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INVESTIGATIONS INTO THE GASTROINTESTINAL CONTROL OF APPETITE AND FOOD INTAKE

A thesis submitted by

Rosalie Vozzo

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Department of Medicine

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intravenous infusion of NO synthase inhibitor NG-nitro-L-arginine methyl ester and intraduodenal infusion of lipid

LIST OF ABBREVIATIONS

5-HT	5-hydroxytriptamine
AN	anorexia nervosa
AVONA	analysis of variance
APD	antropyloroduodenal
ARC	arcuate nucleus
BMI	body mass index
BP	blood pressure
CCK	cholecystokinin
CHO	carbohydrate
CRH	corticotropin releasing hormone
cGMP	cyclic guanidine monophosphate
CNS	central nervous system
DIT	diet-induced thermogenesis
DMN	dorsomedial nucleus
DNA	deoxyribonucleic acid
ENS	enteric nervous system
FBG	fasting blood glucose
FFA	free fatty acid
GI	gastrointestinal
GIP	gastric inhibitory peptide
GLP-1	glucagon-like peptide-1
GTN	glyceryl trinitrate
GTP	guanosine triphosphate
HbA_{1C}	glycosylated haemoglobin
HC	high carbohydrate
HCl	hydrochloric acid
HF	high fat
HF / HP	high fat / high protein
HP	high protein
IPPW	isolated pyloric pressure wave
LiCl	lithium chloride
LH	lateral hypothalamus
L-NAME	NG-nitro-L-arginine methyl ester

L-NMMA	NG-monomethyl-L-arginine
L-NO-Arg	nitro-L-arginine
MONICA	Monitoring trends and determinants in cardiovascular diseases
mRNA	messenger ribonucleic acid
N	no pre-load
NANC	non-adrenergic non-cholinergic
NHANES	National Health and Nutrition Survey
NO	nitric oxide
NOS	nitric oxide synthase
NPY	neuropeptide Y
PFH	perifornical hypothalamus
PROT	protein
PVN	paraventricular nucleus
PYY	peptide YY
SCN	suprachaismatic nucleus
SD	standard deviation
sGC	soluble guanylyl cyclase
SGLT-1	sodium glucose transporter
S. crassicaudata	*Sminthopsis crassicaudata*
TEE	total energy expenditure
TG	triglyceride
T-LNAA	tryptophan-large amino acid
TMPD	transmucosal potential difference
TRH	thyrotropin releasing hormone
VAS	visual analogue scale
VMN	ventromedial nucleus
WHO	World Health Organisation

THESIS SUMMARY

This thesis presents studies relating to the gastrointestinal regulation of appetite and food intake. The two broad areas that have been have been investigated in these studies are 1) the specific effects mediated by different nutrients present in the gastrointestinal tract and 2) the involvement of nitric oxide mechanisms in the peripheral regulation of appetite and food intake. These topics were primarily evaluated in healthy young adult humans, but also in patients with type 2 diabetes.

Obesity is an increasingly prevalent disease the causes of which relate in part to the constant and readily available supply of high fat, energy dense foods. Common dietary approaches to its treatment include a low energy, low-fat, high-carbohydrate diet; however diets with increased protein are becoming increasingly popular. The ability of high protein, rather than carbohydrate and fat pre-loads to produce greater satiety and reduced food intake after a fixed time interval and under spontaneous feeding conditions was investigated in healthy humans. Hunger decreased and fullness increased after both high carbohydrate and high protein pre-loads, relative to no pre-load. Although all nutrient pre-loads delayed the first food request, there was no effect of varying macronutrient ratios on this delay, or on the daily eating frequency. Compensation for over consumption was accurate following high protein but not following high fat or high carbohydrate pre-load, hence total daily food intake was greater after high carbohydrate and high fat pre-loads. These results indicate that the effect of increasing the protein content of the diet is probably to increase satiety and to induce a relative suppression of energy intake at subsequent meals.

Included in the many treatments for overweight and obesity are modifications to specific eating patterns. An inverse relationship has been demonstrated between the number of meals consumed per day and the general state of health. The effects of increased meal frequency on suppression of appetite and food intake were evaluated in healthy humans. Mixed-nutrient meals ingested or infused intragastrically in different frequencies had no significant effect on blood glucose concentrations, on hunger, desire to eat, fullness and satiation, or on *ad libitum* food intake. These findings do not support the promotion of increased meal frequency as a means of reducing food intake.

The interaction between nutrients and mucosal chemoreceptors in the small intestine plays a major role in the regulation of both gastric emptying and appetite. The contribution of the pulsatile nature of gastric emptying to small intestinal feedback mechanisms modulating antropyloroduodenal motility, gastrointestinal hormone release and food intake is unknown. The effects of intraduodenal infusions of a triglyceride mixture either continuously or in a pulsatile fashion on antropyloroduodenal motility, cholecystokinin release and appetite and food intake were evaluated in healthy humans. The two modes of lipid infusion had similar effects on antropyloroduodenal pressures, plasma cholecystokinin concentrations, hunger and fullness ratings and energy intake. These results indicate that the acute effects of intraduodenal lipid on antropyloroduodenal pressures, plasma cholecystokinin concentrations and appetite are not modified by a pulsatile mode of lipid delivery into the duodenum. The pulsatile nature of gastric emptying is therefore unlikely to contribute to any major extent to the nutrient-induced changes in antorplyoroduodenal motility, but hormone release, appetite and food intake.

It is predicted that by 2010 approximately 1.5 million Australians will be affected by non-insulin dependent (type 2) diabetes mellitus. The primary aim of treatment is to reduce blood glucose concentrations, and in the case of many people with type 2 diabetics, who are overweight, to reduce body weight. Fructose has been proposed as an alternative sweetener to glucose in the diet of type 2 diabetics. The relative effects of fructose and glucose on blood glucose, plasma insulin and incretin (glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP)) concentrations, and acute food intake were investigated in patients with diet controlled type 2 diabetes and in non-diabetic, control subjects. Fructose produced smaller post-ingestion blood glucose concentrations than glucose and higher insulin concentrations in diabetics than in non-diabetics. The differences in insulin concentrations were not accounted for by increased incretin (GLP-1 and GIP) concentrations. There was no difference between the effects of fructose and glucose on suppression of food intake in either diabetics or non-diabetics. These results indicate that on the basis of satiating efficiency alone, fructose is unlikely to be useful as a replacement for glucose in the diet of obese patients with type 2 diabetes.

Complex peripheral and central pathways exist, in which a variety of neurotransmitters integrate multiple factors that regulate appetite and food intake. The inhibitory neurotransmitter, nitric oxide (NO) has emerged as a potential regulator of numerous

processes which affect feeding behaviour, including gastrointestinal transit and motility, as well as central and peripheral neural pathways implicated in the control of food intake. Nitric oxide synthase inhibitors reduce food intake in rodents and chickens. The involvement of NO in regulating appetite and food intake pre- and post-pradially was assessed in healthy humans and in an animal model of feeding regulation.

