverpool)
L-phenylalanine for consumption (Scientific Hospital Supplies, Liverpool)
Maltodextrin (free sample from Cerestar Pur, Manchester)
Nesquik (Nestle)
Paracetamol, Panadol tablets (SmithKline Beecham)
Sodium [1-$^{13}$C] octanoic acid (a gift from Professor J. Mathers, University of Newcastle, Newcastle upon Tyne)
Whey protein powder, vanilla flavour (Prolab Nutrition, Bloomfield, Connecticut, USA)

2.2 Methods

2.2.1 Measurement of Appetite

Many of the investigations described in this thesis involve the measurement of appetite. Appetite is a difficult parameter to quantify. There are no tangible factors in the blood that can be assayed, nor physical characteristics that can be measured, as with most subject matter in the field of nutritional science. The multifaceted nature of the regulation of appetite, encompassing psychological as well as physiological influences, is reflected in the difficulty in measuring observable manifestations of
appetite, usually referred to as 'eating behaviour'. The following sections describe the methodology used to measure eating behaviour.

2.2.1.1 Recruitment of subjects

The psychological factors that could influence eating behaviour are multiple and extremely complex. There is a great risk that many experimental designs, aiming to untangle the physiological regulators of appetite, are inherently flawed either because they fail to take account of the psychological profile of an individual or because the sensitivity of design is not great enough to pick up physiological patterns, above the "noise" of psychological influences. It is common practice now for researchers into eating behaviour to employ questionnaires, e.g. the Dutch Eating Behaviour Questionnaire (Van Strien et al., 1986) and the Three Factor Eating Questionnaire (Stunkard & Messick, 1985), to measure a person's psychological profile and eating attitudes before a decision is made on whether to include them as a subject, or to target specific patterns of behaviour.

The Dutch Eating Behaviour Questionnaire (DEBQ) was developed from a factor analysis of large groups of normal and overweight people chosen to reflect eating behaviours of interest (van Strien et al., 1986). It measures three areas of eating behaviour theory – restrained eating, emotional eating and external eating, with a choice of five responses: 'never', 'seldom', 'sometimes', 'often', 'very often', and where appropriate 'not relevant' (see appendix II). During the recruitment process for practical work involving the measurement of appetite responses, every volunteer was required to complete the DEBQ before taking part. Only those scoring an average of less than 3.0 points (min 1, max 5), which would indicate eating behaviour that is likely to be relatively free of restrained, external or emotional influences, were asked to participate.

2.2.1.2 Measurement of food intake

There are numerous methods available to measure eating behaviour, and which methods are used depends on the subject of investigation, setting (laboratory, free-living, etc.) and duration of study. Frequently in nutritional science, investigators want to investigate the effect of a food or food component on subsequent eating behaviour. One way to do this is to measure *ad libitum* food intake.
The most common method is the *ad libitum* buffet test meal, which involves weighing a range of different foods of known energy and macronutrient content before it is offered to the subject as a meal, and then re-weighing what is left when the meal is finished (Hill *et al.*, 1995). The meal is often a selection of palatable foods, served in excess of that normally eaten at one meal, where the subject is free to eat as much of any type of food as they like to feel comfortably full. It is important that the subjects are at the same state of hunger on each occasion, that the subjects like the food, and that they are encouraged not to eat amounts in excess of that required to produce a comfortable level of fullness. This method is expensive and time-consuming in the purchasing and preparation of these test meals. However it is a more naturalistic method than many others available and macronutrient intake data may also be collected.

Preloading techniques are frequently used in the measurement of eating behaviour and appetite. The preload is a controlled meal that is given at a set interval before the test meal, and the subject is usually required to consume it all. This preload is also commonly the manipulated meal and its effects on subsequent behaviour and intake at the *ad libitum* test meal are measured. The interval between preload and test meal can be from a few minutes to five or six hours. The preload can be a liquid or sold meal, highly artificial or normal food.

In the current research, satiety was assessed by the measurement of energy and macronutrient intake 40-90 minutes (see individual methods sections) after consumption of a preload containing the variable of interest. The buffet-style *ad libitum* test meal consisted of a variety of palatable, cold, lunch-type foods on a tray—sandwiches with two types of fillings, salad items, full-fat fruit yoghurt, biscuits, and cake. (See appendix III for a description of the foods used). To ensure that the subjects liked the foods provided but did not eat excessively, they were asked to complete a questionnaire that rated food choices and excluded foods that they did not like (appendix IV). The subject's second choice for each food category was provided in the *ad libitum* test meal. A glass of water was provided with the meal. All foods were weighed before serving and re-weighed after the subjects had eaten to obtain the amount consumed (g) of each food. Energy and macronutrient content of the food consumed was calculated using an Excel spreadsheet with food composition data available from the manufacturers and in McCance and Widdowson's "The
Composition of Foods, 5th Edition” (McCance & Widdowson, 1991). The total energy available from a tray of food was approximately 12,500 kJ. Subjects were instructed to eat to a comfortable level of fullness on each occasion, and to prevent overeating they were informed that they could take the remaining food home with them.

2.2.1.3 Measurement of motivation to eat

Subjective, self-reported measures of hunger, satiety, palatability, mood, and physical well-being are commonly used tools for the study of eating behaviour. A common example of this method is the Visual Analogue Scale (VAS), where a series of 100mm lines appear on a page and words that describe the variable of interest are printed at either end of the scale. The subject is required to mark a point on the line that they feel best describes how they feel at that moment. The scale may be for two extremes of the same adjective, e.g.

Not at all hungry

As hungry as I’ve ever felt

or two adjectives to describe the two extremes of a feeling or sensation, e.g.

Drowsy

Alert

VAS measurements are most often employed in repeated measures, within subject designs and have proved to be reliable indicators in as far as they are sensitive to preload/test-meal manipulations. They also appear to have good validity in that people consistently associate the same sensations with the terms hunger or fullness (Hill et al., 1995). They are useful in tracking changes in appetite over time, (especially in conjunction with the measurement of food intake), and can be used to ensure that there are no other changes in mood or physical well-being that may confound the results. Unfortunately, investigators cannot be certain that when a subject reports an increase in hunger or decrease in fullness, that this does not reflect cognitive cues, which can occur if food is anticipated (Hill et al., 1995). One study
used VAS scores to test the association between subjects’ predictions of how filling a snack food would be (after tasting) and actual intake on a separate occasion (Green & Blundell, 1996). They found that ratings for sweet high-fat foods were higher than other foods but in fact led to a greater energy intake. This could be interpreted as a poor ability on the part of the subjects to predict using VAS scores how filling this particular category of food was, and that subjective scores for hunger and satiety sensations may not always correspond to an actual effect. However, it is more likely a reflection of the fact that sweet high-fat foods are highly palatable, are poorly regulated by internal satiety mechanisms, leading to passive over-consumption. Each subject may have a slightly different interpretation of concepts such as hunger in relation to how they feel, but if the experiment is a within-subject design then some of the disadvantages of self-reported scores for appetite are out of the equation. A further modification to the use of VAS measurements suggested by Green et al (Green et al., 1997) is the satiety quotient (SQ), which uses the VAS ratings of hunger and fullness and food intake data to calculate a quotient that relates intake to the rate of return of motivation to eat in the period after the test meal.

Another subjective measure frequently used in eating behaviour experiments is the food preference checklist. This is a list of different types of food, e.g. ‘a large tomato’, ‘fruit yoghurt’, ‘a bread roll’, etc. and the subject is required to tick each food they feel motivated to eat at that point in time (Blundell & Rogers, 1980). These foods are scored in terms of total energy and macronutrient energy, or total number of foods and number of foods high in a type of macronutrient. Subjects are told that they are not being asked to construct a menu and to consider each item individually. Results from this method are highly correlated with total food intake \( r = 0.64 \) to 0.76 (Blundell & Rogers, 1980). This method is easy to use and inexpensive. Motivation to eat is expressed in “real food” terms and can be determined in terms of macronutrient choice. Disadvantages include the possibility of subjects selecting foods that may reflect motivators other than hunger/satiety, or that may not relate to actual intake (“eyes bigger than the belly” effect!).

In the current research, subjects rated their hunger, desire to eat, and fullness at intervals using VAS ratings. The subjects were asked to rate how they felt at that moment and to regard the ends of the scales as the most extreme feeling they had ever
experienced. The VAS ratings were analysed by measuring the point marked on the
line with a ruler and entering the rating (cm) into an Excel spreadsheet (appendix V).
Food preference checklists were also completed simultaneously as part of some of the
current research. Each subject ticked foods that they would like to eat at that moment
from a list of various sweet and savoury foods, categorised into high protein, high
carbohydrate, high fat, low fat and mixed (high fat and high carbohydrate) food types
but presented in a random uncategorised list (appendix VI). They were instructed that
they were not expected to construct a menu from the list, but just to mark each food
that they felt like eating at that point in time. Again, the number of each type of food,
as well as the energy this would provide in terms of total food and food type (high
protein, high fat etc.) was calculated using Microsoft Excel.

2.2.2 Measurement of Gastric Emptying

2.2.2.1 Scintigraphy

Gastric emptying times were measured using radioangiographic techniques
carried out by Dr Rosemary Morton and Professor Patrick Horton at the Department
of Nuclear Medicine, Royal Surrey County Hospital. The scintigraphy technique
involves the incorporation of radiolabels into a meal and the detection of the
reduction in counts in the stomach by a gamma camera. The gamma camera is a large
detector comprises the collimator, which focuses the gamma rays using a scintillation
crystal into visible light, and then converts the light signals into electronic signals.
These electrical signals are then digitised into counts. Images are taken by the gamma
camera every minute until the stomach has emptied. Subjects were required to
consume a glucose or low/high fat liquid test meal through a wide bore plastic tube,
containing 12 MBq of $^{99m}$Tc-DTPA ($^{99m}$Technetium-diethylenetriaminepentaacetic
acid) label, whilst lying almost fully supine against the camera. DTPA was the
appropriate radiolabel carrier to use in liquid meals. The uniform distribution of the
$^{99m}$Tc-DTPA throughout the test meals was confirmed by imaging the meals in
volumetric tubes in front of the gamma camera, with and without various
concentrations of hydrochloric acid. Immediately following ingestion, gamma camera
imaging was begun using a dual-headed camera (anterior and posterior views) and a
dedicated nuclear medicine computer. The stomach was positioned in the centre of
the field of view. From 0 to 120 minutes after the subject's meal ingestion images were acquired every minute.

2.2.2.2 Electrical Impedance Epigastrography

EIE is a method of measuring gastric emptying using the decrease in conductivity (and therefore increase in impedance) that occurs when a non-conductive liquid meal is ingested and passes through the stomach (Sutton & McClelland, 1983; Pickworth, 1984; Sutton et al., 1985; McClelland & Sutton, 1985). This technique was carried out by Dr Anastasia Giouvanoudi and Dr Behain Amaee from the Department of Physics at the University of Surrey. Six skin-contact electrodes (one pair for inputting the electrical current and two pairs for recording the impedance information) were placed on the upper left abdomen and lower back of the volunteer. The electrodes were positioned according to surface anatomical features. The first was placed adjacent to and below the transpyloric plane (an imaginary plane half-way between the xiphisternum and the uppermost part of the ileum of the pelvis, XP), the second was placed above the transpyloric plane (to target the end of the proximal stomach), and the third electrode lay below and midway between the others over the antral pyloric region. The posterior electrodes were placed parallel to the anterior electrodes. The small (4mA) AC current generated was at a frequency of 100kHz (Sutton et al., 1985) and could not be felt by the subject. Subjects adopted a semi-supine position on the bed and a baseline measurement of impedance before ingestion of a liquid was recorded. Subjects were required to remain immobile if possible as the impedance profile was very sensitive to movement, creating data artifacts. When a satisfactory baseline had been obtained, the change in impedance that occurred as the liquid entered and emptied out of the stomach was measured (deflection). The test meals were in liquid form, non-conductive (< 2 milliSiemens/cm) and 450 ml in volume in order to induce a significant impedance deflection on ingestion. The half emptying time was calculated from the peak of the deflection as a result of ingestion of the meal and the point at which the trace has returned to baseline (figure 2.1).
2.2.2.3 $^{13}$C-octanoic acid breath test

This breath test uses a stable isotope for the indirect measurement of gastric emptying whereby the appearance of $^{13}$CO$_2$ in breath is monitored subsequent to ingestion and metabolism of $^{13}$C-octanoic acid or $^{13}$C sodium octanoate. Octanoic acid is an eight carbon fatty acid which occurs naturally in food. Because it is a medium chain fatty acid it is quickly absorbed in the duodenum and rapidly transported to the liver bound to serum albumin where it is oxidised to CO$_2$. Therefore, the time it takes $^{13}$CO$_2$ to be excreted in the breath reflects the time taken for the meal to empty from the stomach, be absorbed and metabolised. However, the digestion, absorption and metabolism of the labelled test meal is not thought to vary among individuals therefore any difference in this time is assumed to reflect differences in gastric emptying rate.

After an overnight fast, breath samples were taken at -10 and 0 minutes before the labelled test meal to obtain a baseline of $^{13}$CO$_2$ excretion. CO$_2$ production rate was measured using a Deltatrac (Datex, Helsinki, Finland) and ventilated hood with a flow rate of 40 ml/min. The Deltatrac was switched on an hour before use to warm up and was calibrated for atmospheric pressure and gas calibrated using a calibration gas (5% CO$_2$, 95% O$_2$). Measurements of CO$_2$ production (ml/min) were made for 20 minutes before the labelled test meal and then a further 4 sessions for 20
minutes over the next four hours. 50 mg $^{13}$C sodium octanoate (donated by Professor J.C. Mathers, Department of Biological and Nutritional Sciences, University of Newcastle, Newcastle upon Tyne) was incorporated into the liquid test meal. Breath samples were taken in duplicate through a drinking straw into evacuated glass tubes with screw on/off lids with rubber septa. Subjects were counted down and then blew gently for 8-10 seconds through the straw into the tube, which was immediately sealed. Breath samples were taken at frequent intervals for 4 hours following the test meal (see section 4.3.3).

$^{13}$C enrichment of the breath was determined with an isotope ratio mass spectrometer by Dr Chris Seal of the University of Newcastle (Newcastle upon Tyne). $^{13}$C excretion in the breath was expressed as atom percent excess (the percentage of $^{13}$CO$_2$ in total CO$_2$ above natural background of $^{13}$CO$_2$ concentration). The change from baseline of APE after the meal was multiplied by CO$_2$ production in µmol/min by time (time since the last sample, e.g. 5, 10 or twenty minutes), to give $^{13}$CO$_2$ in µmol produced in each time interval. This was summed over the four hours to give a cumulative curve, and then expressed as a percentage of the initial dose cumulatively to give a sigmoidal curve. These values were fitted mathematically using the model equations of Ghoos et al. (1993): $y = m(1-e^{-kt})$. $y$ is the percentage of cumulative $^{13}$C excretion in breath, $t$ is time in minutes, $m$ is an estimate of the total cumulative $^{13}$C recovery when time is infinite, and $k$ and $\beta$ are estimated constants. Half gastric emptying time (T50) and lag phase (Tlag) were calculated from this fitted curve model as:

$$T50 = \frac{-1}{k} \ln\{1-(0.5)^{1/\beta}\} \quad \text{and} \quad Tlag = \frac{-1}{k} \ln\{1-(0.1)^{1/\beta}\}$$

Figure 2.2 shows an example of a fitted curve.
2.2.3 Measurement of circulating metabolites and hormones

2.2.3.1 Blood sampling

Blood samples were taken at regular intervals from subjects via an intravenous cannula inserted into an antecubital vein under local anaesthetic by a qualified medical doctor. Blood samples were taken into tubes containing preservatives. They were kept on ice before centrifugation at 4 °C for ten minutes at 1560 \( g_{(av)} \) to separate the plasma. The plasma was immediately frozen at \(-20 \, ^\circ C\) for analysis at a later date. For the measurement of glucose, blood was transferred to 1 ml fluoride oxalate tubes before centrifugation. 10 ml lithium heparin tubes were used for collection of blood for the measurement of CCK (only one study), amino acids, insulin, and GIP. 5 ml lithium heparin tubes containing added aprotinin (200 KIU/ml blood) were used for the collection of blood for GLP-1. Chilled 5 ml EDTA tubes containing 1 ml sodium acetate buffer (pH 3.6) per 4 ml blood were used to collect blood for the measurement of the plasma CCK. After centrifugation, the separated plasma was transferred into capped LP3 tubes using plastic Pasteur pastettes and frozen at a temperature of \(-20 \, ^\circ C\).

For the measurement of CCK, 0.5 ml of the separated plasma was added to 1 ml 98% (v/v) ethanol in LP4 tubes (duplicates) on the study day. The LP4 tubes were capped and vortexed, then centrifuged at 1560 \( g_{(av)} \) for 15 minutes. The supernatant
was poured into fresh LP4 tubes and the pellet discarded. The supernatant was then either immediately dried for 14 hours in the Savant Speedvac on low vacuum, or stored at -80°C for a maximum of a week before drying. Dried samples were then stored at -80°C until assayed.

2.2.3.2 Analysis of glucose

Plasma glucose was assayed using the Unimate 5 Glucose HK kit supplied by Roche Diagnostic Products Ltd. (Welwyn Garden City, Hertfordshire). The samples were analysed automatically using the Cobas-Mira biochemical analyser (Roche Products Ltd., Hertfordshire), using the hexokinase method outlined below:

\[
\text{hexokinase} \\
\text{D-glucose} + \text{ATP} \rightarrow \text{D-glucose-6-phosphate} + \text{ADP}
\]

\[
\text{glucose-6-phosphate dehydrogenase} \\
\text{D-glucose-6-phosphate} + \text{NAD}^+ \rightarrow \text{D-gluconate-6-phosphate} + \text{NADH} + \text{H}^+
\]

The amount of glucose in any plasma sample will be proportional to the amount of NADH produced. The concentration of NADH was measured spectrophotometrically at 340nm. Quality control samples were inserted into the beginning and end of every assay run, and the interassay coefficient of variation (CV) for the assay was 1.3 %. Intra-assay variation was small, as indicated by a comparison of QC's run at the beginning and end of the assay. If QC values fell outside 2 standard deviations of the mean the assay was repeated.

2.2.3.3 Analysis of amino acids

Plasma free amino acids were analysed using the Waters PICO-TAG™ Amino Acid Analysis System, which involves the pre-column derivatisation of the sample with phenylisothiocyanate (PITC) followed by reversed-phase HPLC (high performance liquid chromatography).

2.2.3.3.1 Standard preparation

The physiological standard was prepared from individual amino acids (Sigma) dissolved in 0.1M HCl.
Prior to analysis of the samples four concentrations of the standard mixture (0.5, 0.25, 0.125 and 0.0625 mM) were run on the HPLC and calibration curves produced for each amino acid. This was in order to confirm that there was a linear relationship in relative peak areas (peak area of amino acid/peak area of internal standard) at each concentration. Norleucine (NLE) was used as the internal standard at a concentration of 0.25 mM in each standard mix. Fig 2.3 shows an example for phenylalanine.

Figure 2.3 Calibration curve for relative peak area of phenylalanine (x/NLE) at four different concentrations (0.5, 0.25, 0.125 and 0.0625 mM).

2.2.3.3.2 Deproteinisation

Plasma samples required preparation before derivatisation and analysis, involving the deproteinisation of plasma to obtain just free amino acids. Norleucine was used as an internal standard and was added to the samples before deproteinisation at the same concentration as that in the standard amino acid mixture (0.526 mM). Three methods were compared ultrafiltration, sulphosalicylic acid and acetonitrile, but the latter yielded very small peaks on the HPLC. The remaining two methods were consequently used to deproteinise the plasma samples.

Firstly, ultrafiltration was used for deproteinising the plasma samples from the aspartame study (section 3.5). This involves placing 100-200 μl of plasma plus
internal standard in an ultrafiltration device containing a Millipore PLGC membrane with a 10,000 molecular weight cut-off limit, and spinning the ultrafiltration device for 10 min at 2122 \(g_{av}\). A small amount of filtrate is recovered (approximately 50 \(\mu l\)) which can then be frozen at -20 °C or immediately derivatised. This is the method recommended by Waters because it protects the column from harmful high molecular weight compounds, eliminates risk of contamination, and produces a filtrate which is "highly reproducible from sample to sample". However, this relies on using a number of ultrafiltration devices, which limits the number of samples that can be deproteinised at one time. In addition, membranes were expensive and only came in packs of 100, which were quickly used when running large numbers of samples. Millipore later discontinued production of the membranes used to fit the ultrafiltration devices in the laboratory.

The alternative method of deproteinising plasma samples, sulphosalicylic acid (SSA) precipitation, was then adopted for the remaining studies. 190 \(\mu l\) of plasma (plus 10 \(\mu l\) internal standard (10 mM) to give a final concentration of 0.526 mM) was vortex mixed with 200 \(\mu l\) of SSA solution (40 \(\mu g/ml\)), and this was spun at 13000 \(g_{av}\) for 15 min. The supernatant was then either frozen at -20 °C or immediately derivatised.

2.5.3.3.3 Derivatisation

Standards, samples, controls and blanks were derivatised using a drying solution of methanol, 1M sodium acetate, and triethylamine (TEA) (2:1:1), and a derivatising solution of methanol, TEA, water, and PITC (7:1:1:1), according to Waters methodology. PITC is the main derivatising agent and produces phenylthiocarbamyl (PTC) amino acid derivatives, which are stable and easily separated by reversed phase HPLC. PITC is also known as Edman's reagent, and is used to degrade peptides in protein sequencing research. See appendix VII for the derivatisation protocol.

2.2.3.3.4 Eluents

Eluents were made in the laboratory at the University of Surrey. Eluent A consisted of Milli-Q water with 70 mM sodium acetate trihydrate + 2.5 % acetonitrile (pH 6.45) and eluent B comprised 45 % acetonitrile + 40 % Milli-Q water + 15 % methanol. Eluent A was filtered under vacuum before the acetonitrile was added and
stored at 4 °C. Both eluents were degassed by bubbling helium gas through the solution for 5-10 minutes prior to use.

The standard Waters gradient used was as follows:

<table>
<thead>
<tr>
<th>Time</th>
<th>Flow rate (ml/min)</th>
<th>% eluent A</th>
<th>% eluent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>1.0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>13.5</td>
<td>1.0</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>24.0</td>
<td>1.0</td>
<td>94</td>
<td>6</td>
</tr>
<tr>
<td>30.0</td>
<td>1.0</td>
<td>91</td>
<td>9</td>
</tr>
<tr>
<td>50.0</td>
<td>1.0</td>
<td>66</td>
<td>34</td>
</tr>
<tr>
<td>62.0</td>
<td>1.0</td>
<td>66</td>
<td>34</td>
</tr>
<tr>
<td>62.5</td>
<td>1.0</td>
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</tr>
<tr>
<td>75.0</td>
<td>1.0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>90.0</td>
<td>1.0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2.1 Reversed phase HPLC gradient used for Waters PICO-TAG™ Amino Acid Analysis System.

A Nova Pak C18 (3.9 x 300 mm I.D.), 60 Å, 4 µl column was used for the separation of the amino acid derivatives. The UV detector operated at a wavelength of 254 nm.

2.2.3.3.5 Processing of chromatograms and analysis of peak areas

Peaks were identified from relative retention times and peak areas were calculated using Maxima software on the PC. Further analysis, specifically the calculation of amino acid concentrations, was carried out using Microsoft Excel. Relative retention times were highly reproducible and closely followed those reported in the Waters manual. Typical chromatograms for a standard and a plasma sample are given in appendix VIII.

2.2.3.3.6 Quality controls

Fasting plasma samples from a volunteer were used as quality controls. Prior to running the study samples the reproducibility of the relative peak areas obtained from these quality control were tested. 7 QCs were prepared and 2 injections were carried out on each sample number. The mean of the 1st and 2nd injection from each sample were used to calculate intra-run CVs. Intra-run CVs were 10 % or less for all amino acids except arginine. Values obtained from the relative peak areas were compared with those obtained in the literature and shown to be in the correct range.
Although tryptophan was included in the standard mix, it degraded quickly and could not always be identified in the chromatograms.

The control samples were deproteinised (by ultrafiltration or sulphosalicylic acid) and derivatised at the same time as the rest of the samples in each run. These were analysed at the beginning and end of each HPLC run to ensure that the values were within range and that there was no big difference in values. The average coefficients of variance for each amino acid measured in the quality control sample within a run and between runs are listed in table 2.2 (n = 38).

<table>
<thead>
<tr>
<th></th>
<th>Inter-run CV (%)</th>
<th>Intra-run CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ultrafiltration</td>
<td>Sulphosalicylic acid</td>
</tr>
<tr>
<td></td>
<td>Ultrafiltration</td>
<td>Sulphosalicylic acid</td>
</tr>
<tr>
<td>asp</td>
<td>25.4</td>
<td>15.6</td>
</tr>
<tr>
<td>glu</td>
<td>17.5</td>
<td>13.3</td>
</tr>
<tr>
<td>ser</td>
<td>18.4</td>
<td>14.4</td>
</tr>
<tr>
<td>gly</td>
<td>15.5</td>
<td>15.9</td>
</tr>
<tr>
<td>tau</td>
<td>18.6</td>
<td>16.7</td>
</tr>
<tr>
<td>thr</td>
<td>21.0</td>
<td>14.8</td>
</tr>
<tr>
<td>ala</td>
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<td>13.1</td>
</tr>
<tr>
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<td>33.5</td>
<td>12.2</td>
</tr>
<tr>
<td>pro</td>
<td>20.1</td>
<td>15.4</td>
</tr>
<tr>
<td>tyr</td>
<td>14.3</td>
<td>16.6</td>
</tr>
<tr>
<td>val</td>
<td>15.5</td>
<td>14.2</td>
</tr>
<tr>
<td>met</td>
<td>23.9</td>
<td>15.1</td>
</tr>
<tr>
<td>ile</td>
<td>17.2</td>
<td>10.4</td>
</tr>
<tr>
<td>leu</td>
<td>17.8</td>
<td>13.1</td>
</tr>
<tr>
<td>phe</td>
<td>14.9</td>
<td>13.0</td>
</tr>
<tr>
<td>orn</td>
<td>22.7</td>
<td>12.7</td>
</tr>
<tr>
<td>Mean</td>
<td>19.7</td>
<td>14.2</td>
</tr>
</tbody>
</table>

Table 2.2. Mean inter-run and intra-run coefficients of variance (%) for HPLC quality control samples using both deproteinisation methods, ultrafiltration and sulphosalicylic acid precipitation.

Intra-run variation was low as most amino acids had mean CVs of less than 10%. However, inter-run variation in the quality control plasma samples was quite large, and noticeably larger when the plasma was deproteinised by ultrafiltration, ranging from 14.3% for tyrosine to 33.5% for arginine. Since intra-run variability was much lower, all samples from an individual subject under one treatment (i.e. all time points from a single study day) were prepared and run together, and data was transformed to change from baseline (x-baseline value) before comparing between conditions.
2.2.3.4 Analysis of paracetamol

Blood samples for paracetamol were collected in 1 ml potassium EDTA tubes. The analysis was performed using a commercial paracetamol assay kit (Cambridge Life Sciences) with the automated Cobas Mira biochemical analyser. It utilises an enzyme specific for the amide bond of acylated aromatic amines, which cleaves the paracetamol molecule, yielding p-aminophenol. This reacts specifically with o-cresol in ammoniacal copper solution to produce a blue colour. The absorbance of the blue colour is measured at 615 nm. Quality controls (QCs) were produced at the University of Surrey by administering a normal paracetamol dose with water to a fasted volunteer and collecting blood 45 min later. The blood was centrifuged and plasma was frozen in 500 µl aliquots. QCs were interspersed throughout all paracetamol assays. The intra- and inter-assay coefficients of variation for QCs at 0.19 mmol/L were calculated to be 4.7 and 8.4 % respectively.

2.2.3.5 Analysis of plasma gut and pancreatic hormones by radioimmunoassay

2.2.3.5.1 Analysis of insulin

Plasma insulin was analysed by radioimmunoassay using iodinated label and polyethylene glycol-accelerated double antibody precipitation by methodology developed by Hampton and Whithey (Hampton & Whithey, 1993) at the University of Surrey. The insulin radiolabel, $^{125}$I conjugated to human insulin, was produced at the University of Surrey, and the insulin antiserum was raised in guinea pigs injected with porcine insulin conjugated to ovalbumin. Normal guinea pig serum (NGPS) and donkey anti-guinea pig (DAGP) serum were used to separate the free insulin from the bound. The non-specific binding (tubes containing assay buffer, sample, radiolabel, NGPS, DAGP, and polyethylene glycol (PEG)) is measured as well as the specific insulin binding (tubes containing all of the above plus guinea pig anti-insulin serum). The charcoal stripped serum (CSS) was produced at the University of Surrey using serum collected from fasted volunteers (see section 2.2.3.5.5).

The insulin radioimmunoassay procedure was as follows:

1) LP3 tubes were set up and labelled in duplicate in racks on a cold tray, where each stage of the assay was carried out.
2) Assay buffer was prepared by adding 0.5 % bovine serum albumin to the stock insulin RIA buffer described in the materials section.

3) Plasma samples were defrosted and centrifuged at 1560 g (4 °C) to spin down fibrin clots.

4) Natural human insulin standards were double diluted to give a standard curve of 1500, 750, 375, 188, 94, 47, and 23 pmol/l.

5) On Day 1, a positive displacement pipette was used to add standards, charcoal stripped serum (CSS), QCs (quality controls), and samples. Insulin antiserum was diluted with assay buffer (1:15000) and added to all tubes except totals and NSB tubes.

6) On Day 2, insulin radiolabel was added to all tubes at 10,000 counts per minute (cpm) per 100 µl (per tube).

7) On Day 3, the bound and free antibody was separated by adding NGPS (1:200 dilution), DAGP (1:16 dilution) and 4 % PEG. Following incubation and centrifugation, the supernatant was aspirated under vacuum and the resultant pellet counted on an automated gamma counter (Wizard 1470) to obtain the cpm of the bound insulin. Insulin concentrations were then automatically calculated from the standard curve using the software linked to the gamma counter.

See table 2.3 for details of the protocol.
<table>
<thead>
<tr>
<th>REAGENTS</th>
<th>TUBES (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DAY 1</strong></td>
<td></td>
</tr>
<tr>
<td>Assay buffer</td>
<td>NSB Standard 250 200 350 250 350 250 350 250</td>
</tr>
<tr>
<td>Standard</td>
<td>NSB Standard 50 - - - - - -</td>
</tr>
<tr>
<td>CSS</td>
<td>NSB QC 50 - - - - - -</td>
</tr>
<tr>
<td>QC plasma</td>
<td>QC 50 - - - - - -</td>
</tr>
<tr>
<td>Sample</td>
<td>Sample 50 - - - - - -</td>
</tr>
<tr>
<td>Antiserum</td>
<td>Antiserum 100 100 - 100 - 100 100 -</td>
</tr>
</tbody>
</table>

Vortex mix all tubes and incubate for 24 hours at 4 °C

| **DAY 2** |               |
| Totals | NSB Standard 100 100 100 100 100 100 100 100 |
| Label | NSB Standard 100 100 100 100 100 100 100 100 |

Vortex mix all tubes and incubate for 24 hours at 4 °C

| **DAY 3** |               |
| Totals | NSB Standard 700 100 100 100 100 100 100 100 |
| NGPS | NSB QC 700 100 100 100 100 100 100 100 |
| DAGP | QC 700 100 100 100 100 100 100 100 |
| 4% PEG | NSB sample 700 100 100 100 100 100 100 100 |

Vortex mix all tubes and incubate for 2 hours at 4 °C. Centrifuge at 1300 g(,,, ) for 30 min, aspirate and count the pellet

Table 2.3 Insulin radioimmunoassay protocol.

Low and high QC samples were run at the beginning and end of an assay. The inter-assay coefficients of variation (CVs) for low, medium and high quality control samples at 62, 230 and 410 pmol/L were calculated to be 8.8, 11.4 and 13.2 % respectively. The laboratory intra-assay coefficients of variation (CVs) for low and high quality control samples were 4.2 % and 5.7 % respectively. If any QC values fell outside 2 standard deviations from the mean the assay was repeated.

2.2.3.5.2 Analysis of GIP

Plasma GIP was measured by double antibody radioimmunoassay using an iodinated label and double antibody precipitation by methodology established by Morgan and colleagues (Morgan et al., 1978) at the University of Surrey. The antiserum was raised in rabbits against porcine GIP conjugated to ovalbumin, and cross reacts 100 % with human GIP. GIP was iodinated (¹²⁵I-GIP) at the University of Surrey and the biosynthetic GIP standard was from Sigma. The charcoal stripped serum (CSS) was produced at the University of Surrey using serum collected from
fasted volunteers (see section 2.2.3.5.5). Donkey anti-rabbit Sac-Cel, a solid phase second antibody suspension, was used to separate the antibody-bound GIP from the free. The GIP radioimmunoassay procedure was as follows:

1) LP3 tubes were set up and labelled in duplicate in racks on a cold tray, where each stage of the assay was carried out.

2) Assay buffer was prepared by adding 0.5% human serum albumin and 50,000 KIU/100 ml aprotinin to the stock GIP RIA buffer described in the materials section.

3) Plasma samples were defrosted and centrifuged at 1560 \( g_{av} \) (4 °C) to spin down fibrin clots.

4) Synthetic human GIP standards were double diluted to give a standard curve of 800, 400, 200, 100, 50 and 25 pmol/l.

5) On Day 1, a positive displacement pipette was used to add standards, charcoal stripped serum (CSS), QCs (quality controls), and samples. GIP antiserum was diluted with assay buffer (1:9000) and added to all tubes except totals and NSB tubes.

6) On Day 2, GIP label (prepared in-house, see section 2.2.3.5.7) was affinity purified (see section 2.2.3.5.8) and then added to all tubes at 5000 counts per minute (cpm) per 100 µl (per tube).

7) On Day 4, the bound and free antibody was separated by adding DAR Sac-Cel (IDS). Following incubation and centrifugation, the supernatant was aspirated under vacuum and the resultant pellet counted on an automated gamma counter (Wizard 1470) to obtain the cpm of the bound GIP. GIP concentrations were then automatically calculated from the standard curve using the software linked to the gamma counter.

See table 2.4 for a detailed protocol.
<table>
<thead>
<tr>
<th>REAGENTS</th>
<th>DAY 1</th>
<th>TUBES (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Totals</td>
<td>NSB Standard</td>
</tr>
<tr>
<td>Assay buffer</td>
<td>-</td>
<td>300</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CSS</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>QC plasma</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Antiserum</td>
<td>-</td>
<td>100</td>
</tr>
</tbody>
</table>

Vortex mix all tubes and incubate for 24 hours at 4 °C

<table>
<thead>
<tr>
<th>DAY 2</th>
<th>Totals</th>
<th>NSB Standard</th>
<th>¢ Standard</th>
<th>Standard</th>
<th>NSB QC</th>
<th>QC</th>
<th>NSB sample</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Label</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Vortex mix all tubes and incubate for 48 hours at 4 °C

<table>
<thead>
<tr>
<th>DAY 4</th>
<th>Totals</th>
<th>NSB Standard</th>
<th>¢ Standard</th>
<th>Standard</th>
<th>NSB QC</th>
<th>QC</th>
<th>NSB sample</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-rabbit Sac-Cel</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Vortex mix all tubes and incubate for 1/2 hour at room temperature. Add 1 ml RO water. Centrifuge at 1100 g(avg) for 30 min, aspirate and count the pellet

Table 2.4 GIP radioimmunoassay protocol.

Low and high QC samples were run at the beginning and end of each assay. The inter-assay coefficient of variation for quality control samples at 383 pmol/L was calculated to be 11.4 %. The laboratory intra-assay coefficients of variation (CVs) for low and high quality control samples were 5.9 % and 4.1 % respectively. If QC’s fell outside 2 standard deviations from the mean the assay was repeated.

2.2.3.5.3 Analysis of GLP-1

Plasma GLP-1 was analysed using radioimmunoassay methods already established at the University of Surrey (Elliott et al., 1993). The GLP-1 (7-36) amide radiolabel was iodinated in-house. Antisera against synthetic human GLP-1(7-36) amide conjugated to bovine serum albumin were raised in rabbits, specific for the C-terminal amidated forms of the peptide. This cross-reacts less than 0.2 % with GLP-1(7-37) amide or GLP-1(1-37) amide, but 100 % with GLP-1(7-36) amide. GLP-1 standards were prepared using synthetic human GLP-1 at the University of Surrey. The charcoal stripped serum (CSS) was produced at the University of Surrey using serum collected from fasted volunteers (see section 2.2.3.5.5). Separation of antibody
bound and free GLP-1 is achieved by the use of Sac-Cel, as with GIP. The GLP-1 radioimmunoassay procedure was as follows:

1) LP3 tubes were set up and labelled in duplicate in racks on a cold tray, where each stage of the assay was carried out.

2) Assay buffer was prepared by adding 0.5 % human serum albumin and 50,000 KIU/100 ml aprotinin to the stock GLP-1 RIA buffer described in the materials section.

3) Plasma samples were defrosted and centrifuged at 1560 g (4 °C) to spin down fibrin clots.

4) Synthetic human GLP-1 standards were reconstituted in assay buffer and then double diluted with CSS to give a standard curve of 160, 80, 40, 20, 10 and 5 pmol/l.

5) On Day 1, a positive displacement pipette was used to add standards, charcoal stripped serum (CSS), QCs (quality controls), and samples. GLP-1 antiserum was diluted with assay buffer (1:6000) and added to all tubes except totals and NSB tubes.

6) On Day 2, GLP-1 label (prepared in-house, see section 2.2.3.5.7) was affinity purified (see section 2.2.3.5.8) and then added to all tubes at 5000 counts per minute (cpm) per 100 µl (per tube).

7) On Day 4, the bound and free antibody was separated by adding DAR Sac-Cel (IDS). Following incubation and centrifugation, the supernatant was aspirated under vacuum and the resultant pellet counted on an automated gamma counter (Wizard 1470) to obtain the cpm of the bound GLP-1. GLP-1 concentrations were then automatically calculated from the standard curve using the software linked to the gamma counter.

See table 2.5 for a more detailed protocol.
**2.2.3.5.4 Analysis of CCK**

Plasma CCK was analysed using radioimmunoassay methods originally established by Professor John Calam (Department of Gastroenterology, Royal Postgraduate Medical School, Hammersmith Hospital, London) (Beardshall et al., 1992) and further developed at the University of Surrey by Samantha Long and Linda Morgan. Plasma was collected into lithium heparin tubes or EDTA tubes containing sodium acetate buffer as described earlier (section 2.2.3.1). Ethanol extraction of the plasma samples was either completed immediately following the separation of the blood (i.e. during the study), or following a period of freezing at -20 °C (details in the methods sections for each chapter). The supernatant obtained from ethanol extraction...
was stored at -80 °C for a short time before drying under vacuum (Savant Speedvac SC210A, Lifesciences International). The dried samples were stored at -80 °C until the assay.

An antibody raised in rabbits (Dino-7 antisera) was obtained from John Calam and Mark Jordinson (Royal Postgraduate Medical School, Hammersmith Hospital). This antibody cross-reacts 100% with human sulphated CCK-8 and larger forms of CCK to a similar extent. Cross-reactivity to synthetic and natural gastrin is less than 1% (Beardshall et al., 1992). Synthetic sulphated CCK-8 (Bachem) was used as standard and sulphated CCK-8 125I-labelled with Bolton and Hunter reagent (Amersham Pharmacia Biotech UK Ltd, Amersham, Bucks) was used as the tracer. Incubation was performed at 4 °C for two further days and separation of free and bound tracer was carried out on day 4 by using anti-rabbit Sac-cel. The assay procedure was as follows:

1. On Day 1, duplicate LP4 tubes containing the dried supernatant from plasma ethanol extraction process were set up along with labelled tubes for the standard curve in racks on a cold tray, where each stage of the assay was carried out. CCK assay buffer was prepared according the recipe (see materials section).

2. Freeze-dried CCK standards (1.6 pmol/ml) were reconstituted in assay buffer and then diluted with buffer to give three dilutions of 1 (1 pmol/ml), 1:10 and 1:100. The three dilutions were added to the standard curve at three different volumes to give 100, 50, 20, 10, 5, 2, 1, 0.5 and 0.1 pmol/l.

3. CCK antiserum was diluted with assay buffer (1:100) and added to all tubes except totals and NSB tubes. CCK radiolabel (Amersham) was diluted to 1000 cpm/400 µl and then added to all tubes.

4. On Day 4, the bound and free antibody was separated by adding DAR Sac-Cel (IDS). Following incubation and centrifugation, the supernatant was aspirated under vacuum and the resultant pellet counted on an automated gamma counter (Wizard 1470) to obtain the cpm of the bound CCK. CCK concentrations were then automatically calculated from the standard curve using the software linked to the gamma counter. Values obtained were subsequently multiplied by 2, since standard curve values were per ml whereas samples contained only 0.5 ml of plasma.
See table 2.6 for the detailed protocol.

<table>
<thead>
<tr>
<th>REAGENTS</th>
<th>TUBES (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:100 dilution</td>
</tr>
<tr>
<td>DAY 1</td>
<td>Tot NSB Std</td>
</tr>
<tr>
<td>Assay buffer</td>
<td>-</td>
</tr>
<tr>
<td>Std</td>
<td>-</td>
</tr>
<tr>
<td>Anti-sera</td>
<td>-</td>
</tr>
<tr>
<td>Label</td>
<td>400</td>
</tr>
</tbody>
</table>

Vortex mix all tubes and incubate for 72 hours at 4 °C.

| DAY 4     | Tot NSB Std | Ø Std | 0.2 | 0.5 | 1 | 2 | 5 | 10 | 20 | 50 | 100 | QC | Sample |
| Anti-rabbit Sac-Cel | - | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

Vortex mix all tubes and incubate for 30 min at room temperature. Add 1 ml RO water. Centrifuge at 1100 g for 30 min, aspirate and count the pellet.

Table 2.6 CCK radioimmunoassay protocol.

Laboratory inter-assay coefficients of variation for low and high quality control samples, used at the beginning and end of each assay, were reported to be 10.6 and 18.2 % respectively (Long, 2000)

2.2.3.5.5 Preparation of charcoal stripped serum

Charcoal stripped serum is used for plasma hormone radioimmunoassays in the standard curve. It is pooled plasma that has been stripped of small proteins and peptide hormones, and is added in order to mimic the serum in the samples. Agarose coated charcoal was previously prepared by mixing 150 g Norit PN.5 charcoal with 500 ml distilled water and leaving to settle. The water was decanted and this process was repeated until the water was clear. This defines the charcoal (removal of fine dust-like particles). The charcoal was left to dry overnight. 25 g agarose was dissolved in 500 ml distilled water and heated to 70 °C. 100 g of the de-fined charcoal was mixed with the agarose and cooled to 50 °C. The solution was poured into 1 litre acetone and stirred before filtering. The charcoal filtrate was dried overnight.

Fasted blood is collected from 3 to 5 volunteers (to accumulate 500 ml or more) and the blood is left standing in Kilner jars at room temperature for 2-3 hours in order to obtain a clot. The blood is then stored at 4 °C overnight. The next day, serum is decanted from the blood clot and pooled. The prepared charcoal is added to
the pooled serum at 25 g per 200 ml of plasma and the resulting slurry is left to mix overnight at 4 °C. The slurry is then centrifuged at 6500 g_{av} for 60 minutes. The supernatant is removed and filtered. A 0.25 ml aliquot of the filtered serum is centrifuged at 13,000 g_{av} and checked for remaining fines of charcoal. The filtering process is repeated until no charcoal fines remain. Charcoal stripped serum is stored in aliquots of 10 ml at −20 °C.

2.2.3.5.6 Quality controls

Pooled plasma obtained from several volunteers who donated blood was used for quality controls for GIP, GLP-1, insulin and CCK radioimmunoassays. Volunteers either gave blood after an overnight fast or 45 to 60 minutes following a high fat, high carbohydrate meal (3 assorted doughnuts) to produce the low and high quality controls (QCs). Plasma QCs for plasma paracetamol analysis on the Cobas Mira were from the blood of a fasted volunteer who had consumed 1.5 g paracetamol 45 minutes earlier. All QCs were stored at −20 °C in 1 ml aliquots.

2.2.3.5.7 Iodination

\(^{125}\)I-GIP and \(^{125}\)I-GLP-1 were produced at the University of Surrey using the same method for both peptides. 3 g of Sephadex G-15 was left overnight in 15 ml of 0.1 M sodium acetate pH 5.0 at room temperature. On the day of the iodination the swollen Sephadex was poured into a plastic chromatography column (35 micron filter) with a yellow pipette tip and a length of 1 mm PVC tubing attached. 15 ml of protein elution buffer (0.5 % HSA and 5000 KIU/ml aprotinin in 50 ml of 0.1 M sodium acetate pH 5.0) was allowed to drip through the column.

5 µg of GIP or 3 µg of GLP-1 (7-36) amide had previously been weighed out on the microbalance, and then dissolved in 10 µl of 0.4 M phosphate buffer. The column, peptide and other equipment were transferred to the hot lab where the \(^{125}\)I sodium iodide (10 µl containing 37 MBq) was added to the peptide. 10 µl of chloramine T solution (7.5 mg in 5 ml 0.4 M phosphate buffer pH 7.4) was added to the radiolabel and peptide, and the reagents were mixed with the displacement pipette tip for 15 seconds. 20 µl of sodium metabisulphite solution (10 mg in 5 ml 0.4 M phosphate buffer pH 7.4) was immediately added to stop the reaction. 200 µl of protein elution buffer was added to the iodinated peptide, and then the whole solution was transferred to the top of the Sephadex column. The labelled peptide solution was
allowed to drip through, while protein elution buffer was gradually added to the top of the column, and the drips were collected into LP4 tubes. Ten drips were counted per fraction, and a 10 µl aliquot of each fraction was counted on the gamma counter to produce an elution profile with two peaks. The fractions of the first peak were kept as they contained the iodinated peptide. The second peak was the free iodine. The chosen fraction (usually the highest or the one after) was aliquoted into 20 µl aliquots in LP3 tubes containing 100 µl of 10 % HSA, and the aliquots were frozen and stored at -20 °C. See figure 2.4 for an example of an elution profile.

![Figure 2.4 Elution profile of a GLP-1 iodination (counts per minute in 10 µl of fraction).](image)

### 2.2.3.5.8 Affinity purification of GIP and GLP-1

The label was added on day 2 of the GIP and GLP-1 radioimmunoassays, and affinity purification was carried out immediately beforehand. Designated GIP and GLP-1 affinity columns had been prepared previously and were stored in bicarbonate buffer at 4 °C. Both affinity purification columns contained a solid phase (silica beads) of rabbit anti-GLP-1 or anti-GIP antibodies.

The bicarbonate buffer was allowed to drain out of the clamped column. The column was washed with 10 ml 0.0825M HCl (passed through under pressure). Then 20 ml of GIP/GLP-1 assay diluent (1:10 stock buffer + HSA and aprotinin) was allowed to drip through to equilibrate the column. The GIP/GLP-1 radiolabel was reconstituted with 3 ml assay diluent and layered on the stoppered column, which was
then capped and roller-mixed for 30 minutes. The column was then un-stoppered and the assay diluent was left to drain out. The column was washed with 20 ml RO water to remove more free iodine in addition to that removed on the day of iodination. 5 ml of 0.0825M HCl was gradually added to the column and the drips containing the iodinated peptide were collected in a 5 ml glass vial that had previously been rinsed in assay diluent. An aliquot of the eluted label was then counted and the dilution calculated to add approximately 5000 cpm/100 µl to each assay tube. The column was further washed with 10 ml water and 10 ml bicarbonate buffer, and then stored in bicarbonate buffer at 4 °C.

2.2.4 Statistical Analysis

Food intake was analysed using one factor repeated measures ANOVA using a statistical package for the PC (STATISTICA for Windows, Statsoft, Inc. (1997) Tulsa, OK, USA). This software was also applied for the analysis of subjective appetite responses and plasma hormone and metabolite data using two-way analysis of variance with repeated measures (ANOVA). Two-way, repeated measures ANOVA on \( x - b \) values (\( x \) minus baseline value) or two-way, repeated measures ANCOVA (analysis of covariance), with baseline values designated as covariates, were carried out where the baseline data were very variable. For example, these analyses were used where there was high inter- and intra-individual variation in VAS ratings, variable baseline ratings in the case of plasma amino acid data (see section 2.2.3.3.6), or where sample sizes were small. Paired t-tests were used where appropriate. Pearson's correlation coefficient was used to test the association between two sets of data; Spearman rank order correlation was used if the data was not normally distributed. Spearman rank order correlation is a non-parametric test that calculates the relationship between two sets of data by determining the number of concordant ranking pairs minus the number of discordant ranking pairs. Modifications and additional statistical procedures are described in the methods sections for each chapter.
2.3 Reproducibility of satiety methodology: the measurement of food intake using *ad libitum* test meals and visual analogue scales for the subjective assessment of appetite.

2.3.1 Aims

The purpose of this study was to investigate the reproducibility of the methodology used in the current research to measure eating behaviour. Subjects were given identical preloads under the same conditions on three separate occasions in order to assess variation in food intake and the reproducibility of the visual analogue scales.

2.3.2 Subjects

Eighteen healthy volunteers (10 male, 8 female) aged between 20 and 28 years were recruited from the University. The volunteers were all non-dieting, normal weight, non-smoking, and had no special dietary requirements (although two vegetarians were included) or eating habits. The volunteers were also screened using the DEBQ (van Strien *et al.*, 1986). Only those men and women scoring less than 3.0 overall were asked to participate. To ensure that the subjects liked the foods provided but did not eat excessively, they were asked to complete a questionnaire that rated food choices and excluded foods that they did not like (appendix IV). The subject's second choice for each food category was provided in the *ad libitum* test meal. Finally, all volunteers signed a consent form that stated they had read the Information for Volunteers sheet and agreed to the conditions of the study. The subjects were told that the study was investigating mood and food choice. The study protocol was approved by the local ethics committee.

2.3.3 Study protocol

A single-blind, within-subjects, repeated measures design was used for the experiment, with each subject serving as his or her own control. Each subject was required to attend the Clinical Investigation Unit at the University of Surrey on 3 occasions – each occasion for an identical liquid milkshake-type meal, the composition of which remained unknown to all but the investigators.

On the day of a study, subjects ate their breakfast (which they were required to keep consistent on each study day), after which they were required to drink only water and eat only what was provided for them on the study. They came to the
Clinical Investigation Unit between 1200 and 1300 h for a set lunch. This was to ensure that they were at a standardised level of hunger/satiety when they arrived for the study in the evening. Lunch was a commercially available pasta based ready-meal (the same type was consumed each study day) providing 1456-1644 kJ, 14.5-20.9 g protein, 11.0-13.5 g carbohydrate, and 4.3-6.4 g fat (mean 1623 kJ, 17.5 g protein, 12.3 g carbohydrate, 5.0 g fat). The subjects had been instructed to eat a light evening meal the day before and their customary (cereal-based) breakfast, and also to avoid strenuous exercise and alcohol from 2000 h the day before until the study. On the day of the study they were required to drink only water and eat only what was provided for them from breakfast onwards.

The subjects arrived at the Unit between 1715 and 1730 h and completed their first VAS ratings (appendix V). They then consumed the milkshake-type meal preload and then sat quietly together watching light television programmes. They filled out further VAS ratings 5, 20, 40, and 55 min after the preload, and were separated after 60 min to eat the buffet-style ad libitum test meal (see appendix III). The subjects were partitioned so that they could not see others eating, and they were instructed not to talk to each other in order to minimise external influences on their food intake. They were asked to eat as much as they liked until they felt comfortably full, and informed that if they would like more of the food on their tray they could take home the remainder. This was designed to prevent over-consumption beyond the state of comfortable fullness. Final VAS sheets and questionnaires were completed when they had finished eating. There were at least 7 days between sessions.

2.3.4 Test meals

An identical milkshake-type liquid meal was used on each study day as the preload. The subject was given a choice of strawberry or banana flavour, but this was kept consistent on each study day. The ingredients were blended with an electrical blender and made up to 450 ml with water (table 2.7):
Table 2.7 Composition of liquid preload used to test the reproducibility of satiety methodology

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double Cream</td>
<td>46 g (22 g water, 22 g fat, &lt;1 g protein, 1.2 g CHO, 828 kJ)</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>50 g (50 g CHO, 837 kJ)</td>
</tr>
<tr>
<td>Nesquik</td>
<td>20 g (20 g CHO, 326 kJ)</td>
</tr>
<tr>
<td>Total energy</td>
<td>1992 kJ</td>
</tr>
</tbody>
</table>

2.3.5 Statistical analysis

Tests of repeatability were performed on VAS ratings according to Bland and Altman (Bland & Altman, 1986) and Raben et al. (Raben et al., 1995). Thus coefficients of repeatability (CR), i.e. 2 x standard deviation (2 SD), on the differences between meal 1 and 2, meal 2 and 3, and meal 1 and 3 were calculated for fasting values, mean scores over the 5 to 55 min range, and post-meal values. The CR is defined so that 95% of the difference is expected to be less than 2 SD (British Standards Institution, 1979). The coefficient of variation (CV) was also calculated: CV (%) = (SD/mean of three meals) x 100. Correlation coefficients were calculated on subjects’ ratings between study sessions (r²). Lastly, the mean differences in VAS scores between sessions (between meal 1 and 2, meal 2 and 3, and meal 1 and 3) were tested by t-test for unpaired data assuming unequal variances for significant differences from zero (Bland and Altman, 1986) in order to check for bias.

2.3.6 Results

2.3.6.1 Energy and food intake

Energy intakes are illustrated according to study session order (first, second and third occasion) below (figure 2.5).
Figure 2.5 Energy intake following repeated identical 1992 kJ liquid meal preloads on three separate test occasions (mean ± SEM, n = 18). Intake was lower during the third meal compared to both the first (p = 0.004) and second meals (p = 0.01), but there was no difference between energy intake during the first and second study sessions.

One factor repeated measures ANOVA showed a clear order effect for ad libitum test meal energy intake (p = 0.006) (see figure 2.5). Post hoc comparison of the means for energy intake indicated that subjects consumed significantly less food on the third study session compared to the first session (p < 0.005) and the second session (p < 0.05).

One factor repeated measures ANOVA of the macronutrient data (figure 2.6) showed that the reduction in energy intake was distributed evenly, rather than a selection of one type of macronutrient, with significant differences in protein (p = 0.002), carbohydrate (p = 0.006) and fat (p = 0.03).
Figure 2.6 Macronutrient intake following repeated identical 1992 kJ liquid meal preloads on three separate test occasions (mean ± SEM, n = 18). The post hoc Duncan test showed that intake was lower during the third meal compared to both the first (protein and CHO p = 0.003, fat p = 0.02) and second meals (protein p = 0.003, CHO p = 0.02, fat p = 0.03), but there was no difference between intake during the first and second study sessions.

Coefficients of variance (CVs) of energy intake for each subject were calculated as a measure of within-subject variance for energy intake. The mean CV in ad libitum test meal intake over the three study sessions was 13.9 %, ranging from 6.7 % to 25.8 %, showing that subjects differed greatly in their ability to respond consistently to an identical preload. Further analysis of the distribution of within-subject CVs showed that 11 out of 18 of the subjects had inter-meal CVs between 6 and 15 %. There were no significant differences between men and women in the magnitude of within-subject variability in energy intake (mean CVs 15.3 % men, 12.1 % women), but the women had a smaller range of CVs (6.7-25.8 % men, 7.8-18.5 % women).

2.3.6.2 Subjective appetite ratings

The results for hunger, desire to eat and fullness are illustrated (see figures 2.7, 2.8 and 2.9). Repeated measures two-way ANOVA on hunger, change in hunger, desire to eat, change in desire to eat, fullness, and change in fullness showed no differences between study sessions (meal order), or meal order x time interactions. As expected, there was a highly significant time effect on all appetite scores, p <0.00001. It can be seen from the graphs that the profiles for hunger and desire to eat were very similar on each occasion, and that fullness ratings did not differ significantly.
Figure 2.7 VAS ratings (cm) for hunger following repeated identical 1992 kJ liquid meal preloads on three separate test occasions (mean ± SEM, n = 18).

Figure 2.8 VAS ratings (cm) for desire to eat following repeated identical 1992 kJ liquid meal preloads on three separate test occasions (mean ± SEM, n = 18).

Figure 2.9 VAS ratings (cm) for fullness following repeated identical 1992 kJ liquid meal preloads on three separate test occasions (mean ± SEM, n = 18).

See appendix XXX for repeatability tables. Mean differences in ratings (including baseline, post-preload (1 hour, means), and post-ad libitum test meal ratings) between meals (meal 2-1, meal 3-1, and meal 3-2) were not significantly different from zero using a t-test for unpaired data assuming unequal variances (Blond & Altman, 1986; Raben et al., 1995). The correlation coefficients ($r^2$) over all baseline and post-preload VAS ratings ranged between 0.14 and 0.78, and the
Figure 2.8 VAS ratings (cm) for desire to eat following repeated identical 1992 kJ liquid meal preloads on three separate test occasions (mean ± SEM, n = 18).

Figure 2.9 VAS ratings (cm) for fullness following repeated identical 1992 kJ liquid meal preloads on three separate test occasions (mean ± SEM, n = 18).

See appendix IX for repeatability tables. Mean differences in ratings (including baseline, post-preload (1 hour means), and post-\textit{ad libitum} test meal ratings) between meals (meal 2-1, meal 3-1, and meal 3-2) were not significantly different from zero using a t-test for unpaired data assuming unequal variances (Bland & Altman, 1986; Raben \textit{et al}., 1995). The correlation coefficients ($r^2$) over all baseline and post-preload VAS ratings ranged between 0.14 and 0.78, and the
majority were statistically significant. However, the statistical significance of these correlation coefficients dropped considerably following the *ad libitum* test meal (range $r^2$ 0.00 – 0.36). CVs (%) were calculated for each subject for their baseline, post-preload (1 hour means), and post- *ad libitum* test meal VAS ratings to see how their scores varied between study sessions (see figure 2.10). Baseline and post-preload fullness ratings appeared to be more variable than those for hunger and desire to eat. Post- *ad libitum* test meal scores were less variable for fullness ratings compared to hunger and desire to eat.

![Figure 2.10 Mean within-subject CVs (%) on VAS ratings following repeated identical 1992 kJ liquid preloads (baseline, post-preload, and post- *ad libitum* test meal) for hunger, desire to eat (DTE) and fullness VAS ratings. Within-subject CVs (%) were increased for hunger and DTE ratings after the *ad libitum* test meal, compared to fullness, whereas CVs were increased for baseline fullness ratings and post-preload compared to hunger and DTE ratings.]

### 2.3.7 Discussion

#### 2.3.7.1 Food intake

Administering an identical preload on three separate occasions led to a non-significant decrease in subsequent mean food intake on the second session, and a statistically significant reduction by the third session.

The food intake results may reflect a sensory-specific satiety process. Sensory-specific satiety is a term usually used to describe a decrease in the
pleasantness of the food during the course of a meal, leading to the termination of eating. It is generally accepted that this process is a food-specific satiety, unrelated to post-prandial effects and entirely a result of sensory influences (Hetherington et al., 1989). In the present study it seems likely that the sensory properties of the buffet-style test meal are having a psychological effect on appetitive response. The most obvious explanation for the decrease in intake is that the attractiveness and perceived pleasantness of the meal decreased on the second and third occasions, which could be termed "sensory apathy". Another explanation may be that the subjects knew they could leave the investigation unit once they had eaten, and the circumstances were no longer novel on the second and third study sessions, which may have led to them rushing the *ad libitum* test meal in order to leave the study earlier.

The majority of subjects (11 out of 18) did not have very high inter-meal variability in food intake (<15%). This suggests that it is the minority of subjects that may yield unreliable results in a study using an *ad libitum* food intake experimental design to measure the effect of a nutritional variable on appetite control. It is possible that this minority comprise individuals that slipped through the net during the screening process, i.e. they deviate from the "normal" eating behaviour pattern but this was not detected by the DEBQ, nor were they excluded by screening out smokers, dieters, and high-level exercisers. Consideration must be given to whether volunteers were truthful when these questions were asked. Since these individuals may always get through, despite efforts to exclude them by questionnaires and other screening tools, it further serves to emphasise the importance of large subject numbers and randomisation when carrying out this type of study.

2.3.7.2 Visual analogue scales

Subjective ratings of hunger, desire to eat and fullness showed no differences between the three study sessions. Our results agree with those of Raben et al. (1995) in that VAS scores may produce similar mean responses over time following a test meal (in our case a liquid preload) but the within-subject differences between the study sessions were large as shown by the coefficients of variance. The results serve to emphasise the large amount of variability in hunger and satiety, or at least perception of hunger and satiety, from day to day under seemingly identical environmental conditions, which is then added to by methodological variation.
Mean differences between study sessions were small, but the coefficients of repeatability showed that they were spread over a large range. 95% of the difference is within 2 SD, denoted by the CR, and this ranged from 2.20 to 4.15 cm for fasting scores, 1.81 and 3.28 cm for post-preload scores, and 2.44 and 4.11 cm for post-ad libitum test meal scores. These are quite large CRs for a scale of 1 to 10 cm but they are comparable to those reported by Raben and colleagues (1995) (CRs of 2.9 to 5.2 cm for fasting scores). A more recent study using the same statistical methods to assess the reproducibility of appetite scores reported slightly lower values (Flint et al., 2000). Flint and colleagues obtained CRs of 2.7 to 3.0 cm for fasting scores and 1.6 to 2.0 cm for post-preload scores, but they only measured two repeated study sessions whereas three sessions were included in the present study. This is important as the upper level CRs obtained in the present study were mainly a result of comparison with the third session. They also used a much greater number of subjects (n = 55). Correlation coefficients of subjects' ratings between sessions were moderately high following the preload, and all statistically significant, although the association was not as close between the second and third session (average $r^2$ of 0.4). Baseline correlations were fairly high. There were no significant correlations of subjects' ratings between sessions post-ad libitum test meal, except for desire to eat between the second and third sessions. This means that subjects who were scoring higher in one session after the ad libitum test meal, did not necessarily score higher in another session. Therefore, subjects were less consistent in their VAS ratings once they had consumed the ad libitum test meal. This may reflect the fact that they were not eating the same amount of food on each occasion, or that they enjoyed the meal less on consecutive occasions, or maybe they took less care over their scoring as it was the last thing they did before they left the study.

An important pattern to emerge out of the VAS ratings results was observed from the coefficient of variance (CV %) figures. Within-subject variability over the three study sessions was higher for hunger and DTE, but lower for fullness, after the ad libitum test meal. Inversely, baseline and post-preload ratings were more variable for fullness and considerably less variable for hunger/DTE. This observation directs our attention to a design weakness in the VAS paradigm. It can be interpreted as a reflection of the fact that subjects can judge their subjective feelings of satiety more reliably and reproducibly when there is a stimulus to satiety (i.e. they have eaten a meal). Concomitantly, subjective feelings of hunger or DTE can be expressed more
reliably when the stomach is not full. Even after the liquid preload, hunger and DTE was less variable within subjects, so a “drink”-type meal does not suppress motivation to eat as much as a solid buffet-style meal does. There may be a case for changing the design of the scales to a continuum of ‘hungry’/‘full’, rather than the separate ‘not at all hungry’/‘as hungry as I’ve ever felt’ and ‘not at all full’/‘as full as I’ve ever felt’ models used in this thesis, and in many other publications. Although these concepts are treated as two different sensations for the purposes of the study of appetite, it may be that the human subjects used in these experiments exhibit an improved response when hunger and satiety are treated as two extremes of the same sensation.

The present study further underlines the importance and complexity of the psychological effects of food and the influence of psychological state on food intake and appetite. The reduction in food intake over the three study sessions under identical conditions has serious implications for all experimental studies that measure satiety, and show that the measurement of the effect of any given physiological variable on satiety must minimise psychological “noise” so as not to overwhelm the physiological effects. It also suggests that any physiological effects on satiety would have to be extremely marked to be picked up above the psychological influences. The variability shown in baseline VAS ratings is of concern since there is no nutritional stimulus and so this should stand a better chance of reproducibility between sessions. However, this may be rectified during analysis by standardising post-preload scores (y) to baseline values (x), i.e. change from baseline (y-x). Z scores can also be used to transform the data so that the number of SD from the mean of subject ratings over the time points for each rating is analysed, which was recommended for the processing of VAS data when there is large variation (Rogers, informal communication).

With hindsight, the food intake measurement in this experiment could have been improved by the measurement of ratings of pleasantness, palatability, and sensory properties of the ad libitum test meal by VAS scores. This would have shed light on the reasons behind the reductions in food intake. The importance of randomisation is underscored by these results. It is also important to use a large number of subjects when studying appetite. The inclusion of meal order as a covariant in repeated measures ANOVA calculations may be a way round the problem of reduced food intake with more than two repeated meals. Setting up a
familiarisation session with the *ad libitum* test meal and using VAS ratings may have removed the novelty factor and improved reproducibility scores. However, this may also carry the risk of accentuating the “sensory apathy” already mentioned which could lead to a worsening of reproducibility. Another improvement to the experimental protocol would have been if all the subjects had been informed that they would be required until a later, set time, which may have reduced the risk of the subjects rushing the test meal in order to leave earlier. These suggestions are, however, suitable improvements for future work on appetite and food intake.
Chapter Three
Chapter 3

THE EFFECT OF PHENYLALANINE ON APPETITE AND POSSIBLE PHYSIOLOGICAL MECHANISMS

3.1 General introduction

In studying the role of protein and amino acids in appetite regulation, it was initially decided to investigate the effect of a single amino acid, phenylalanine, on satiety mechanisms.

Phenylalanine is an amino acid with an aromatic side chain (figure 3.1). It is one of the nutritionally essential amino acids, and of the total body phenylalanine content, a very small fraction is free in the plasma (Garlick & Reeds, 1993).

![Figure 3.1. The molecular structure of a) phenylalanine and b) L-aspartyl-L-phenylalanine methyl ester (aspartame)](image)

Thirty years ago it was shown that duodenal perfusion with phenylalanine, unlike many other individual amino acids, increased CCK secretion compared to saline, although not to the same extent as a duodenal perfusion of a mixture of essential amino acids (Go et al., 1970). This observation was supported by findings that showed intestinal perfusion of phenylalanine led to a strong CCK secretory response in dogs (Meyer & Grossman, 1972). Gibbs and Smith had noted that CCK induced satiety responses in rats and monkeys, and went on to test the hypothesis that phenylalanine ingestion may therefore suppress food intake (Gibbs & Smith, 1977). As expected, L-phenylalanine preloads (1g/kg body weight) led to a strong and rapid suppression of food intake in rhesus monkeys. Phenylalanine was also found to delay gastric emptying in human beings (0.1 M in 600 cc water) (Byrne et al., 1977). Weight of food ingested was reduced in a separate human study on obese volunteers
where 8 g of encapsulated amino acids (phenylalanine, valine, methionine, and tryptophan) were ingested half an hour before the meal (Butler et al., 1981). In addition, Muurahainen and colleagues (1988) reported that encapsulated phenylalanine (10g), consumed simultaneously with tomato soup, significantly reduced *ad libitum* food intake at a meal presented twenty min after the preload.

Phenylalanine is widely ingested as the sweetener, aspartame (L-aspartyl-L-phenylalanine methyl ester). Aspartame is a high intensity, non-nutritive sweetener added to a large variety of foods but most commonly in low-calorie beverages and desserts, and as a tabletop sweetener added to tea or coffee. When aspartame enters the intestine following ingestion it can produce a number of possible combinations of metabolites. Enzymes may hydrolyse it to aspartate, phenylalanine and methanol, which are then absorbed into the circulation. Aspartame may also undergo esterase hydrolysis into aspartylphenylalanine and methanol in the intestinal lumen. This dipeptide is then absorbed and hydrolysed to the two constituent amino acids within the enterocyte. Aspartame is not thought to enter the blood stream (Pardridge, 1986).

There have been a number of human studies carried out that show aspartame increases satiety (Birch *et al.*, 1989; Rogers *et al.*, 1990; 1991; 1995). Rogers and colleagues (1990) found that 234 mg of encapsulated aspartame plus 200 ml water significantly reduced energy intake one hour later by 732 kJ, whereas 234 mg of aspartame dissolved in 200 ml water and 2 placebo capsules containing cornflour only reduced energy intake by 205 kJ, a non-significant effect. The control preload was 200 ml water and 2 placebo capsules. A second experiment revealed that the aspartame dissolved in water led to a greater decrease in self-rated desire to eat and hunger, but it was the encapsulated aspartame that led to a significant reduction in food intake. Rogers *et al.* (Rogers *et al.*, 1991) also investigated whether the amino acid constituents of aspartame, phenylalanine and aspartate, would have individual effects on food intake in an attempt to identify the source of aspartame’s anorectic effects. Again, food intake was reduced at the meal one hour after the preload capsule containing 400 mg of aspartame, but neither the phenylalanine (200 mg), nor aspartate (200 mg) had any effect compared to the control (cornflour in a capsule). The anorectic effect could only be stimulated by aspartame ingested without tasting. Rogers and Blundell (Rogers & Blundell, 1992) could not produce a comparable reduction in food intake in rats when aspartame was injected intraperitoneally with
water, or administered by gavage. The authors explained this as the result of a species difference; an argument backed up by the fact that rats do not taste aspartame as sweet. Another study from the same laboratory discovered that the time interval between preload and test meal was important in whether food intake would be significantly reduced. In this case, there was a reduction after 60 min, but not after 30 or 5 min (Rogers et al., 1995).

There are also a few studies that do not support the assertion that aspartame reduces subsequent food intake on a short-term basis. Encapsulated phenylalanine had no effect on food intake, macronutrient selection and self-rated scores for appetite and mood in human volunteers in a study by Ryan-Harshman and colleagues (Ryan-Harshman et al., 1987). The same authors also carried out an experiment to test the effects of encapsulated aspartame at two large doses and another dose of phenylalanine on measures of appetite and again, there were no significant differences in food intake or subjective appetite scores. However, Rogers and Blundell presented a paper containing re-analysis of the raw data from the first experiment by Ryan-Harshman and colleagues (Rogers & Blundell, 1994). They pointed out that the placebo used was another amino acid, alanine, which may well also suppress food intake. The differences between 3 doses of phenylalanine (0.84, 2.52 and 5.04 g), and 5.04 g alanine was marginally non-significant (p = 0.052) in 13 subjects. A post hoc test showed that the main difference was between the highest and lowest doses of phenylalanine (p = 0.008), and there was even a trend towards suppressed food intake following alanine compared to the lowest dose of phenylalanine (p = 0.057). Therefore, not only was there a dose-dependent suppression of food intake by phenylalanine, but food intake following the lowest dose of phenylalanine (0.84 g) was probably closer to the true control level than alanine. Rogers and Blundell (1994) concluded that phenylalanine induced a dose-dependent suppression of food intake and alanine suppressed food intake in this instance. Similar conclusions were drawn from further inspection of the results from experiment two.

Anderson et al. (Anderson et al., 1989) looked at the effects of aspartame on food intake on children but found different results. They carried out two experiments. The first compared aspartame plus carbohydrate with sodium cyclamate (amounts in equivalent sweetness to the aspartame) and alanine in amino acid equivalent amounts
in a fruit-flavoured drink. The second experiment compared aspartame with sucrose. Neither experiment produced any differences between conditions at the *ad libitum* test meal 90 min later (food intake and macronutrient selection), or subjective appetite ratings. However, it could be argued that 90 min was too long a delay between the preload and the test meal to detect the behavioural outcome from possible hormonal or direct effects triggered in the gut, and also that alanine was not an appropriate choice for a control.

Twenty-four weeks of aspartame treatment (75 mg/kg body weight per day) had no effects on body weight in over one hundred human subjects (Leon et al., 1989), but then previous experiments only showed that aspartame influenced short-term satiety. Rodin (1990) failed to show an effect of aspartame-sweetened lemonade compared with fructose-sweetened, glucose-sweetened lemonade or plain water on food intake 40 min later. Aspartame also had no effect on food intake one hour later in a study using diet soda as the vehicle (Black et al., 1990). These experiments used aspartame dissolved in a beverage, whereas Rogers and colleagues (1990, 1991, 1992, 1995) had demonstrated an effect for encapsulated aspartame only.

From these studies, it is difficult to form any clear idea of what is happening to appetite mechanisms following aspartame or phenylalanine ingestion. The conflicting results are likely to be the result of differences in experimental design, (age/sex, preload, dose administered, the *ad libitum* test meal, and time elapsed between the preload and test meal). There is sufficient evidence to suspect that aspartame, or phenylalanine alone, does have a satiety effect, and the following chapter aims to confirm or refute this assumption and to address possible physiological mechanisms. Since CCK and GLP-1 are widely believed to be important satiety peptides in human beings (Ballinger et al., 1995; Lieverse et al., 1995; Flint et al., 1998; Gutzwiller et al., 1999), and CCK has already been shown to be secreted in response to large doses of phenylalanine (Ballinger & Clark, 1994), the involvement of these hormones will be investigated. Effects on gastric emptying rate, which is inhibited by these gut peptides and is intrinsically linked to satiety, will also be explored.
3.2 The effect of phenylalanine supplementation on food intake and subjective appetite ratings

3.2.1 Aims and hypothesis

This study tested the hypothesis that phenylalanine induces short-term satiety mechanisms in human subjects, and that there is a dose-response relationship between phenylalanine consumption and on both subjective ratings of appetite and subsequent short-term food intake.

3.2.2 Subjects

Nine healthy volunteers (4 male, 5 female) aged 22 to 30 years (mean 24, SD 2.6 years) were recruited from students and staff at the University of Surrey. None of the volunteers accepted on to the study were dieting, had a BMI of >25 kg/m², had special dietary requirements or unusual eating habits, were phenylketonurics, or took regular medication (except oral contraceptives or non-prescription analgesics). The volunteers were also screened using the Dutch Eating Behaviour Questionnaire (van Strien et al., 1986) shown in appendix II, which measures dietary restraint, externality and emotionality. Only those men and women scoring less than 3.0 overall (mean score = 2.4, SD = 0.3) were asked to participate. A 3-day food diary was completed prior to acceptance on the study in order to highlight any unusual dietary habits.