No previous studies have evaluated the possibility that NO regulates appetite and feeding behaviour in humans. NG-nitro-L-monomethyl arginine (L-NMMA) and NG-nitro-L-arginine methyl ester (L-NAME), non-selective inhibitors of NO synthase (NOS), were administered intravenously in two separate studies, to evaluate the role of NO in the short-term regulation of appetite in healthy subjects. Neither drug had any effect on energy intake or sensations of hunger or fullness. Consistent with a systemic effect both L-NMMA and L-NAME decreased heart rate and blood pressure. It is unlikely that peripheral NO has a role in the regulation of normal human appetite and food intake.

To evaluate the role of NO mechanisms in mediating the effects of small intestinal nutrients on antropyloroduodenal motility and appetite in healthy humans, intravenous L-NAME was administered prior to and concurrent with intraduodenal lipid infusion. NG-nitro-L-arginine methyl ester (L-NAME) increased diastolic blood pressure, decreased heart rate and had no effect on antropyloroduodenal pressures or food intake. Intravenous administration of the systemic NO synthase inhibitor L-NAME, in a dose that affects cardiovascular function in humans, does not modify the antropyloroduodenal motor and appetite responses to intraduodenal lipid infusion. Despite having significant effects on cardiovascular function in the doses used, neither L-NMMA nor L-NAME, had any effect on feeding or appetite and antropyloroduodenal motor responses to intraduodenal lipid. These results suggest that NO does not affect short-term appetite, food intake or antropyloroduodenal motor function in humans.

The studies reported in this thesis provide new information on the regulation of appetite food intake by gastrointestinal mechanisms in healthy and type 2 diabetics humans. These observations will contribute to advances in basic appetite physiology and clinically to dietary interventions in the treatment of obesity and type 2 diabetes mellitus.

STATEMENT OF ORIGINALITY

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution (unless otherwise indicated) and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

Part of the study described in Chapter 7 was submitted towards an Honours degree by J Mudge, University of Adelaide, (2000). This study was the forerunner to the study described in Chapter 8, and inclusion of this work in this thesis was necessary. Similarly, part of the work (using L-NMMA) described in Chapter 12 was submitted as towards the candidates own Honours degree, University of Adelaide, (1997). It was logical to include it in this thesis, as results from both this, and the follow up study (using L-NAME) were used together to draw the conclusions described in Chapter 12.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Rosalie Vozzo

June 2002

DEDICATION

I dedicate this thesis to my dear parents Virginia and Cosimo Vozzo.

For your unconditional and unfailing support, I am forever grateful.

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The following individuals and organisations made the studies in this thesis possible and made my years as a postgraduate student a valuable and rewarding learning experience.

All of the studies reported in this thesis were conducted in the Department of Medicine and Gastrointestinal Investigation Unit, Royal Adelaide Hospital (1997-2001).

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The following co-investigators provided technical support on the studies indicated:

Ms Jane Mudge (Department of Medicine, Royal Adelaide Hospital) conducted much of the work reported in Chapter 7 and submitted this work as part of her Honours thesis (University of Adelaide, 2000).

Prof Charles Malbert (Institut National de la Recherche Agronomique, France) wrote the software used to regulate the timing of intraduodenal infusions in Chapter 10.

Prof Arthur Shulkes (Department of Surgery, University of Melbourne) conducted the cholecystokinin assay reported in Chapter 10.

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Mrs Selena Doran and Dr Yu-Chung Su (Department of Medicine, Royal Adelaide Hospital) co-conducted the work reported in Chapter 14.

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PUBLICATIONS

The material in this thesis formed the basis for the publications listed below:

Vozzo R, Wittert G, Cocchiaro C, Tan, W C, Fraser R, Chapman, I. High protein, high carbohydrate and high fat yoghurt pre-loads and their effect on subsequent food intake in subjects who are free to choose when as well as how much they eat. *Appetite* (Submitted).

Mudge J, Vozzo R, Luscombe N, Clifton P, Chapman I, Wittert G. The effects of increased dietary protein on satiation, thermogenesis, and metabolism are attenuated by the addition of fat. *Appetite* (Submitted).

Vozzo R, Baker B, Wittert G, Wishart J, Morris H, Horowitz M, Chapman I. Glycemic, hormone and appetite responses to monosaccharide ingestion in patients with type 2 diabetes. *Metabolism* (Accepted for publication).

Vozzo R, Su Y-C, Fraser R, Wittert G, Horowitz M, Malbert C, Shulkes A, Vollombello T, Chapman I. Antropyloroduodenal, cholecystokinin and feeding responses to pulsatile and non-pulsatile intraduodenal lipid infusion. *J Neurogastroenterol and Motility* 14; 25-33, 2002.

Su Y-C, Vozzo R, Doran S, Leelakusolvong S, Rayner C, Chapman I, Jones K, Horowitz M. Effects of the nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME) on antropyloroduodenal motility and appetite in response to intraduodenal lipid infusion in humans. *Scand J Gastroenterol* 36(9); 948-54, 2001.

Vozzo R, Wittert G, Horowitz M, Morley J, Chapman I. Effect of nitric oxide synthase inhibitors on short-term appetite and food intake in humans. *Am J Physiol* 276; R1562-8, 1999.

Vozzo R, Wittert G, Chapman I, Fraser R, Hope P, Horowitz M, Alshaher M, Kumar V, Morley J. Evidence that nitric oxide stimulates feeding in the marsupial *Sminthopsis crassicaudata*. *Comp Physiol Biochem* 123; 145-51, 1999.

Chapter 1

APPETITE AND DISEASE

1.1 INTRODUCTION

The regulation of appetite and food intake is highly complex and very variable. Humans eat for a variety of reasons, including to meet energy requirements, for cultural purposes, time of day, social setting, stress, boredom, palatability / reward, and food availability. Furthermore, the characteristics of an individual meal are altered not only by external factors but also by endogenous influences determined by the nature and quantity of nutrients already present in the gastrointestinal tract, and nutrients stored in other locations (Campfield and Smith, 1998a; Morley, 1987; Morley, 1990b; Read *et al.*, 1994). Consequently, energy intake at any given meal is highly variable both between and within individuals. The body's response to different food types and patterns of exposure and their interaction with subsequent food intake plays a pivotal role in regulating mammalian feeding behavior, and accordingly body weight. A chronic imbalance between the types and quantities of nutrients consumed and the utilization of those nutrients by the body ultimately will lead to significant and possibly critical weight gain or loss.