To ensure that the subjects liked the foods provided but did not eat excessively, they were asked to complete a questionnaire that rated food choices and excluded foods that they did not like (appendix IV). The subject's second choice for each food category was provided in the ad libitum test meal. The subjects were also given empty capsules of the type used in the experiment in advance to make sure that they could swallow them without any difficulty. Finally, all volunteers signed a consent form at the beginning of their first session, which stated that they had read the Information for Volunteers sheet. The subjects were told that the study was investigating the effect of amino acids on mood and food choice, but they were not told explicitly what the capsules contained. The study protocol was approved by the local ethics committee.
3.2.3 General procedure

A within-subjects, repeated measures single blind design was used for the experiment, with each subject serving as his or her own control. Every subject received each of the following: 5 g phenylalanine, 2 g phenylalanine, 400 mg phenylalanine and 5 g cornflour (placebo) in random order. Each dose was administered in 10 opaque gelatine capsules; the intermediate doses were made up to 5 g with cornflour. The capsules were ingested with 300 ml of cool water (10-15 °C), followed by 200 ml of warm water (40-45 °C) (to facilitate the dissolving of the capsules). Each subject was required to attend the Clinical Investigation Unit at the University of Surrey on four occasions – each occasion for one treatment.

The subjects had been instructed to eat a light evening meal and their normal (cereal-based) breakfast, and to avoid alcohol from 2000 h the evening before until the study. They were also to abstain from any heavy or vigorous exercise for 24 hours before the study. On the day of the study they were required to drink only water and eat only what was provided for them from breakfast onwards. Subjects were provided with their choice of standard pasta-based ‘ready-meal’ for lunch (at between 1200 h and 1300 h), which was kept the same on each study day (mean 1623 kJ, 17.5 g protein, 12.3 g carbohydrate, 5.0 g fat). They were then free to go until the start of the study. Table 3.1 shows the study protocol. In addition to visual analogue scales (VAS) for hunger, desire to eat and fullness, subjects were also required to rate how well they felt (headache, stomachache, nausea, heart pounding, feeling flushed, faint, jittery, refreshed and light-headed) in order to monitor any side-effects of phenylalanine consumption.
<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Action</th>
<th>Visual analogue scales (VAS) and food preference checklist</th>
</tr>
</thead>
<tbody>
<tr>
<td>1730</td>
<td>Arrive at the investigation unit</td>
<td></td>
</tr>
<tr>
<td>1755</td>
<td></td>
<td>✅</td>
</tr>
<tr>
<td>1800</td>
<td>Treatment (consumed capsules + water)</td>
<td></td>
</tr>
<tr>
<td>1815</td>
<td></td>
<td>✅</td>
</tr>
<tr>
<td>1835</td>
<td></td>
<td>✅</td>
</tr>
<tr>
<td>1855</td>
<td></td>
<td>✅</td>
</tr>
<tr>
<td>1900</td>
<td>Consume <em>ad libitum</em> buffet-style test meal.</td>
<td>✅</td>
</tr>
<tr>
<td>~1920</td>
<td></td>
<td>✅ (after meal termination)</td>
</tr>
</tbody>
</table>

Table 3.1 Study protocol for the investigation of the effect of phenylalanine supplementation on food intake and subjective appetite ratings.

Whilst eating the *ad libitum* test meal, the subjects were partitioned in order to minimise social interaction and external influences on their eating behaviour. Final visual analogue scale (VAS) ratings (appendix V) and food preference checklists (appendix VI) were completed when the subjects finished eating. When the final ratings and questionnaires had been completed the subjects were free to go home. There were at least three days between sessions.

### 3.2.4 Subjective measures of appetite

Subjects rated their hunger, desire to eat, and fullness and completed food preference checklists as described in section 2.2.1.3.

### 3.2.5 Treatment doses and meals

*Capsules:* The gelatine capsules were filled with L-phenylalanine (Scientific Hospital Supplies) and/or cornflour, and weighed on a microbalance to ensure that each capsule contained 500 mg exactly. Ten capsules made up a 5 g dose. The kilojoule, carbohydrate and phenylalanine contents of each dose is shown in table 3.2.
<table>
<thead>
<tr>
<th>Approximate Energy (kJ)</th>
<th>Cornflour (g)</th>
<th>Phenylalanine (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>84</td>
<td>5</td>
</tr>
<tr>
<td>400 mg PA</td>
<td>84</td>
<td>4.6</td>
</tr>
<tr>
<td>2 g PA</td>
<td>84</td>
<td>3</td>
</tr>
<tr>
<td>5 g PA</td>
<td>84</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.2. Approximate energy, carbohydrate and phenylalanine content of each treatment.

*Ad libitum test meal:* The self-selection meal consisted of a variety of palatable, cold, lunch-type foods – sandwiches with two types of fillings, salad items, full-fat fruit yoghurt, biscuits, and cake. See appendix III for a description of the foods used. All foods were weighed before being served and re-weighed after the subjects had eaten to record the amount consumed (g) of each food. A glass of water (300 ml) was provided with the meal. On average the total energy available from a tray of food was approximately 12,552 kJ.

3.2.6 Results

3.2.6.1 Energy and food intake

Results show a trend towards reduced food intake following increasing doses of phenylalanine (see figure 3.2). One way repeated measures analysis of variance (ANOVA) comparing energy intake (kJ) during the *ad libitum* test meal across all four doses produced no statistically significant differences. Standardising energy intake for body weight (intake in kJ/weight in kg) did not produce any significant differences either. Comparing weight of food intake (g) across all doses by one way repeated measures ANOVA produced a difference that was not quite significant (p = 0.066), as did weight of food intake standardised for body weight, p = 0.061 (see figure 3.2). A paired t-test between energy intake following the control and 5 g phenylalanine (PA) conditions showed that there was a significant difference in energy intake (p <0.05).

One way, repeated measures ANOVA was also carried on energy intake at each study session to test for any order effects, since previous work had revealed that energy intake is reduced on the third study session with repeated identical preloads and *ad libitum* test meals (see section 2.3). There were no differences in energy intake between study sessions (p = 0.532) and mean intakes appeared similar for each session (4942, 5276, 4766 and 5021 kJ in the 1st, 2nd, 3rd and 4th study sessions respectively).
Repeated measures ANOVA of protein intake (g) showed a significant treatment effect between the four doses, $p < 0.02$. The same effect was found for protein intake standardised for body weight (see table 3.3), $p < 0.02$. Post hoc analysis of protein intake (g) standardised for body weight revealed that the subjects consumed significantly less protein following 5 g of phenylalanine compared to the control treatment ($p < 0.002$), the 400 mg phenylalanine ($p < 0.02$) and the 2 g phenylalanine ($p < 0.05$). No significant differences were found in fat or carbohydrate consumption when intake was analysed in terms of weight (g) or weight (g) standardised for body weight.

Figure 3.2 The effect of phenylalanine dose (control, 400 mg, 2 g or 5 g) on food intake (weight ingested (g) per kg body weight). Mean ± SEM, $n = 9$, *$p < 0.05$.
<table>
<thead>
<tr>
<th>Treatment (phe)</th>
<th>Energy intake (kJ) per kg/bw</th>
<th>Food intake (g) per kg/bw</th>
<th>Protein intake (g) per kg/bw</th>
<th>Fat intake (g) per kg/bw</th>
<th>CHO intake (g) per kg/bw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>77 ± 7</td>
<td>9.0 ± 0.7</td>
<td>0.60 ± 0.05</td>
<td>0.77 ± 0.08</td>
<td>2.20 ± 0.17</td>
</tr>
<tr>
<td>400 mg</td>
<td>76 ± 7</td>
<td>8.8 ± 0.7</td>
<td>0.56 ± 0.05</td>
<td>0.77 ± 0.09</td>
<td>2.18 ± 0.18</td>
</tr>
<tr>
<td>2 g</td>
<td>72 ± 4</td>
<td>8.3 ± 0.6</td>
<td>0.54 ± 0.05</td>
<td>0.73 ± 0.04</td>
<td>2.08 ± 0.15</td>
</tr>
<tr>
<td>5 g</td>
<td>67 ± 5</td>
<td>7.6 * ± 0.4</td>
<td>0.48** ± 0.04</td>
<td>0.66 ± 0.05</td>
<td>1.91 ± 0.15</td>
</tr>
</tbody>
</table>

*Significantly different from control dose and 400 mg phenylalanine, p < 0.05; ** significantly different from control dose, p < 0.002.

Table 3.3 Food intake by energy (kJ) and weight (g) per kg body weight (per kg/bw), and macronutrient intake by weight (g) per kg/bw following the control condition and increasing doses of phenylalanine (400 mg, 2 g, and 5 g).

Ad libitum test meal intake was also analysed in terms of type of food consumed – savoury, sweet or condiments. Repeated measures ANOVA of intake of savoury foods in terms of weight (g) showed a main effect of dose that was just short of significance, p = 0.061. Post-hoc analysis indicated that the savoury food intake after 5 g of phenylalanine was significantly less than that after the control dose (p < 0.02). There were no differences in intake of sweet foods or condiments, however.

**3.2.6.2 Subjective ratings of appetite**

The results showed no significant differences in subjective appetite ratings between doses. See figures 3.3, 3.4 and 3.5.

Two way, repeated measures ANOVA of the VAS scores for hunger, desire to eat, and fullness, and the change from baseline (x-b) for hunger, desire to eat and fullness, showed no significant main effects of dose. ANOVA of the raw data excluded the after meal ratings, as you would expect the subjects to eat to the same level on each occasion, and ANOVA of the change from baseline excluded the baseline ratings in addition. There was a significant main effect for time for the raw data for hunger (p < 0.02), desire to eat (p < 0.05), and fullness (p < 0.05), and for the change from baseline (at t +15, +35 and +55 min) for hunger (p < 0.005) and desire to eat (p < 0.005). There were no dose x time interactions for hunger, desire to eat or fullness.

Pearson Product Moment correlation analysis of the change in score at t +15 min (t +15 min minus baseline) with ad libitum test meal intake found that only the change in fullness correlated significantly with subsequent energy intake (r = -0.765,
p = 0.016) and weight of food intake (r = -0.820, p = 0.007) following treatment with 5 g phenylalanine. There were no correlations for hunger and desire to eat, or for any other dose treatment.

Figure 3.3 Hunger VAS ratings (cm) following consumption of encapsulated phenylalanine at doses of 400 mg, 2 g, or 5 g compared to control. Mean ± SEM, n = 9. There were no significant differences in hunger response.
Figure 3.4 Desire to eat VAS ratings (cm) following consumption of encapsulated phenylalanine at doses of 400 mg, 2 g, or 5 g compared to control. Mean ± SEM, n = 9. There were no significant differences in desire to eat.

Figure 3.5 Fullness VAS ratings (cm) following consumption of encapsulated phenylalanine at doses of 400 mg, 2 g, or 5 g compared to control. Mean ± SEM, n = 9. There were no significant differences in fullness response.

Satiety quotients (SQs) were calculated for hunger, desire to eat and satiety ratings but one way repeated measures ANOVA revealed no significant differences between treatments (p = 0.246, 0.684 and 0.838 respectively). There were no significant side effects resulting from phenylalanine consumption.
3.2.6.3 Food preference checklist

Two way, repeated measures ANOVA on the change from baseline of the total number and total energy of all foods, protein foods, high fat foods, high carbohydrate foods, mixed carbohydrate/fat foods and low fat foods showed no main effect of dose on type of food selected, but there were consistent significant main effects for time (ANOVA of t +15, +35 and +55 min) for change in total energy and total number of foods. See figure 3.6. There was also a dose x time interaction for change from baseline (x-b) of carbohydrate food preference (p <0.05). Post-hoc analysis showed that this was due to a decrease in carbohydrate food selection after 2 g of phenylalanine compared to an increase for the other 3 treatments, especially at t +55 min.

![Figure 3.6 Number of food items selected from the food preference checklist following consumption of encapsulated phenylalanine at doses of 400 mg, 2 g, or 5 g compared to control. Mean ± SEM, n = 9. There were no significant differences in the total number of food items selected.](image)

3.2.7 Discussion

3.2.7.1 Food intake

The results show that energy intake was not affected by phenylalanine supplementation in doses of up to 5 g in young healthy adults. There was, however, a dose-related suppression of food intake in terms of food weight per kg body weight, which approached significance, and post hoc analysis revealed that intake after the 5
g phenylalanine dose was significantly lower compared to the control and 400 mg phenylalanine conditions.

The data suggest that the bulk or volume of the food rather than the energy content was the major determinant of satiety and that phenylalanine may act by amplifying the short-term satiation process that occurs while the food is passing from the stomach to the duodenum. Furthermore, it is possible that phenylalanine may promote a delay in gastric emptying, therefore curtailing the volume of food that may be comfortably ingested. Phenylalanine may act on specific amino acid receptors in the duodenum that trigger a neural reflex to delay gastric emptying, possibly via a vago-vagal pathway as amino acid-specific afferent vagal receptors have been identified as being located superficially in the upper intestinal mucosa (Jeanningros, 1982). One could also speculate that vagal afferent signals could trigger the stimulation of CCK release from the CCK-secreting cells in the upper small intestine, either through autonomic nervous transmission to the enteric nerve plexus or via a long neural reflex. There is some evidence that the phenylalanine may also be acting directly on CCK cells that border onto the small intestine lumenal wall to stimulate CCK secretion, possibly via a calcium channel opening mechanism (Liddle, 1997).

There were no effects on subjective appetite ratings prior to the ad libitum test meal, and a gastric load is known to potentiate the inhibiting actions of CCK on gastric emptying. Therefore, these results support the theory that duodenal phenylalanine immediately triggered CCK secretion. It is hypothesised that circulating CCK acted on receptors on vagal afferent fibres sensitive to gastric distension during the test meal thereby amplifying their basal firing. This would increase the subsequent delay in gastric emptying (Schwartz et al., 1991).

3.2.7.2 Macronutrient selection

Protein intake (g) was significantly suppressed with increasing dose, with post hoc comparisons showing intake after the 5 g dose to be significantly lower than intake after the other three conditions. Suppression of protein intake might be expected since 5 g is a large amount of phenylalanine to ingest at once, with the daily estimated intake of these subjects averaging 2.3 g per day (from their food diaries). An excessive intake of protein can have adverse metabolic effects, especially if there is an imbalance in essential amino acids as in this case (Jackson, 1999), and so this may reflect a post-absorptive central mechanism to protect against amino acid
toxicity. The possible routes for central aminostatic regulation of protein intake are via amino acid sensors in the brain or by conversion to the neurotransmitter dopamine via tyrosine. However, the evidence for a significant increase in dopamine synthesis and release following administration of amino acid precursors is not convincing (see section 1.3.2). In addition, although a few studies have investigated the existence of amino acid sensors in the brain that may regulate food intake (Rogers & Leung, 1973; Anderson et al., 1994; Currie et al., 1995), the mechanistic evidence is sparse and therefore not conclusive as yet.

There is another possible explanation. Since satiation is increased following 5 g phenylalanine, the reduction in protein may be linked to a hedonistic decision by the subjects to reduce their selection of savoury foods, in order to leave more room for the highly palatable sweet/high fat foods. This is supported by the dose-related suppression in savoury items of food whereas the same relationship is not observed for sweet foods or condiments. The reduction in protein intake was not predicted by any reduction in high protein food selection in the pre-meal food preference checklist scores. A Pearson Product Moment correlation failed to show any correlation between food preference checklist selection of high protein foods over t +15, t +35 and t +55 min, and actual protein intake (g/kg body weight) at the ad libitum test meal for any of the dose conditions. There was, however, a significant correlation between intake of savoury foods (g) after the 5 g dose of phenylalanine and protein energy selection in the food preference checklists at t +55 min, (r = 0.692, p = 0.039). More work is required to disentangle the role of food types versus macronutrients in satiety, but this is outside the scope of the present research.

3.2.7.3 Subjective appetite

Subjective feelings of hunger, desire to eat and fullness were unaffected by phenylalanine treatment. These results would therefore lend support to the hypothesis suggested by Rogers et al. (1995) for aspartame that phenylalanine is amplifying the satiating effect of food in the stomach and small intestine, and that phenylalanine treatment does not affect pre-meal feelings of hunger and satiety. Only the change in fullness immediately following capsule ingestion showed any correlation with food intake (per kg/bw) at the ad libitum test meal, which suggests that hunger and desire to eat may be under a different mechanism of control to fullness and that hunger and desire to eat may be poor predictors of subsequent intake. With hindsight, the
experimental protocol could have been improved by carrying on the VAS ratings beyond the *ad libitum* test meal, perhaps every hour until their next meal, so that any post-meal effects on satiety could have been discerned.

### 3.7.2.4 Food preference checklists

These results show that pre-meal food choices may be affected by increased doses of phenylalanine, with a dose-response suppression of selection of carbohydrate foods and low fat foods in terms of energy. When low fat food preference is standardised for the change from baseline, there are no significant effects for dose, time or a dose x time interaction at t +15, t +35 and t +55 min. It cannot be assumed, therefore, that treatment was affecting preference for low fat foods. The effect of 2 g of phenylalanine on carbohydrate food preference (change from baseline) is difficult to explain, since the 5 g phenylalanine treatment produced similar results to the control and 400 mg phenylalanine treatments. The greater risk of statistical type 1 errors in a study of this size may explain this result.

In conclusion, it has been demonstrated that there is a weak dose-dependent effect of phenylalanine on food intake, with a significant effect after 5 g of phenylalanine. It is probable that the influence of this amino acid on food intake would have been more marked if there had been more subjects in the study. Since *pre-ad libitum* test meal subjective appetite scores were unaffected, phenylalanine may have reduced food intake by amplifying the satiating effect of the *ad libitum* test meal, possibly via CCK-mediated mechanisms.

### 3.3 The effect of phenylalanine supplementation on circulating metabolite and hormone status, gastric emptying and satiety

#### 3.3.1 Aims and hypothesis

A large dose of phenylalanine has been shown to reduce subsequent food intake with an *ad libitum* test meal. It is hypothesised that the mechanisms for this satiety effect may involve an increase in the secretion of the satiety peptides, CCK or GLP-1, and possibly delayed gastric emptying. Potential effects on other hormones, GIP and insulin were investigated, and levels of plasma metabolites, glucose and amino acids, were measured.
3.3.2 Subjects

Six lean healthy volunteers (4 females and 2 males) between 20 and 30 years were recruited from the University. The volunteers were all non-dieting, with no special dietary requirements or eating habits (as assessed by the completion of a three day food diary), and a BMI <25 kg/m². They had no significant current or previous medical history (including phenylketonuria) and took no medication apart from the oral contraceptive pill. They were selected on the basis of their responses to the Dutch Eating Behaviour Questionnaire (van Strien et al., 1986) shown in appendix II, and their self-reported habitual protein intake and physical activity levels. Subjects were non-smokers and did not consume more than 20 units of alcohol per week. All subjects were interviewed and a venous blood sample taken for haematological and biochemical screening to confirm their suitability to participate in the study. The volunteers signed a consent form before they were accepted onto the study, which included a statement that they understood that they would be giving some blood as part of the study. Their GP was informed that their patient was going to participate in this study, and given the opportunity to notify as to any medical reason why their patient should not take part.

To ensure that the subjects liked the foods provided but did not eat excessively, they were asked to complete a questionnaire to rate their food preferences and to exclude foods that they did not like (appendix IV). The subject’s second choice for each food category was provided in the ad libitum test meal. The subjects were also given empty capsules of the type used in the experiment in advance to make sure that they could swallow them without any difficulty. The subjects were told that the study was to investigate the effects of phenylalanine on mood and food choice. The study protocol was approved by the local ethics committee.

3.3.3 General procedure

A within-subjects, repeated measures design was used for the experiment, with each subject serving as his or her own control. Subjects were asked to refrain from drinking alcohol the evening before. On the day of the study they were required to consume their normal breakfast, and not eat anything else until their standard lunch. Lunch was provided as a standard pasta dish that the subject has previously
indicated a liking for, and eaten between 1145 and 1230 h. For the rest of the day the
subjects were required to eat or drink nothing except water until two hours before the
study.

Subjects arrived at the Clinical Investigation Unit at 1730 h (2 people per
study day), and an intravenous cannula was inserted into an antecubital vein in the
forearm under local anaesthetic. Blood samples were taken for the measurement of
plasma amino acids, glucose, insulin, GLP-1, GIP and CCK according to the
methodology detailed in section 2.2.3.1. Six electrodes were positioned over the
stomach and lower back to measure gastric emptying by electrical impedance
epigastrography (EIE). A baseline blood sample was taken, and the first VAS ratings
(appendix V) completed by the subject. Although the main purpose of the study was
to investigate changes in plasma hormones and metabolites and effects on gastric
emptying, VAS ratings were completed during the study, as well as the measurement
of food intake. Subjects lay in a semi-supine position on a bed and were instructed to
stay as motionless as possible in order to obtain a steady EIE trace. Once a
satisfactory ten minute baseline reading had been obtained for the EIE recordings, the
subjects consumed 5 g of encapsulated phenylalanine or placebo (cornflour) with 300
ml of cool water (10-15 °C), followed by 150 ml of warm water (40-45 °C) in order to
hasten the dissolution of the 10 gelatine capsules in the stomach. Further blood
samples (15 ml) were taken at 15, 25, and 35 min, and VAS ratings/food preference
checklists were completed by the subjects at 10, 20 and 30 min after the capsules. 40
min after ingestion of the capsules the EIE recordings were stopped and the ad
libitum test meal was offered to the subject. An interval of 40 min was chosen
because it was hypothesised that the phenylalanine-induced satiety mechanisms were
short-term processes involving a direct effect of phenylalanine on intestinal receptors
or luminal gastrointestinal hormone-secreting cells. Since it was assumed that the
capsules would dissolve within 30 min of ingestion, the optimal time for a short-term
augmentation of satiety was theorised to be 40 min after phenylalanine ingestion.

The subjects were screened off from the other participants and instructed to
eat until they were comfortably full, and to the same level of fullness on both
occasions. They were also told that they could take the remaining food home with
them if they wished. The duration of the meal was noted. Immediately following the
termination of eating a last blood sample was obtained and the last set of subjective appetite questionnaires completed; then the volunteers were free to go.

3.3.4 Treatments and meals

Capsules. The 10 capsules were filled with 5 g L-phenylalanine (Scientific Hospital Supplies) or 5 g cornflour. It was decided to administer 5 g phenylalanine as this was the amount previously shown to produce a clear satiety effect in human volunteers (see section 3.2.6.1).

Ad libitum test meal: This was the same meal as described in section 3.2.5 (appendix III).

3.3.5 Subjective measures of appetite

Subjects rated their hunger, desire to eat, and fullness as detailed in section 2.2.1.3.

3.3.6 Results

3.3.6.1 Gastric emptying

Gastric emptying rates were slower following treatment with phenylalanine compared to the control (see table 3.4).
Table 3.4 Gastric half-emptying times (T50s) following 5 g of encapsulated phenylalanine or cornflour (control), *p < 0.05.

Results were analysed using a two-tailed paired t-test, and it was shown that the T50s following the phenylalanine treatment were significantly longer in duration than the control treatment (p = 0.017).

3.3.6.2 Blood metabolite and hormone profiles

3.3.6.2.1 Plasma glucose

Plasma glucose concentrations were unaffected by phenylalanine treatment (figure 3.7).

![Plasma glucose concentrations](image)

Figure 3.7 Plasma glucose concentrations (mean ± SEM, n = 6) following either 5 g phenylalanine or 5 g cornflour (control). There were no significant differences in plasma glucose response.

3.3.6.2.2 Plasma phenylalanine

Plasma levels of phenylalanine were measured in order to estimate maximum time taken for capsules to dissolve and their contents to reach the duodenum. Plasma
levels of phenylalanine started to increase 35 min after ingestion of the capsules. Furthermore, by the time the subjects had finished eating their *ad libitum* test meal, the increased plasma levels were approximately four times higher than the meal-induced increase following ingestion of the control. Mean plasma phenylalanine concentrations are shown for 5 subjects in figure 3.8. Two way, repeated measures ANOVA on the change from baseline (x-b) showed the difference in phenylalanine concentrations between treatments to be nearly significant overall (p = 0.056), and there was a significant treatment x time interaction (p <0.01) mostly due to the large difference in plasma phenylalanine levels after the test meal. A paired t-test between treatments at t +35 min showed a significant difference (p <0.05) due to increased concentrations after the phenylalanine treatment.

![Figure 3.8](image)

**Figure 3.8 Plasma phenylalanine concentrations (mean ± SEM, n = 5) following either 5 g phenylalanine or 5 g cornflour (control).** The difference in plasma phenylalanine levels were significant at t +35 min (paired t-test, p <0.05) and highly significant following the *ad libitum* test meal, resulting in a significant treatment x time interaction (p <0.05) over all time points (post hoc Duncan test shows a significant difference, p <0.005, after the test meal) and a near significant treatment x time interaction (p = 0.074) before the *ad libitum* test meal (post hoc Duncan test shows a significant difference, p <0.05, at t +35 min).

### 3.3.6.2.3 Plasma tyrosine

Since phenylalanine is a precursor for tyrosine synthesis, plasma tyrosine concentrations were analysed to discover whether a high dose of phenylalanine could significantly increase plasma tyrosine levels. Plasma levels of tyrosine started to
increase slightly 35 min after ingestion of the capsules. Following the *ad libitum* test meal, the increased mean plasma levels were approximately 1.6 times higher than the meal-induced increase following ingestion of the control. Mean plasma tyrosine concentrations are shown for 5 subjects in figure 3.9. Two way, repeated measures ANOVA on the change from baseline (x-b) showed the difference in tyrosine concentrations between treatments to be nearly significant overall (p = 0.063). There was a significant treatment x time interaction (p <0.01) due to the large difference in plasma tyrosine levels after the test meal. A paired t-test between treatments at t +35 min showed that there were not yet any significant differences in plasma levels of tyrosine (p = 0.122), and that all the difference occurred following the *ad libitum* test meal.

**Figure 3.9 Plasma tyrosine concentrations (mean ± SEM, n = 5) following either 5 g phenylalanine or 5 g cornflour (control).** The difference in plasma tyrosine change from baseline values were not significant at t +35 min but highly significant following the *ad libitum* test meal resulting in a treatment x time interaction (p <0.01).

### 3.3.6.2.4 Plasma leucine

Plasma leucine levels are shown in figure 3.10 to illustrate the response of other plasma amino acids that are not linked to phenylalanine intake. There was no difference in plasma levels of leucine between treatments.
Figure 3.10 Plasma leucine concentrations (mean ± SEM, n = 5) following either 5 g phenylalanine or 5 g cornflour (control). There were no differences in plasma leucine concentrations between treatments.

3.3.6.2.5 Plasma GIP

There were no differences in plasma GIP concentrations before the ad libitum test meal, but the postprandial rise in GIP was clear for both treatments. The mean increase in plasma GIP concentrations following the ad libitum test meal (n = 5) was larger following the phenylalanine treatment but this was not statistically significant. See figure 3.11.
3.3.6.2.6 Plasma GLP-1

There were no differences in plasma GLP-1 concentrations before the _ad libitum_ test meal was eaten; plasma GLP-1 levels increased markedly for both treatments following the meal (figure 3.12).

Figure 3.12 Plasma GLP-1 concentrations (mean ± SEM, n = 6) following an encapsulated treatment of either 5 g phenylalanine or 5 g cornflour (control). There were no significant differences in plasma GLP-1 response.
3.3.6.2.7 Plasma insulin

Plasma insulin concentrations are illustrated in figure 3.13. There were no significant differences between the profiles following the treatment, but as expected there was an increase in plasma insulin following the *ad libitum* test meal.

![Figure 3.13 Plasma insulin concentrations (mean ± SEM, n = 6) following an encapsulated treatment of either 5 g phenylalanine or 5 g cornflour (control). There were no significant differences in plasma insulin response.](image)

3.3.6.2.8 Plasma CCK

Plasma CCK concentrations were higher following the phenylalanine treatment compared to control before the *ad libitum* test meal (see figure 3.14). Two way, repeated measures ANOVA showed that there were no overall significant differences in plasma CCK levels between treatments. However, there was a significant treatment x time interaction before the *ad libitum* test meal when baseline CCK concentrations were included as a covariate (ANCOVA, p <0.05), due to the cross-over in CCK response profiles. There was also a significant treatment x time interaction immediately before (t +35 min) and after the *ad libitum* test meal when baseline CCK concentrations were included as a covariate (ANCOVA, p <0.05).
Figure 3.14 Plasma CCK concentrations (mean ± SEM, n = 6) following an encapsulated treatment of either 5 g phenylalanine or 5 g cornflour (control). There was a treatment x time interaction when comparing phenylalanine to control due to the opposing time-courses in plasma CCK levels before (p <0.05) and after the ad libitum test meal (p <0.05).

3.3.6.3 Energy and food intake

Energy and food intake was almost identical following phenylalanine or the control, and repeated measures ANOVA between the two treatments showed no significant differences. See table 3.5. In accord with this, there were no differences in protein, fat or carbohydrate intake in terms of weight in grams.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Energy intake (kJ)</th>
<th>Food intake (g)</th>
<th>Protein intake (g)</th>
<th>Fat intake (g)</th>
<th>CHO intake (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4631</td>
<td>401</td>
<td>535</td>
<td>50</td>
<td>34</td>
</tr>
<tr>
<td>Control</td>
<td>4577</td>
<td>395</td>
<td>510</td>
<td>50</td>
<td>33</td>
</tr>
</tbody>
</table>

Table 3.5 Food intake by energy (kJ) and weight (g), and macronutrient intake by weight (g) following an encapsulated treatment of either 5 g phenylalanine or 5 g cornflour (control).

There were no significant differences in the duration of the ad libitum test meal between conditions.
3.3.6.4 Subjective ratings for appetite

Two way, repeated measures ANOVA of the VAS ratings for hunger, desire to eat, and fullness, and the change from baseline (x-b) for hunger, desire to eat and fullness, showed no significant main effects of treatment. See figures 3.15, 3.16 and 3.17.

Figure 3.15 Hunger VAS ratings (mean ± SEM, n = 6) following consumption of 5 g encapsulated phenylalanine or cornflour (control). There were no significant differences in hunger response.

Figure 3.16 Desire to eat VAS ratings (mean ± SEM, n = 6) following consumption of 5 g encapsulated phenylalanine or cornflour (control). There were no significant differences in desire to eat.
Figure 3.17 Fullness VAS ratings (mean ± SEM, n = 6) following consumption of 5 g encapsulated phenylalanine or cornflour (control). There were no significant differences in fullness response.

Satiety quotients (SQs) were calculated for hunger, desire to eat and satiety ratings but a two-tailed paired t-test revealed no significant differences between treatments (p = 0.211, 0.276 and 0.560 respectively).

3.3.7 Discussion

Previous studies have shown phenylalanine reduces food intake and that it also induces a strong CCK secretory response (Meyer & Grossman, 1972; Gibbs & Smith, 1977; Owyang et al., 1986; Muurahainen et al., 1988; Ballinger & Clark, 1994). The present study aimed to investigate the nature of the physiological response to phenylalanine consumption that may explain its effects on satiety, especially in regard to gastric emptying rate and the satiety peptides CCK and GLP-1.

Gastric emptying rate was clearly shown to be slower following treatment with phenylalanine compared to the control, as demonstrated by longer half-emptying times of a water load obtained by electrical impedance epigastrography. This supports a role for delayed gastric emptying in phenylalanine-induced satiety.

The measurement of plasma phenylalanine concentrations was carried out as an indicator of the maximum time taken for the capsules to dissolve in the stomach and their contents to reach the intestine. Previous research has shown that the pharmacokinetic responses to aspartame capsules differ from those observed when
the substance is ingested in a solution (Stegink et al., 1987). Peak plasma phenylalanine concentrations were significantly higher and reached significantly earlier when 3 g aspartame was consumed in a liquid rather than swallowed in capsules, which also increased plasma phenylalanine:LNAA (large neutral amino acid) ratios. As the initial divergence between plasma phenylalanine profiles over time in this study occurred at t +35 min, it can be safely assumed that the capsules had at least started to dissolve and that the intestinal mucosa was in direct contact with a significant quantity of dissolved phenylalanine. The relatively late appearance of plasma phenylalanine is probably due in part to a low solubility (2.965 g in 100 ml water), and also the fact that free amino acids are mainly absorbed in the ileum by specific amino acid transporters (Kutchai, 1998). The very large increase in plasma phenylalanine levels after the ad libitum test meal following treatment with 5 g phenylalanine compared to 5 g cornflour suggests the ingestion of the meal coincided with the time when the majority of the phenylalanine dose was absorbed.

Oral ingestion of a relatively large dose of phenylalanine has a marked effect on plasma tyrosine concentrations. Tyrosine is synthesised from phenylalanine, and is a precursor in the brain for the catecholamine dopamine, which is involved in the regulation of food intake. Tyrosine transport into the brain involves the same large neutral amino acid carrier as tryptophan; therefore a tyrosine/LNAA supply-limited mechanism of dopamine synthesis may conceivably be involved in appetite regulation. Unfortunately the tyrosine/LNAA ratio could not be calculated here, since it was not possible to measure plasma levels of tryptophan successfully. Since plasma leucine was unchanged by phenylalanine supplementation it can be assumed that the plasma tyrosine/LNAA ratio was increased. However, as discussed in section 1.3.2, an enhanced entry of tyrosine into the brain cannot be taken to imply increased rates of tyrosine hydroxylation (forming intermediates for dopamine synthesis), and the duration of the study was rather short for such a process, in the unlikely event that it did take place, to make a difference to short-term appetite control.

Phenylalanine treatment was shown to produce differing plasma CCK responses to the control but this was not a simple increased level following one treatment compared to the other. Following the control treatment, mean plasma CCK levels appeared to increase by 1 pmol/L at t +15 min, whereas they remained steady
following phenylalanine. However, this difference did not contribute to the significant treatment x time interaction shown for pre-meal values (2 way, repeated measures ANOVA with individual values expressed as change from baseline: x-b). In fact the difference between plasma CCK concentrations at t +35 min, where levels were increased following phenylalanine compared to control, were significant (p <0.05) after a post hoc Duncan test and the sole contributory factor to the treatment x time interaction. This difference coincides with the time point when plasma phenylalanine concentrations start to increase, supporting the hypothesis that phenylalanine ingestion increases CCK secretion. Since some phenylalanine was absorbed and CCK secretion was also increased by t +35 min, then it can be inferred that lumenal phenylalanine was interacting with intestinal receptors or CCK-secreting cells between t +25 and t +35 min.

The difference between CCK levels after the ad libitum test meal, where plasma CCK is decreased following phenylalanine treatment, is less robust. A paired t-test on the post-meal values (x-b) was not significant (p = 0.145), nor on the raw data with either t +35 min or baseline values as covariates. Although this difference contributes significantly to the treatment x time interaction over all time points when 2 way, repeated measures ANOVA (post hoc Duncan test, p <0.05) is carried out on the raw data or x-b data, the analysis only includes 5 subjects due to 1 missing post-meal time point. Therefore, the reliability of this result must be questioned. The individual data shows that 3 out of 5 subjects showed a decrease in plasma CCK concentrations after the meal following phenylalanine treatment. If this is a real effect, it could be speculated that the CCK-secreting cell becomes desensitised to the stimulation of food as a result of previous exposure to high doses of phenylalanine. Further studies would be required before this result could be confirmed.

The results show that phenylalanine in relatively high doses has no effect on plasma levels of glucose, insulin, GIP or GLP-1, either during the fasting state or post-prandially. Amino acids to have relatively poor insulinaemic effects following ingestion, and there is nothing in the literature to suggest that phenylalanine may be a potent secretagogue for GIP or GLP-1.

In this study, no concurrent differences in satiety were observed, either in terms of food intake or subjective ratings for hunger, desire to eat and fullness.
However, this is not surprising since past studies have shown that inter- and intra-subject variation is large and in this investigation there were only six subjects. It is also possible that the physiological measurements may have been an important factor in obscuring any effect on satiety, since cannulation, observing the decanting of blood in the same room, and wearing electrodes on the stomach and lower back are hardly a natural setting for a meal.

It can be concluded so far that the phenylalanine ingestion led to a delay in gastric emptying and an increase in CCK secretion. This provides support for a CCK-mediated mechanism for short-term phenylalanine-induced satiety. However, a direct causal relationship between phenylalanine-induced CCK secretion and CCK-induced satiety cannot be assumed. Failure to block satiety with CCK-A antagonists following an amino acid mixture (equivalent to the amino acid pattern of albumin) (Trigazis et al., 1997) or intraduodenal phenylalanine (Yox et al., 1992) in rats suggests that CCK-A receptors are responsive to CCK released by protein but not CCK released by amino acids in suppressing food intake. One possibility is that protein-stimulated CCK acts directly on vagal afferent CCK-A receptors to cause satiety, whereas amino acids may act indirectly to increase satiety by delaying gastric emptying thereby amplifying gastric distension sensations, since one of the major actions of CCK is in delaying gastric emptying.
3.4 The effect of alanine supplementation on food intake and subjective appetite ratings

3.4.1 Aims and hypothesis

Alanine has been used by previous researchers as a control treatment when investigating the influence of phenylalanine or aspartame on hunger and satiety (Ryan-Harshman et al., 1987; Anderson et al., 1989). However, Rogers & Blundell (Rogers & Blundell, 1994) have suggested that alanine itself may have an effect on appetite. On this basis it is hypothesised that alanine, administered as a preload, will enhance satiation at an ad libitum test meal compared to a control preload of cornflour. Subjective visual analogue scales (VAS) and food preference checklists will be used to detect any pre-meal changes in appetite.