The studies reported in this thesis deal with the control of appetite, which is clinically significant due to the high prevalence of appetite related disorders. This chapter briefly reviews the more common appetite-related disorders present in the adult populations of developed countries. Obesity, anorexia nervosa and the 'anorexia of ageing' are discussed in the context of impaired appetite and food intake regulation. Epidemiological data related to recent trends in prevalence are reviewed, together the relationship between feeding behaviour and the development of these diseases, and the medical complications associated with obesity, such as type 2 diabetes.

1.2 OBESITY

Obesity is a condition associated with excess body fat. The most commonly used assessment of overweight and obesity is the body mass index (BMI); weight (kg) divided

by the square of the height (m). Individuals with a BMI less than 19 kg / m^2 are considered underweight, 19 - 25 kg / m^2 healthy weight, and 25 - 30 kg / m^2 overweight. Individuals over 30 kg / m^2 are termed clinically obese (National Task Force on the Prevention and Treatment of Obesity, 2000). However, this measurement reflects body size not fatness, as BMI does not allow one to distinguish between lean mass and fat mass, although the two are closely related, except at the extremes of body weight. Nor does it give any information on the distribution of fat around the body. This is particularly important in relation to diseases associated with increased body fat mass where the distribution of fat is important. For example, abdominal fat, particularly visceral fat, has a greater detrimental effect on health, than non-abdominal (eg. lower body) fat. Waist circumference measures, skin-fold thickness and bioelectrical impedance and other measures of body composition (eg. DEXA, hydrostatic weighing, CT scan) all provide this additional information.

Obesity is a medical problem affecting a significant proportion of the global population (Kopelman, 2000). Paradoxically, it now co-exists with under nutrition as an escalating global epidemic (Section 1.3). Increasingly obesity affects virtually all age and socioeconomic groups in both developed and developing countries. It is now so common that it may be the most significant contributor to all ill health, principally because it both predisposes to and exacerbates many other diseases. In particular it is a major factor in the development of diabetes mellitus, coronary artery disease, some forms of cancer, arthritis and sleep-apnoea disorders (Despres, 1993; Despres, 1994; Kelley, 1998). Obesity has a multifactorial nature with genetic background, environmental factors, energy intake and expenditure, culture and foetal nutrition among the factors central to its development (Kopelman, 2000).

1.2.1 Epidemiology

The WHO Monitoring trends and determinants in cardiovascular diseases (MONICA) study provided a comprehensive worldwide assessment of overweight and obesity during the period 1983-1986 (Molaris *et al.*, 1999). The BMI distributions in 48 MONICA populations for men and women aged 35-64 y were compared. Data show that in all but one male population and in the majority of female populations, between 50% and 75% of the adults surveyed were either overweight or obese. In almost one third of the female populations, over 75% of individuals were overweight. Further, between the years 1983 and 1986, 15% of adult men and 22% of adult women surveyed were obese (Molaris *et al.*,

1999). In Australia, an investigation performed as part of a National Heart Foundation's Trends in Cardiovascular Risk Factors in Australia revealed that amongst adults (25-64 y) 55.6% of males and 38.3% of females were overweight or obese in 1989 (Bennett and Magnus, 1994).

Perhaps the most disturbing characteristic of this obesity epidemic is it's increasing prevalence. Between the years 1980-1989, the proportion of overweight or obese Australian males rose 12%, and females rose 35% (Bennett and Magnus, 1994). Between 1976-1980 and 1988-1994, the prevalence of overweight increased from 46% to 54% in the United States (Flegal *et al.*, 1998). During the same period, the prevalence of obesity increased from 14.5% to 22.5% (Flegal *et al.*, 1998). Extrapolations suggest that if current trends continue, between 25% and 50% of the adult population in countries including Australia, England, Mauritius, Brazil and the USA will be obese by 2025 (Kopelman, 2000).

In countries where obesity has customarily been uncommon, its incidence is now also increasing. Thus, in developing countries such as in Africa, rural adults with a traditional lifestyle showed no or little gain in body weight until the mid 1980's (WHO, 1997). However, the rise in socioeconomic status and rapid urbanization of these populations has been associated with rapid rises in the prevalence of obesity, especially in women (WHO, 1997). Although Japanese populations have maintained relatively low obesity rates, in males there has been a doubling of the prevalence of obesity from 0.7% of adult males over the age of 20 years in 1976, to 1.8% in 1993 (Hodge *et al.*, 1995).

1.2.2 Etiology of overweight and obesity

Numerous factors regulate body weight and hence potentially contribute to the development of the overweight state and obesity. Specific areas included in this discussion are the interactions between genetic, environmental and psychosocial factors acting through the physiological mediators of energy intake and expenditure.

1.2.2.1 Genetic contribution to weight disturbance

The precise effect of genetic influences on weight control is unclear. Epidemiological, genetic and molecular studies have shown that, independent of lifestyle or environmental conditions, some individuals are more susceptible to obesity than others. In support of a

significant genetic contribution to the regulation of body weight is the finding that longer-term dietary interventions affecting body weight, show individuals tend to return the body to their initial weight (Leibel *et al.*, 1995). Changes in involuntary energy expenditure probably exist to serve as a homeostatic mechanism to limit weight loss or gain. This infers the existence of a 'set-point' for body weight, where significant deviations from the genetically pre-determined body weight are strongly resisted. Whether a similar process applies to food intake is unknown.

Also supporting a role for the heritability of obesity is the finding that obesity tends to run in families (eg. obese children usually have obese parents (Rice *et al.*, 1999)), although the contributors to this finding may be a combination of environmental, cultural, and genetic factors. The heritability estimates for BMI are in the range from 25% to 40% (Faith *et al.*, 1999; Pietilainen *et al.*, 1999). Fat distribution, on the other hand, shows a significant heritability level in the order of about 50% of the total human variation (Bouchard, 1997). The concept of genetic susceptibility is best highlighted by results from twin studies. Identical twins exposed to periods of positive or negative energy balance, showed greater similarity within pairs than between pairs in the differences in the rate of weight gain (Bouchard, 1996), suggesting that genetic susceptibility predicts those, who under certain environmental circumstances, will develop obesity. Apart from very rare obesity-associated disorders in humans (Section 1.2.2.2), the genotype of an individual alone does not appear to determine the development of obesity.

Against a major role for genetic causes in the development of obesity is the rapid rate at which the prevalence of obesity has risen in populations over recent years. It is likely that genetic factors operate mainly through susceptibility genes, which increase the risk of developing obesity, but are not essential for its development. The candidate genes for susceptibility to obesity relate to those affecting body composition (i.e. anatomical distribution of fat), food intake and energy expenditure.