3.4.2 Subjects

Twelve healthy volunteers (7 male, 5 female) aged 18 to 25 years (mean 21, SD 2.1 years) were recruited from students at the University of Surrey. BMI ranged from 20.5 to 28.5 kg/m² (mean 23.5, SD 2.7 kg/m²). Volunteers accepted on to the study were non-smokers, non-dieting, had no special dietary requirements or unusual eating habits, and did not take regular medication (except oral contraceptives or non-prescription analgesics). The volunteers were also screened using the Dutch Eating Behaviour Questionnaire (van Strien et al., 1986) shown in appendix II, which measures dietary restraint, externality and emotionality. Only those men and women scoring less than 3.0 overall (mean score 2.4, SD 0.4) were asked to participate. A 3-day food diary was completed prior to acceptance on the study in order to highlight any unusual dietary habits.

To ensure that the subjects liked the foods provided but did not eat excessively, they were asked to complete a questionnaire that rated food choices and excluded foods that they did not like (appendix IV). The subject's second choice for each food category was provided in the ad libitum test meal. The subjects were also given empty capsules of the type used in the experiment in advance to make sure that they could swallow them without any difficulty. Finally, all volunteers signed a consent form prior to the first study day that stated they had read the Information for Volunteers sheet. The subjects were told that the study was investigating the effect of
amino acids on mood and food choice, but they were not told explicitly what the capsules contained. The study protocol was approved by the local ethics committee.

3.4.3 General procedure

A within-subjects, repeated measures single blind design was used for the experiment, with each subject serving as his or her own control. Every subject received each of the following: 5 g L-alanine (Scientific Hospital Supplies) and 5 g cornflour (control) in random order. Each dose was administered in 10 opaque gelatine capsules, and the capsules were ingested with 400 ml of warm water to facilitate the dissolving of the capsules. Subjects were required to attend the Clinical Investigation Unit at the University of Surrey on two occasions – each occasion for one treatment (test or control).

The subjects had been instructed to eat a light evening meal and their normal (cereal-based) breakfast, and to avoid alcohol from 2000 h the evening before until the study. They were also requested to abstain from any heavy or vigorous exercise for 24 hours before the study. On the day of the study they were required to drink only water and eat only what was provided for them from breakfast onwards. Subjects were instructed to come to the Clinical Investigation Unit at the same designated time on both occasions (between 1115 h and 1145 h). Table 3.6 shows the study protocol.
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>-20</td>
<td>Arrive at the investigation unit</td>
</tr>
<tr>
<td>-5</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Treatment (consumed capsules + water)</td>
</tr>
<tr>
<td>+5</td>
<td></td>
</tr>
<tr>
<td>+20</td>
<td></td>
</tr>
<tr>
<td>+40</td>
<td></td>
</tr>
<tr>
<td>+60</td>
<td>Consume <em>ad libitum</em> buffet-style test meal.</td>
</tr>
<tr>
<td>+75</td>
<td>(after meal termination)</td>
</tr>
</tbody>
</table>

Table 3.6 Study protocol for the effect of alanine on food intake and subjective appetite ratings.

Whilst eating the *ad libitum* test meal, the subjects were partitioned to minimise social interaction and external influences on eating behaviour. They were instructed to eat until they were comfortably full. Subjects could take leftover food home if they wished. VAS ratings were not used for well-being ratings (headache, stomachache nausea etc.) as there was a risk that too many ratings might cause subjects to take less care over their appetite scores. Instead, subjects were asked if they felt well throughout the study period with the intention of noting any side effects of alanine consumption. Subjects noted the time of their next eating episode following the *ad libitum* meal. There was a one week interval between sessions.

3.4.4 Subjective measures of appetite

Subjects rated their hunger, desire to eat, and fullness and completed food preference checklists as described in section 2.2.1.3.

3.4.5 Treatments and meals

*Capsules:* The gelatine capsules were filled with L-alanine or cornflour, weighed on a microbalance to ensure that each capsule contained 500 mg exactly. Ten capsules made up a 5 g dose.

*Ad libitum test meal:* This was the same meal as described in section 3.2.5 (appendix III).
3.4.6 Results

3.4.6.1 Energy and food intake

Mean energy (kJ), food (g) and macronutrient (g) intakes at the *ad libitum* test meal following alanine and control treatments are shown in table 3.7. A paired, two-tailed t-test comparing energy, food and macronutrient intake showed no statistically significant differences.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Energy intake (kJ)</th>
<th>Food intake (g)</th>
<th>Protein intake (g)</th>
<th>Fat intake (g)</th>
<th>CHO intake (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Mean: 4526, SEM: 305</td>
<td>Mean: 501.7, SEM: 29.3</td>
<td>Mean: 33.5, SEM: 2.6</td>
<td>Mean: 46.5, SEM: 3.0</td>
<td>Mean: 132.4, SEM: 9.8</td>
</tr>
<tr>
<td>Control</td>
<td>Mean: 4205, SEM: 408</td>
<td>Mean: 463.8, SEM: 36.5</td>
<td>Mean: 31.6, SEM: 3.2</td>
<td>Mean: 42.8, SEM: 4.5</td>
<td>Mean: 123.3, SEM: 11.8</td>
</tr>
</tbody>
</table>

Table 3.7 Food intake by energy (kJ) and weight (g), and macronutrient intake by weight (g) following consumption of 5 g encapsulated alanine or cornflour (control).

There were no significant differences in the time intervals to the next eating episode following the *ad libitum* test meal between conditions, with the average intermeal interval being 3h 17 min (SEM 37 min) and 4 h 27 min (SEM 27 min) for alanine and the control respectively.

3.4.6.2 Subjective ratings of appetite

There was a slight early decrease in desire to eat and an early increase in fullness following alanine compared to the control. Two way, repeated measures ANOVA showed few significant overall main effects of treatment. Desire to eat was decreased at t +5 min following treatment with alanine (p <0.05) compared to control. This difference was not significant for hunger (p = 0.110). ANCOVA of fullness (with baseline values as covariate) showed a significant effect of treatment over t +5 and t +20 min (p <0.05), with levels of fullness being greater following alanine compared to control. This suggests that alanine increased satiety immediately following ingestion of the capsules. See figures 3.18, 3.19 and 3.20.
Figure 3.18 Hunger VAS ratings (mean ± SEM, n = 12) following consumption of 5 g encapsulated alanine or cornflour (control). There were no significant differences in hunger response.
Figure 3.19 Desire to eat VAS ratings (mean ± SEM, n = 12) following consumption of 5 g encapsulated alanine or cornflour (control). Desire to eat (x-b values) was decreased at t +5 min following treatment with alanine (paired t-test, p <0.05) compared to control.

Figure 3.20 Fullness VAS ratings (mean ± SEM, n = 12) following consumption of 5 g encapsulated alanine or cornflour (control). Fullness ratings (ANCOVA with baseline values as covariate) were significantly increased following alanine compared to control at time points t +5 and t +20 min (p <0.05).
Satiety quotients (SQs) were calculated for hunger, desire to eat and fullness ratings but a paired t-test revealed no significant differences between treatments (p = 0.607, 0.444 and 0.914 respectively).

3.4.6.3 Food preference checklist

Two way, repeated measures ANOVA on the total number of all foods, protein foods, high fat foods, and high carbohydrate foods were analysed. A significant main effect of treatment was found for total number of foods selected (x-b), with more foods being selected over all time points (including post-meal selections) following the control compared to alanine treatment (p <0.05). See figure 3.21. The opposite relationship was found for number of high protein foods selected but this was not statistically significant (figure 3.22). There were no differences in number of high carbohydrate items selected between treatments (figure 3.23). A paired t-test at t +5 min revealed a near significant difference for number of high fat foods selected (x-b), as more items were selected following control compared to alanine treatment (p = 0.096). See figure 3.24.
Figure 3.21 Change in number of food items selected from the food preference checklist (mean ± SEM, n = 12) following consumption of encapsulated alanine compared to control. More food items (change from baseline values: x-b) were selected following the control treatment compared to alanine treatment (p <0.05).

Figure 3.22 Change in number of high-protein food items selected from the food preference checklist (mean ± SEM, n = 12) following consumption of encapsulated alanine compared to control. There were no significant differences between treatments.
Figure 3.23 Change in number of high-carbohydrate food items selected from the food preference checklist (mean ± SEM, n = 12) following consumption of encapsulated alanine compared to control. There were no significant differences between treatments.

Figure 3.24 Change in number of high-fat food items selected from the food preference checklist (mean ± SEM, n = 12) following consumption of encapsulated alanine compared to control. There were no significant differences between treatments, but a paired t-test at t +5 min revealed a near significant difference for number of high fat foods selected (x-b), (p = 0.096).

Side effects such as nausea or headache were not reported by any subject following alanine consumption.
3.4.7 Discussion

The aim of this study was to determine whether alanine could exert a similar suppressive influence on food intake to phenylalanine. This would clarify whether alanine is a suitable nutrient to use as a control treatment when investigating the satiating properties of other amino acids or aspartame, as used by previous researchers (Ryan-Harshman et al., 1987; Anderson et al., 1989). Various tools were used to measure appetite, including food intake assessment at an ad libitum test meal, VAS ratings of hunger, desire to eat and fullness, and food preference checklists.

The food intake data showed that alanine had no effect on energy, weight of food or macronutrient intake at the ad libitum test meal 60 min later. These results are at odds with those reported by Rogers & Blundell (1994), when they reanalysed the negative data from a study on the effect of phenylalanine on food intake that used alanine as a control (Ryan-Harshman et al., 1987). Rogers & Blundell concluded that alanine suppressed food intake in that study, although less potently than phenylalanine. Based on this evidence, it is surprising that the hypothesis for the present study is not supported. In addition to this, there was no difference in the duration of the inter-meal interval following the ad libitum test meal.

However, the lack of effect of alanine on food intake was not reflected by the subjective appetite measurements. Early appetite responses to the treatments appeared to be affected, with desire to eat reduced by alanine at t +5 min and fullness increased by alanine at t +5 and t +20 min. In addition to this, a significant main effect of treatment was uncovered for change from baseline in total number of food items selected from the food preference checklists; alanine ingestion resulted in fewer food items being selected overall. This suggests that, although food intake was unaffected, pre-meal subjective hunger and satiety were influenced by alanine ingestion. Ryan-Harshman et al. (1987) did not find any statistically significant treatment effects from their VAS data for hunger but this can be explained by the fact that they were filled out too infrequently, i.e. immediately before treatment, immediately before the ad libitum test meal, and after the ad libitum test meal. Alanine may influence appetite but this is likely to be through a different mechanism to phenylalanine. Although phenylalanine has no effects on pre-meal subjective appetite, it causes a dose-dependent reduction in food intake (Rogers & Blundell, 1994; see section 3.2), and it has been postulated that this may be because phenylalanine enhances the satiating
capacity of food. However, alanine reduced hunger and decreased fullness before the meal but did not affect food intake. Thus it can be theorised that the influence of alanine on motivation to eat is overruled by the presence of other nutrients in the gastrointestinal tract.

3.5 The effect of aspartame and aspartame constituents on gastric emptying, plasma cholecystokinin and glucagon-like peptide-1 response

3.5.1 Aims and hypothesis

Aspartame has previously been found to increase satiety in human subjects (Leung et al., 1968). The hypothesis for this study is that aspartame affects satiety by potentiating CCK secretion in the presence of nutrients in the intestine, and consequently delaying gastric emptying. This study will determine whether aspartame has any such physiological effects. The possibility that aspartame may alter the secretion of other gut hormones will be examined. Whether the whole and the amino acid constituents, phenylalanine and aspartic acid, have the same effects when administered in equivalent amounts will also be explored.

3.5.2 Subjects

Six lean healthy volunteers (4 female and 2 male) were recruited from the University, aged 24 to 31 years (mean 27.7, SD 2.9 years). The volunteers were all non-dieting, with no special dietary requirements, and a BMI <25 kg/m² (mean 20.1, SD 1.5 kg/m²). They had no significant current or previous medical history (including phenylketonuria) and took no medication apart from the oral contraceptive pill and occasional minor analgesics. They were selected on the basis of their responses to the Dutch Eating Behaviour Questionnaire (mean score 2.4, SD 0.2) (van Strien et al., 1986) shown in appendix II, and their physical activity levels. Subjects did not consume more than 20 units of alcohol per week.

All subjects were interviewed and a venous blood sample taken for haematological and biochemical screening to confirm their suitability to participate in the study. The volunteers signed a consent form before they were accepted onto the study, which included a statement that they understood that they would be donating some blood as part of the study. Their GP was informed of their participation in the
study, and was given the opportunity to notify the researcher of any reason why they should not take part. The study protocol was approved by the local ethics committee.

3.5.3 General procedure

A within-subjects, repeated measures design was used for the experiment, with each subject serving as his or her own control. Subjects were asked to refrain from consuming alcohol, caffeine and products containing aspartame or paracetamol the evening before, and until the study. On the day of the study they were required to consume nothing except water until 90 min before the study, and then no water until the study began.

Subjects arrived at the Clinical Investigation Unit at the University of Surrey between 0730 h and 0900 h (two people per study day), and an intravenous cannula was inserted into an antecubital vein in the forearm under local anaesthetic by a qualified doctor or nurse. Blood samples were taken according to the methodology detailed in section 2.2.3.1. Six electrodes were positioned over the stomach and lower back to measure gastric emptying by EIE, as detailed in section 2.2.2.2. Once a satisfactory ten minute baseline reading had been obtained for the EIE recordings, the subjects consumed one capsule containing either the placebo, aspartame or aspartic acid + phenylalanine, together with 450 ml water containing 3 dissolved crushed paracetamol tablets (1.5 g paracetamol). Blood samples were taken for the measurement of plasma glucose, amino acids, CCK, GLP-1, GIP and insulin, and VAS sheets (appendix V) were completed according to the protocol shown in table 3.8. Sixty min after ingestion of the capsule and water the EIE recordings were stopped and the subject was given the opportunity to move about. Immediately on their return the EIE recordings were restarted, and when a steady baseline had been attained (approximately 5 min later) a liquid test meal was offered to the subject. Another hour of blood samples and VAS questionnaires followed.
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Blood sample</th>
<th>Gastric emptying (EIE)</th>
<th>VAS</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>-10</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
<td>✓</td>
<td>Consume aspartame capsule + water + paracetamol</td>
</tr>
<tr>
<td>+5</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>+10</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
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<tr>
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<td></td>
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<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>+60</td>
<td></td>
<td></td>
<td>✓</td>
<td>Consume liquid test meal</td>
</tr>
<tr>
<td>+65</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
</tr>
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<tr>
<td>+120</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.8. Study protocol for the effect of aspartame and constituent amino acids on gastric emptying, plasma metabolite and hormone response and subjective appetite ratings.

3.5.4 Treatments and meals

Capsules: The capsules were filled with 400 mg aspartame (donated by NutraSweet Powder, Forum Products Ltd, Redhill) or 176 mg of L-aspartic acid + 224 mg L-phenylalanine (Scientific Hospital Supplies, Liverpool) (the same amounts as 400 mg aspartame), or 400 mg cornflour (control), and they were weighed out on a microbalance.

Test meals: A liquid test meal containing 1992 kJ was consumed by the subject on each of the 3 study days. The liquid test meal was mixed using an electric blender and was formulated using double cream, maltodextrin and strawberry- or banana-flavoured Nesquik and were made up to 450 ml in volume with water (see table 3.9).
Table 3.9 Composition of liquid test meal used to test the physiological effects of ingesting aspartame or aspartame constituents (400 mg).

3.5.5 Subjective measures of appetite

Subjects rated their hunger, desire to eat, fullness, and thirst as detailed in section 2.2.1.3.

3.5.6 Results

3.5.6.1 Gastric emptying

3.5.6.1.1 Electrical Impedance Epigastrography

Figure 3.25 shows that gastric half-emptying times (T50s) following the liquid test meal were shorter following the constituent amino acids:
Figure 3.25 Mean gastric emptying T50s (± SEM, n = 6) following aspartame or its amino acid constituents compared to the control treatment. Gastric emptying times following the liquid test meal were significantly different between treatments ($p < 0.005$), with shorter T50s following the constituent amino acids.

Results were analysed using repeated measures ANOVA, and it was shown that there were no differences between the treatments following the water load but that they were significantly different following the liquid test meal ($p = 0.002$), with the post-hoc Duncan test showing that T50s were shorter following the amino acids compared to the control and aspartame. The aspartame and control gastric emptying times were very similar for five out of six of the subjects.

The percentage volume emptied following each liquid load is illustrated in figure 3.26. Repeated measures two way ANOVA showed that there was a significant overall treatment effect ($p < 0.0005$), and the post-hoc Duncan test showed that there was a greater percentage volume retained following the control compared to the aspartame ($p < 0.05$) and amino acids ($p < 0.0005$), and following the aspartame treatment compared to the amino acids ($p < 0.005$).
Liquid test meal

![Graph showing percentage volume of liquid test meal remaining in the stomach consumed one hour after ingestion of aspartame, constituent amino acids of aspartame or the control (mean ± SEM, n = 6). The rate of gastric emptying significantly decreased firstly with aspartame, and to a greater extent following the constituent amino acids.]

3.5.6.1.2 Paracetamol absorption

Plasma paracetamol concentrations were measured for 60 min following ingestion of the treatment (aspartame, constituent amino acids or control), 1.5 g paracetamol and the water load (figure 3.27). Time until peak concentration (t_{max}), peak concentration (c_{max}) and area under the curve (AUC) were calculated for each subject's paracetamol results for the first sixty min until the liquid test meal. There were no significant differences between treatments.

Table 3.10 Gastric emptying parameters for paracetamol absorption following consumption of aspartame or constituent amino acids compared to control. Mean (± SEM) n = 6. Repeated measures ANOVA between the treatments show no significant differences.

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Figure 3.27 Plasma paracetamol concentrations following consumption of aspartame or constituent amino acids compared to control (mean ± SEM, n = 6). There were no significant differences in plasma levels between treatments.

The parameters of gastric emptying of the water load were as follows (table 3.10):

Table 3.10 Gastric emptying parameters for paracetamol absorption following consumption of aspartame or constituent amino acids compared to control. Mean (± SEM) n = 6. Repeated measures ANOVA between the treatments show no significant differences.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Aspartame</th>
<th>Phenylalanine + aspartic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>tmax (min)</td>
<td>Mean 30.0 7.8</td>
<td>Mean 27.5 4.6</td>
<td>Mean 32.5 7.2</td>
</tr>
<tr>
<td></td>
<td>SEM 7.8</td>
<td>SEM 4.6</td>
<td>SEM 7.2</td>
</tr>
<tr>
<td>cmax (mmol/L)</td>
<td>0.19 0.02</td>
<td>0.17 0.01</td>
<td>0.18 0.02</td>
</tr>
<tr>
<td>AUC (mmol/L by min)</td>
<td>7.47 0.94</td>
<td>7.69 0.66</td>
<td>7.69 0.94</td>
</tr>
<tr>
<td>AUC t0 to t+15 min (mmol/L by min)</td>
<td>1.85 0.50</td>
<td>1.67 0.28</td>
<td>1.52 0.44</td>
</tr>
</tbody>
</table>

3.5.6.2 Blood metabolites and hormones

3.5.6.2.1 Plasma glucose

Figure 3.28 shows plasma glucose concentrations. As expected, plasma glucose levels increased significantly following the liquid test meal, and ingesting aspartame or phenylalanine + aspartic acid had no effect on plasma glucose concentrations compared to baseline. Two way repeated measures ANOVA revealed
no significant differences in glucose response either before or after the liquid test meal.

Figures 3.28 Plasma glucose concentrations following consumption of aspartame or constituent amino acids compared to control (mean ± SEM, n = 6). There were no significant differences in plasma levels between treatments.

3.5.6.2.2 Plasma phenylalanine

Figure 3.29 shows plasma phenylalanine concentrations (n = 5). Phenylalanine levels increased following aspartame and constituent amino acids ingestion compared to the control. Two way, repeated measures ANOVA (values as change from baseline, x-b) revealed a main treatment effect (p < 0.05) on plasma levels, with a post hoc Duncan test between treatments showing the greatest differences to lie between the constituent amino acids and control treatments (p < 0.05), followed by aspartame compared to control (p = 0.06). If the plasma phenylalanine profiles are separated into pre-meal and post-meal levels, there was a main treatment effect before the liquid test meal that was marginally non-significant (p = 0.067), but this difference is more marked following the liquid test meal (p < 0.05). Plasma phenylalanine levels appeared to increase more quickly following the aspartame compared to the phenylalanine + aspartic acid treatment.
Figure 3.29 Plasma phenylalanine concentrations (x-b) following consumption of aspartame or constituent amino acids compared to control (mean ± SEM, n = 5). There was a significant main effect of treatment on overall phenylalanine levels (p <0.05). Phenylalanine levels were significantly different from control following phenylalanine + aspartic acid (p <0.05), and aspartame (p <0.05) following the liquid test meal.
3.5.6.2.3 Plasma aspartic acid

Plasma aspartic acid concentrations (n = 5) are shown in figure 3.30. There were no significant main effects of treatment, time or treatment x time interactions on plasma aspartic acid levels.

![Figure 3.30](image)

Figure 3.30 Plasma aspartic acid concentrations (x-b) following consumption of aspartame or constituent amino acids compared to control (mean ± SEM, n = 5). There were no significant differences in plasma aspartic acid levels between treatments.
3.5.6.2.4 Plasma tyrosine

Plasma tyrosine concentrations (n = 5) are shown in figure 3.31. There were no significant main effects of treatment, time or treatment x time interactions on plasma tyrosine levels.

Figure 3.31 Plasma tyrosine concentrations (x-b) following consumption of aspartame or constituent amino acids compared to control (mean ± SEM, n = 5). There were no significant differences in plasma tyrosine levels between treatments.

3.5.6.2.5 Plasma CCK

Plasma CCK concentrations are illustrated in figure 3.32. Plasma CCK remained at baseline levels following the treatment until the liquid test meal, when there was a rise in CCK levels following all treatments. There were no significant differences between CCK responses to treatment, nor were there any treatment x time interactions.
3.5.6.2.6 Plasma insulin

Mean plasma insulin concentrations are shown in figure 3.33. There were no significant effects of treatment either before or after the liquid test meal on plasma insulin response.

3.5.6.2.7 Plasma GIP

Mean plasma GIP concentrations are shown in figure 3.34. There were no significant differences between treatments either before or after the liquid test meal. The plasma GIP value at 90 min appears to be reduced following the control pre-treatment compared to the aspartame and amino acids, although this difference is not statistically significant by repeated measures ANOVA at that time point (p = 0.19).
Figure 3.33 Plasma insulin concentrations following consumption of aspartame or constituent amino acids compared to control (mean ± SEM, n = 6). There were no significant differences in plasma insulin levels between treatments.

Figure 3.34 Plasma GIP concentrations following consumption of aspartame or constituent amino acids compared to control (mean ± SEM, n = 6). There were no significant differences in plasma GIP levels between treatments.

3.5.6.2.8 Plasma GLP-1

Mean plasma GLP-1 concentrations are shown in figure 3.35. The plasma GLP-1 response to the liquid test meal was higher following the control compared to the aspartame and the constituent amino acids. Results were analysed using repeated measures two-way ANCOVA (with baseline values as covariate), and it was shown...
that there was a main treatment effect on plasma GLP-1 concentrations (p <0.05), with the post-hoc Duncan test showing this to be the product of lower GLP-1 concentrations following the aspartame compared to control at t +75 and t +90 min (p <0.00005 and p <0.005 respectively), and amino acids at t +75 and t +90 mins (p <0.0001 and p <0.005 respectively).

Figure 3.35 Plasma GLP-1 concentrations following consumption of aspartame or constituent amino acids compared to control (mean ± SEM, n = 6). Significant differences were found between treatments overall (p <0.05). GLP-1 plasma concentrations were strongly suppressed following the liquid test meal due to prior ingestion of aspartame and phenylalanine + aspartic acid.

3.5.6.3 Subjective ratings of appetite

Desire to eat was suppressed following the liquid test meal as a result of treatment with the constituent amino acids. Figures 3.36, 3.37 and 3.38 show hunger, desire to eat and fullness respectively following the aspartame and constituent amino acids.
Figure 3.36 Hunger VAS ratings (mean ± SEM, n = 6) following consumption of aspartame or constituent amino acids compared to control. There were no significant differences in hunger response.

Hunger

Although there appeared to be a trend for reduced hunger ratings following the constituent amino acids, two way repeated measures ANCOVA (with baseline as covariate) and ANOVA (x-b values) did not uncover any significant differences between treatments, either overall time points, pre-meal or post-meal.
Figure 3.37 Desire to eat VAS ratings (mean ± SEM, n = 6) following consumption of aspartame or constituent amino acids compared to control. Desire to eat was significantly suppressed following the liquid test meal as a result of prior ingestion of the constituent amino acids, phenylalanine + aspartic acid (p <0.05).

**Desire to eat**

Desire to eat was significantly reduced following the liquid test meal as a result of prior ingestion of the constituent amino acids.

Two way repeated measures ANCOVA (with baseline as covariate) over the entire time course of desire to eat ratings revealed no main treatment effects but a treatment x time interaction (p <0.05). Repeating this procedure over the pre-meal ratings failed to produce any significant differences. There was a significant treatment effect on change in desire to eat from the pre-liquid test meal baseline (60 min) (p <0.05), due to the suppression of desire to eat following pre-treatment with the constituent amino acids, phenylalanine and aspartic acid.
Figure 3.38 Fullness VAS ratings (mean ± SEM, n = 6) following consumption of aspartame or constituent amino acids compared to control. There were no significant differences in fullness between treatments.

Fullness

There were no significant main treatment effects or treatment by time interactions for fullness ratings.
3.5.6.3.1 Correlations between desire to eat and plasma phenylalanine/gastric emptying times following the liquid test meal

Figure 3.39 shows that the change in plasma phenylalanine at t +75 min (x-b) and the change in desire to eat were significantly correlated following the constituent amino acids (r = -0.9774, p = 0.004). There were no significant associations between gastric half-emptying times (T50s) and change in desire to eat (t +70 – t +60 min).

![Graph showing correlation between change in plasma phenylalanine and change in desire to eat.](image)

Figure 3.39 The association between individual ratings of change in desire to eat (cm) following ingestion of the liquid test meal (t +70 – t +60 min) and change in plasma phenylalanine concentrations at t + 75 min (mmol/L). There was a significant correlation between these parameters following the constituent amino acids (p <0.005), but the association was marginally non-significant following the aspartame treatment (p = 0.056).
3.5.7 Discussion

The hypothesis for the present study was made up of three parts:

A) aspartame increases CCK secretion,
B) aspartame delays gastric emptying (via CCK),
C) aspartame increases satiety at a meal by these two linked mechanisms.

The results did not support any part of this hypothesis for the following reasons:

A) CCK secretion was unaffected by ingestion of 400 mg aspartame or equivalent constituent amino acids (figure 3.32),
B) gastric emptying times were slightly shorter following aspartame and markedly shorter by equivalent constituent amino acids (figures 3.25 and 3.26),
C) food intake at a meal was not measured but aspartame did not affect subjective hunger, desire to eat or fullness either before or after a liquid meal. However, subjective ratings of desire to eat post-meal were reduced by prior ingestion of the constituent amino acids (figures 3.36, 3.37, and 3.38).

Unexpected effects were also observed:

A) GLP-1 secretion was attenuated following the test meal when encapsulated aspartame or its constituent amino acids are consumed 60 min earlier,
B) the time course of plasma levels of phenylalanine differed between aspartame and the constituent amino acids.

It was also demonstrated that ingestion of 400 mg aspartame or its constituent amino acids had no effects on gastric emptying of water before the ingestion of a fat/carbohydrate-containing liquid meal. In addition, there were no differences in plasma glucose, aspartic acid, tyrosine, insulin, or GIP following any treatment.

The lack of effect of aspartame and the constituent amino acids on plasma CCK concentrations does not support the theory that aspartame induces satiety by potentiating the release of CCK in response to food (Rogers & Blundell, 1992). Previous work in human subjects showing the release of CCK in response to phenylalanine has involved much larger doses of up to 10 g (Ballinger & Clark, 1994; section 3.2). This is approximately 50 times the amount of phenylalanine used in this experiment and therefore it is not surprising that an increase in plasma CCK levels
was not demonstrated here. Rogers & Blundell had identified CCK as a likely mediator for phenylalanine- and aspartame-induced satiety because both CCK and aspartame require a gastric stimulus to reduce appetite – gastric loads amplify satiety produced by CCK infusions (Schwartz et al., 1991) and aspartame fails to affect pre-meal VAS ratings but strongly reduces food intake. Rogers & Blundell (1992) also highlighted that the C-terminal dipeptide of CCK (and gastrin) contains the amino acid sequence Asp-Phe. The possibility of aspartame acting as a kind of CCK receptor agonist, however, is remote due to the fact that aspartame is hydrolysed to aspartic acid and phenylalanine before it can enter the circulation and that C-terminal CCK fragments with a sequence less than 4 amino acids do not show significant activity at CCK receptors (Ranney et al., 1976; Rehfeld et al., 1980; Opperman, 1984; Cherner et al., 1988). Aspartame may well induce an increase in CCK secretion at higher doses of 2-5 g, but not at the low levels of intake previously shown to reduce food intake (Rogers et al., 1990; Rogers et al., 1991).

Since no impact was made on plasma CCK levels by 400 mg aspartame or constituent amino acids, it is not unexpected that gastric emptying was not delayed either. If CCK had been elevated, a slower gastric emptying rate would have been a likely outcome. However, the apparent acceleration in gastric emptying rate following the liquid meal, shown by % volume of meal retained in the stomach following aspartame and phenylalanine + aspartic acid, was not anticipated. This effect was stronger following the constituent amino acids, as shown by the lack of significance for the shorter T50s following aspartame. It is indicated by the data, therefore, that aspartame weakly accelerated gastric emptying, and the constituent amino acids were a stronger stimulus of emptying rate.

The finding that gastric emptying is accelerated following the liquid test meal as a result of pre-treatment with aspartame or the constituent amino acids is open to criticism regarding the methodology. Gastric emptying in the second half of the experiment was measured by EIE alone, and the discovery that the half-emptying times obtained using this method are clearly shorter than physiologically possible for a 1992 kJ meal raises some important questions. Since this method measures electrical conductivity and thus impedance, then gastric secretions are a source of impedance error since they may increase the conductivity of gastric contents, reducing total impedance and apparent emptying times. A non-conductive meal such
as the fat/carbohydrate liquid meal used in the present study would appear to empty faster than the true rate of emptying. Does the apparent acceleration in gastric emptying rate following the aspartame and amino acids result from a stronger stimulation of gastric acid secretion?

Administration of large amounts of mixed amino acids, phenylalanine, tryptophan and histidine strongly stimulates gastrin and gastric acid secretion as shown by many researchers (Elwin, 1974; Cieszkowski et al., 1975; Walsh et al., 1975; Konturek et al., 1976; Richardson et al., 1976; Byrne et al., 1977; Konturek et al., 1978a; Taylor et al., 1982). The administration of phenylalanine in the present study did not seem to affect gastric acid secretion in the first hour, or if it did the effect was not strong enough to be reflected in the gastric impedance results. We know that gastric emptying was unaffected during this time from the paracetamol data. Plasma phenylalanine levels began to increase at t +15 min and were significantly raised by t +60 min following the constituent amino acids, indicating that ingested phenylalanine had been in contact with gastric and intestinal mucosa during the first hour but failed to increase gastric acid secretion by a significant enough amount to affect impedance results. The studies that had shown increased gastric acid secretion following oral or gastric infusion of amino acids used larger amounts than used in the present study, typically 10 g or more (Cieszkowski et al., 1975; Walsh et al., 1975; Konturek et al., 1976; Richardson et al., 1976; Byrne et al., 1977; Konturek et al., 1978a; Taylor et al., 1982). Therefore, it cannot be inferred from these publications that the small amounts of phenylalanine (224 mg) used in this study increased pre-meal gastric acid secretion, especially as there was no evidence from the impedance data to suggest this.

It has previously been shown, however, that intravenous administration of amino acids can increase gastric acid secretion via a gastrin-independent mechanism (Konturek et al., 1978a; Konturek et al., 1978b). This raises the possibility that the absorbed plasma phenylalanine in the latter half of the experiment (60-120 min) may have been stimulating gastric acid secretion during the gastric emptying of the fat/carbohydrate liquid test meal. During this time, faster emptying rates were shown following the constituent amino acids (when larger increases in plasma phenylalanine were observed), and to a lesser extent following the aspartame. Do these apparent shorter emptying times represent an increase in acid secretion and therefore a
reduction in impedance, or are they really a reflection of accelerated emptying rates? The work of Konturek and colleagues was further supported by other researchers who used physiological plasma levels of the aromatic amino acids (McArthur et al., 1983; Lenz et al., 1988). McArthur and colleagues intravenously infused graded doses of phenylalanine and tryptophan at physiological plasma levels over 4 hours in human subjects. Intravenous L-phenylalanine (at 3.13 mmol/h, or 65 mg/h in 125 ml) stimulated gastric acid secretion to 39% of the response to pentagastrin (maximal acid response) independently of gastrin while plasma phenylalanine concentrations were only raised to 0.09 mmol/L from a baseline of 0.05 mmol/L. Plasma phenylalanine in the present study rose from a mean baseline of 0.07 mmol/L to a mean peak of 0.107 mmol/L at t +60 min following the constituent amino acids treatment; this elevation in plasma phenylalanine is certainly of a scale comparable to those observed by McArthur et al. (1983), and therefore one which you would expect to stimulate gastric acid secretion if similar physiological responses were occurring.

However, the present study differs from the McArthur study. A major difference, apart from the route of administration, is that the subjects in this study also consumed a fat/carbohydrate-containing liquid test meal at the time of peak plasma phenylalanine levels. The meal itself was neutral (pH 6.6) and the double cream would have had a buffering effect on the acidity of the stomach contents. Furthermore, research has shown that 20 g intraduodenal fat completely inhibits the gastric acid response to intravenous amino acids + glucose; moreover intravenous fat nearly completely inhibits it (Varner et al., 1980). The liquid test meal used in the present study contained 22 g fat. It was shown in another study that intravenous infusion of glucose to maintain hyperglycaemia (plasma glucose 15 mmol/L) also completely abolished the gastric acid response to intravenous amino acids infused at 10 g/hour (Lam et al., 1995). Plasma glucose levels only peaked at a mean concentration between 7.7 and 9.2 mmol/L in the present study (t +120 min), but systemic concentrations may have been raised enough to contribute to an inhibitory effect on gastric acid secretion. Furthermore, a study that looked at intravenous amino acids infusion using a solution that imitated physiological postprandial amino acid patterns found that basal gastric acid secretion was barely increased; they concluded that the findings of McArthur et al. (1983) were probably physiologically irrelevant as there may have been an unknown potentiating effect of the infusion of
phenylalanine and tryptophan together without any other amino acids present (Schulte-Frohlinde et al., 1993).

No measurements of gastric acid secretion were made in this study and therefore no firm conclusions can be drawn regarding the validity of these gastric emptying results. Gastric acid secretions were not detected by EIE during the first half of the study when the water load was emptied, and usually gastric distension prompts an increase in gastric acid secretion (Richardson et al., 1976). Possibly a large increase in gastric acid secretion is required to alter epigastric impedance and confound the gastric emptying results. If the post-prandial rise in plasma phenylalanine was likely to elicit a greater increase in gastric acid secretion above control levels following the liquid test meal, it is likely that this would have been completely inhibited by the entry of fat and carbohydrate into the duodenum and their consequent absorption. In considering the facts, it can be concluded that EIE gastric emptying times in this experiment are probably quantitatively inaccurate, but any differences identified are likely to represent genuine changes in gastric emptying rates. With hindsight it would have been preferable to use the paracetamol absorption method with the liquid test meal but it was not known whether there would be any effect on gastric emptying nor when these effects would occur.

The reasons why phenylalanine and aspartic acid, and to a lesser extent aspartame, stimulated gastric emptying in this study are not obvious. Initially, these finding seem to contradict previous observations (see section 3.3) where ingestion of phenylalanine delayed gastric emptying. However, this study only used 224 mg phenylalanine + 176 mg aspartic acid unlike the previous study which used 5 g phenylalanine (22 times more). It is likely that these greatly differing doses of phenylalanine provoked two different physiological responses. Another major difference is that the 5 g phenylalanine delayed gastric emptying of a water load, whereas 224 mg phenylalanine + 176 mg aspartic acid did not affect of emptying of a water load but did accelerate gastric emptying of a fat/carbohydrate-containing load. The interaction of the nutrients with the intestine therefore, may have modified the response to the amino acids. The most obvious difference between these studies is the aspartic acid component. Although the majority of aspartic acid (>99 %) is consumed in energy metabolism pathways in the intestinal mucosa (Kutchai, 1998; Wu, 1998), a fact borne out by the failure to increase plasma aspartic acid concentrations at any
time following ingestion, it may have interacted with receptors or enteroendocrine cells in the lumen before absorption. However, there is little in the literature to suggest that aspartic acid has any effects on gastric emptying rates, apart from a study where aspartic acid relaxed guinea pig fundic muscle cells and this would inhibit emptying (Huizinga, 1980). It is an excitatory amino acid and when localised in nerve terminals in the CNS is classed as a neurotransmitter, but the relevance of this to lumenal aspartic acid is questionable.

The reduction in desire to eat observed after the liquid test meal following prior ingestion of the capsule containing the constituent amino acids supports previous findings to a limited extent. The lack of effect of treatment following the water load on all three appetite variables (hunger, desire to eat and fullness) supports the original assertion that aspartame does not influence pre-meal motivation to eat (Rogers et al., 1995). However, the absence of any significant differences in hunger or fullness following the liquid test meal is difficult to explain. The change in hunger following the test meal is also reduced as a result of prior constituent amino acid ingestion, an observation that supports the desire to eat findings even though non-significant. The lack of effect on fullness may be just a weakness in the methodology. The fact that the meal was in liquid form may have reduced its satiating properties and therefore it proved difficult for the subjects to rate how full they felt without a strong satiety stimulus.

The suppression of desire to eat following the constituent amino acids but not following the aspartame during the second part of the study is an ambiguous finding. However, the primary purpose of this study was to discover the endocrine and metabolic effects of small doses of aspartame and constituent amino acids, as the satiety effects had already been clearly demonstrated on a number of occasions (Rogers et al., 1990; 1991; 1995). The small subject number used in this study design was adequate to detect physiological changes but typically studies designed to determine appetite effects use a much larger number of subjects. The problem of sample size was exacerbated by the invasive nature of the protocol, with the electrodes and wires from the EIE equipment and the cannulation for blood samples possibly impinging on the ability of the subjects to judge their motivation to eat consistently.
The attenuation of GLP-1 secretion following aspartame and constituent amino acids after ingestion of the liquid test meal is an unexpected finding, since the GLP-1 response is reduced under the same conditions where food intake is usually suppressed (Rogers et al., 1990; 1991; 1995), and where post-prandial desire to eat is reduced. The reduction in desire to eat was only shown following the constituent amino acids, whereas the suppression of GLP-1 levels occurred following both the aspartame and the constituent amino acids. It thus appears that GLP-1 levels are not linked to the appetite responses to aspartame and constituent amino acids, and that the attenuation of GLP-1 secretion is a side effect of administration of these amino acids and dipeptides.

Reduced GLP-1 secretion may have reduced the level of inhibition of gastric emptying, and indeed it has been shown that the gastric emptying rate of the liquid test meal is accelerated following the two treatments – markedly following the amino acids, and less so following the aspartame. Whether there is a cause and effect relationship between these observations or that they were simply a result of another unknown factor remains to be established.

Plasma levels of phenylalanine, tyrosine and aspartic acid were measured in order to obtain some idea of how soon the amino acids were absorbed and whether there were any differences in plasma profiles between aspartame and the constituent amino acids. The results for plasma phenylalanine were interesting. Plasma phenylalanine following the control remained at basal levels until the liquid test meal, when concentrations dropped considerably. This was expected, as the carbohydrate in the liquid test meal would have induced insulin secretion, which would enhance amino acid uptake into the tissues. Following aspartame, plasma concentrations were already slightly increased by t +15 min (non-significant p = 0.088, n = 5) and then remained at a fairly constant level until t +90 min, when concentrations began to decline. Following phenylalanine + aspartic acid, plasma levels of phenylalanine did not increase significantly until t +60 min (p <0.005, n = 5), at which point the increase was rapid and very distinct. Concentrations remained elevated at t +75 min and then dropped to previous levels at t +90 min. These pharmacokinetic differences cannot be the result of the rate of capsule dissolution as conditions were identical for each treatment. Therefore, the aspartame must have dissolved or been absorbed in the intestine at a different rate to the constituent amino acids. Aspartame is relatively low
in solubility (~3 g in 100 ml water), and phenylalanine and aspartic acid have even an even lower combined solubility (2.965 g and 0.778 g in 100 ml water respectively), and therefore it is probable that aspartame was absorbed earlier and at a slower rate than the constituent amino acids. This probability is supported by the fact that the rate of absorption of di- and tripeptides across the small peptide membrane transport system in the brush border is faster than the rate of individual amino acid transport. In addition, most of the amino acids that are absorbed in the jejunum are transported in to the epithelial cells as di- and tripeptides. The majority of free amino acids are consequently absorbed in the ileum via specific amino acid transport systems (Kutchai, 1998). Therefore, the blunted increase in plasma phenylalanine concentrations following aspartame is probably due to low solubility and the time required for its methyl group to be hydrolysed by intestinal esterases to yield methanol and aspartylphenylalanine; the earlier appearance of higher concentrations of phenylalanine may be because the dipeptide aspartylphenylalanine was absorbed more rapidly than the free amino acids and a greater proportion of dipeptides are absorbed in the jejunum. Some of the aspartylphenylalanine dipeptide may have been further broken down to free amino acids before absorption. The comparatively late appearance of plasma phenylalanine following the constituent amino acids, although also relatively low in solubility, is probably due to the fact that free amino acids are mainly absorbed in the ileum. Plasma tyrosine was unaffected by the treatments, probably because the amount of phenylalanine consumed was not sufficient to increase tyrosine synthesis.

The striking difference in plasma profiles of phenylalanine following the constituent amino acids compared to the aspartame reflects the pattern of gastric emptying. Aspartame produced a modest steady increase in plasma phenylalanine during the first half of the study and a weak acceleration in gastric emptying rate following the liquid test meal, whereas the constituent amino acids caused a sudden, relatively large peak in plasma phenylalanine and a stronger increase in gastric emptying rate following the liquid test meal. The magnitude and temporal association between these two physiological processes suggests that they may be directly linked, although the mechanism for this is unclear. Whether this is a direct inhibitory effect of the amino acids on humoral or neural reflexes that normally delay the rate of gastric emptying cannot be determined with any certainty from this study, but the observed reduction in GLP-1 levels may provide a clue.
It is postulated that the amino acids escaped absorption for far enough along the intestine to either directly inhibit the GLP-1-secreting L cells, or stimulate an intermediate paracrine factor or neural reflex that could inhibit GLP-1 secretion. The bulk of the constituent amino acids were absorbed relatively late (t +60 and +75 min) compared to when the capsules must have dissolved in the stomach (t +15 min according to the initial absorption of aspartame) giving them time to travel a good distance down the intestine. The absorption of the aspartame began earlier but persisted for as long as the constituent amino acids absorption, which could account for the weaker stimulus for faster gastric emptying rate, as proportionally less of the aspartame was in contact with intestinal mucosa at the time of the liquid meal. The fact that gastric emptying of the water load was unaffected by the earlier absorption of the aspartame amino acids can therefore be explained by the involvement of GLP-1 secretion, which would not be stimulated until the liquid meal was ingested. Therefore the restriction of GLP-1 release following the meal by the lumenal amino acids attenuated the inhibitory influence of this peptide on the rate of gastric emptying of the meal. Possible mechanisms for the inhibition of GLP-1 secretion by direct or paracrine effect, or neural reflex on the L cells can only be speculated on here.

It is doubtful whether the lower GLP-1 levels are wholly responsible for the faster rate of gastric emptying. GLP-1 definitely delays gastric emptying and the decreased availability of this peptide probably did reduce the inhibition of gastric emptying in this case. However, there was probably another factor involved in addition to lower GLP-1 levels because aspartame and the constituent amino acids reduced GLP-1 secretion to the same extent, whereas the increase in gastric emptying rate was greater following the constituent amino acids compared to the aspartame. Therefore, in addition to the reduced delay in gastric emptying following aspartame and its constituent amino acids due to the attenuation of an inhibitory influence (GLP-1), a direct pathway for stimulation of gastric emptying by the individual constituent amino acids is proposed. Mechanisms are unknown but it could be hypothesised that circulatory phenylalanine was acting on chemoreceptors in the stomach which may have initiated stimulation of gastric motility either via local neural reflexes or via vagal afferents and efferents.
Although we have shown these physiological consequences of ingesting small amounts of aspartame and constituent amino acids, the question of how aspartame/constituent amino acids increase satiety remains. The mechanisms for aspartame-induced satiety are difficult to discuss in the context of this study since no differences in subjective appetite ratings were shown (although food intake may have been reduced as previously shown, had we measured this parameter). However, a significant reduction in desire to eat was shown following the constituent amino acids and consequently we can speculate on mechanisms based on the current findings. The increased rate of gastric emptying is unlikely to have reduced desire to eat since it is normally a delay in gastric emptying that causes fullness sensations due to distension of the stomach. The faster delivery of nutrients to the intestine and more rapid absorption may have reduced desire to eat but through what mechanism? The effects of CCK and GLP-1 on satiety have been ruled out in this study since CCK was unaffected and GLP-1 secretion was diminished. Faster absorption of phenylalanine after the constituent amino acids was not a result of faster gastric emptying since this occurred just before the liquid test meal.

It is hypothesised therefore, that the reduction in desire to eat was a consequence of the differences in pharmacokinetics of phenylalanine absorption following the two treatments. Due to differing rates and sites of absorption, plasma phenylalanine appeared slowly and earlier following aspartame whereas there was a sudden relatively large rise in plasma levels following the constituent amino acids. It can be inferred from this that the peak in plasma phenylalanine at t +60 and t +75 min following the constituent amino acids reduced feelings of desire to eat at the same time as the meal was ingested through a postabsorptive mechanism. This hypothesis is supported by statistical correlations between various parameters, although these should be regarded with caution due to the small sample number. Whereas there were no significant associations between gastric half-emptying times (T50s) and change in desire to eat (t +70 - t +60 min), there was a highly significant correlation for the constituent amino acids between the change in plasma phenylalanine at t +75 min (x-b) and the change in desire to eat (r = -0.9774, p = 0.004). This means that those subjects who had higher plasma phenylalanine concentrations (relative to baseline) after ingestion of the liquid test meal also had larger reductions in desire to eat. This relationship was marginally non-significant following the aspartame (r = -0.8681, p = 0.056), suggesting that even though the VAS ratings did not pick up any reduction in
desire to eat following aspartame treatment, the relationship between increased plasma phenylalanine and motivation to eat might still exist.

In conclusion, these results show that aspartame did not increase CCK secretion, nor did it delay gastric emptying. The different responses to phenylalanine in this study compared to the previous study in this chapter, "The effect of phenylalanine supplementation on circulating metabolite and hormone status, gastric emptying and satiety" (section 3.3), may be a reflection of a dose effect. Small amounts of amino acids may be distinct from large amounts (5 g or more) in their physiological consequences. The differing absorption rates of phenylalanine between treatments may therefore have caused the divergent ratings of desire to eat. A sudden rise in plasma phenylalanine, together with the ingestion of fat and carbohydrate, reduced subjective ratings of desire to eat. Free amino acids from the two treatments may have inhibited GLP-1 secretion in the jejunum and ileum, and these lower GLP-1 levels contributed to the accelerated gastric emptying rates. Either lumenal aspartic acid or high concentrations of circulating phenylalanine may have also directly added to the faster gastric emptying of the liquid meal, in addition to the attenuated GLP-1 response. GLP-1 levels had no effect on appetite in this study, which suggests it is not an important satiety peptide in this situation. Gastric emptying rates were also unimportant in appetite regulation under these circumstances.
Chapter Four
A COMPARISON OF ELECTRICAL IMPEDANCE EPIGASTROGRAPHY WITH OTHER TECHNIQUES FOR THE MEASUREMENT OF GASTRIC EMPTYING

4.1 General introduction

Electrical impedance epigastrography (EIE), a non-invasive technique for measuring gastric emptying (Sutton & McClelland, 1983; Pickworth, 1984; Sutton et al., 1985; McClelland & Sutton, 1985), was used in the previous chapter to detect differences in gastric emptying rates following treatment with encapsulated phenylalanine and aspartame. EIE relies on the principle that the resistance (impedance) between electrodes placed on the stomach and lower back is increased by the ingestion of a non-conductive meal. This is recorded as epigastric impedance, which is assumed to decrease in proportion with the meal leaving the stomach. This method has been previously shown to compare well with the dye-dilution method (McClelland & Sutton, 1985) and scintigraphy (Sutton et al., 1985) when using a liquid test meal of orange squash. It was already suspected that gastric secretions might influence electrical impedance (Mangnall, 1989), but EIE was chosen to measure gastric emptying in the phenylalanine study (section 3.3) because a water load was used (which would not generate significant gastric secretion). An alternative method, paracetamol absorption, was also included in addition to EIE for the aspartame study (section 3.5), although this could not be used both before and after the liquid test meal. The main advantages of EIE were portability, reduced costs, ease of operation and its relatively non-invasive nature, unlike the majority of available methods for the measurement of gastric emptying.

It was noted that the gastric half emptying times (T50) calculated for the fat/carbohydrate liquid test meal used in the aspartame study (section 3.5.6.1.1) were shorter than expected for a 1992 kJ load. The average T50 following the 1992 kJ liquid test meal (control treatment) was 25.2 min (SD 5.4), a value that is clearly too low when compared to those values in the literature. For half of the meal to be emptied by 25 min, the amount of energy delivered to the duodenum would have been 40 kJ/min, whereas other studies have found a fairly constant rate of 8-13 kJ/min during the first hour of gastric emptying (Hunt et al., 1985; Carbonnel et al.,
1994). It appeared that EIE was underestimating the time taken for nutrient-containing liquid meals to empty from the stomach, possibly due to interference from gastric secretions. Gastric secretions are highly conductive due to their ionic composition, and will reduce the impedance of the stomach contents, giving an erroneous rate of emptying. However, with regard to within-subject studies, where differences in repeated measures of gastric emptying rate in response to manipulated liquid meals were of interest, the quantitative capabilities of this method were less important than being able to discriminate between longer or shorter gastric emptying times. In other words, as long as the influence of gastric secretions on the change in impedance of the stomach contents was a minor influence on apparent emptying rates of different meals, then it could still be used as a tool in repeated measures nutritional studies.

It was decided to compare the EIE method with other methods of measuring gastric emptying. Firstly, EIE was compared to scintigraphy (Department of Nuclear Medicine, Royal Surrey County Hospital), a “gold standard” for the measurement of gastric emptying but disadvantaged for routine use by the cost of equipment and the use of radioactive isotopes (Urbain & Charkes, 1995). Although scintigraphy is considered to be a “gold standard” method, it is not without its technical and interpretational difficulties. For example, the distal end of the duodenum sometimes loops up behind the stomach, potentially leading to an overestimation of the remaining volume in the stomach when calculating the counts from an image. On the whole though, the use of a dual-headed gamma camera and calculating the geometric mean of the anterior and posterior counts in the stomach eliminates many of the errors associated with scintigraphy (septal penetration and downscatter). Secondly, EIE was compared to two other indirect measurements of gastric emptying, the paracetamol absorption and $^{13}$C-octanoic acid absorption methods (see section 2.2.2 for methodology overview). Both studies compared methods using liquid meals of differing fat composition and plasma concentrations of glucose, insulin and gut hormones were measured to see how they varied with gastric emptying rate.
4.2 Comparison of electrical impedance epigastrography with the “gold standard” method, scintigraphy, using liquid test meals of differing energy content.

4.2.1 Aims and hypothesis

Although EIE seems to generate shortened T50s for liquid meals containing fat and carbohydrate, it was hypothesised that these would still correspond in rank to those obtained by scintigraphy. Three liquid test meals of differing composition were used to test the agreement between the two methods, and levels of blood hormones and metabolites were measured in order to relate these to the gastric emptying results.

4.2.2 Subjects

Six volunteers (2 female, 4 male) were recruited at the Royal Surrey County Hospital, Guildford. Only subjects that were over the age of 45 years were accepted on to the study due to the use of radioactivity for the scintigraphy measurements. Subjects were all in the normal weight range (mean BMI 22.1 kg/m², SD 1.9). All subjects were free from any medical history that may affect gastrointestinal motility. Standard haematology and biochemistry screening was conducted at the RSCH to confirm suitability to participate in the study, and a preliminary gastric emptying test of a water load using the EIE equipment was carried out to ensure accurate placement of the electrodes and to check that a clear trace could be obtained. The study protocol was approved by the local ethics committee. Subjects were asked to consume a light evening meal and refrain from consuming alcohol and caffeine the evening before the study, and on the study day. On the day of the study, after an overnight fast, they were required to consume nothing except water for six hours before the study, and then no water until the study was due to start.

4.2.3 General procedure

The study design was a randomised single-blind cross-over. Subjects arrived at the Clinical Investigation Unit at the Royal Surrey County Hospital at 1515 h, whereupon an intravenous cannula was inserted into a forearm vein by a qualified doctor; they were then escorted to the Nuclear Medicine department. Blood samples were taken for the measurement of plasma glucose, insulin, GLP-1 and GIP according to the methodology detailed in section 2.2.3.1. The EIE electrodes were attached to the stomach and lower back, and a comfortable supine position was
established between the horizontal detectors of the dual-headed gamma camera. The gamma camera detectors were positioned over and under the subject, and a weak radioactive source (Co-57), held over the xiphisternum, was used to position the stomach centrally in the field of view. EIE recordings were carried out for 10 min to enable a stable baseline impedance measurement to be obtained. Blood samples (15 ml) were also taken at t – 10 min and t +0 min (just before the meal). At around 1600 h (0 min) the subject ingested either the glucose meal or one of the two fat/carbohydrate liquid test meals containing the radioactive label (12MBq of $^{99m}$Tc-DTPA) via a wide bore plastic tube, whilst still lying in a supine position. At this point the imaging was initiated on the gamma camera and lasted up to two hours. Continuous EIE measurements were recorded simultaneously. The geometric mean (anterior counts x posterior counts)$^{1/2}$ of both anterior and posterior views were determined to correct for changes in tissue attenuation. Blood samples were obtained at 15 minute intervals following the meal. A schematic diagram of the protocol is below.

Table 4.1 Outline of protocol for comparison of scintigraphy and EIE in the measurement of gastric emptying.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>EIE</th>
<th>Scintigraphy</th>
<th>Blood Samples</th>
<th>Meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>-10</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0</td>
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<td></td>
</tr>
<tr>
<td>+5</td>
<td></td>
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<tr>
<td>+15</td>
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<td>+30</td>
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<tr>
<td>+120</td>
<td></td>
<td></td>
<td>*</td>
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</tr>
</tbody>
</table>

* Measurements only taken at +120 min for the milkshakes

Note:

1. The first three study sessions (one individual’s glucose and low fat test meals and another’s glucose meal) positioned the subject in a semi-supine position rather than almost fully supine. The detectors had been aimed at an angle to the stomach and duodenum rather than parallel, and a parallel view facilitated a more
accurate detection of the counts left in the stomach by the gamma camera. The change in position may have led to an alteration in gastric emptying time, and, if so, a slower emptying rate would be expected supine compared to semi-supine. In addition, the first four study sessions (the same two individuals’ glucose and low fat test meals) took place in the morning whereas the rest of the study sessions took place late afternoon. It is possible that this may have had a minor bearing on gastric motility. Nausea may also have an effect on gastric motility but this was not reported by any of the subjects.

2. Due to technical difficulties whilst working in the Nuclear Medicine department of the Royal Surrey County Hospital, blood samples were not kept in ideal conditions following centrifugation. The internal temperature of the designated centrifuge was warm and there was no freezer available in the vicinity. GIP and GLP-1 contained in the plasma samples were in an advanced stage of degradation by the time the plasma was assayed, since these hormones are not stable unless stored on ice and frozen immediately after separation of the plasma. Consequently, plasma GIP and GLP-1 concentrations are not reported here due to doubts over the accuracy of the data.

3. The constraints of time and money limited the study to 6 subjects. This sample size was clearly not ideal for a validation study such as this, especially when correlating gastric half-emptying times between the methods. For that reason, additional studies are required before the current results can be confirmed as definitive.

4.2.4 Meals

A different liquid test meal was ingested by the subject on each of the three occasions – a glucose meal, a low fat meal and a high fat meal. The glucose meal was 450 ml in volume, with a 10 % concentration of glucose and an energy content of 753 kJ. The fat/carbohydrate liquid test meals were formulated using double cream, maltodextrin and Nesquik and were also made up to 450 ml in volume with water (see table 4.2). The conductivity of the low and high fat test meals were 0.29 and 0.54 milliSiemens/cm respectively. Therefore a very simple meal containing 10 % glucose (45 g) was tested between methods, and also two meals that contained the same amount of carbohydrate (70 g) but differing amounts of fat (4 g and 44 g):
<table>
<thead>
<tr>
<th></th>
<th>Glucose test meal</th>
<th>Low fat test meal</th>
<th>High fat test meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double Cream</td>
<td>45 g</td>
<td>8g (4g fat, 151 kJ)</td>
<td>92g (44g fat, 1657 kJ)</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>50g (50g CHO, 837 kJ)</td>
<td>50g (50g CHO, 837 kJ)</td>
<td></td>
</tr>
<tr>
<td>Nesquik (flavouring)</td>
<td>20g (326 kJ)</td>
<td>20g (326 kJ)</td>
<td></td>
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<tr>
<td>Glucose</td>
<td>753 kJ</td>
<td>1314 kJ</td>
<td>2820 kJ</td>
</tr>
<tr>
<td>(Fat: 0% energy)</td>
<td>(Fat: 12% energy)</td>
<td>(Fat: 59% energy)</td>
<td></td>
</tr>
<tr>
<td>CHO: 100% energy)</td>
<td>CHO: 88% energy)</td>
<td>CHO: 41% energy)</td>
<td></td>
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</table>

Table 4.2. Composition of glucose, low and high fat liquid test meals used for the comparison of scintigraphy and EIE in the measurement of gastric emptying.

4.2.5 Statistical analysis

Results were analysed by one factor repeated measures ANOVA or two way repeated measures ANCOVA (with baseline values as covariates) for statistical differences as detailed in section 2.2.4. Incremental area under the curve was also employed to compare differences in plasma glucose or insulin responses between the low and high fat test meals by a two-tailed, paired t-test. Agreement between the gastric emptying methods was assessed by Spearman rank order correlation.

4.2.6 Results

4.2.6.1 Gastric emptying times

4.2.6.1.1 Electrical Impedance Epigastrography (EIE)

Repeated measures ANOVA showed that there were clear differences in gastric half-emptying times following the three different meals for EIE (n = 6, p = 0.0001), with the Duncan post-hoc test revealing the differences to lie between glucose and high fat meals, and between low fat and high fat meals, but not between glucose and low fat meals. The average gastric emptying half times (T50s) using the EIE method are shown in figure 4.1.
All subjects  
Glucose test meal  
Low fat test meal  
High fat test meal

Figure 4.1 Gastric half emptying times (T50s) obtained by the electrical impedance epigastography method (mean ± SEM, n = 6). Gastric emptying times were significantly longer following the high fat test meal (2820 kJ) compared to the low fat test meal (1314 kJ) and the glucose test meal (753 kJ). This difference between meals was highly statistically significant (p <0.0005).

4.2.6.1.2 Scintigraphy

The geometric means for scintigraphy were also significantly different (n = 6, p = 0.015) and the post-hoc test showed that the differences followed the same pattern as for EIE. Gastric half-emptying times were longer for the high fat milkshake, as expected. The average gastric emptying half times (T50s) using the scintigraphy method are shown in figure 4.2.

Figure 4.2 Gastric half emptying times (T50s) obtained by the scintigraphy method (mean ± SEM, n = 6). Gastric emptying times were significantly longer following the high fat test meal (2820 kJ) compared to the low fat test meal (1314 kJ) and the glucose test meal (753 kJ). This difference between meals was statistically significant (p <0.05).
4.2.6.1.3 Comparison of the two methods

Paired two tailed t-test between the EIE and scintigraphic T50s showed significant differences for the glucose meal (p <0.0005), low fat meal (p <0.000005) and high fat meal (p <0.05). The percentage volume of meal emptied from the stomach was also analysed by two-way repeated measures ANOVA for each meal over the first 60 min in order to ascertain whether the actual percentages were significantly different between methods. There was a significant main difference in gastric emptying methods for the glucose meal (p <0.05), the low fat meal (p <0.000005), and the high fat meal (p <0.0005), with the overall percentage emptied values being higher for EIE compared to scintigraphy.

Using Spearman’s rank correlation coefficients it was shown that there were no significant correlations within any of the meal types, although there was a trend towards significance for the correlation between EIE and scintigraphy half emptying times following the low fat test meal (Spearman \( r = 0.771 \), p = 0.072). Figure 4.3 illustrates the relationship between the sets of EIE and scintigraphy data.

![Figure 4.3 Scatterplot showing the association between gastric half emptying times (T50) from the two methods, EIE and scintigraphy, following each of the liquid test meals (glucose 753 kJ, low fat 1314 kJ, and high fat 2820 kJ). No significant associations between methods were shown by Spearman rank correlation.](image-url)
Table 4.3. Mean gastric half-emptying times and descriptive statistics following each of the liquid test meals (glucose 753 kJ, low fat 1314 kJ, and high fat 2820 kJ).

4.2.6.2 Plasma glucose and insulin

Plasma glucose and insulin concentrations were measured simultaneously with gastric emptying for each meal. The low fat and high fat meals contained the same amount of carbohydrate but differing amounts of fat and so these were compared to see how these blood parameters changed with the addition of fat. Plasma levels of these substances were correlated with gastric emptying T50s as measured by EIE and scintigraphy following all three liquid test meals.

4.2.6.2.1 Plasma glucose

The results for plasma glucose concentrations following the three meals are illustrated below (figure 4.4). Two way repeated measures ANCOVA between the two meals with a constant carbohydrate content (low and high fat test meals) showed that there were no meal-related significant differences over time between the meals, nor was there a meal x time interaction.

Paired t-tests (2-tailed) for the incremental area under the curve (IAUC) revealed no significant differences between the low and high fat test meals, either overall, t + 0-45 min or t + 45–90 min. There were no significant correlations
between IAUC or tmax (time until peak concentration) for plasma glucose and gastric half emptying times.

![Graph showing plasma glucose concentrations following liquid test meals]

Figure 4.4 Plasma glucose concentrations (mean ± SEM, n = 6) following each of the liquid test meals (glucose 753 kJ, low fat 1314 kJ, and high fat 2820 kJ). No significant differences were found between the low and high fat liquid test meals.

4.2.6.2.2 Plasma insulin

Plasma insulin concentrations are shown in figure 4.5. The two test meals (low and high fat) led to a significant main effect of treatment (p < 0.05); the highest overall insulin levels followed the low fat milkshake. There was also a treatment x time interaction (p < 0.005), with the post-hoc test revealing that the differences lay mainly between t + 30 and 90 min.
Figure 4.5 Plasma insulin concentrations (mean ± SEM, n = 6) following each of the liquid test meals (glucose 753 kJ, low fat 1314 kJ, and high fat 2820 kJ). Plasma insulin levels were significantly higher following the low fat test meal compared to the high fat test meal (p <0.05).

Paired t-tests (2-tailed) for the incremental area under the curve (IAUC) revealed a significant difference between the low and high fat test meals, t + 0-45 min (p = 0.013). There were no significant correlations between IAUC or tmax (time until peak concentration) for plasma insulin and gastric half emptying times.

4.2.7 Discussion

The aim of this study was to compare EIE as a tool for measuring gastric emptying with the “gold-standard” scintigraphy. Both methods achieved statistical significance in the difference in gastric half-emptying times for the low and high fat test meals. However, the actual half-emptying times reported were very different (see table 4.3). It can be seen that the data ranges do not overlap between the two methods, and the ranges and standard deviations are much larger for the scintigraphic data – characteristics that become increasingly marked with increasing fat content of the meals. The longest gastric half-emptying time recorded by scintigraphy (high fat test meal) was nine times longer than the longest recorded by EIE.

Scintigraphy is the accepted “gold standard” method for measuring gastric emptying (Urbain & Charkes, 1995), although it also has its interpretational
problems. It is therefore assumed that these scintigraphic T50s are accurate. This assumption is supported by a closer look at the emptying rates in terms of energy content. Studies employing both dye-dilution and scintigraphic methods have found gastric emptying rates of a liquid meal to be fairly constant at 8-13 kJ/min during the first hour of gastric emptying (Hunt et al., 1985; Carbonnel et al., 1994), with an average rate of 11.3 kJ/min over two hours for a 400 ml, 4.2 kJ/ml polycose liquid test meal (Hunt et al., 1985). Calculation of energy emptied per min from the scintigraphic T50s yields values of 7.4, 10.7, and 8.8 kJ/min for the glucose, low fat and high fat test meals respectively. However, the corresponding values using the EIE T50s generates emptying rates of 21.8, 32.5 and 48.1 kJ/min for glucose, low fat and high fat test meals respectively; these emptying rates are clearly nonsensical when compared to the scintigraphic emptying rates and the results obtained in previous studies (Hunt et al., 1985; Carbonnel et al., 1994).

Although the actual half-emptying times obtained in the present work are extremely different, similarities in subject ranking order between the methods would uphold EIE as a useful method where discerning differences in gastric emptying rates are a priority. Using a non-parametric correlation test, it is clear that there is no relationship between the gastric half-emptying times for the two methods within meal-type. It could be argued that this may be because the sample size is small, and the low fat milkshake did show a near significant correlation. There may have been a weak relationship between the two sets of data if the sample size had been larger, especially as a positive result had already been reported in one of the original papers for an orange squash drink (Sutton et al., 1985). It should be noted, however, that two out of six subjects in this paper had EIE gastric half-emptying times that were only two-thirds of that recorded by scintigraphy, and another subject had a scintigraphic emptying time that went beyond the duration of the gamma camera recording (Sutton et al., 1985). Based on the present results, the inescapable conclusion is that EIE is not an accurate method for measuring actual gastric emptying time compared to the "gold standard" scintigraphy. The large degree of variance that can be observed in the data produced by the so-called "gold standard" shows that this method is hardly ideal, but the smaller variance obtained by EIE may be an artifact of the apparent limitation of all gastric emptying times to below 35 min.
It may be that EIE and scintigraphy are simply not measuring the same physiological process. EIE is measuring the change in electrical impedance and this is assumed to be a direct reflection of the stomach contents emptying into the duodenum. It is suspected that the impedance recordings are also picking up changes in conductivity due to gastric secretions, especially with the introduction of fat into the meals. The high fat test meal may be reducing conductivity by buffering the gastric secretions and inhibiting further gastric secretions via enterogastrone feedback, e.g. CCK, GLP-1 (Lloyd et al., 1997), thereby increasing impedance and appearing to prolong gastric emptying time compared to the low fat test meal. This effect is presumed to be additive to the real gastric emptying differences occurring in terms of reduction in impedance, causing an apparently smaller T50. It is not known what proportion of the EIE T50s was a reflection of gastric emptying changes and what proportion was due to differences in gastric secretions. Another possible source of impedance error is the changing resistance on the surface of the skin where the electrode is attached due to sweating, although you would expect this type of error to be fairly consistent for each meal, unless there was a large change in room temperature from one occasion to another.

The discrepancies in gastric emptying time between methods seem to be magnified by the increasing complexity of the meal, and so it would be useful to carry out the same comparison on six subjects simply with a water load. This has not yet been done due to financial restraints and difficulties in organising sessions on the gamma camera at the Royal Surrey County Hospital. In order to investigate the possibility of gastric and pancreatic secretions altering the impedance recordings via changes in conductivity, it has been suggested that a meal with the same conductivity as the basal stomach tissue and contents is administered, as then it could be assumed that any changes in impedance would be due to secretions in the gut. Preliminary investigations into a possible correction factor for the effects of gastric secretions on gastric emptying times have been produced by colleagues in the Department of Physics (Amaee et al., 1999).

As expected, the addition of fat to a carbohydrate-constant meal (low fat and high fat test meals) significantly decreased plasma concentrations of insulin over the course of two hours, although plasma glucose levels were not significantly affected. Plasma glucose levels did at least appear to follow the same profile as plasma insulin
as illustrated by the charts, and there was a significant treatment by time interaction from t +45 to t +90 min (p <0.005) due to the late plasma glucose peak following the high fat test meal. The reduced plasma insulin and later plasma glucose peak following the high fat test meal are likely to be a result of a fat-induced slowing down of gastric emptying rate.

In order to investigate the importance of gastric emptying in plasma levels of glucose and insulin the relationship between these parameters was analysed using non-parametric correlation techniques. Gastric emptying T50s from both methods were matched by rank order against time until peak concentration (tmax) and incremental area under the curve (IAUC) for the plasma constituents. In other words, do the subjects with longer gastric emptying times have higher or lower levels of insulin and glucose? Predictably, EIE T50s did not correlate with plasma glucose or insulin, as you might expect since this method was a poor evaluator of gastric emptying times. However, scintigraphic T50s showed no significant correlations either.

In conclusion, the two methods for measuring gastric emptying, EIE and scintigraphy, yield differing results for gastric emptying times and do not seem to relate to each other. Scintigraphy is costly, impractical and is a poor “gold standard”, but it appears from these results that EIE may not be a valid alternative.
4.3 Comparison of electrical impedance epigastrography with paracetamol absorption and the $^{13}$C-octanoic acid breath test, using low and high fat liquid test meals.

4.3.1 Aims and hypothesis

EIE gastric emptying times have been shown to be artificially short and they failed to correspond in subject rank to those of scintigraphy. Although scintigraphy is regarded as the "gold standard" for gastric emptying, it is highly subject to error. It was hypothesised that EIE gastric emptying times may therefore relate to other indirect methods of measurement. The aim of this study was to compare EIE with two different methods for gastric emptying measurement, paracetamol absorption and the $^{13}$C-octanoic acid breath test. The same two liquid test meals of differing fat content were used to test the agreement between the two methods, and levels of blood hormones and metabolites were measured in order to relate these to the gastric emptying results.

4.3.2 Subjects

Ten volunteers (9 females, 1 male), mean age 25 years (SD 2.2) were recruited for the study from students at the University of Surrey, Guildford. Subjects were in the normal weight range (mean BMI 20.6 kg/m$^2$, SD 2.5). All subjects were free from any significant medical history and standard haematology and biochemistry screening was conducted at the RSCH to confirm suitability to participate in the study. A letter was sent to their GP to inform them about the study and to give their GP an opportunity to confirm their patient's suitability. Subjects were asked to refrain from consuming alcohol and caffeine the evening before the study, and on the study day. They were also requested to avoid taking any products containing paracetamol for forty-eight hours before the study. On the day of the study, after an overnight fast, they were required to consume nothing except water until one hour before the study, and then no water until the study was due to start. The study protocol was approved by the local ethics committee.

4.3.3 General procedure

Subjects were studied on two occasions (at least one week apart), one day for the low fat meal and one day for the high fat meal. The order of the meals was randomised. Subjects arrived singly at the Clinical Investigation Unit at the
University of Surrey at 0900 h, whereupon a qualified medical professional inserted an intravenous cannula into an antecubital vein under local anaesthetic. Blood samples were taken for the measurement of plasma glucose, insulin, GLP-1, GIP and CCK according to the methodology detailed in section 2.2.3.1. The six EIE electrodes were attached to the stomach and lower back, and the subjects found a comfortable semi-supine position on the bed. EIE recordings were carried out for 10-15 min to enable a stable baseline impedance measurement to be obtained. Baseline blood samples (15 ml) and breath samples were also taken at t−10 and t +0 min (just before the meal).

Volume of CO₂ production was determined by the use of a metabolic monitor (Deltatrac) and ventilated hood with a flow rate of 40 ml/min. The Deltatrac was switched on 1 h before the study to warm up and calibrated for barometric pressure and gas using a standard gas mixture of 5 % CO₂ and 95 % O₂. Breath samples were collected at intervals into gas-tight glass tubes with screw lids for the determination of ¹³C enrichment of the breath with an isotope ratio mass spectrometer at the Department of Biological and Nutritional Sciences, University of Newcastle (Dr Chris Seal). ¹³CO₂ in the breath samples was expressed as atom percent excess (APE), i.e. the percentage of ¹³CO₂ in the total CO₂ above natural background of ¹³CO₂. Five measurement sessions of CO₂ production rate were carried out (one measurement per min) and the data (ml/min) was averaged over each session to give continuous values for the whole 4 h. APE and CO₂ production in ml/min was entered into this formula to give μmoles of ¹³CO₂ in each time interval:

\[
\text{CO}_2 \text{ (ml/min) x APE x 44.6}^\text{y} \times \text{time interval of breath samples (min)}
\]

¹³CO₂ (µmol) was then summed over 4 h to give a cumulative curve. Each cumulative time point was then expressed as a percentage of the initial dose to give a cumulative recovery curve. This was then fitted using the equations given in section 2.2.2.3.