Specific genetic abnormalities causing obesity -leptin

A number of rare monogenic causes of obesity, account, for only a tiny minority of all obese individuals, of these, genetic leptin-deficiency is the best characterized. Leptin is the protein product of the *ob* gene. It is expressed primarily, but not exclusively, in adipose tissue in proportion to the size of adipose tissue stores. The discovery of the *ob* gene, in

1994 (Zhang *et al.*, 1994) renewed interest in the possibility of a single gene underlying obesity. Individuals with extreme, early-onset obesity due to congenital leptin deficiency (Montague *et al.*, 1997) respond predictably to exogenous leptin administration. Subcutaneous leptin administration in a child with severe obesity produced significant weight loss in the absence of altered energy expenditure, indicating that a suppressive effect on food intake mediates the effect of leptin to reduce body weight (Farooqi *et al.*, 1999). These data are consistent with animal data; mice homozygous for the *ob* mutation are overweight, have larger body fat stores, and are hyperphagic and hyperinsulinaemic. Peripheral leptin administration reverses these abnormalities, and intracerebroventricular leptin administration effectively reduces weight, indicative of a centrally mediated effect, (Campfield *et al.*, 1998b). In fact, circulating leptin levels are elevated in obese individuals, indicative of a leptin-resistant state. It is possible that the leptin gene is important in cases of extreme obesity (Clement *et al.*, 1996; Reed *et al.*, 1996), but unlikely that this gene is responsible for the majority of obesity cases.

1.2.2.2 Energy expenditure

Changes in energy expenditure are an important means of controlling weight. Total energy expenditure (TEE) includes three components: basal metabolic rate (~60%), diet-induced thermogenesis (~10%) and energy used for physical activity (highly variable ~20-50%). The first two of these components are largely involuntary and regulated by a combination of genetic and environmental factors such as physical activity level and dietary intake. Physical activity is voluntary, and therefore a major target in the treatment of obesity. There is very little evidence to suggest that changes in dietary intake have major effects on either basal metabolic rate or diet-induced thermogenesis.

Metabolic rate

There is a strong association between low metabolic rate and an increased relative risk of gaining weight. Surprisingly though, obesity is associated with an increased absolute basal and 24 h metabolic rate (Leibel *et al.*, 1995; Ravussin *et al.*, 1982). In studies utilizing the double-labelled water technique to measure TEE in free-living subjects, the TEE was elevated in obese compared to lean individuals (Leibel *et al.*, 1995). When the specific basal energy needs of obese individuals were assessed using a respiration chamber (continuous measurement of gaseous exchange) they were reportedly higher than those of lean individuals (Ravussin *et al.*, 1982), indicating that the basal energy needs of obese

individuals are greater than those of lean persons. However, when adjusted for fat-free mass, lean and obese individuals have similar basal metabolic rates (Ravussin *et al.*, 1982). In a prospective study in 5-year-old girls, energy expenditure correlated negatively with subsequent BMI at adolescence (Griffiths *et al.*, 1990). In adult Pima Indians, a low relative metabolic rate (resting and 24 h) adjusted for differences in fat-free mass, age and gender was a risk for body weight gain (Ravussin *et al.*, 1988). Obesity therefore cannot be attributed to a low absolute metabolic rate as is often proposed, but it is possible that decreased energy expenditure per unit lean body mass may predispose to the development of obesity in some individuals. Whilst a low metabolic rate does not necessarily cause persons to become overweight, in the appropriate environment, including low physical activity and abnormal regulation of dietary intake, it is likely to be a major contributing factor.

Diet-induced thermogenesis

The conversion of energy to heat is termed thermogenesis (which has two components, obligatory and facultative) (Rothwell and Stock, 1979). The basal metabolic rate accounts largely for obligatory thermogenesis, whereas facultative thermogenesis consists of non-shivering thermogenesis and diet-induced thermogenesis (DIT). Non-shivering thermogenesis is activated to maintain body temperature in response to a cold environment, and involves the generation of heat without muscular activity (Rothwell and Stock, 1979). Diet-induced thermogenesis (thermic effect of feeding) is the elevation in metabolic rate that occurs after food intake (Dulloo and Girardier, 1989). In non-human mammals it is believed to allow the dissipation of excess calories as heat, thereby resisting obesity (Landsberg and Young, 1993). In rodents, access to a highly palatable, high-fat diet stimulates dietary-induced thermogenesis (DIT) in brown adipose tissue (Rothwell and Stock, 1979). This does not appear to be the case in humans, where the thermogenic response to excess energy consumption is limited to about 25% of the energy consumed (Ravussin *et al.*, 1985). This implies that the remaining 75% of the excess energy consumed is stored. The reason for the apparent threshold for DIT in humans is unclear, although in man brown adipose tissue is non-functional. Obese subjects have a blunted diet-induced thermogenesis compared with lean subjects, although the precise clinical importance of this finding is uncertain (Segal *et al.*, 1992).

Role of physical activity

Physical activity is the most variable component of TEE and represents 20-50% of energy expenditure. The importance of activity in weight control is demonstrated by, 1) the well recognized phenomena that a combination of physical activity and energy restriction is more effective for weight loss (and possibly maintenance of weight loss) than either alone, 2) physical activity affects body composition favorably during weight loss by preserving or increasing lean mass while promoting fat loss, and 3) physical activity affects the rate of weight loss in a dose-response manner that is based on both frequency and duration of physical activity(Stefanick, 1993; Wilmore, 1983).

A number of studies (Ching *et al.*, 1996; DiPietro, 1995; French *et al.*, 1994; Williamson *et al.*, 1993; Ching *et al.*, 1996) report lower body weights and body fat indices (including skinfold measures and BMI) among individuals with self-reported high levels of physical activity or fitness. In developed countries, there is a negative relationship between levels of physical activity and obesity (Kopelman, 2000). For instance, individuals reporting physical activity three or more times per week, lost weight between surveys (median interval 5.7 y), whilst, those who reported undertaking little activity gained weight (Rissanen *et al.*, 1991). In contrast, cross-sectional studies show higher habitual energy expenditure in obese compared to lean individuals (Prentice *et al.*, 1996). A possible explanation for this is the greater energy requirement as a result of their greater total and lean body mass. Although the most direct effect of physical activity on energy balance is through the actual energy expended during activity, there are additional benefits. For example, the resting metabolic rate (which, when low, is a predictor of obesity) is elevated for several hours after an exercise session (Ravussin and Swinburn, 1993). The results of these studies support increasing voluntary physical activity as a way of effectively curbing the positive energy balance which results in obesity.

1.2.2.3 Energy intake

Dietary factors have a strong influence on energy balance, and together with energy expenditure are the major modifiable factors through which external forces promote weight gain. Increased energy intake may be a factor that has contributed to the rapid rise in obesity over the past 50 years. However, a direct correlation has not been reported between the prevalence of obesity and increased energy intake in developed countries. These data could reflect under reporting of intake (it has been suggested that food intake is under-

reported by approximately 30%) (Heitmann and Lissner, 1995; Schoeller, 1990). Alternatively, it appears more likely that there has been a failure to reduce food intake appropriately in response to the decreased energy expenditure by most communities over recent years (see above).