Volunteers received a high or low fat liquid test meal at t +0 min, which contained 50 mg of ¹³C-labelled Na-octanoate and 1.5 g of paracetamol (3 x 500 mg

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*y Conversion factor from CO₂ (ml) to CO₂ (µmol). 1mol gas occupies 22.414 L at STP therefore 1 µmol gas occupies 0.022414 ml. 1/0.022414 = 44.6.
Blood samples, EIE recordings and breath test samples were taken at intervals thereafter. A schematic diagram of the protocol is below.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Deltatrac Breath sample (duplicates)</th>
<th>Blood sample (15 ml)</th>
<th>EIE</th>
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<tbody>
<tr>
<td>-20</td>
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<td></td>
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<tr>
<td>-10</td>
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<td>+240</td>
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Table 4.4. Outline of protocol for comparison of EIE, paracetamol absorption and $^{13}$C-octanoic acid breath test in the measurement of gastric emptying.

See section 2.2.3.1 for blood sampling protocol.
4.3.4 Meals

Two different liquid test meals were ingested by the subject on each of the two occasions – a low fat meal and a high fat meal identical to those in the previous study. These were formulated using double cream, maltodextrin and Nesquik and were made up to 450 ml with water (see table 4.5) containing 1.5 g of paracetamol. Carbohydrate content was constant whereas fat content differed. The conductivity of the low and high fat test meals were 0.29 and 0.54 milliSiemens/cm respectively.

<table>
<thead>
<tr>
<th></th>
<th>Low fat test meal</th>
<th>High fat test meal</th>
</tr>
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<tbody>
<tr>
<td>Double Cream</td>
<td>8g (4g fat, 151 kJ)</td>
<td>92g (44g fat, 1657 kJ)</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>50g (50g CHO, 837 kJ)</td>
<td>50g (50g CHO, 837 kJ)</td>
</tr>
<tr>
<td>Nesquik (flavouring)</td>
<td>20g (326 kJ)</td>
<td>20g (326 kJ)</td>
</tr>
<tr>
<td>Total energy</td>
<td>1314 kJ (Fat: 12 % energy</td>
<td>2820 kJ (Fat: 59 % energy</td>
</tr>
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<td></td>
<td>CHO: 88 % energy)</td>
<td>CHO: 41 % energy)</td>
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</table>

Table 4.5 Composition of low and high fat liquid test meals used for the comparison of EIE, paracetamol absorption and $^{13}$C-octanoic acid breath test in the measurement of gastric emptying.

50 mg of $^{13}$C-labelled Na-octanoate was dissolved in the double cream in a measuring jug before the maltodextrin, Nesquik, and water (containing paracetamol – see below) was added, and the whole mixture was blended.

Note:

1. The first 4 subjects were administered soluble paracetamol in the milkshake. Soluble paracetamol was used because this was the method of choice used by previous authors (Maddern et al., 1985) and it was necessary to ensure that the paracetamol was evenly dispersed throughout the test meal. It was observed however, that the half emptying times recorded by the EIE equipment were unusually long (approximately 40 to 50 min for the high fat test meal instead of the more usual 30 min), and they did not differentiate between the low and high fat milkshakes. It was confirmed that this is not due to individual variation as two subjects were tested again with the same milkshake, omitting the paracetamol, and their half-emptying times were shorter and in the range that would be expected. It is unlikely that paracetamol was directly delaying gastric emptying as this was a well-established method used by many researchers in the past.
(Heading et al., 1973; Maddern et al., 1985; Petring & Flachs, 1990; van Wyk et al., 1993; van Wyk et al., 1995). Additionally, one subject received soluble and non-soluble paracetamol on two separate occasions with the high fat liquid test meal, which gave very similar plasma paracetamol results for both conditions. This suggests that the different forms of paracetamol did not directly affect gastric emptying rate or the absorption kinetics of paracetamol in this individual.

The most likely explanation was that the soluble paracetamol altered the conductive properties of the liquid test meal. Soluble paracetamol increased the conductivity of the low fat meal from 0.29 to 4.16 milliSiemens/cm and the high fat meal from 0.54 to 3.69 milliSiemens/cm. It was discovered from the manufacturers of this brand of soluble paracetamol (Sterwin Medicines, Guildford) that 47% of the tablet weight was sodium bicarbonate (for effervescence), and therefore a total of 4.026 g sodium bicarbonate was being added to the test meal (in 3 tablets of soluble paracetamol). Increased conductivity of the stomach contents would indeed be expected to decrease impedance from the outset and perhaps appear to artificially lengthen gastric emptying time. It was decided, therefore, to use tablets of paracetamol (without effervescence) for the remaining six subjects. These were crushed in a pestle and mortar, added to water and then blended in with the test meal ingredients using an electric hand whisk.

In summary, three subjects received low and high fat test meals with soluble paracetamol, one subject received two high fat test meals with soluble or crushed tablet-form paracetamol, and six subjects (whose results are reported here) received low and high fat test meals with crushed non-effervescent paracetamol tablets. Two of the subjects that had received soluble paracetamol also underwent one more session (EIE alone) with a paracetamol-free high fat test meal, to see what their gastric half-emptying times should have been. There were no reports of nausea during any study session.

2. Unfortunately one subject's impedance trace (n = 6 non-effervescent paracetamol tablets group) for the high fat test meal had to be excluded because the trace contained numerous movement artifacts and the data was uninterpretable.

3. Plasma CCK concentrations were analysed from t +0 to t +120 min rather than from t -10 to t +240 min due to a shortage of antiserum.
4. Although 10 subjects were used in the study, the problems encountered using EIE with soluble paracetamol resulted in a smaller sample size of 6 subjects. As for the last study, this sample size was not ideal when calculating correlations. Again, additional studies are required before the current results can be confirmed as definitive.

4.3.5 Statistical analysis

Results were analysed by two-tailed paired t-test or two way repeated measures ANCOVA for statistical differences as detailed in section 2.2.4. Time until peak concentration (tmax) and incremental area under the curve (IAUC) were also employed to compare differences in plasma hormone or metabolite response between low and high fat test meals. Plasma paracetamol response was expressed as tmax, peak concentration (cmax) and (IAUC). EIE and 13C-octanoic acid gastric emptying parameters were expressed as gastric half emptying times (T50). Agreement between gastric emptying methods was assessed by Spearman rank order correlation.

4.3.6 Results

4.3.6.1 Gastric emptying times

4.3.6.1.1 Electrical impedance epigastrography (EIE)

The average gastric emptying half times (T50s) using the EIE method are illustrated in figure 4.6. A two-tailed, paired t-test showed that the longer T50s following the high fat liquid meal were significantly different to the low fat test meal T50s (n = 6, p <0.05).
Figure 4.6 Gastric half emptying times (T50s) obtained by the electrical impedance epigastrography method (mean ± SEM). Gastric emptying times (in the non effervescent paracetamol group) were significantly longer following the high fat test meal (2820 kJ) compared to the low fat test meal (1314 kJ) (p <0.05). The chart also illustrates the misleading EIE T50s obtained following the addition of soluble paracetamol rather than non-effervescent tablets to the liquid test meal (n = 3), and the shorter, more typical EIE T50s for two of these subjects when a paracetamol-free high fat liquid test meal was ingested.

4.3.6.1.2 $^{13}$C-octanoic acid breath test

The breath test T50s were significantly different between test meals for the whole sample group (n = 9, p <0.005) and for the smaller sub-group of subjects that could be measured by EIE (n = 6, p <0.05). Gastric emptying times were longer following the high fat test meal, as illustrated in figure 4.7.
Figure 4.7 Gastric half emptying times (T50s) obtained by the $^{13}$C-octanoic acid breath test method (mean ± SEM). Gastric emptying times were significantly longer following the high fat test meal (2820 kJ) compared to the low fat test meal (1314 kJ) (in all subjects, p <0.005, and in the non-effervescent paracetamol subgroup, p <0.05).

Figure 4.8 shows the mean cumulative $^{13}$C percentage dose recovery following the low and high fat test meals.
Figure 4.8 Cumulative $^{13}$C(CO2) recovery as a percentage of dose administered following low (1314 kJ) and high fat (2820 kJ) liquid test meals (mean ± SEM, n = 9).

4.3.6.1.3 Paracetamol absorption test

Paracetamol concentrations in the plasma following the two liquid test meals are shown in figure 4.9. No significant differences were found between meals for mean plasma paracetamol levels over 4 hours ($p = 0.099$), but there was a highly significant meal by time interaction ($p = 0.000005$).
Figure 4.9 Plasma paracetamol concentrations (mmol/L) following low fat (1314 kJ) and high fat (2820 kJ) liquid test meals (mean ± SEM, n = 6). There was a highly significant meal by time interaction (p < 0.00001).

Time to reach peak plasma paracetamol concentrations (tmax) and incremental area under the curve (IAUC), 0-120 min, were significantly different between test meals (see figure 4.10). Peak concentrations (cmax) were not significantly different between test meals (see table 4.6).

<table>
<thead>
<tr>
<th>Low fat test meal</th>
<th>High fat test meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.106 (0.021)</td>
<td>0.127 (0.013)</td>
</tr>
<tr>
<td>0.497 (0.039)</td>
<td>0.333 (0.017)</td>
</tr>
</tbody>
</table>

Table 4.6 Plasma paracetamol gastric emptying parameter, mean (SEM), following low fat (1314 kJ) and high fat (2820 kJ) liquid test meals (mean ± SEM). Results are given for all subjects (n = 9) and subjects who took non-effervescent paracetamol tablets only (n = 6). There were no significant differences in cmax, following the test meals.
Figure 4.10 Plasma paracetamol gastric emptying parameters, $t_{\text{max}}$ (min) and incremental AUC, 0-120 min (mmol/L by time (min)), following low fat (1314 kJ) and high fat (2820 kJ) liquid test meals (mean ± SEM). Results are given for all subjects ($n = 9$) and subjects who took non-effervescent paracetamol tablets only ($n = 6$). * $p <0.05$, ** $p <0.01$. $T_{\text{max}}$ was significantly longer for the whole group and the sub-group following the high fat test meal. Incremental AUC, 0-120 min was significantly larger in the sub-group that took non-effervescent paracetamol tablets only.

Table 4.6 Plasma paracetamol gastric emptying parameter, $c_{\text{max}}$ (mmol/L), following low fat (1314 kJ) and high fat (2820 kJ) liquid test meals (mean ± SEM). Results are given for all subjects ($n = 9$) and subjects who took non-effervescent paracetamol tablets only ($n = 6$). There were no significant differences in $c_{\text{max}}$ following the test meals.

<table>
<thead>
<tr>
<th>$c_{\text{max}}$ (mmol/L)</th>
<th>Mean (SEM)</th>
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<tbody>
<tr>
<td></td>
<td>Non-effervescent tablets only ($n = 6$)</td>
</tr>
<tr>
<td>Low fat test meal</td>
<td>0.108 (0.010)</td>
</tr>
<tr>
<td>High fat test meal</td>
<td>0.127 (0.013)</td>
</tr>
</tbody>
</table>

4.3.6.1.4 Comparison of the three methods

4.3.6.1.4.1 EIE and paracetamol absorption test

EIE and the paracetamol absorption test produce different gastric emptying parameters and so they are not directly comparable by a paired t-test. Gastric emptying parameters from the paracetamol absorption method ($t_{\text{max}}$, IAUC 0-60 min and IAUC 0-120 min) and EIE T50s were plotted against each other and the
Spearman’s rank correlation was computed. There were no significant correlations between methods. See figure 4.11.

Figure 4.11 Plasma paracetamol gastric emptying parameters, tmax (min), IAUC 0-120 min (mmol/L by time) and IAUC 0-60 min (mmol/L by time), plotted against EIE T50s (min) following low fat (1314 kJ) and high fat (2820 kJ) liquid test meals. Spearman rank order correlation between methods for each meal produced no significant associations.

4.3.6.1.4.2 EIE and $^{13}$C-octanoic acid breath test

Two-tailed, paired t-tests between EIE and breath test T50s revealed that gastric emptying times were significantly different for these methods (low fat p <0.001, high fat p <0.0005), with the breath test T50s being very much longer than the EIE T50s. EIE and breath test T50s were plotted against each other to assess the strength of their association (see figure 4.12) and the Spearman rank correlation was computed. No significant correlations were found between the two methods (r = 0.486, p = 0.329 low fat milkshake; r = 0.700, p = 0.188 high fat milkshake).
Figure 4.12 $^{13}$C octanoic acid breath test T50s (min) plotted against EIE T50s (min) following low fat (1314 kJ) and high fat (2820 kJ) liquid test meals. Spearman rank order correlation between methods for each meal produced no significant associations.

4.3.6.1.4.3 Paracetamol absorption and $^{13}$C-octanoic acid breath tests

Gastric emptying parameters from the paracetamol absorption method ($t_{max}$, IAUC 0-60 min and IAUC 0-120 min) and $^{13}$C-octanoic acid breath test T50s were plotted against each other, and the Spearman's rank correlation was computed. There were no significant correlations between methods. See figure 4.13.
Figure 4.13 Plasma paracetamol parameters of gastric emptying, tmax (min), IAUC 0-120 min (mmol/L by time) and IAUC 0-60 min (mmol/L by time), plotted against $^{13}$C-octanoic acid breath test T50s (min) following low fat (1314 kJ) and high fat (2820 kJ) liquid test meals. Spearman rank order correlation between methods for each meal produced no significant associations.

4.3.6.1.4.4 Discriminatory power of the gastric emptying parameters between the low and high fat liquid test meals

The difference between the low fat and high fat results for each gastric emptying parameter (GET) was calculated for each subject, and this was converted to a coefficient (%) of difference in order to standardise the large variation between the different methods:

\[
\text{Difference coefficient} \, (\%) = \left( \frac{\text{low fat GET} - \text{high fat GET}}{\text{average} \, (\text{low fat GET, high fat GET})} \right) \times 100
\]

The largest difference coefficients within the subgroup of 6 subjects were produced by the paracetamol tmax method (mean -108.1 %, SEM 22.3), and the smallest by the breath test T50s (mean -26.6 %, SEM 9.4). Figure 4.14 illustrates the discriminatory power of each method for the whole group (n = 9) and the non-effervescent paracetamol tablet sub-group (n = 6). Paracetamol IAUC, 0-120 min,
also showed a greater level of discrimination between the meals compared to the breath test and EIE T50s.

**Figure 4.14** The magnitude of the differences in gastric emptying times between the low and high fat test meals by each of the three different methods (± SEM), EIE T50, \(^{13}\)C-octanoic acid breath test T50 and paracetamol t\(_{\text{max}}\) (min). The difference coefficient is \((\text{low fat GET} - \text{high fat GET})/\text{average (low fat GET, high fat GET)}) \times 100\%\). Coefficients are given for the whole group (n = 9) and for the subjects that consumed the non-effervescent paracetamol tablets only (n = 6).

4.3.6.2 Plasma metabolites and hormones

4.3.6.2.1 Plasma glucose

The results for plasma glucose concentrations following the low and high fat meals are illustrated below (figure 4.15). Two way repeated measures ANCOVA between the low and high fat test meals showed that there were no meal-related significant differences over time between the meals, but there was there a significant meal x time interaction (p <0.00005).

Plasma glucose IAUC (0-120 min), cmax and t\(_{\text{max}}\) were correlated by Spearman rank order correlation with gastric emptying parameters (n = 6 due to missing data). There was a significant positive correlation between glucose IAUC (0-120 min) and paracetamol IAUC (0-120 min) (Spearman \(r = 0.943, p <0.005\))
following the high fat test meal, but there were no significant relationships between plasma glucose and gastric emptying parameters following the low fat test meal.

Figure 4.15 Plasma glucose concentrations (mean± SEM, n = 9) following differing liquid test meals (low fat 1314 kJ and high fat 2820 kJ). There was a highly significant treatment x time interaction between test meals (p <0.00005).

4.3.6.2.2 Plasma insulin

The results for plasma insulin concentrations following the low and high fat meals are illustrated below (figure 4.16). Two way repeated measures ANCOVA between the low and high fat test meals showed that there was a significant main effect of meal on overall plasma insulin levels (p <0.05).

Plasma insulin IAUC (0-120 min), cmax and tmax were correlated by Spearman rank order correlation with gastric emptying parameters (n = 6 due to missing data). There were no significant relationships between plasma insulin and gastric emptying parameters following either meal.
Figure 4.16 Plasma insulin concentrations (mean ± SEM, n = 9) following differing liquid test meals (low fat 1314 kJ and high fat 2820 kJ). There was a significant treatment effect between test meals (p <0.05).

4.3.6.2.3 Plasma GIP

The results for plasma GIP concentrations following the low and high fat meals are illustrated below (figure 4.17). Two way repeated measures ANCOVA between the low and high fat test meals showed that there was a significant main effect of meal on overall plasma GIP levels (p <0.05).

Plasma GIP IAUC (0-120 min), cmax and tmax were correlated by Spearman rank order correlation with gastric emptying parameters (n = 6 due to missing data). There was a significant positive correlation between GIP cmax and EIE T50s (Spearman r = 0.900, p <0.05), and between GIP tmax and breath test T50s (Spearman r = 0.899, p <0.05) following the high fat test meal. There were no significant correlations following the low fat test meal.
Figure 4.17 Plasma GIP concentrations (mean± SEM, n = 9) following differing liquid test meals (low fat 1314 kJ and high fat 2820 kJ). There was a significant treatment effect between test meals (p <0.05).

4.3.6.2.4 Plasma GLP-1

The results for plasma GLP-1 concentrations following the low and high fat meals are illustrated below (figure 4.18). Two way repeated measures ANCOVA between the low and high fat test meals showed that there was no significant main effect of meal on overall plasma GLP-1 levels. However, from t +60 to t +240 min there was a significant meal by time interaction (p <0.05).

Plasma GLP-1 IAUC (0-120 min), cmax and tmax were correlated by Spearman rank order correlation with gastric emptying parameters (n = 6 due to missing data). There were significant correlations between GLP-1 cmax and paracetamol IAUC (0-120 min) (Spearman r = 0.886, p <0.05) following the low fat test meal. There were also significant correlations following the high fat test meal between GLP-1 tmax and EIE T50s (Spearman r = 0.975, p <0.005) and breath test T50s (Spearman r = 0.897, p <0.05).
Figure 4.18 Plasma GLP-1 concentrations (mean ± SEM, n = 9) following differing liquid test meals (low fat 1314 kJ and high fat 2820 kJ). There was a significant treatment by time interaction from t +60 to t +240 min (p <0.05).

4.3.6.2.5 Plasma CCK

The results for plasma CCK concentrations (t +0 to t +120 min only) following the low and high fat meals are illustrated below (figure 4.19). Two way repeated measures ANCOVA between the low and high fat test meals showed that there was a significant main effect of meal on overall plasma CCK levels (p <0.05).

Plasma CCK IAUC (0-120 min), cmax and tmax were correlated by Spearman rank order correlation with gastric emptying parameters (n = 6 due to missing data). The only gastric emptying parameter to significantly correlate with CCK was the breath test T50, which had a negative relationship with CCK cmax following the low fat test meal (Spearman r = -0.826, p <0.05).
Figure 4.19 Plasma CCK concentrations (mean± SEM, n = 9) following differing liquid test meals (low fat 1314 kJ and high fat 2820 kJ). There was a significant main effect of meal on overall plasma CCK levels (p <0.05).

4.3.7 Discussion

The aim of this study was to compare three methods of measuring gastric emptying, two of which relied on the relationship between absorption of a substrate and gastric emptying rate, and the other employing the change in electrical impedance that occurs when a non-conductive meal enters and leaves the stomach.

Using the same low and high fat liquid test meals as in the previous study (section 4.2), it was shown that EIE T50s, breath test T50s and paracetamol parameters tmax and IAUC 0-120 min were all significantly different between the two meals. Therefore, in a research setting, where the object of the study was to discern differences in the gastric emptying rate of a liquid meal, all the methods tested would apparently be adequate. However, the three methods differed in their discriminatory power in assessing the difference between low and high fat meals. A difference coefficient was used to assess the discriminatory power of the methods. This showed that the paracetamol absorption method was the best discriminator and that the breath test T50s showed the least difference. EIE T50s were closer to breath T50s than paracetamol tmax in their discriminatory power between meals. When the
paracetamol tmax means are compared to the scintigraphy T50 means obtained in the previous study for identical test meals (see section 4.2.6.1.2), it is striking how similar they seem (low fat: scintigraphy T50 61 min, SEM 4, paracetamol tmax (n = 6) 63 min, SEM 25; high fat: scintigraphy T50 160 min, SEM 38, paracetamol tmax (n = 6) 175 min, SEM 17). Scintigraphic T50s from the previous study (section 4.2.6.1.2) also showed a similar magnitude of discrimination between the test meals (scintigraphy -75.8 %, SEM 17.9; paracetamol tmax (n = 6) -76.9 %, SEM 23). Although this is a comparison across two different studies and therefore different subjects, the test meal composition was identical. Thus the similarities between the values obtained by scintigraphy and paracetamol tmax suggest that the latter method could be investigated as a cheaper, safer method of estimating the T50s that would be obtained if the "gold standard" was used. Heading et al. (1973) found a significant relationship between scintigraphic T50s and peak plasma paracetamol concentrations (r = -0.77, p <0.005), as did Maddern et al. (1985) for plasma paracetamol tmax (r = 0.62, p <0.05), both following liquid meals. Therefore, there is some reason to believe that paracetamol absorption (tmax) was quantitatively the better method in this study, despite the fact that the values of the other methods, EIE and 13C-octanoic acid breath test, were expressed as gastric half emptying times, whereas tmax is merely the time until peak concentration.

Again, EIE T50s were artificially short, with mean values of 21 min (low fat) and 28 min (high fat) leading to expected average emptying rates of 32 (low fat) and 50 kJ/min (high fat) over the whole emptying period; emptying rates that are not physiologically plausible, repeating the findings of previous studies using this method (see sections 3.5.7 and 4.2.7). Breath test T50s (208 min, SEM 26, and 272 min, SEM 33, for low and high fat meals respectively, n = 6) produce expected emptying rates of 3.2 and 5.2 kJ/min respectively. These emptying rates appear rather slow and so whilst EIE is underestimating gastric emptying times, the 13C-octanoic acid breath test may be over-estimating emptying times. As already discussed, the reasons for the underestimated EIE T50s may involve the increasing conductivity of the stomach contents during the emptying phase, possibly the result of gastric secretions.

The explanations for overestimated breath test T50s can be ascertained from published debates in 13C-octanoic acid breath test papers. The original experiment by Ghoos et al. (Ghoos et al., 1993) involved a 1046 kJ meal containing scrambled egg.
2 slices of white bread, 5 g margarine and 150 ml water. The egg yolk had already been spiked with the labelled octanoic acid sodium salt and baked separately. The mean T50 was 72 min, a great deal shorter than the gastric half emptying times obtained in the present work, even if you take the low energy content into account, with an average estimated emptying rate of 7 kJ/min. These authors had shown that the gastric emptying rate is the main rate-limiting step for appearance of $^{13}$C enriched CO$_2$ in the breath. However, the breath test T50s reported in the present work include the amount of time taken for the $^{13}$C-sodium octanoate to be absorbed, metabolised and excreted from the body’s CO$_2$ pool. Ghoos et al. (1993) argued that this delay is constant between and within individuals. When the closely related breath test T50s and scintigraphy T50s were regressed against each other, the regression line intersected with the y axis at 66 min rather than the origin, and this value of 66 min was subtracted from breath test T50 results (obtained from the mathematical formula used here) as an estimate of scintigraphic T50s. This value of 66 min was said to correspond to the T50 for absorption and oxidation of octanoic acid after intraduodenal intubation (62 min).

The values reported in the present study can actually be considered as being half processing times (Delbende et al., 2000). The subtraction of 66 min cannot be applied to the data reported in this thesis as a different type of test meal was used and half the amount of $^{13}$C-octanoic acid was added to the meal. To apply the same correction factor to these results would necessitate a validation study against scintigraphy for the low and high fat liquid test meals. However, just out of interest, the correction factor of 66 min would yield T50s of 142 and 206 min for the low and high fat meals respectively, and estimated average emptying rates over the 4 h would be 5 and 7 kJ/min respectively. These rates correspond more closely to those obtained in the literature (Ghoos et al., 1993; Maes et al., 1996). As pointed out in the original paper however (Ghoos et al., 1993), it was shown that the preparation methods of the separately baked and spiked egg yolk ensured good retention of the tracer, whereas it is not known whether separation of fat and aqueous phases occurred in the high fat liquid meal of the current experiment, or how this would have affected appearance rates of enriched CO$_2$.

Choi et al. (Choi et al., 1997) repeated the methods of Ghoos et al. (1993) to test their hypothesis that a uniform correction factor could be applied across
individuals and within repeated measures on an individual. Although they did find good repeatability within individuals, equal to that obtained using scintigraphy, they found that the duration of the collection period (4, 5 or 6 h) introduced large variability, and that the T50s did not correlate with scintigraphic T50s, and so they argued that a correction factor of -66 min for breath test T50s was not valid. They asserted that the inter-individual variations in absorption and oxidation were large and that a sampling period of 6 h with more frequent sampling was crucial for accurate gastric emptying breath test T50s. These conclusions were disputed by Perri et al. (Perri et al., 1998), who repeated the experiment again and showed a good correlation between scintigraphic and breath test T50s ($r = 0.74$, $p = 0.00003$), with the regression line intercepting the y axis at 61 min (very close to the original 66 min). Their whole study (with a greater number of subjects, $n = 88$) was reported in a recent paper (Delbende et al., 2000), where the same significant correlation remained and the cross-section between the y axis and the regression line was at 67 min, 1 min more than the value obtained by Ghoos et al. (1993). Objections to Choi and colleagues' point of view were also raised by Maes et al. (Maes et al., 1998), who thought that longer collection periods improved curve fitting but did not greatly affect the T50 in normal subjects, which according to the mathematical formula depends on the instantaneous slope and shape of the curve, not the total amount of $^{13}$CO$_2$ recovered in the breath. They said that scintigraphy also benefits from more frequent sampling time points, and they maintained that the time taken for absorption, metabolism and excretion was extremely constant.

A fairly constant level of $^{13}$CO$_2$ in baseline breath samples is important, since the increase above baseline in $^{13}$C enrichment is determined to calculate percentage dose recovered. Therefore, a similar diet the day before each study (so that $^{13}$C-rich foods are not consumed before one study and not the other) and a stable metabolic rate (i.e. strict control over levels of physical activity before and during the study) is of the utmost importance in carrying out $^{13}$C-substrate breath test studies. Both of these controls were observed in carrying out the present study thus it is assumed that excretion rate was not underestimated, especially as CO$_2$ production rates were measured directly, as suggested by Amarri and colleagues (Amarri et al., 1998), rather than estimated using body surface area and a value of 5 mmol/m$^2$ per min as previous investigators had done (Ghoos et al., 1993; Maes et al., 1995; Maes et al., 1996; Choi et al., 1997). Also, a frequent rate of breath sampling was included in the
experimental protocol, increasing the accuracy of the curve fitting and achieving correlation coefficients >0.95 between the raw data and fitted data (mean r = 0.997).

It is likely that the results obtained by the $^{13}$C-octanoic acid breath test method are precise, but not accurate due to the absence of a validated correction factor for the liquid test meals used in this study. Perhaps using high energy liquid test meals required the longer sampling time of 6 h suggested by Choi et al. (1997), especially since many individuals' cumulative curves had not yet reached 50 % percentage dose recovered by the 4 h time point following the high fat test meal. The weak discriminatory power between low and high fat liquid meals compared to the paracetamol absorption test may have been a result of the long gastric emptying times (due to increased energy and fat content), and perhaps the breath test method is more suited to reduced energy loads with shorter T50s that fall within the 4 h. Regarding the present work, the subject of volunteer compliance is pertinent here as it is unlikely that they would have been willing to remain motionless for 6 hours.

When the three gastric emptying methods were correlated against each other for each meal using non-parametric methods it was found that none of the tests significantly correlated against any of the other tests. This is probably partly due to small sample size as often the actual R values were moderately high. Fairly strong associations were found between paracetamol IAUC (0 to 60 min) and EIE T50 (Spearman r = -0.71) and paracetamol IAUC (0 to 120 min) and breath T50 (Spearman r = -0.77) following the low fat meal, and between breath test T50 and EIE T50 following the high fat meal (Spearman r = 0.70). It is difficult to discuss the significance of these findings with a sample size of 6 subjects, but it appears that there may have been a trend for the breath test and paracetamol parameters to agree following the low fat meal.

Unlike the previous experiment (section 4.2.6.2.1) using identical meals, plasma glucose levels were significantly different over time, as shown by a significant treatment by time interaction (p <0.00005). This is due to the fact that the sampling time of the present study was twice as long as the previous one. If the same time interval is taken (2 h) then no significant differences are found as before. As in the previous study, plasma insulin concentrations were significantly decreased following the high fat test meal compared to the low fat test meal, and the plasma
glucose and insulin levels followed a similar profile. The increased plasma insulin concentrations and earlier mean glucose peak following the low fat test meal compared to the high fat test meal are likely to be due to the faster gastric emptying rate of the low fat meal. In support of this it can be observed from figure 4.9 that the time course of plasma paracetamol concentrations were reflected by the plasma glucose and insulin profiles.

Plasma GIP concentrations were significantly increased following the high fat test meal compared to the low fat during the whole 4 h sampling time. There was also a significant treatment by time interaction in plasma GLP-1 response, with higher levels of GLP-1 following the high fat meal. The results for plasma GIP and GLP-1 agree with those previously reported for low and high fat meals (Long, 2000). Plasma CCK concentrations were significantly increased following the high fat meal compared to the low fat meal, an effect that is well known and agrees with earlier findings (Hopman et al., 1985; Liddle et al., 1985; Drewe et al., 1992). It can also be noted that the plasma CCK response following the high fat test meal was biphasic, with the second mean peak occurring in parallel to the plasma GIP mean peak. It could be hypothesised that the initial peak (which occurred in parallel to the low fat test meal peak) was mainly gastric distension-induced, with the second peak a consequence of increasing intestinal fat load, which is why it emerges simultaneously with peak plasma GIP concentrations, levels of which closely reflect rate of fat absorption (Koop et al., 1990).

A number of correlations were found between plasma hormones and gastric emptying parameters. Slower EIE gastric emptying of the high fat test meal was associated with higher GIP peaks and longer times to reach peak GLP-1 concentrations. Longer breath test T50s for the high fat test meal were associated with extended times to reach peak GLP-1 and GIP concentrations, and faster breath test emptying of the low fat test meal was associated with increased peak CCK concentrations. Increased paracetamol concentrations were associated with increased levels of plasma glucose following the high fat test meal, and with increased levels of GLP-1 following the low fat test meal. None of the gastric emptying parameters correlated with plasma insulin concentrations.
The relationship between longer gastric emptying times and an extended time to reach peak GIP and GLP-1 concentrations, and between delayed gastric emptying and decreased CCK levels following the low fat meal, suggests that gastric emptying rate determines the secretion rate of these hormones. Although a greater amount of fat ingestion will delay gastric emptying and increase hormone secretion, there is variability between individuals in their gastric emptying times and so those individuals who empty more slowly for a given test meal will also show a longer time to reach peak plasma concentrations compared to those who empty the meal at a faster rate. Interestingly, although insulin IAUC (0 to 120 min) correlated with glucose IAUC (0-120 min) following the high fat meal there were no correlations between parameters of insulin secretion and parameters of incretin secretion for either test meal, and thus it appears that differing insulin profiles were directly influenced by circulating levels of absorbed glucose rather than the incretins, GIP and GLP-1.

In conclusion, EIE T50s are quantitatively inadequate for clinical and research use, but in terms of ranking order with other methods and with plasma glucose and hormone parameters, the EIE methodology performed better than it did in the previous study. This may be explained by the fact that the physicists who operated the equipment had improved their techniques for positioning the electrodes to obtain a stronger deflection with the ingestion of the meal. The breath test method was a poor discriminator between meals and may have been hampered by the choice of test meal. However, it correlated well with paracetamol parameters following the low fat meal (when breath T50s were shorter). The best method, both quantitatively (although tmax is not equal to T50) and in terms of discriminating power between low and high fat test meals seemed to be the paracetamol absorption method, especially tmax. Paracetamol IAUC correlated well with other methods and with plasma glucose and hormonal parameters. The paracetamol absorption method is fairly invasive since it involves taking blood, but could be the easiest and least expensive method for measuring the emptying rate of liquid test meals in a research setting, especially where blood samples are frequently taken for the measurement of other variables anyway. It is also safe compared to scintigraphy, as ionising radiation is not involved, and is suitable for studies where subjects may be overweight or obese (a limiting factor for EIE) or where it is not practical to remain still for long periods (a limiting factor for EIE and 13C-octanoic acid breath test).
Chapter Five
5.1 Introduction

It is a well-established fact that protein is more satiating, kilojoule for kilojoule, than carbohydrate or fat (Booth et al., 1970; De Castro, 1987; Barkeling et al., 1990; Hill & Blundell, 1990; Johnstone et al., 1996; Vandewater & Vickers, 1996; Stubbs et al., 1996; Porrini et al., 1997; Latner & Schwartz, 1999). However, there is some evidence that different protein sources may differ in their satiating capacity, as it was shown that satiety was greater after a meal of fish compared to a beef or chicken meal (Uhe et al., 1992). Little is yet known how this may happen and not all observers report differences. Lang and colleagues (Lang et al., 1998) tested satiety and food intake using 6 different protein sources, but there were no differences in 8-hour satiety ratings or 24-hour food intake. A subsequent study, using smaller quantities of protein (casein, gelatin, and soy protein), showed a slight difference in hunger, although it was confirmed that varying the protein type in a mixed meal had no effect on 24 hour food intake (Lang et al., 1999).

Of interest in this chapter is the concept of "fast" and "slow" proteins introduced by Boirie et al. (Boirie et al., 1997) to describe the differences between whey and casein in their digestion and absorption. They showed that casein, which coagulates in the stomach due to precipitation by gastric acid, had a slower rate of absorption and exerted less of an increase in plasma amino acids compared with the non-coagulating whey protein. Acidification of casein in the stomach creates a curd, thereby delaying the gastric emptying of this protein and also causing a resistance to digestion by pepsin (Billeaud et al., 1990; Miller et al., 1990). On the basis of Melinkoff's original aminostatic concept relating plasma amino acids to satiety (Melinkoff et al., 1956), whey as a "fast" protein, might be expected to be more satiating than casein. When ingested as liquid test meals, whey reduced short-term food intake to a greater extent than casein (Long et al., 2000), (see figure 5.1), suggesting that any positive influence on satiety mediated by the longer stomach retention time of casein was overwhelmed by the postprandial metabolic responses.
Figure 5.1 Ad libitum test meal energy intakes (n 16) 90 min after isoenergetic liquid preloads containing 48 g casein or whey protein (1674 and 1695 kJ respectively). Maltodextrin and double cream were added, giving a final % energy ratio of 50:25:25 for protein:CHO:fat. Values are means with their standard errors represented by vertical bars. Mean energy intakes were significantly different, (paired two-tailed t-test; p = 0.034,). (From Long et al., 2000).

This implies that large rises in post-prandial plasma amino acid levels have a strong effect on short-term appetite responses, but that measurement of satiety over a longer period (Lang et al., 1999) may not detect the influence of postprandial amino acid kinetics. The importance of plasma amino acid levels over time following a meal requires further investigation.
5.2 Investigation into the effect of casein and whey protein on circulating metabolite and hormone status, gastric emptying and subjective appetite ratings

5.2.1 Aims and hypothesis

Previous work carried out by Samantha Long at the University of Surrey showed that a whey protein liquid meal reduced food intake to a greater extent than the same meal containing casein (Long et al., 2000). It is hypothesised that these different milk proteins, administered as a liquid preload, exert differing effects on short-term metabolite and hormonal responses before and after a standard meal. Since, whey protein is more satiating than casein protein, the elevation of satiety gut peptides GLP-1 and CCK might be expected. A randomised within-subject experimental design was used to examine the metabolic responses and pancreatic/gut hormone profiles following casein or whey liquid test meals.

5.2.2 Subjects

Nine lean healthy volunteers (8 female and 1 male) were recruited from the University, mean age 25 years (range 22 – 30). The volunteers were all non-dieting, with no special dietary requirements, and a BMI <25 kg/m² (mean 22.6, SD 1.5 kg/m²). They had no significant current or previous medical history and took no medication apart from the oral contraceptives. Volunteers who consumed more than 20 units of alcohol per week or consumed more than 20% of their energy intake as protein were excluded (as assessed by a 7 day food diary), as were smokers and heavy exercisers/athletes. All volunteers completed the Dutch Eating Behaviour Questionnaire (van Strien et al., 1986) as shown in appendix II, and those whose mean score was >3.0 were excluded (mean score 2.4, SD 0.5). Prospective subjects were interviewed and gave written consent to participate in the study, including a statement that they understood that they would be giving some blood as part of the study. A venous blood sample was taken for haematological and biochemical screening (full blood count, liver and renal function) to confirm their suitability to participate in the study. A letter was sent to their GP to inform them about the study, and to give their GP an opportunity to confirm their patient’s suitability. The study protocol was approved by the local ethics committee.
5.2.3 General procedure

Subjects were studied on two occasions (at least 3 days apart), one day for the whey protein meal and one day for the casein protein meal. They were instructed to avoid alcohol and high protein meals the evening before, and until the study. It was also requested that they withhold from taking any paracetamol tablets or paracetamol-containing products for the previous 24 hours. On the day of the study they consumed a standard high carbohydrate cereal and skimmed milk breakfast (delivered to the volunteer in advance) before 0800 hours, and then consumed nothing except water until 60 min before the study, and then no water until the study.