The overall processes that govern food intake are complex (Chapter 2). The amount of energy ingested over 24 h depends on three major variables: the energy and / or macronutrient composition of the food eaten, the size of individual meals and the frequency with which meals are ingested. Two distinct mechanisms regulate the latter two processes –satiation and satiety. Satiation, describes the series of sensations that regulate meal termination. This is primarily responsible for determining the size of individual meals. Satiety, on the other hand, describes the absence of hunger, the sensation that prevents an individual from commencing a meal (Blundell and Halford, 1994; Rolls and Hammer, 1995). Food already present in the gastrointestinal tract influences both of these processes. Recent experimental evidence suggests that different food types (i.e the macronutrients carbohydrate, fat and protein) may differ in the effects they exert on the regulation of mammalian food intake. In particular their effects on satiety, and therefore on subsequent food intake appear to differ (See Table 1.1). Many of these differences are directly related to factors such as macronutrient storage capacity, palatability and energy density (these are described below). Much of this evidence remains controversial and these differences are examined in Chapters 7, 8 and 11.

Data suggest that the macronutrient composition of the diet (particularly its fat content) is also an important determinant of daily food intake and consequently body weight. Changes in body weight, are driven by changes in energy balance, and it is likely that dietary macronutrient composition is important since it influences energy intake (though differential effects on appetite via different satiety effects) and hence energy balance. For example, the diet of obese individuals has been reported to contain a higher proportion of fat than that of lean individuals (Lissner and Heitmann, 1995). Furthermore, healthy normal weight young adult subjects eat 30% more energy per day when presented with food items with a high fat content compared with when they are presented with foods with a high carbohydrate content (Dreon *et al.*, 1988). A further fact is that the excess energy ingested on a high-fat diet, is consumed as both a smaller volume, and a smaller weight of food than when high-carbohydrate or high-protein diets are consumed, because high-fat

foods have a higher energy density of (9 v 4 kcal / g) (Bouchard, 1996). From the point of view of factors that affect appetite and satiety, the storage capacity, energy density, palatability, oxidation, metabolic transfer, stimulation of hormones and neurotransmitters and daily intake of the three macronutrients all differ considerably, this is dealt with in detail in Chapter 3.

It should be noted that whilst in theory, low-fat diets should promote weight loss, there is evidence to counter this claim. In fact, controlled trials in free-living populations have not supported this idea (Katan *et al.*, 1997). Further, there is evidence demonstrating that low-fat, high carbohydrate diets decrease plasma high-density lipoprotein cholesterol concentrations (Mensick and Katan, 1992), which is known to increase the risk of coronary artery disease. Thus, the promotion of a high-carbohydrate, low-fat diet for weight loss is controversial, and particularly for individuals prone to coronary artery disease should be approached with caution.

Table 1.1 Summary of characteristics of macronutrients (WHO, 1997)

	Protein	**Carbohydrate**	**Fat**
Ability to bring eating to an end (Satiation)	high	intermediate	low
Ability to suppress hunger (Satiety)	high	high	low
Contribution to daily energy intake	low	high	high
Energy density	low	low	high
Storage capacity in the body	low	low	high
Metabolic pathway to transfer excess intake to another compartment	yes	yes	no
Autoregulation (ability to stimulate own oxidation on intake)	excellent	excellent	poor

Storage capacity

Dietary macronutrient composition has a major influence on the extent of nutrient storage when energy intake exceeds immediate needs. Macronutrients with a low storage capacity are preferentially oxidized when intake exceeds requirements. For example, protein has a limited storage capacity and therefore amino acid metabolism is tightly regulated to ensure the oxidation of any excess. Similarly, carbohydrate has a restricted capacity for storage as glycogen, and consequently its intake and oxidation are very tightly regulated. On the

other hand, the capacity for fat storage is virtually unlimited. Hence, excess dietary fat does not acutely increase fat oxidation. Instead, this excess is readily stored in adipose tissue depots with very high efficiency. Such differences suggest that carbohydrate and protein balance is more highly regulated than fat balance (Acheson *et al.*, 1988; Flatt, 1987; Horton *et al.*, 1995; Zurlo *et al.*, 1990).

Palatibility

The palatability of foods plays an important role in determining consumption. An increase in palatability tends to promote over-consumption and thus a positive rather than negative energy balance. Highly preferred foods often have a high fat content, and the preference for these foods appears to be linked to the influence of fat on their sensory properties. Both odours and flavours depend on fat-soluble volatile flavour molecules (Mela, 1990). Fat gives foods a characteristic, highly palatable texture (Mela, 1990). Sweetness is also a very recognizable, powerful and pleasurable taste and increasing the sugar content of foods is one of the easiest ways to increase their palatability. Foods usually combine two or more of these characteristics, for instance, a preference for sweet-fat mixtures has been observed in obese women, which may be a factor in promoting excess energy consumption (Drenowski, 1994). These findings support a role for taste in promoting fat and simple carbohydrate intake.

Energy density

Energy density is an important determinant of food intake (Poppitt and Prentice, 1996; Suubbs *et al.*, 1996; Prentice and Poppitt 1996). Recent experimental evidence suggests that many individuals eat by volume, rather than energy content (Rolls *et al.*, 1999). Dietary fat provides approximately 9 kcal / g compared to 4 kcal / g for carbohydrate and protein (Bell *et al.*, 1998). If the volume or weight of food consumed is maintained at a constant level, then the relatively high energy density of fat compared with protein and carbohydrate could be a factor in its over-consumption. Thus, a high energy intake is potentially a passive response to high fat consumption rather than an active decision to overeat.

Satiating capacity

The ability of food already present in the gastrointestinal system, or indeed nutrients already present in the body, to suppress further food intake is an important factor in

determining energy intake. There is considerable evidence to suggest that fat is less satiating than either carbohydrate or protein (Porrini *et al.*, 1995; Stubbs *et al.*, 1996). The reason for this may include factors such as the effects of stomach distension, nutrient absorption, hormone release and oxidation of nutrients (Morley, 1987; Morley, 1995; Read *et al.*, 1994) (Chapter 2). While post-ingestive factors such as those affecting the mechanics of the upper gastrointestinal tract are likely to influence satiation, post-absorptive factors probably have the strongest effect on the satiating capacity of macronutrients. For example, dietary fat is metabolized more slowly after meals than carbohydrate and protein (Schutz *et al.*, 1989), which may limit its satiating capacity. On the other-hand, some types of dietary fat stimulate cholecystokinin release upon entering the intestine (McLaughlin *et al.*, 1999), and this putative satiety hormone could, in theory, lead to early satiety. It is very possible that much of the controversy that exists concerning the specific satiating effects of dietary macronutrients relates to the sub-type of macronutrient administered. For example, there is evidence that not all monosaccharides (Rodin, 1990; Spitzer and Rodin, 1987) or fats (French *et al.*, 1998; Lawton *et al.*, 2000) are equal with respect to their effects on satiety. (Chapters 4 and 10). In considering the effects of macronutrients on appetite and food intake, it is therefore important to consider the specific sub-type of macronutrient administered.

Neurotransmitter release

Many of the physiological complexities associated with satiety control arise from multiple interactions between and within the 'satiety centre' located in the ventromedial hypothalamus and the gastrointestinal system (Chapter 2). The relay of information between these two regions depends on a variety of hormonal and neural connections. In recent years it has been suggested that the inhibitory neurotransmitter, nitric oxide (NO) may play a pivotal role in the integration of signals affecting appetite and food intake.

Endogenous NO may modulate mammalian food intake by acting centrally as a neurotransmitter in feeding-related pathways (Choi *et al.*, 1995) and peripherally by regulating gastrointestinal motility (Burleigh, 1992; Desai *et al.*, 1991). Inhibition of NO production suppresses feeding in a variety of non-human animal species including mice (Morley and Flood, 1991), rats (Squadrito *et al.*, 1993) and chickens (Choi *et al.*, 1994). Studies with genetically obese rodents indicate that obesity is associated with an increased sensitivity to the appetite-suppressant actions of NO synthase inhibition, (Squadrito *et al.*,

1994). It is unknown whether NO release is stimulated by luminal nutrients and whether this regulates subsequent food intake (Chapter 5 and 13). The potential application of these data to new treatment strategies to treat obesity makes these findings especially interesting.

1.2.3 Obesity and associated health risks

Obesity is a chronic disease characterized by slow progression throughout adult life or by periods of weight stability or short-term weight loss followed by relapse. Chronic obesity is associated with common causes of morbidity and mortality, such as type 2 diabetes mellitus (Chan *et al.*, 1994), coronary heart disease (CHD) (Rabkin *et al.*, 1977), hypertension (MacMahon *et al.*, 1987), and dyslipidemia (Denke *et al.*, 1993; Denke *et al.*, 1994). Obesity is also associated with a greater prevalence of gallbladder disease, gout and osteoarthritis and may be associated with an elevated incidence of certain types of cancer, particularly hormone-dependent cancers such as those of breast and prostate (Ekman, 1999). In addition, obesity compromises reproductive function (Tataranni *et al.*, 1997). Many of these associations follow a dose-response relationship in which risk increases as the degree of obesity increases.

1.2.3.1 Type 2 diabetes mellitus

There is strong correlation between obesity and glucose intolerance in type 2 diabetics. An estimated 15.6 million North American adults (8% of those over the age of 20 years) have diabetes (National Task Force on the Prevention and Treatment of Obesity, 2000), with type 2 diabetes (non-insulin dependent diabetes mellitus or adult-onset diabetes) accounting for between 90-95% of all diagnosed cases (National Task Force on the Prevention and Treatment of Obesity, 2000).

Body mass index correlates strongly with risk of developing type 2 diabetes. Data suggest that more than 80% of individuals with type 2 diabetes are obese (Chan *et al.*, 1994). In the Nurse's Health Study (Carey *et al.*, 1997) women with a BMI of greater than 31 kg / m^2 had a 40-fold greater risk of developing diabetes 40 compared to women with a BMI of less than 22 kg / m^2. In the Male Health Professionals Study, males with a BMI greater than 35 kg / m^2, were more than 40 times at risk than males with a BMI less than 23 kg / m^2 of developing type 2 diabetes (Chan *et al.*, 1994). Data from the National Health and Nutrition Survey III (NHANES III) indicate that 67% of diagnosed diabetics have BMI of

greater than 27 kg / m^2, and 46% have a BMI of greater than 30 (Harris *et al.*, 1998). Furthermore, significant weight gain during the adult years is strongly and independently correlated with the risk of developing type 2 diabetes (Chan *et al.*, 1994; Ferrannini and Camastra, 1998b). After adjusting for age, BMI is a dominant predictor of diabetes risk; risk increased 5-fold for those with a BMI of 25 kg / m^2, 28-fold for those with a BMI of 30 kg / m^2, and over 90-fold with a BMI of 35 kg / m^2 or greater, when compared with women with a BMI less than 21 kg / m^2 (Kopelman, 2000). Furthermore, epidemiological data indicate that the increase in the prevalence of type 2 diabetes parallels the dramatic increase in weight over the past decade (Kopelman, 2000).

Although a number of theories have been proposed explaining the link between obesity and type 2 diabetes, most interest has focused on the cascade of metabolic abnormalities triggered by insulin resistance, and the lipolytic properties of adipocytes (particularly abdominal adipocytes). Obesity is characterized by elevated fasting plasma glucose concentrations and an exaggerated plasma insulin response to an oral glucose load (Kolterman *et al.*, 1980). In the obese state the rate of lipolysis (fat breakdown) is elevated, leading to an increased release of free fatty acids (FFA). This has detrimental effects on hepatic insulin uptake and results in increased hepatic gluconeogenesis (breakdown of amino acids and conversion to glucose) contributing to the systemic hyperinsulinaemia, as the pancreatic β-cells produce compensatory insulin. As plasma FFA concentrations increase the insulin-resistant individual can no longer maintain this state of hyperinsulinaemia and hyperglycemia occur (Sniderman and Cianflone, 1995).

Weight loss of 5% to 10% of initial body weight improves glucose tolerance in persons with obesity (Ferrannini and Camastra, 1998a). This effect seems to be most prominent in obese patients with newly diagnosed diabetes. Patients with more severe hyperglycemia and longer-duration type 2 diabetes may respond more slowly and achieve smaller improvements in blood glucose than persons with newly diagnosed or less severe type 2 diabetes (Rippe *et al.*, 1998). Dietary modification therefore has the potential to improve both glycemic control and to reduce food intake and, as a result, body weight in diabetics (Chapter 11).

1.3 ANOREXIA NERVOSA

Abnormal eating patterns are a characteristic of patients with anorexia nervosa. It is now clear that these patients have problems in both experiencing and expressing hunger, suggesting both satiation and satiety mechanisms may be impaired. Although the precise steps involved in the development of anorexia nervosa (AN) are unclear, impaired nutrient-induced gastrointestinal feedback appears to perpetuate the disorder once they are present. This syndrome is associated with multiple endocrine and psychological abnormalities (Stoving *et al.*, 1999), which are discussed here in terms of the release of appetite-related peptides, and perceptions of food and eating behaviour.