Subjects arrived at the Clinical Investigation Unit at the University of Surrey at 1100 h (2 to 4 people per study day), and an intravenous cannula was inserted into an antecubital vein in the forearm under local anaesthetic by a qualified doctor or nurse. Blood samples were taken for the measurement of plasma amino acids, glucose, insulin, GLP-1, GIP and CCK according to the methodology detailed in section 2.2.3.1. Baseline blood samples were taken and the initial visual analogue scale (VAS) ratings (appendix V) of hunger, desire to eat and fullness were completed by the subject (-10 and 0 minute time points). The subject consumed a liquid test meal containing either 50% of the energy content as whey or casein protein (see table 5.1). The liquid test meal was presented in a black opaque beaker with a lid and was drunk through a straw so that any difference in the appearance of the preloads was not visible to the subject. The milkshake also contained 1.5 g of dissolved paracetamol (three crushed paracetamol tablets) in order to measure gastric emptying. Blood samples were taken at intervals for 90 min before the standard lunch was provided. VAS ratings were completed by subjects immediately before each blood sample was taken. Subjects were required to consume all the food provided at the standard lunch. The standard lunch contained sandwiches, yoghurt and cake, and the amount of energy provided was standardised to the subject’s body weight (42 kJ per kg body weight). Two further VAS ratings and blood samples were taken following the standard lunch.
### Table 5.1 Study protocol for the effect of casein and whey liquid test meals on plasma metabolite and hormone response, gastric emptying and subjective appetite ratings.

#### 5.2.4 Meals

Isoenergetic, high protein liquid test meals containing 1674/1695 kJ were consumed by the subject each study day. The milkshake meal was formulated using commercially available whey or casein protein formulas (see appendix XI for amino acid composition), double cream, and maltodextrin, so that protein provided 50% of the energy, fat 25% and carbohydrate 25%, plus 1.5 g paracetamol (3 x 500 mg paracetamol tablets). They were made up to 450 ml with water (see table 5.2):

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Blood sample</th>
<th>VAS</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>-10</td>
<td>✔</td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>✔</td>
<td>✔</td>
<td>Consume casein or whey liquid test meal +1.5 g paracetamol</td>
</tr>
<tr>
<td>+5</td>
<td>✔</td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td>+15</td>
<td>✔</td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td>+25</td>
<td>✔</td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td>+35</td>
<td>✔</td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td>+45</td>
<td>✔</td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td>+60</td>
<td>✔</td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td>+75</td>
<td>✔</td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td>+90</td>
<td>✔</td>
<td>✔</td>
<td>Consume standard lunch (42 kJ/kg body weight)</td>
</tr>
<tr>
<td>+120</td>
<td>✔</td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td>+180</td>
<td>✔</td>
<td>✔</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2 Composition of liquid test meals used to test the effect of different protein types, casein and whey, on plasma metabolite and hormonal response.

The standard cold lunch was calculated to supply each subject with 42 kJ/kg body weight, with each component of the meal making up the same proportion of total energy intake for each individual. This was calculated using a Microsoft Excel worksheet linked to other worksheets containing nutritional compositions of the foods (appendix X). The meal was based on the subjects’ food choices (obtained during...
recruitment): turkey or ham sandwich, lettuce, almond or lemon cake, and “thick and creamy” yoghurt.

5.2.5 Subjective measures of appetite

Subjects rated their hunger, desire to eat, and fullness as detailed in section 2.2.1.3. Results were analysed by two way repeated measures ANCOVA as raw data with baseline values as covariate. The t −10 and t +0 min values were expressed as a mean and designated as covariate (or t +90 min when analysing post-standard lunch scores).

5.2.6 Results

5.2.6.1 Gastric emptying

Plasma paracetamol concentrations following the casein and whey liquid test meals are shown in figure 5.2.

Two-way repeated measures ANOVA on plasma paracetamol concentrations showed that there was no overall treatment effect, but there was a treatment x time effect (p <0.05), significant at t +35 min according to the post hoc Duncan test. This reflects the cross over where plasma paracetamol concentrations following the casein showed a greater increase compared to the whey until t +60 min, at which point concentrations levelled off and then dropped below whey levels.
Figure 5.2 Plasma paracetamol concentrations (mean ±SEM, n = 9) following isoenergetic ~1700 kJ liquid test meals containing either casein or whey protein (50 % energy). There were no significant differences in plasma levels between treatments but there was a treatment x time interaction (p <0.05).

Time until peak concentration (tmax), peak concentration (cmax) and area under the curve (AUC) were calculated for each subject’s paracetamol results for the first 90 min until the standard lunch. There were no significant differences. The mean results were as follows (table 5.3):

<table>
<thead>
<tr>
<th></th>
<th>Casein (SEM)</th>
<th>Whey (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tmax (min)</td>
<td>56.7 (9.17)</td>
<td>73.3 (8.82)</td>
</tr>
<tr>
<td>Cmax (mmol/L)</td>
<td>0.08 (0.01)</td>
<td>0.08 (0.01)</td>
</tr>
<tr>
<td>Incremental AUC 0-90 min (mmol/L by min)</td>
<td>4.25 (0.34)</td>
<td>4.03 (0.53)</td>
</tr>
</tbody>
</table>

Table 5.3 Mean gastric emptying parameters for paracetamol absorption following isoenergetic ~1700 kJ liquid test meals containing 50 % energy either casein or whey protein, t +0 – t +90 min (before the standard lunch). Mean ± SEM, n = 9. Paired t-test between the conditions showed no significant differences.

5.2.6.2 Blood metabolite and hormone profiles

5.2.6.2.1 Plasma glucose

Plasma glucose concentrations are shown in figure 5.3. Ingesting both the high casein and high whey liquid test meal led to a slight rise in plasma glucose concentrations but there were no differences between treatments.
Figure 5.3 Plasma glucose concentrations (mean ±SEM, n = 9) following isoenergetic ~1700 kJ liquid test meals containing either casein or whey protein (50 % energy). There were no significant differences in plasma levels between treatments.

5.2.6.2.2 Plasma amino acids

Plasma amino acid concentrations were analysed as change from baseline values (x-b) by two way repeated measures ANOVA.

Total plasma amino acids, expressed as change from baseline (x-b), are shown in figure 5.4. Two way repeated measures ANOVA revealed no overall significant differences in total plasma amino acid levels between liquid test meals (p = 0.158), although there was a significant treatment x time interaction (p = 0.005). Two way repeated measures ANOVA on post-standard lunch values (t +90 to t +180 min) revealed a nearly significant increase in total plasma amino acids concentrations following the whey test meal (p = 0.051).
Figure 5.4 Change in mean total plasma amino acids (x-b mmol/L, ± SEM) following isoenergetic ~1700 kJ liquid test meals containing either casein or whey protein (50 % energy). There was a significant treatment x time interaction (p = 0.005) due to increasing concentrations during the 2nd and 3rd hours following the whey liquid test meal compared to the casein.

Results for individual amino acids (x-b averaged over 180 min, derived from two way repeated ANOVA calculations) are shown in table 5.4.

The branched chain amino acids (valine, leucine and isoleucine) and threonine were among those amino acids that showed the uppermost statistical significance in their differences in postprandial plasma profiles between the two test meals. See figure 5.5.
<table>
<thead>
<tr>
<th>Amino acid (mmol/L)</th>
<th>Casein test meal (mean x-b, 0-180 min)</th>
<th>Whey test meal (mean x-b, 0-180 min)</th>
<th>Main treatment effect (p value)</th>
<th>Treatment x time interaction (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid (asp)</td>
<td>0.004</td>
<td>0.009</td>
<td>&lt;0.005</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Glutamic acid (glu)</td>
<td>0.014</td>
<td>0.029</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Glycine (gly)</td>
<td>0.035</td>
<td>0.027</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Threonine (thr)</td>
<td>0.070</td>
<td>0.119</td>
<td>&lt;0.05</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td>Alanine (ala)</td>
<td>0.113</td>
<td>0.140</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Arginine (arg)</td>
<td>0.050</td>
<td>0.053</td>
<td>NS</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Proline (pro)</td>
<td>0.211</td>
<td>0.126</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Tyrosine (tyr)</td>
<td>0.053</td>
<td>0.043</td>
<td>NS</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Valine (val)</td>
<td>0.166</td>
<td>0.207</td>
<td>NS (p = 0.082)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Methionine (met)</td>
<td>0.036</td>
<td>0.037</td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Isoleucine (ile)</td>
<td>0.090</td>
<td>0.164</td>
<td>&lt;0.005</td>
<td>&lt;0.00005</td>
</tr>
<tr>
<td>Leucine (leu)</td>
<td>0.160</td>
<td>0.244</td>
<td>&lt;0.001</td>
<td>&lt;0.0000001</td>
</tr>
<tr>
<td>Phenylalanine (phe)</td>
<td>0.040</td>
<td>0.033</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 5.4 Mean plasma amino acid values (mmol/L) over t +0 to t +180 min expressed as change from baseline (x-b), derived from two-way repeated measures ANOVA, following isoenergetic ~1700 kJ liquid test meals containing 50% energy either casein or whey protein. NS: non-significant. Plasma concentrations were significantly increased overall following the whey liquid test meal for asp, glu, thr, ile, and leu. Plasma concentrations were significantly increased following the casein liquid test meal for pro. There were no differences for gly, ala, arg, tyr, val, met and phe. There were significant treatment x time interactions due to higher concentrations following whey in the 2nd and 3rd hours for asp, glu, thr, arg, val, met, ile, and leu. There were significant treatment x time interactions due to higher concentrations following casein in the 1st hour for tyrosine. There was a significant treatment x time interaction for proline due to higher concentrations following casein throughout the whole study.

The branched chain amino acids (valine, isoleucine and leucine) and threonine were among those amino acids that showed the uppermost statistical significance in their differences in postprandial plasma profiles between the two test meals. See figure 5.5.
Figure 5.5 Change in mean total plasma branched chain amino acids (x-b mmol/L, ± SEM) following isoenergetic ~1700 kJ liquid test meals containing either casein or whey protein (50 % energy). Valine, isoleucine and leucine plasma profiles were all clearly divergent with high levels of statistical significance following the two test meals.

Two-way repeated measures ANCOVA on the raw data showed that overall plasma GLP-1 concentrations were significantly lower following the whey liquid test meal (p = 0.001). There was also a significant treatment effect (p = 0.020), which reflected the divergence experienced after the 180 min time point.

3.2.6.2 Plasma GLP-1

Plasma GLP-1 concentrations are illustrated in Figure 5.6. Two-way repeated measures ANCOVA on the raw data, t = 40-150 min was used to examine the difference (p = 0.191). However, while valine, valine, isoleucine and leucine baseline (x-b) significant differences were due primarily to the isoenergetic nature of the test meals (t = 40-90 min (p = 0.041)). Analysis of potential differences following the standard lunch was carried out to observe that an increased GLP-1 (change from t = 180), and a significant increase in GLP-1 (p = 0.004), due to the large increase in GLP-1 levels at t = 90 min compared to the casein.
5.2.6.2.3 Plasma GIP

Plasma GIP concentrations are illustrated in figure 5.6.

![Graph](image)

**Figure 5.6** Plasma GIP concentrations (mean ±SEM, n = 9) following isoenergetic ~1700 kJ liquid test meals containing either casein or whey protein (50% energy). Plasma GIP concentrations were significantly higher following the whey test meal compared to the casein (p < 0.005).

Two way repeated measures ANCOVA on the raw data, t +0 – 180 min, showed that overall plasma GIP concentrations were significantly higher following the whey liquid test meal (p = 0.001). There was also a significant treatment x time effect (p = 0.020), which reflected the divergence in plasma GIP levels after the +25 min time point.

5.2.6.2.4 Plasma GLP-1

Plasma GLP-1 concentrations are illustrated in figure 5.7. Two way repeated measures ANCOVA on the raw data, t +0 – 180 min, did not produce any significant differences (p = 0.191). However, when values were calculated as change from baseline (x-b), significant differences were shown between the casein and whey liquid test meals t +0 – 90 min (p = 0.041). Analysis of plasma GLP-1 concentrations following the standard lunch was carried out as change from pre-lunch baseline (change from +90 min), and a significant treatment x time effect was shown (p = 0.004), due to the large increase in GLP-1 levels at +120 min following the whey compared to the casein.
Figure 5.7 Plasma GLP-1 concentrations (mean ±SEM, n = 9) following isoenergetic ~1700 kJ liquid test meals containing either casein or whey protein (50 % energy). Plasma GLP-1 concentrations (x-b) were slightly increased following the whey test meal compared to the casein but this difference was not quite significant (p = 0.057).

5.2.6.2.5 Plasma Insulin

Plasma insulin concentrations are illustrated in figure 5.8. There were no significant differences in plasma insulin concentrations following casein or whey liquid preloads.

5.2.6.2.6 Plasma CCK

Plasma CCK concentrations are illustrated in figure 5.9. Two way repeated measures ANCOVA on the raw data, 0-180 min, showed that overall plasma CCK concentrations were significantly higher following the whey liquid test meal (p = 0.017).
**Figure 5.8** Plasma insulin concentrations (mean ±SEM, n = 9) following isoenergetic ~1700 kJ liquid test meals containing either casein or whey protein (50 % energy). Plasma insulin concentrations were not significantly different.

**Figure 5.9** Plasma CCK concentrations (mean ±SEM, n = 9) following isoenergetic ~1700 kJ liquid test meals containing either casein or whey protein (50 % energy). Plasma CCK concentrations were significantly increased following the whey test meal (p <0.05).
5.2.6.3 Subjective ratings of appetite

Desire to eat and hunger was reduced by whey protein compared to casein following the standard lunch, and fullness was increased by whey compared to casein for the duration of the 3 h. Hunger, desire to eat and fullness after the casein and whey liquid test meals are shown (figures 5.10, 5.11 and 5.12 respectively).

Two way repeated measures ANCOVA of the hunger ratings showed that there were no overall or pre-standard lunch treatment effects. However, there was a trend towards a treatment effect following the standard lunch (p = 0.061), taking t +90 min as covariate. Desire to eat ratings were also significantly different following the standard lunch (p = 0.020). Two way repeated measures ANCOVA of fullness ratings showed that there was a significant treatment effect on fullness t +0 to t +180 min (p = 0.037) and t +90 to t +180 min (p = 0.031), and a trend towards increased fullness from t +0 to t +90 min (p = 0.070).

Figure 5.10 VAS ratings (cm) for hunger (mean ± SEM, n = 9) following isoenergetic ~1700 kJ liquid test meals containing either casein or whey protein (50 % energy). The whey liquid test meal reduced hunger to a greater extent following the standard lunch compared to the casein liquid test meal. This effect was marginally non-significant (p = 0.06).
Figure 5.11 VAS ratings (cm) for desire to eat (mean ± SEM, n = 9) following isoenergetic ~1700 kJ liquid test meals containing either casein or whey protein (50 % energy). The whey liquid test meal reduced desire to eat to a greater extent following the standard lunch compared to the casein liquid test meal (p <0.05).

Figure 5.12 VAS ratings (cm) for fullness (mean ± SEM, n = 9) following isoenergetic ~1700 kJ liquid test meals containing either casein or whey protein (50 % energy). The whey liquid test meal increased overall fullness to a greater extent compared to the casein liquid test meal (p <0.05).
5.2.7 Discussion

The main interest that lay behind this investigation was the evidence for and against the differing satiating capacity of various protein sources (Uhe et al., 1992; Lang et al., 1998; 1999). Boirie et al. (1997) had previously introduced the concept of "fast" and "slow" proteins to describe the differences between whey and casein in their digestion and absorption. Casein, as a coagulating protein, had a slower rate of absorption and exerted less of an increase in plasma amino acids compared with whey, which does not coagulate. Based on the aminostatic theory that a larger rise in plasma amino acids would increase satiety, a study by Long and colleagues (2000) at the University of Surrey found that whey was more satiating than casein. The purpose of this study was to identify the physiological processes behind these differences in satiating capacity, specifically the endocrine and metabolic processes.

Firstly, the demonstration of different gastric emptying rates was attempted using the paracetamol absorption method. The results showed that paracetamol absorption was faster following the casein until t +35 min but then levelled out for the remainder of the study period. Whey, on the other hand, continued to produce an increase in plasma paracetamol concentrations, overtaking casein concentrations at 75 min and reaching a mean peak at 120 min. The initially faster absorption of paracetamol following the casein probably represented the solubilised paracetamol in the liquid compartment of the meal, as the casein protein had precipitated into curds in the stomach. The removal of the protein compartment (through forming solidified curds) of the liquid meal might have accelerated initial liquid gastric emptying compared to the whey liquid meal, which presumably contained evenly dispersed lipid, carbohydrate and protein throughout each unit volume of the meal emptied. The comparatively greater proportion of protein and fat reaching the duodenum following the whey meal would have induced a greater inhibition of initial gastric emptying. Therefore, the differing intragastric properties of the two test meals might have altered the pharmacokinetics of paracetamol absorption. Casein resulted in an initial faster gastric emptying rate followed by a plateau in plasma levels due to slower emptying of the solid casein curds. Whey, on the other hand resulted in a steady, initially more linear emptying rate which resulted in a higher mean plasma paracetamol peak and then a decline.
Therefore the paracetamol absorption method is unsuitable for calculating established gastric emptying parameters such as tmax, cmax and AUC with these meals because the casein meal effectively changes from a liquid meal to a solid/liquid meal through precipitation in the stomach. The pharmacokinetics of paracetamol absorption can, however, still be used to illustrate the differing gastrointestinal effects of the two protein sources, proven by the significant treatment by time interaction produced by repeated measures, two-factor analysis of variance (p < 0.05).

Despite the obviously differing gastrointestinal absorption rates occurring in some components of the meal, plasma glucose concentrations were remarkably similar following both liquid test meals. Neither plasma insulin and glucose responses were significantly different following the two meals, in contrast to previous findings for different protein types (Hubbard et al., 1989; Gannon et al., 1988; Lang et al., 1999). However, plasma insulin concentrations followed a similar pattern to plasma paracetamol concentrations, with plasma insulin levels appearing higher until t +60 min following the casein liquid test meal, and then decreasing below whey levels after t +60 min. To some extent this was similar to plasma glucose concentrations, with plasma glucose response appearing slightly higher following the casein meal up until 60 min. Plasma glucose, insulin and paracetamol all followed time courses that were very similar to each other, and that were very different from the time courses taken by plasma gastrointestinal hormones.

Just under half of the plasma amino acids measured (5 out of 13) had an overall greater plasma response to the whey meal, whereas the rest showed no significant difference from t +0 to t +180 min. The only exception was proline, which significantly increased following the casein meal. A further 3 amino acids were significantly increased in the plasma from t +60 to t +180 min following whey (making a total of 8 out of 13). Examination of the plasma amino acid profiles of the whey and casein protein formulas (appendix XI) reveals that the largest differences in composition (g per 100g) between the two products are in arginine, methionine, phenylalanine, proline and tyrosine, where these amino acids are increased by more than 50 % in the casein formula. Three of these amino acids, phenylalanine, tyrosine and proline are among the five amino acids that were not increased in the plasma after whey, and all in fact show an increase in levels during the first hour following the
casein. Arginine and methionine were still increased following whey from \( t +60 \) to \( t +180 \) min, despite their larger relative content in the casein test meal.

Analysis of plasma total amino acids confirmed that the majority of amino acids were increased in the plasma following the whey liquid test meal relative to the casein liquid test meal, but that the difference was mainly apparent after the first hour. Individual amino acids varied widely in the size of this divergence in plasma levels; the branched chain amino acids (valine, isoleucine and leucine) and threonine showed the most marked differences. Phenylalanine, whose effects on satiety are reported more than any other amino acid, showed no significant differences in plasma levels following the two test meals, probably due to the greater amount in the casein formula. Tryptophan is another amino acid that has been frequently linked with appetite regulation, but unfortunately tryptophan concentrations could not be analysed using this methodology.

If the whey protein component of the test meal was emptied from the stomach more rapidly than the casein protein component, we can hypothesise that the protein-induced CCK response will be larger following the whey test meal. This indeed appears to be the case although the degree to which the higher plasma CCK concentrations are a result of the fat component or the protein component of the whey meal remains unknown. If it is assumed that the mixture emptied into the duodenum for the duration of the pre-standard lunch study period (90 min) contained a greater proportion of protein per ml following the whey meal compared to the casein, then it is likely that the higher CCK levels in the plasma were due to the greater secretory effect of hydrolysed whey protein. However, the greater GIP response following the whey, usually regarded as a marker for fat absorption, suggests that a greater proportion of fat was being emptied and absorbed as well, certainly from \( t +35 \) min until \( t +90 \) min. The increase in plasma GLP-1 levels following the whey meal supports this assumption, and also provides an additional mechanism for the greater satiating properties of whey compared to casein according to the current literature supporting GLP-1 as an important satiety peptide (Gutzwiller et al., 1999; Flint et al., 1998).

The visual analogue scales for subjective measurement of hunger, desire to eat and fullness supported previous work in that whey appeared to reduce hunger and
desire to eat following the standard lunch, and increase overall fullness compared to casein. Clear differences can be discerned between the responses to the two liquid test meals and the present findings confirm that due to the differing nutrient availability, endocrine responses and metabolic kinetics of these two protein sources, satiety is greater following the faster-absorbed whey protein compared to casein. The greater satiating capacity of whey may be a result of CCK- and GLP-1- mediated satiety mechanisms as well as the large increase in plasma amino acid concentrations; indeed the latter process may well be insufficient to induce such a marked satiety response following mixed meals without the additional support of the gut peptides that are involved in appetite regulation.

The results are consistent with previous findings that casein and whey liquid test meals emptied from the stomach at different rates. The whey meal emptied homogeneously at a steady rate, whereas the casein meal emptied in a biphasic manner due to its transformation to a solid/liquid meal, with the liquid component emptying earlier. The metabolite and hormonal profiles provide a clue to the contents of these components. Carbohydrate emptied at a uniform rate with the liquid component of the casein meal, since there were no significant differences in plasma insulin or glucose between meals. The divergent profiles of plasma CCK and GIP (and to a lesser extent GLP-1) following t +25 min suggest that fat, as well as protein, emptied more slowly after the casein meal. This may have been a result of incorporation of fat into the casein curds or possibly a generally stronger inhibition of gastric emptying through a neural reflex response to the retention of solid matter in the fundus.

In conclusion, it was shown that the different protein types, casein and whey, exert differing effects on plasma amino acid, GIP, GLP-1 and CCK responses before and after a standard meal. It is difficult to attribute the greater satiety response to a whey meal to the large increase in plasma amino acid concentrations exclusively, since the increase in satiety-gut hormones (CCK and GLP-1) that resulted from the difference in gastric emptying may have contributed to the reduction in food intake observed in the previous experiment (Long et al., 2000). In reality, it is difficult to separate these hormonal and metabolite responses since they will always occur together in a natural context, and their influences on satiety probably complement each other.
Chapter Six
The three strands of experimental work described in the preceding chapters were carried out with the purpose of uncovering the short-term physiological mechanisms behind the protein-related regulation of appetite. The methodology used in order to do this was investigated in terms of the repeatability of tools used to measure appetite, and also the accuracy of the technique chosen to measure gastric emptying. An individual amino acid that had previously been implicated in the induction of satiety, phenylalanine, was investigated in terms of its effect on appetite, gastrointestinal hormones, metabolites and gastric emptying, administered both as a large dose, and also as a small dose as part of aspartame. Finally, two protein types were investigated as part of a mixed macronutrient liquid test meal in terms of their effect on plasma levels of amino acids, gastrointestinal and pancreatic hormones, and gastric emptying, and how these variables related to appetite response. The relevance and importance of these results will be discussed in the following chapter.

6.1 Methodology

6.1.1 The measurement of appetite

Preloads and ad libitum test meal intakes have frequently been used to measure food intake following the manipulation of macronutrients or energy, but the reproducibility of food intake measurement has not, to the author's knowledge, so far been addressed. In the work described in this thesis, energy intake at the ad libitum buffet-style meal on repeated occasions was reduced on the third study session, despite the fact that the liquid preload and experimental conditions were identical on each study day. This raises an important issue for studies using ad libitum food intake as a measure of changes in appetite, and underlines the considerable influence of cognitive processes on eating behaviour. The value of randomisation, use of psychological screening tools such as the DEBQ, minimisation of external and emotional influences, and uniformity in experimental conditions (environmental setting, presentation of the meal, time of day etc.) is highlighted by this work. In the context of this thesis, there was only one study carried out that measured food intake on more than two occasions: "The effect of phenylalanine supplementation on food
intake and subjective appetite ratings" (see section 3.2). The results were organised in terms of study session number order and analysed by repeated measures ANOVA, but mean food intake was equal across each study session thus the reduction in food intake observed with phenylalanine intake was not confounded by a meal order effect, although the strength of the phenylalanine-induced satiety may have been dissipated by a repeated meals effect. The ad libitum buffet-style meal is limited as a method for measuring appetite changes for the reason that it will discern very large effects but more subtle changes in food intake may be masked by the "sensory apathy" that occurs with repeated identical meals. Therefore, it is suggested that studies aiming to discern small changes in food intake following a certain treatment limit the number of study sessions to two, if possible, and leave at least a week "wash-out" period between study sessions.

This "sensory apathy" effect on food intake with repeated identical meals is a symptom of the fact that laboratory food intake research by definition is a poor model for free-living eating patterns. The very fact that researchers want to isolate one variable and measure its effect on food intake means that all the other factors that normally influence food intake are removed, and this distorts the true eating response to the variable of interest. The problems associated with laboratory measurement of food intake have recently been reviewed by de Castro (De Castro, 2000). This author points out that the cost of food, the lack of external restraints such as a work schedule on the timing and duration of a meal, the emphasis on lunch-time meals during the week rather than evening-time meals and weekends (when many people consume the majority of the day's energy intake), prior beliefs and predetermined ideas about foods, and social facilitation are all factors that increase or decrease food intake and are removed in the laboratory. The removal of these influences can lead to the over-emphasis of the importance of a variable of interest, especially as appetite studies typically last for a few hours only. For example, palatability appears to be a major influence on food intake from laboratory studies whereas in fact free-living people rarely select foods low in palatability and so the influence of palatability on overall food intake is relatively small (de Castro, 2000). In the context of the present research work, free-living people would rarely sit down to a meal far larger than they could possibly eat as they do with the ad libitum buffet-style meal. Thus, the methodology removes one important real-life mechanism for the control of food intake — the perceived wastefulness and gluttony normally implicit in this kind of eating.
behaviour. Further thought should be applied to designing a method for the measurement of food intake that simulates eating patterns in real-life, but that keeps constant but does not remove the other influences on food intake (reduces "noise"), in order that the true significance of any nutritional variable can be judged. This would present an extremely challenging and lengthy task for any researcher in the field of appetite.

Visual analogue scales are important tools for the measurement of appetite, not just as a proxy for the measurement of food intake, but often in detecting changes in motivation to eat that are not reflected by food intake, as demonstrated by the results reported in this thesis for alanine treatment (see section 3.4) and in the work of other research groups (Gielkens et al., 1999; Lang et al., 1999). They are, however, highly dependent on the individual's interpretation of bodily sensations and strongly influenced by cognitive factors. Visual analogue scales and their reproducibility were also investigated simultaneously with food intake following the same three repeated identical liquid preloads and ad libitum buffet-style meal. Although no significant differences were found between mean scores for hunger, desire to eat and fullness on each occasion, this does not adequately show the reproducibility of VAS ratings. The statistical methods of Bland and Altman (1986), as used by Raben et al. (Raben et al., 1995) in their study of the reproducibility of VAS ratings on two occasions, were applied to the results obtained here. Raben and colleagues found reasonably large repeatability coefficients (CR) in their study, and the CRs reported in this thesis were of a similar magnitude. Subsequently smaller CRs were reported in a more recent study by the same group (Flint et al., 2000), but the measurements were only repeated on two occasions, and the upper level CRs obtained in the present study were mainly a result of comparison with the third session.

The relatively poor reproducibility of VAS ratings on repeated study days with identical preloads limits the usefulness of this methodological tool for the detection of more subtle changes in appetite. Although it has been shown in this thesis (see sections 3.4, 3.5 and 5.2) and in many other research papers (Silverstone & Stunkard, 1968; Hill & Blundell, 1990; Flint et al., 1998; Kahler et al., 1999) that the subjective measurement of appetite using VAS ratings can be used successfully to detect changes in response to pharmacological and nutritional variables, the risk of missing small changes in motivation to eat is great. VAS measurements require care
when interpreting the results and when analysing statistically. Different people tend to use the scales in different ways – some may regard the extreme ends of the scale as, for instance, 'as hungry as I've ever felt' in any day, and mark their scales at the ends more often. Others may regard the ends of the scale as 'as hungry as I've ever felt' in their lifetime, and mark their scales around the middle. It is very important that the subjects are informed what is expected of them, i.e. that the ends of the line are extreme feelings, to mark the extent of their feelings according to that exact moment, and to emphasise the importance of these scales to subjects or they may succumb to tedium and take less care over the accuracy of their marks on the line. The simultaneous use of food preference checklists can occasionally reflect motivation to eat more robustly than VAS ratings, possibly because it presents the individual with a way of thinking about their appetite sensations in real terms, i.e. how many of these foods do you feel like eating? Some people will respond with more accuracy to one method more than another and perhaps the best way of detecting appetite changes is by spreading the net wide and using VAS ratings, food preference checklists and ad libitum food intake simultaneously. Bearing this in mind, it can be concluded that VAS ratings show relatively poor reproducibility in the measurement of appetite but can be a useful accessory to other within-subject, repeated measurements of food intake; the method is sensitive to variables that have potent effects on motivation to eat (those sufficient to override cognitive and learned influences on eating behaviour). The high risk of type II errors should be considered within some experimental paradigms when using VAS ratings.

Power calculations were made based on the data according to the method of Gore & Altman (1982) using a nomogram. Appendix XII shows the results. The number of subjects needed to detect a 10 % difference in fasting VAS scores in a repeated measures design with a power of 0.8 would be between 25 to 34. These numbers are slightly higher for the post-preload mean (27 to 45) and a good deal lower for the post-ad libitum test meal values (11 to 25). The number of subjects required to detect a 10 % difference (418 kJ) in ad libitum test meal intake with a power of 0.8 would be 73; however, only 32 subjects would be needed for a 15 % difference in intake. These sample sizes seem quite large compared to the numbers used in the appetite studies here (section 3.2, n = 9; section 3.4, n = 12). However, our sample sizes are comparable to those used in the literature. The power calculations carried out by Flint et al. (2000) on pooled data (n = 55) showed that 32 subjects were
needed to test all VAS appetite parameters, with a power of 0.8 and using a paired
design – a figure that is not that far from the subject number of 45 calculated here.
Running 32 to 45 subjects in an experimental study involving an *ad libitum* test meal
would be expensive, and investigators must balance the risk of type I and II errors
against the cost of running that many subjects. If the variable of interest is expected to
have a large effect on appetite then fewer subjects (10 – 20) could be used, and it
must be considered whether the variable is of any relevance to appetite regulation if
the effect is expected to be small. Nevertheless, stringent statistical procedures for
appetite studies are extremely important as the results of studies that use small sample
sizes may be misleading, or at the very least need to be regarded with caution.

### 6.1.2 The measurement of gastric emptying

Gastric emptying was measured by EIE (a technique developed and conducted
by colleagues in the Department of Physics at the University of Surrey) during two of
the studies described in this thesis (sections 3.3 and 3.5), not including the
comparative methodological studies. The validity of the EIE technique was called
into doubt when abnormally short half emptying times compared to those reported in
the scientific literature were obtained for the liquid test meal used in the aspartame
study (section 3.5). EIE was compared with the “gold standard” scintigraphy in the
first comparative study, and then with the paracetamol absorption test and the $^{13}\text{C}$-
octanoic acid breath test in the second comparative study. EIE and scintigraphy
yielded differing results for gastric emptying times, and they related poorly to each
other in terms of rank order. Ranking order and agreement in values remained poor
between EIE and the paracetamol absorption and $^{13}\text{C}$-octanoic acid breath tests. The
second comparative study showed that slower gastric emptying rates of the high fat
test meal, as measured by EIE, were associated with longer times to reach peak
plasma GIP and GLP-1 concentrations; however, the EIE/scintigraphy study found no
such relationships for EIE and metabolites.

The fact that EIE discerned differences in gastric emptying rate of a water
load following 5 g phenylalanine, and in the gastric emptying rate of a liquid test
meal following aspartame or the constituent amino acids, suggests that it could
reliably detect a slower or faster gastric emptying rate. However, it was realised that
the gastric emptying values were shorter than expected, and that the validity of this
method was not sound as originally thought (McClelland & Sutton, 1985; Sutton *et
The reason for the abnormally short gastric emptying times is the premature return to baseline. This is thought to be due to gastric acid secretions increasing the conductivity of the stomach contents, thereby reducing the magnitude of the difference in conductivity between the stomach tissue and the stomach contents (a non-conductive meal). Since the differences obtained in EIE T50s are mainly a result of the initial trajectory of the reduction in impedance before this is truncated by the postprandial flattening of the trace due to the increased conductivity of the stomach contents, the proportion of the value that is determined by gastric acid secretion can be assumed to be minor but enough to prevent a correlation with other methods of gastric emptying. The problems associated with using EIE do not lend support for its future use in nutritional or clinical studies, but for the purpose of the work detailed in section 3.3 and 3.5, this method adequately detected differences in gastric emptying rates despite yielding erroneous values.

6.2 Phenylalanine and appetite

The objectives behind this line of enquiry included the exploration of the physiological mechanisms behind the reduction in satiety following aspartame ingestion previously demonstrated by Peter Rogers and colleagues. To recap, it had been shown that aspartame (but not phenylalanine or aspartic acid alone) ingested without tasting reduced food intake whereas aspartame dissolved in water had no effects (Rogers et al., 1990; 1991). In addition, aspartame reduced food intake when the interval between capsule and ad libitum test meal was one hour, but not with intervals of 5 and 30 min (Rogers et al., 1995). The authors had speculated that the physiological mechanism might involve the augmentation of CCK secretion in response to nutrients, since phenylalanine in large doses had previously been shown to induce CCK secretion (Ballinger & Clark, 1994; section 3.3) and also due to the fact that aspartame did not affect pre-meal subjective ratings of appetite. The main aims in the present research work was to discover if CCK or GLP-1 were involved, since GLP-1 is another gastrointestinal hormone involved in satiety, and whether this involved a modification of gastric emptying.

Large doses of phenylalanine (5 g) reduced food intake but did not influence pre-meal subjective ratings of appetite. There was a discernible dose-dependent effect on food intake (weight of food) but smaller doses did not cause a significant effect. The same large dose of phenylalanine increased pre-meal plasma CCK concentrations
and also overall plasma levels of tyrosine. It was also shown that alanine, used by previous authors as a control when testing the effect of phenylalanine on appetite (Ryan-Harshman et al., 1987), did not affect food intake but did reduce hunger and increase fullness before the *ad libitum* buffet-style meal. It appears that whilst large doses of phenylalanine did not influence appetite when there were no other nutrients in the gastrointestinal tract, the treatment did amplify the satiating effect of food; in contrast, the equivalent dose of alanine did increase pre-meal satiety but this was obscured by the satiating effect of a meal. More complicated results were unearthed when this line of enquiry was extended to small doses of aspartame, which had previously been shown to potently reduce food intake (Rogers et al., 1990; Rogers et al., 1991). Aspartame did not increase CCK concentrations in the plasma, but both its constituent amino acids, and to a lesser extent aspartame itself, accelerated gastric emptying rates. Subjective ratings of desire to eat were reduced following a standard liquid test meal (one hour after the constituent amino acids treatment), and this was associated with larger plasma peaks in plasma phenylalanine. Plasma GLP-1 secretion was suppressed by aspartame and the constituent amino acids. Although the reasons for this effect are not clear from the present results, the lower GLP-1 levels may partially account for the acceleration in gastric emptying rate, since one of the main functions of GLP-1 is the inhibition of gastric emptying.