1.3.1 Definition and epidemiology

Anorexia nervosa (AN) is a medical condition characterized by: 1) loss of weight to less than 85% of the expected weight for age and height, 2) intense fear of obesity, 3) disturbed body image, and 4) in post-menarchal women, amenorrhea for at least 3 months (Irwin, 1993a; Irwin, 1993b).

Worldwide, the prevalence of AN is reported to be no greater than 0.5% of the female population over 15 years of age, but this may be an under estimate. The highest incidence occurs among 15-19 y old women (Joergensen, 1992; Nielsen, 1990). Two studies of prevalence have been conducted in Australia: one reported a prevalence of 0.1% among a large group of schoolgirls, (Ben and Morton, 1990) and the other reported a lifetime prevalence of 0.4% among a large group of twins (Wade *et al.*, 1996). Although female teenagers are afflicted at 5 times the rate of women in their 20's and 30's (Pawluck and Gorey, 1998), the incidence of AN particularly among very young women, at greatest risk, does not appear to be increasing. However, the incidence among women in their 20's and 30's appears to be increasing (Pawluck and Gorey, 1998). Anorexia nervosa is considerably less prevalent amongst men (female: male incidence ratio 8.2) (Pawluck and Gorey, 1998).

1.3.2 Perceptions of food in anorexia nervosa

Differences in the way patients perceive foods are major psychological determinants of appetite. Stoner et al, (Stoner *et al.*, 1996) assessed the ratings of liking and desire to eat various foods, and whether ratings differ according to the energy or macronutrient content of foods in patients with AN, and in non-anorexic controls. The anorectic group rated their desire to eat high-energy foods significantly lower than their desire to eat low-energy

foods, whereas controls rated their desire to eat high- and low-energy foods equally. After a salad pre-load (720 kJ), patients with AN were more likely than non-anorexics, to choose low- as opposed to high-energy foods, and in general ate a smaller proportion of fat than the non-anorexics (Rolls *et al.*, 1992), reflecting potential differences in the orosensory responses to these foods in anorexics. Consistent with this, the palatability of low energy food is reported to be significantly higher in anorexic patients when compared to controls. After 8 weeks of hospital treatment in the patients, palatability of low energy food had decreased, although the dislike for high-energy food remained stable (Bossert *et al.*, 1991). Whether this is a conditioned psychological response or reflects a gastrointestinal dysfunction, due to the differential release of satiety hormones in response to the ingestion of high- and low-energy foods in patients with AN, or a combination of these is unknown.

1.3.3 Appetite-related symptoms in anorexia nervosa

Lower food intake by patients with AN may stem from impaired sensory feedback following meal ingestion; patients with AN frequently report exaggerated sensations associated with eating. Common gastrointestinal complaints in AN include bloating, constipation, vomiting and diarrhea (Waldholtz and Andersen, 1990). Moreover, in response to mixed nutrient pre-loads, anorexics consistently rate hunger and desire to eat lower and fullness greater than non-anorexic control subjects. This has been suggested to reflect hypersensitivity to the products of digestion. As a result, these patients consume significantly less at a test meal, than their body-weight-matched controls (Hetherington and Rolls, 1991). Although this has not been directly assessed, a study evaluating the gastric emptying of a solid (poached egg) meal, a glucose solution and a saline solution in anorexic patients supports this hypothesis; emptying of nutrients, but not saline, was significantly delayed in anorexics, compared to controls (Robinson *et al.*, 1988). Impaired feedback therefore appears to relate directly to the presence of nutrient, rather than mechanically induced responses. Not surprisingly, when nutritional rehabilitation occurs, symptoms of heightened sensory responses to food subside (Waldholtz and Andersen, 1990). The occurrence of amplified appetite-related symptoms may thus be secondary to the presence of the disorder, rather than a factor in its development.

1.3.4 Appetite-regulating peptides

Endocrinological studies suggest that hypothalamic dysfunction may be involved in the development and therefore the potential treatment of AN. In particular, the basal and / or

nutrient-induced release of a small number of appetite-regulating peptides (Chapter 2) acting at central sites may be disrupted in AN, which may explain their persistent satiety.

There are few reported studies on the circulating basal and / or post-prandial concentrations of appetite-regulating peptides in anorexic patients. Women with AN have increased plasma vasoactive intestinal peptide (VIP) concentrations and decreased gastrin, cholecystokinin (CCK) and somatostatin (Baranowska *et al.*, 1997; Ferron *et al.*, 1997). While information on the basal serum concentrations of the satiety-inducing peptide CCK in AN is contradictory (Harty *et al.*, 1991), a small number of studies demonstrate a more rapid and pronounced rise in post-prandial CCK concentrations in anorexics than in non-anorexic subjects (Harty *et al.*, 1991; Phillipp *et al.*, 1991), leading to the suggestion that CCK may be an etiologic factor in AN. However, after weight normalisation in six patients with AN, both fasting and postprandial plasma CCK concentrations were similar to those in six healthy control subjects (Geracioti *et al.*, 1992).

In anorexic patients, serum leptin levels are lower than in their non-anorexic counterparts (Baranowska *et al.*, 1997; Ferron *et al.*, 1997). However, this appears to reflect their lower body fat stores rather than be an etiologic factor. Plasma NPY and galanin concentrations in women with AN reportedly do not differ from levels in the non-anorexic controls (Baranowska *et al.*, 1997).

Although these data indicate that the basal and nutrient-stimulated release of a number of gastrointestinal peptides are disrupted in AN, there is no evidence that endocrine abnormalities are primary causes of AN. However, it is still possible that endocrine dysfunction exaccerbates the ongoing gastrointestinal symptoms associated with the disorder (Stoving *et al.*, 1999).

1.4 ANOREXIA OF AGEING

The age-related decline in appetite and energy intake occurring in healthy, ambulant, non-institutionalised older people has been termed the 'anorexia of ageing' (Morley, 1990a). 'Anorexia of ageing' may predispose individuals to pathological weight loss and malnutrition, which represent major causes of morbidity and mortality in the elderly. While much of the observed decrease in energy intake can be attributed to the decline in

energy expenditure that occurs during normal ageing (Morley, 1990a), healthy elderly (65-94 y) subjects eat less energy, particularly from fat, than healthy younger (21-35 y) individuals (Wurtman *et al.*, 1988). Healthy older persons are less hungry and more rapidly satiated after eating a standard meal than younger persons (Clarkston *et al.*, 1997). The multiple factors that contribute to the physiological 'anorexia of ageing' have recently been reviewed (MacIntosh *et al.*, 2000). Among the physiologic causes are those that directly relate to impaired gastrointestinal system function, including a deterioration of taste sensation and the decline in nutrient-induced sensory and motor function (Murphy, 1993).