Since reduced levels of GLP-1 did not decrease subjective satiety in this study and, plasma CCK concentrations were unaffected, it can be concluded that these gastrointestinal hormones are not involved in the reduction in food intake previously observed one hour after ingestion of encapsulated aspartame. The increase in gastric emptying rate following the standard liquid test meal with aspartame and constituent amino acids treatment is probably not relevant to satiety mechanisms either. The lack of effect of aspartame on post-meal subjective ratings of appetite is puzzling and does not support the findings of Rogers and colleagues (1990; 1991; 1995), since you would expect post-meal subjective ratings of appetite to reflect food intake. However, this may be because VAS results probably reflect a mainly cognitive, motivational aspect of appetite plus post-absorptive metabolic satiety signals, whereas food intake is a combination of responses to feelings of distension, the elusive post-absorptive metabolic satiety signals, as well as cognitive influences. The most direct satiety message during a meal is when the stomach begins to get full, and most people will stop eating before this sensation becomes uncomfortable unless the food is highly
palatable and is eaten infrequently. Since the meal was a standard liquid test meal of medium energy content (1992 kJ), maybe the increased fullness sensations experienced with solid, higher energy ad libitum test meals (Rogers et al., 1990; 1991; 1995) were not triggered by the liquid, medium energy test meal used in the present work (Hulshof et al., 1993). The aspartame was amplifying the satiating effect of food in Rogers and colleagues’ studies, possibly through increasing distension-induced nervous transmissions to the brain, which could not take hold with a standard liquid meal, perhaps because most of the liquid meal is held in the antrum and emptied rapidly rather than stored in the fundus before liquefaction (Collins et al., 1991; Urbain & Charkes, 1995). The fact that reductions in subjective desire to eat ratings were observed in the present study suggests that the constituent amino acids affected appetite via other mechanisms, reflected by the widely differing plasma phenylalanine profiles following aspartame and the constituent amino acids. The time point where the greatest reduction in desire to eat occurred coincided with the peak plasma phenylalanine concentrations and the ingestion of the standard liquid test meal. Whereas, plasma phenylalanine concentrations following the aspartame stayed moderately elevated throughout the study, there was no increase in concentrations then a sudden peak following the constituent amino acids. This occurred due to differences in intestinal absorption for dipeptides and individual amino acids as already discussed (section 3.5). In summary, the contrast in appetite results following encapsulated aspartame compared to Rogers and colleagues’ studies can be attributed to the use of a smaller standard liquid test meal rather than a large ad libitum solid test meal, i.e. lack of a strong stimulus for gastric distension. The additional finding that constituent amino acids reduced desire to eat following a standard liquid test meal is probably related to the absorption of individual amino acids in the distal small intestine (ileum) and the sudden plasma phenylalanine peak that coincided with the ingestion of the meal, i.e. post-absorptive signals.

Therefore, if it is hypothesised that aspartame may have previously increased satiety by enhancing distension-induced neural signals in the presence of a substantial gastric stimulus, but not with the involvement of gastrointestinal hormones CCK and GLP-1, nor gastric emptying, what is the relevance of this to free-living human beings? Previous findings that encapsulated aspartame does not reduce food intake when ingested up to 30 min before a meal (Rogers et al., 1995), and only when ingested without tasting (Rogers et al., 1990), suggest that the likelihood of aspartame
ingestion affecting anyone's normal eating behaviour is remote. In theory there are still possibilities for the therapeutic treatment of overweight, but the reduction in energy intake involved might not be effective in cases of obesity (~730 kJ at the *ad libitum* test meal (Rogers et al., 1990)), and the obtrusive and obsessive nature of this intervention in eating behaviour would not be regarded by experts in the field as conducive to re-educating the patient towards normal healthy meal patterns.

If it is hypothesised that the constituent amino acids of aspartame, phenylalanine and aspartic acid, reduced desire to eat in this study by large increases in plasma phenylalanine concentrations, this is of more interest with regard to the original aminostatic theory discussed in section 1.1.4.1. It suggests that an increase in plasma phenylalanine concentrations can reduce motivation to eat independently from pre-absorptive factors such as the satiety gut peptides, CCK and GLP-1, and distension satiety signals (this can be assumed since gastric emptying was accelerated, which would reduce intragastric pressure). If the kinetics of post-prandial increases in plasma amino acids can affect motivation to eat, this suggests that there is an aminostatic sensor either in the periphery or the central nervous system that transmits satiety signals to the appetite centres in the brain. Since peak phenylalanine concentrations were still in the normal postprandial range following the constituent amino acids treatment containing 224 mg phenylalanine (mean 0.107 mmol/L, SEM ± 0.021 at t+60 min), this suggests that the amino static sensor is very sensitive to small imbalances in plasma amino acid profiles. The satiety response observed in the earlier studies, where food intake was reduced in response to a 5 g dose of phenylalanine, clearly represented a separate mechanism involving delayed gastric emptying and increased CCK production. This cannot be regarded as a physiological response, as the likelihood of any individual ingesting 5 g of phenylalanine, especially with no other amino acids to balance this, is extremely slight.

In conclusion, it is hypothesised that the relevance of phenylalanine ingestion to normal everyday appetite regulation is minor. Previous findings that phenylalanine increases satiety via the increased secretion of CCK did not apply to physiological phenylalanine intakes. Physiological levels of phenylalanine intake affected satiety by reducing motivation to eat immediately after ingestion of a nutrient-containing liquid test meal, but this was probably because there was a sudden imbalance of amino acids in the plasma over a short time period (i.e. a high phenylalanine/total remaining
amino acids ratio). The only instance where this might occur in real life is by ingestion of "diet" drinks sweetened with aspartame while in the fasted state, followed by the consumption of a protein-free meal. However, it has been shown in the present work that plasma phenylalanine concentrations following aspartame were constant and elevations were slight due to differences in absorption of the dipeptide compared to free phenylalanine.

6.3 Physiological mechanisms behind the differing satiating effects of two proteins, casein and whey

The phenylalanine chapter established that small physiological imbalances in plasma amino acid concentrations could influence motivation to eat, but that in practice this was unlikely to occur in free-living human beings. The main aims of the casein and whey chapter (section 5.2) were to investigate the physiological mechanisms behind the different satiating properties of the two milk proteins, and the importance of the biokinetics of plasma amino acids following different types of whole protein sources was of particular interest. Was the increased satiating capacity of whey protein due to the larger increases in plasma amino acids that would result from the faster gastric emptying rate compared to casein protein? The results showed that there was a divergence in plasma amino acid levels after the first hour of the study, with total plasma levels increased relative to casein following the whey test meal. We were unable to prove that the differences in the time course of plasma amino acids were wholly responsibly for the differences in satiety, since plasma levels of the satiety gut peptides, GLP-1 and CCK, were also higher following the whey protein meal. This requires further studies with a modified protocol to investigate the importance of postprandial amino acid levels in the short-term satiety response. Carbohydrate-free and fat-free test meals, which would simplify the gut-mediated hormonal response, would be highly unpalatable, unrepresentative of a normal mixed meal, and would not remove the influence of differing protein-induced CCK secretion on satiety. The other alternative would be to separate the components of a mixed meal to eradicate the gut-mediated, gastric emptying-linked response, i.e. a liquid test meal of fat and carbohydrate could be ingested simultaneously with intravenous or intraduodenal infusion of an amino acid mixture simulating a normal protein source, for example beef. If the amino acid mixture was infused at different rates, replicating the plasma profiles seen when the casein and whey liquid test meals

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have been consumed, then any differences in satiety responses could be examined against a background of hopefully constant gut peptide secretion.

Of course, plasma amino acids profiles are not independent from gut-mediated mechanisms during the course of digestion and absorption of a normal-mixed meal. In addition, casein is the only protein to exhibit this property of precipitating in stomach acid to form curds and thereby delay gastric emptying, and therefore is limited as a model for appetite responses to normal protein-containing meals. In a normal meal, there is often a variable quantity of vegetable and cereal fibre which will affect gastric emptying and absorption rates of amino acids, as well as the differing digestibility properties of different protein sources (meats, dairy, egg albumin, Quorn (Burley et al., 1993)). The fat and carbohydrate content of a meal will affect gastric emptying rate through the negative feedback of gut hormones, thereby influencing the absorption rate of amino acids. It is only when we try and unravel all these physiological responses to food to discover the importance of one particular mechanism, such as the aminostatic control of food intake, that the extent to which all of these processes are inextricably linked can be truly understood.

6.4 Concluding remarks

This thesis has addressed the short-term physiological mechanisms behind amino acid-induced satiety in human beings. The effect of a single amino acid, phenylalanine, on short-term satiety and gastrointestinal responses has been investigated. It is concluded from these studies that phenylalanine does lead to an increase in satiety, but this may be a result of two different physiological mechanisms depending on whether consumed as a large dose (pre-absorptive gastrointestinal response) or a small dose (post-prandial increases in plasma phenylalanine concentrations). The suppressive effect of aspartame on food intake is not a result of CCK or GLP-1 response, nor is it due to delayed gastric emptying, but it has been hypothesised here that perhaps the dipeptide methyl ester stimulates sensory nerve endings in the intestine that enhance the sensations of gastric distension. The direct stimulation of sensory nerve terminals near the lumen of the GI tract is an important area of research for protein/amino acid-induced satiety. A greater understanding of these neuronal signals is essential if we are not going to remain ignorant of the mechanisms for the short-term regulation of appetite. The investigation into the physiological mechanisms behind the differing satiating properties of casein and
whey has shown that the difference in gastric emptying rates of meals incorporating these proteins result in distinct responses for a range of gastrointestinal hormones, in addition to a large rises in plasma amino acid concentrations following the whey compared to the casein meal. The research shows that short-term changes in appetite as a result of protein intake may involve acute increases in postprandial plasma amino acid profiles. These post-prandial changes are not exclusive of pre-absorptive satiety mechanisms such as gastric distension, delayed gastric emptying, and increased secretion of satiety gut peptides in their influence on food intake and subjective appetite.

6.5 Recommendations for future work

The work that has been carried out for this thesis has yielded some interesting results, but has raised many more questions than it was able to answer. These questions may form the basis of future research, and hypotheses for further studies are suggested below.

1) Aspartame reduces food intake but does not increase pre-meal subjective ratings of appetite. Since this has been shown to be independent of an increase in plasma CCK levels, it is hypothesised that aspartame may act directly on gastrointestinal afferent nerve terminals to enhance the stimulation of distension-induced feelings of fullness. Further physiological studies in animals (not the rat since there may be a species difference in responses to aspartame (Rogers & Blundell, 1992)), possibly using the techniques of Jeanningros (1982), are needed to test whether specific vagal afferent neurons in the stomach and intestine respond to perfusion with aspartame.

2) The short-term satiety response to large doses of phenylalanine (5 g) involves increased secretion of CCK and delayed gastric emptying. Using the same dose of phenylalanine compared to a control dose (cornflour), but ingested simultaneously with a fat/carbohydrate preload would determine its effects on satiety when consumed as part of a meal. Measurement of plasma CCK and gastric emptying rate would establish whether phenylalanine affects these variables additionally to the fat-induced effects, or whether a fat-induced delay in gastric emptying and increase in CCK secretion would overwhelm any phenylalanine-induced effects.
3) Intraduodenal and intravenous infusion of an amino acid mixture simulating the profile of a commonly ingested food protein, e.g. beef, should be carried out in human subjects to determine the importance of postprandial flux in circulatory amino acids in appetite regulation. The mixture would be infused at physiological concentrations but at different rates (slow and steady versus rapid and acute) over 3-4 hours, and subjective appetite ratings and *ad libitum* food intake would be measured during that time. This would clarify whether the kinetics of post-absorptive changes in plasma amino acids are directly related to satiety or whether they are just a consequence of pre-absorptive changes in gastric emptying and hormonal response.
Publications
Publications


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Appendices
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<th>Method</th>
<th>Details</th>
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<tr>
<td><strong>13C-octanoic acid breath test</strong></td>
<td>13C-octanoic acid is a carbon-labelled medium chain fatty acid, which undergoes rapid absorption in the duodenum and oxidation in the liver. 13CO₂ excretion in the breath over time was found to closely reflect gastric emptying rate, the main rate-limiting step. 13C enrichment is measured by an isotope ratio mass spectrometer and results are expressed as % recovery of administered dose. It is preferable to measure CO₂ production simultaneously rather than use an estimate based on body surface area. Based on the assumption that the octanoic acid is digested, absorbed, metabolised and excreted at the same rate between individuals and within individuals with repeated measures. Non-invasive.</td>
<td>(Ohoos et al., 1993; Maes et al., 1994; Maes et al., 1995; Maes et al., 1996; Choi et al., 1997; Swart &amp; van den Berg, 1998; Deltende et al., 2000)</td>
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<tr>
<td><strong>Applied potential tomography</strong></td>
<td>Non-invasive, less portable than EIE. 16 electrodes are placed in a circular array around the trunk. An alternating current of 5mA at 50kHz is passed between pairs of adjacent electrodes and the potential difference between the remaining 13 adjacent electrode pairs are measured. Gastric emptying profiles are obtained by circling the image of the stomach and calculating the change in resistivity with ingestion of a liquid meal. Reduces problem with movement artifacts seen for EIE. Compares well with scintigraphy and dye dilution techniques.</td>
<td>(Avill et al., 1987; Mangnall, 1989)</td>
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<td><strong>Dye dilution</strong></td>
<td>Uncomfortable and invasive. A nasogastric tube is passed into the empty stomach, which is then washed out, and the test meal, labelled with phenol red, is administered. Samples are removed from the stomach at intervals and the volume of food remaining in the stomach and volume of gastric secretions is measured using the concentration of phenol red. The sample is then returned to the stomach until the next sampling point.</td>
<td>(George, 1968; Beckers et al., 1992; Maughan &amp; Leiper, 1996).</td>
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<td><strong>Electrical impedance epigastrotomography (EIE)</strong></td>
<td>Non-invasive, portable. 6 electrodes are placed on the stomach and lower back. An alternating current of 4mA at 100kHz is passed through one set of electrodes and the impedance is recorded by the remaining 4. Half-emptying time is calculated from the peak of the initial impedance deflection to the half way point where the trace returns to baseline. Non-conductive liquid meals used. Subject remains very still due to movement artifacts.</td>
<td>(Pickworth, 1984; McClelland &amp; Sutton, 1985; Sutton et al., 1985; Boston, 1995; Mangnall, 1989)</td>
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<td><strong>Magnetic resonance imaging (MRI)</strong></td>
<td>Non-invasive, but expensive. Can be used to measure gastric emptying, gastric motility and visualise the stomach in 3D. Consume a non-toxic meal containing gadolinium tetrazacyclododecane tetracetic acid (Gd-DOTA). Transaxial slices (10mm wide) taken over the upper abdomen every 15 min to measure emptying. Total volume of gastric contents at each time point calculated by summing the gastric volumes of all slices of each sequence, and calculated according to signal intensity. Avoids errors of overlap seen in scintigraphy. Disadvantages: have to hold breath in expiration during transaxial scans to reduce movement artefacts.</td>
<td>(Schwizer et al., 1992; Lehmann et al., 1996; Schwizer et al., 1996; Schwizer et al., 1997)</td>
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<tr>
<td><strong>Paracetamol absorption</strong></td>
<td>Paracetamol is quickly absorbed from the duodenum and this was found to relate to gastric emptying. 1.5g of soluble paracetamol is administered orally, and the appearance of paracetamol in the blood plasma is used to measure the rate of emptying of a liquid meal. Liquid meals only. The area under the curve, time until peak plasma concentrations, and value of peak plasma concentrations are used as indicators of gastric emptying.</td>
<td>(Heading et al., 1973; Maddern et al., 1985; Petring &amp; Flachs, 1990; van Wyk et al., 1993; van Wyk et al., 1995)</td>
</tr>
<tr>
<td><strong>Scintigraphy</strong></td>
<td>Considered to be the „gold standard“. Use a gamma camera (often with anterior and posterior detectors) to show the passage of a radiolabelled meal through the stomach and into the duodenum, and to produce scintigraphic images of the counts per minute. % retention of meal can be calculated from the number of counts in the defined region of interest (the full stomach at completion of ingestion). Can be used for solid and liquid meals. Expensive, equipment in demand, involves radioactivity so repeated studies limited. Subject to correction for radioactive decay, downscatter for dual isotope studies and septal penetration, intestinal overlap, and movement of the meal towards the camera (need to use the geometric mean).</td>
<td>(Harvey et al., 1970; Heading et al., 1971; Meyer et al., 1976; Tothill et al., 1978; Meyer et al., 1983; Donovan &amp; Harding, 1986; Urbain &amp; Charleux, 1995; Harding &amp; Potgieter, 1998).</td>
</tr>
<tr>
<td><strong>Ultrasonography</strong></td>
<td>Non-invasive. Uses an ultrasound instrument and records gastric emptying on videotape. Can measure solid-liquid and liquid meal emptying. Gastric emptying time calculated by measuring the cross-section of the gastric antrum corresponding to the sagittal plane passing through the superior mesenteric vein (reference point) (time for the antral area to return to baseline). Compares well with scintigraphy.</td>
<td>(Takaoa et al., 1993; Cucchiara et al., 1995; Okamoto et al., 1997)</td>
</tr>
</tbody>
</table>
APPENDIX II  THE DUTCH EATING BEHAVIOUR QUESTIONNAIRE

Subject number __________________________ Date __________________________

Please answer each question by circling the most appropriate alternative.

1) If you have put on weight, do you eat less than you usually do?
   Never   Seldom   Sometimes   Often   Very often   Not relevant

2) Do you have a desire to eat when you are irritated?
   Never   Seldom   Sometimes   Often   Very often   Not relevant

3) If food tastes good to you, do you eat more than usual?
   Never   Seldom   Sometimes   Often   Very often

4) Do you try to eat less at mealtimes than you would like to eat?
   Never   Seldom   Sometimes   Often   Very often

5) Do you have a desire to eat when you have nothing to do?
   Never   Seldom   Sometimes   Often   Very often   Not relevant

6) Do you have a desire to eat when you are depressed or discouraged?
   Never   Seldom   Sometimes   Often   Very often   Not relevant

7) If food looks and smells good, do you eat more than usual?
   Never   Seldom   Sometimes   Often   Very often

8) How often do you refuse food or drink offered because you are concerned about your weight?
   Never   Seldom   Sometimes   Often   Very often

9) Do you have a desire to eat when you are lonely?
   Never   Seldom   Sometimes   Often   Very often   Not relevant

10) If you smell something delicious, do you have a desire to eat it?
    Never   Seldom   Sometimes   Often   Very often

11) Do you watch exactly what you eat?
    Never   Seldom   Sometimes   Often   Very often

12) Do you have a desire to eat when someone lets you down?
    Never   Seldom   Sometimes   Often   Very often   Not relevant

13) If you have something delicious to eat, do you eat it straight away?
    Never   Seldom   Sometimes   Often   Very often

14) Do you deliberately eat foods that are slimming?
    Never   Seldom   Sometimes   Often   Very often

15) Do you have a desire to eat when you are cross?
    Never   Seldom   Sometimes   Often   Very often

16) Do you have a desire to eat when you are expecting something unpleasant to happen?
    Never   Seldom   Sometimes   Often   Very often
17) If you walk past the baker, do you have a desire to buy something delicious?
   Never   Seldom   Sometimes   Often   Very often
18) When you have eaten too much, do you eat less than usual the following days?
   Never   Seldom   Sometimes   Often   Very often   Not relevant
19) Do you have a desire to eat when you are anxious, worried or tense?
   Never   Seldom   Sometimes   Often   Very often
20) If you walk past a snack bar or café, do you have a desire to buy something delicious?
   Never   Seldom   Sometimes   Often   Very often
21) Do you deliberately eat less in order not to become heavier?
   Never   Seldom   Sometimes   Often   Very often
22) Do you have a desire to eat when things are going against you, or when things have gone wrong?
   Never   Seldom   Sometimes   Often   Very often
23) If you see others eating, do you also have a desire to eat?
   Never   Seldom   Sometimes   Often   Very often
24) How often do you try not to eat between meals because you are watching your weight?
   Never   Seldom   Sometimes   Often   Very often
25) Do you have a desire to eat when you are frightened?
   Never   Seldom   Sometimes   Often   Very often   Not relevant
26) Can you resist eating delicious foods?
   Never   Seldom   Sometimes   Often   Very often
27) How often in the evening do you try not to eat because you are watching your weight?
   Never   Seldom   Sometimes   Often   Very often
28) Do you have a desire to eat when you are disappointed?
   Never   Seldom   Sometimes   Often   Very often   Not relevant
29) Do you eat more than usual when you see others eating?
   Never   Seldom   Sometimes   Often   Very often
30) Do you take your weight into account when you eat?
   Never   Seldom   Sometimes   Often   Very often
31) Do you have a desire to eat when you are emotionally upset?
   Never   Seldom   Sometimes   Often   Very often   Not relevant
32) When preparing a meal, are you inclined to eat something?
   Never   Seldom   Sometimes   Often   Very often
33) Do you have a desire to eat when you are bored or restless?
   Never   Seldom   Sometimes   Often   Very often
### AD LIBITUM TEST MEAL COMPOSITION

<table>
<thead>
<tr>
<th>Food</th>
<th>Amount served (g)</th>
<th>Energy density (kJ/g)</th>
<th>Macro nutrients (g per g food)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td>Turkey sandwiches OR</td>
<td>298</td>
<td>10</td>
<td>0.10</td>
</tr>
<tr>
<td>Ham sandwiches OR</td>
<td>298</td>
<td>11</td>
<td>0.10</td>
</tr>
<tr>
<td>Cheese sandwiches OR</td>
<td>285</td>
<td>13</td>
<td>0.12</td>
</tr>
<tr>
<td>Tuna sandwiches¹</td>
<td>310</td>
<td>10</td>
<td>0.13</td>
</tr>
<tr>
<td>Potato crisps</td>
<td>26</td>
<td>23</td>
<td>0.06</td>
</tr>
<tr>
<td>Sweet biscuits²</td>
<td>96</td>
<td>15-16</td>
<td>0.04-0.06</td>
</tr>
<tr>
<td>Cakes</td>
<td>107-140</td>
<td>17</td>
<td>0.03-0.07</td>
</tr>
<tr>
<td>“Thick and creamy”</td>
<td>200</td>
<td>4</td>
<td>0.03</td>
</tr>
<tr>
<td>fruit yoghurt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lettuce</td>
<td>50</td>
<td>&lt;1</td>
<td>0.01</td>
</tr>
<tr>
<td>Cucumber</td>
<td>84</td>
<td>&lt;1</td>
<td>0.01</td>
</tr>
<tr>
<td>Tomato</td>
<td>75</td>
<td>&lt;1</td>
<td>0.01</td>
</tr>
<tr>
<td>Mayonnaise³</td>
<td>-</td>
<td>30</td>
<td>0.01</td>
</tr>
<tr>
<td>English mustard</td>
<td>-</td>
<td>8</td>
<td>0.08</td>
</tr>
</tbody>
</table>

¹ Subjects had a choice of two kinds of sandwich.

² Subjects had a choice of type of biscuit, cake, and yoghurt.

³ Mayonnaise and English mustard were offered in jars with the rest of the food. The jars were weighed before and after the meal.
APPENDIX IV  LUNCH AND AD LIBUTUM TEST MEAL PREFERENCE
QUESTIONNAIRE

<table>
<thead>
<tr>
<th>ID number</th>
<th>Date</th>
<th>Time</th>
</tr>
</thead>
</table>

Standard lunch

As part of this study a standard lunch is provided in the Clinical Investigation Unit kitchen which you can come and eat at some time between 11.30 am and 1 pm. There is a choice of three lunches. Please tick whichever you prefer or cross out what you will not eat:

- Chicken pasta bake
- Cheese, pasta and tomato pasta bake
- Taglietelli carbonara
- Jelly

*Ad libitum* test meal

Please rank these sandwich fillings from 1 to 4 in terms of preference (with 1 being the one you most prefer and crossing out any you definitely do not like):

<table>
<thead>
<tr>
<th>Turkey</th>
<th>Tuna</th>
<th>Cheese</th>
<th>Ham</th>
</tr>
</thead>
</table>

Please cross out any salad items that you do not like:

- Lettuce
- Cucumber
- Tomato

Do you like ready salted crisps? If not please indicate which flavour you prefer.

Please rank these biscuits in terms of preference and cross out any you do not like:

<table>
<thead>
<tr>
<th>Lincoln</th>
<th>Tesco All Butter</th>
<th>Tesco Almond Shortie</th>
<th>Tesco All Butter Fruit</th>
<th>Rich Tea</th>
</tr>
</thead>
</table>

Please rank which yoghurt flavours you prefer or cross out what you do not like:

<table>
<thead>
<tr>
<th>Morello Cherry</th>
<th>Strawberry</th>
<th>Brazil pineapple</th>
<th>Williams pear</th>
<th>White peach</th>
</tr>
</thead>
</table>

Please rank which cakes you prefer or cross out what you do not like:

<table>
<thead>
<tr>
<th>Almond slice</th>
<th>Lemon slice</th>
<th>Mini rolls</th>
</tr>
</thead>
</table>

Thank you.
APPENDIX V  VISUAL ANALOGUE SCALES (VAS) USED TO MEASURE SUBJECTIVE APPETITE

ID number ______________  Date ______________  Time ______________

Answer the questions by placing a vertical mark through the line for each question. Mark the lines according to how you feel at this moment. Regard the ends of the lines as indicating the most extreme sensations you have ever felt.

How hungry do you feel?
Not at all hungry ──────────────────────── Extremely hungry

How strong is your desire to eat?
Not at all strong ──────────────────────── Extremely strong

How full do you feel?
Not at all full ──────────────────────── Extremely full

How thirsty do you feel?
Not at all thirsty ──────────────────────── Extremely thirsty
APPENDIX VI       FOOD PREFERENCE CHECKLIST

ID number __________________  Date __________________  Time ________________

Please indicate which of the food items below you would like to eat at this moment. Do this by placing a cross on the line next to the item or items. Consider each item in turn and independently from the other items — you are not being asked to construct a menu form these foods. What we are interested in is, if you were presented with the item, would you like to eat it now. Therefore, you might mark some of the items, none of them or all of them.

____   A medium sized roast chicken breast
____   A currant bun
____   A small pancake (sweet)
____   A medium sized peach
____   A small packet of roasted, salted peanuts
____   A large grilled cod fillet (no batter)
____   A large tomato
____   A small baked potato
____   A small slice of rich cheesecake
____   A small slice of apple pie
____   A small gammon steak
____   A medium portion of boiled rice
____   A slice of melon
____   A plain omelette (2 eggs)
____   Fruit yoghurt (thick and creamy type, 150 g pot)
____   A large bread roll
____   A large portion of prawns
____   A medium chunk of cheddar cheese
____   A medium sized apple
____   A plain croissant
____   A large portion of tuna (canned in brine)
____   A medium sized bowl of cornflakes with semi-skimmed milk and sugar
____   A small cream-filled chocolate éclair
____   A large raw carrot (peeled)
____   A cheese scone
____   Two slices of salami
____   Grilled, lean beef rump steak (5oz)
____   A green salad (no dressing)
____   A large banana
____   Three grilled fish fingers (coated in breadcrumbs)

Other(s)  ________________________________________________________________
APPENDIX VII  DERIVATISATION PROCEDURE FOR PLASMA AMINO ACID ANALYSIS USING WATERS PICO-TAG SYSTEM FOR THE HPLC

Solutions to be made up:

A. Drying solution

200 µl Methanol
200 µl Sodium acetate (1M)
100 µl Triethylamine (TEA)

B. Derivatising agent (with PITC)

20 µl PITC
140 µl Methanol
20 µl TEA
20 µl Water

PROCEDURE:

1. Vacuum dry sample/standard (20 µl) using the Pico-Tag work station
2. Add drying solution (10 µl), vortex and vacuum dry again
3. Coupling step: derivatise by adding derivatising agent (20 µl), vortex, leave for 10 min at room temperature and vacuum dry again
4. Add methanol (10 µl), vortex and dry thoroughly
5. Reconstitute in 100 µl eluent A (70 mM sodium acetate trihydrate, pH 6.45 + 2.5 % acetonitrile) and inject 20 µl.

NB. Allow PITC to come to room temperature before opening to prevent condensation.
TEA should be blanketed with nitrogen gas and stored in the refrigerator for a maximum of 3 months.
APPENDIX VIII

EXAMPLE OF A STANDARD AND PLASMA AMINO ACID SAMPLE CHROMATOGRAPH USING WATERS PICO-TAG METHODOLOGY FOR THE HPLC

[Graph showing chromatograms with labeled peaks for various amino acids, including ASP, SER, GLY, TRP, VAL, ILE, LEU, NLE, PRE, TRP, and others. The chromatograms are labeled as 'Standard solution' and 'Plasma sample'.]
### APPENDIX IX

**REPEATABILITY OF APPETITE VAS RATINGS WITH REPEATED IDENTICAL LIQUID TEST MEALS**

<table>
<thead>
<tr>
<th>BASELINE</th>
<th>1st and 2nd study session</th>
<th>1st and 3rd study session</th>
<th>2nd and 3rd study session</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean difference (CR)</td>
<td>R²</td>
<td>Mean difference (CR)</td>
</tr>
<tr>
<td>Hunger (cm)</td>
<td>0.28 (2.55)</td>
<td>0.42**</td>
<td>0.25 (3.90)</td>
</tr>
<tr>
<td>Desire to eat (cm)</td>
<td>0.16 (2.20)</td>
<td>0.60**</td>
<td>0.28 (4.15)</td>
</tr>
<tr>
<td>Fullness (cm)</td>
<td>-0.69 (3.50)</td>
<td>0.23*</td>
<td>-0.52 (3.84)</td>
</tr>
</tbody>
</table>

Differences in baseline VAS ratings following repeated identical 1992 KJ liquid preloads on three occasions. CR: coefficient of repeatability = 2 x SD of mean differences. *p <0.05, **p <0.01.

<table>
<thead>
<tr>
<th>MEAN 0-60 min</th>
<th>1st and 2nd study session</th>
<th>1st and 3rd study session</th>
<th>2nd and 3rd study session</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean difference (CR)</td>
<td>R²</td>
<td>Mean difference (CR)</td>
</tr>
<tr>
<td>Hunger (cm)</td>
<td>0.01 (2.29)</td>
<td>0.64**</td>
<td>-0.02 (3.11)</td>
</tr>
<tr>
<td>Desire to eat (cm)</td>
<td>0.02 (1.81)</td>
<td>0.78**</td>
<td>0.02 (3.28)</td>
</tr>
<tr>
<td>Fullness (cm)</td>
<td>0.05 (2.25)</td>
<td>0.68**</td>
<td>0.33 (3.05)</td>
</tr>
</tbody>
</table>

Differences in post-preload VAS ratings following repeated identical 1992 kJ preloads on three occasions. CR: coefficient of repeatability = 2 x SD of mean differences. *p <0.05, **p <0.01.

<table>
<thead>
<tr>
<th>POST AD LIBUTUM-TEST MEAL</th>
<th>1st and 2nd study session</th>
<th>1st and 3rd study session</th>
<th>2nd and 3rd study session</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean difference (CR)</td>
<td>R²</td>
<td>Mean difference (CR)</td>
</tr>
<tr>
<td>Hunger (cm)</td>
<td>0.02 (3.32)</td>
<td>0.00</td>
<td>0.24 (2.48)</td>
</tr>
<tr>
<td>Desire to eat (cm)</td>
<td>0.40 (3.32)</td>
<td>0.00</td>
<td>0.41 (2.10)</td>
</tr>
<tr>
<td>Fullness (cm)</td>
<td>-0.54 (2.92)</td>
<td>0.04</td>
<td>-0.43 (3.61)</td>
</tr>
</tbody>
</table>

Differences in average post-ad libitum test meal VAS ratings following repeated identical 1992 KJ preloads on three occasions. CR: coefficient of repeatability = 2 x SD of mean differences. **p <0.01.
## APPENDIX X

**EXAMPLE OF MICROSOFT EXCEL WORKSHEET USED TO CALCULATE STANDARD LUNCH PORTIONS TO PROVIDE 42 KJ PER KG BODY WEIGHT**

<table>
<thead>
<tr>
<th>Food</th>
<th>Portion</th>
<th>Amount to give</th>
<th>Amount (g)</th>
<th>Protein (g)</th>
<th>Fat (g)</th>
<th>CHO (g)</th>
<th>Weight (g)</th>
<th>Amount to provide 42 kJ in total</th>
<th>42 kJ</th>
<th>Body weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkey Sandwich</td>
<td>quarter</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0</td>
<td>0.06667</td>
<td>0</td>
<td>66</td>
</tr>
<tr>
<td>Ham Sandwich</td>
<td>quarter</td>
<td>4.40022</td>
<td>1351.194124</td>
<td>12.9</td>
<td>11.9</td>
<td>41.1</td>
<td>130.907</td>
<td>0.08667</td>
<td>20.4726</td>
<td></td>
</tr>
<tr>
<td>Almond Slice</td>
<td>per half slice</td>
<td>0</td>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0</td>
<td>0.032</td>
<td>0.0</td>
<td>9.4</td>
</tr>
<tr>
<td>Lemon Slice</td>
<td>per half slice</td>
<td>2.112</td>
<td>618.56256</td>
<td>1.2</td>
<td>5.2</td>
<td>18.6</td>
<td>37.3824</td>
<td>0.053</td>
<td>0.32635</td>
<td></td>
</tr>
<tr>
<td>Lettuce</td>
<td>per gram</td>
<td>39.6</td>
<td>21.539232</td>
<td>0.3</td>
<td>0.1</td>
<td>0.8</td>
<td>39.6</td>
<td>0.6</td>
<td>0.32635</td>
<td></td>
</tr>
<tr>
<td>yoghurt</td>
<td>per gram</td>
<td>198</td>
<td>828.432</td>
<td>6.1</td>
<td>5.5</td>
<td>31.1</td>
<td>198</td>
<td>3</td>
<td>12.552</td>
<td></td>
</tr>
</tbody>
</table>

**Totals**

<table>
<thead>
<tr>
<th>Protein (% energy)</th>
<th>Fat (% energy)</th>
<th>CHO (% energy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.0</td>
<td>31.0</td>
<td>55.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Food</th>
<th>Portion</th>
<th>Amount to give</th>
<th>Bread (g)</th>
<th>Marg (g)</th>
<th>Filling (g)</th>
<th>Cake (g)</th>
<th>Lettuce (g)</th>
<th>Yoghurt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkey Sandwich</td>
<td>quarter</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ham Sandwich</td>
<td>quarter</td>
<td>4.40022</td>
<td>81</td>
<td>11</td>
<td>39</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Almond Slice</td>
<td>per half slice</td>
<td>0</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lemon Slice</td>
<td>per half slice</td>
<td>2.112</td>
<td></td>
<td></td>
<td>37</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lettuce</td>
<td>per gram</td>
<td>39.6</td>
<td></td>
<td></td>
<td>37</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>yoghurt</td>
<td>per gram</td>
<td>198</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**TOTAL WEIGHT (g)**

<table>
<thead>
<tr>
<th>Bread</th>
<th>Marg</th>
<th>Filling</th>
<th>Cake</th>
<th>Lettuce</th>
<th>Yoghurt</th>
</tr>
</thead>
<tbody>
<tr>
<td>81</td>
<td>11</td>
<td>39</td>
<td>37</td>
<td>40</td>
<td>198</td>
</tr>
</tbody>
</table>
### COMPOSITION OF WHEY AND CASEIN PROTEIN LIQUID TEST MEALS

<table>
<thead>
<tr>
<th>Per 100g of formula</th>
<th>WHEY</th>
<th>CASEIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kJ)</td>
<td>1727</td>
<td>1498</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>76.2</td>
<td>85.0</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>8.73</td>
<td>1.2</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>4.76</td>
<td>1.5</td>
</tr>
<tr>
<td>Amino acids (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>4.8</td>
<td>3.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.3</td>
<td>3.8</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>10.2</td>
<td>7.3</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>17.2</td>
<td>22.3</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>8.4</td>
<td>5.8</td>
</tr>
<tr>
<td>Leucine</td>
<td>10.5</td>
<td>10.1</td>
</tr>
<tr>
<td>Lysine</td>
<td>9.1</td>
<td>8.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.6</td>
<td>3.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.1</td>
<td>5.4</td>
</tr>
<tr>
<td>Proline</td>
<td>6.1</td>
<td>10.5</td>
</tr>
<tr>
<td>Serine</td>
<td>5.2</td>
<td>6.3</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.2</td>
<td>4.6</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2.1</td>
<td>1.4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.4</td>
<td>5.8</td>
</tr>
<tr>
<td>Valine</td>
<td>6.0</td>
<td>7.4</td>
</tr>
</tbody>
</table>
APPENDIX XII

POWER CALCULATIONS USING THE DATA FROM THE
REPEATABILITY STUDY FOR AD LIBITUM FOOD
INTAKE AND VAS RATINGS

<table>
<thead>
<tr>
<th>Detectable difference (mm):</th>
<th>Fasting</th>
<th>Post-preload (1 h mean)</th>
<th>Post-ad libitum test meal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Hunger</td>
<td>34</td>
<td>14</td>
<td>27</td>
</tr>
<tr>
<td>Desire to eat</td>
<td>25</td>
<td>11</td>
<td>43</td>
</tr>
<tr>
<td>Fullness</td>
<td>33</td>
<td>14</td>
<td>45</td>
</tr>
</tbody>
</table>

Power calculations for visual analogue scales (VAS). Number of subjects needed to show the detectable difference of appetite scores with a power of 0.8 (p <0.05) using a repeated measures design. Calculations based on data from Chapter 2, “Reproducibility of satiety methodology: the measurement of food intake using ad libitum test meals and visual analogue scales for the subjective assessment of appetite” (section 2.3), n = 18.

<table>
<thead>
<tr>
<th>Detectable difference (kJ):</th>
<th>418</th>
<th>628</th>
<th>837</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy intake</td>
<td>73</td>
<td>32</td>
<td>18</td>
</tr>
</tbody>
</table>

Power calculations for energy intake at an ad libitum test meal. Number of subjects needed to show the detectable difference of energy intake with a power of 0.8 (p <0.05) using a repeated measures design. Calculations based on data from Chapter 2, “Reproducibility of satiety methodology: the measurement of food intake using ad libitum test meals and visual analogue scales for the subjective assessment of appetite” (section 2.3), n = 18.