1.4.1 Epidemiology

The proportion of people living into 'old' age is increasing in both developed and developing countries and it is estimated that in the US, the elderly population will increase by 8% over the next 20 years, so that by the year 2020, approximately 25% of the population will be older than 65 years (MacIntosh *et al.*, 2000). Similar figures have been predicted for Australia where the proportion of individuals over the age of 65 y is predicted to jump from 12% (in the year 2000), to 24% in the year 2051 (MacIntosh *et al.*, 2000).

1.4.2 Sensory perception

Gastrointestinal sensation plays an important role in the regulation of satiation and early satiety (Chapter 2). There is evidence that older people experience reduced sensitivity to gastrointestinal tract distension. For example, during proximal gastric distension perceptions of fullness, abdominal discomfort and bloating were less in older than young subjects (MacIntosh, 2001). It is unlikely that this is responsible for the development of 'anorexia of ageing', as reduced sensitivity to stomach contents would, if anything, diminish symptoms of early satiety, fullness and bloating associated with eating, and promote greater intake of food.

1.4.3 Gut-peptide release

The release of a number of hormones, in response to the presence of food in the lumen of the gastrointestinal tract is responsible, in part, for eliciting satiety (Chapter 2). There are data to show that ageing is associated with an enhanced endogenous release of such hormones, specifically cholecystokinin (CCK), but also glucagon-like peptide-1 (GLP-1), peptide YY (PYY) and amylin (MacIntosh, 2001; Sandstrom and El, 1999).

1.4.3.1 Cholecystokinin

Cholecystokinin is the most widely studied of the satiety hormones, considered to have a role in the 'anorexia of ageing'. There is evidence that under both basal (fasting) conditions, and in response to lipid and glucose meals, circulating CCK concentrations are higher in elderly, compared to young adult subjects (MacIntosh, 2001). Increased numbers of CCK immunoreactive cells have been reported in the duodenum of older as opposed to younger subjects (Sandstrom and El, 1999). In addition, suppression of food intake by exogenous CCK infusions is greater in older than in young subjects (31% versus 15% of control day energy intake) (MacIntosh *et al.*, 1999). These observations support a significant role for CCK in the 'anorexia of ageing', with the potential for intervention.

1.4.3.2 Amylin

Amylin is a 37 amino acid peptide hormone manufactured, stored and released with insulin from the pancreatic β-cells. Although the role of amylin as a satiety hormone in humans is yet to be evaluated, studies in rodents demonstrate that peripheral administration of amylin decreases food intake (Morley *et al.*, 1997). In the 'anorexia of ageing' a single study has reported that both basal and nutrient-induced release of amylin increases between middle and old age (MacIntosh *et al.*, 2000). However, in a separate study, Mitsukawa *et al.*, (1992) showed no difference in basal and glucose-stimulated amylin concentration between young and older adults. The reason for this discrepancy is unclear. Further studies will be necessary to establish a role from amylin in the 'anorexia of ageing'.

1.4.3.3 Other gut peptides

There are few studies on the effects of ageing on glucagon-like peptide-1 (GLP-1) (Chapter 2.3.5.2) concentrations, and those that are available provide conflicting data. Both fasting and plasma GLP-1 concentrations in response to a 100 g oral carbohydrate load were reported to be higher in post-menopausal than in pre-menopausal women (Ranganath *et al.*, 1998). MacIntosh *et al.*, (1999) observed no difference in plasma GLP-1 concentration between young and older adults either after an overnight fast, or after intraduodenal infusions of lipid and glucose. In addition, no difference was found in basal, glucose- or lipid-stimulated plasma PYY concentrations between healthy young and older adults (MacIntosh *et al.*, 1999). Further investigation into this area is therefore necessary.

1.4.4 Leptin

Circulating leptin (Section 1.2.2.1) concentrations reflect body fat stores, and may have some role in the long-term regulation of food intake (Campfield and Smith, 1998a) (Chapter 2). Plasma leptin concentrations reportedly increase with age (MacIntosh, 2001), as does percent body fat. A single study indicates that the ageing-associated increase in plasma leptin levels may be independent of the increase in body fat (Baumgartner *et al.*, 1999). Whether human ageing is associated with a decrease in sensitivity to the satiating effects of leptin is unknown. Despite this, it is unlikely that impaired the leptin release or response to endogenous leptin has a major effect on the etiology of the anorexia of ageing (Chapter 2).

1.4.5 Central appetite-regulating pathways

Studies in animals have established that endogenous opioids may modulate feeding by stimulating central feeding sites (Morley, 1987). Peripheral administration of naloxone (opioid antagonist) suppresses food intake in healthy young human subjects, and also in healthy older subjects (MacIntosh *et al.*, 2001). Healthy older individuals thus appear to retain their sensitivity to the suppressive effects of naloxone on energy intake and it is unlikely that impaired opioid activity contributes to the 'anorexia of ageing'.

Nitric oxide may play a role in the modulation of food intake by acting at central sites, such as those in the hypothalamus (Choi *et al.*, 1994; Choi *et al.*, 1995) and there is limited evidence that NO may be more important with advancing age. The NO synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME) decreased food intake in 12- and 24-month-old mice more efficiently than in the 3-month-old mice (Morley *et al.*, 1996). Further studies are required to establish the importance of NO mechanisms in the regulation of healthy appetite (Chapters 5, 12-14) as well as a potential role in the 'anorexia of ageing'.

1.5 CONCLUSION

This chapter has focused on the more common disorders associated with impaired regulation of appetite. Appropriate regulation of appetite and food intake is central to maintaining optimal health. A major goal of appetite physiologists is to identify processes involved in the normal control of mammalian feeding behaviour and define the abnormalitites in obesity (and other appetite-related disorders) to allow the development of

strategies to restore normal eating behaviour. With the exception of Chapters 10 (Study in type 2 diabetics) and 11 (Study in animals), the studies reported in this thesis are concerned with the physiology of mammalian appetite regulation in healthy human subjects.

Chapter 2

REGULATION OF APPETITE AND FOOD INTAKE

2.1 INTRODUCTION

Mammalian appetite is an elaborate mixture of physiological and psychosocial phenomena. The physiological complexities associated with appetite control arise from interactions between and within multiple central and peripheral sites. Until recently a central system termed the 'feeding centre', located in the lateral hypothalamus and 'satiety centre' in the ventromedial hypothalamus, with input from satiety signals from the periphery, were thought to regulate the overall drive to eat. Work published in the field of appetite physiology over the past decade has demonstrated that this view of mammalian feeding control is over-simplistic. Nutrient supply to the brain, the release of gastrointestinal hormones and neurotransmitters, gastrointestinal transit of partly digested nutrients, metabolic products and nutrient stores (Blundell and Halford, 1994; Mantzoros, 1999; Morley, 1987; Read *et al.*, 1994) all interact to influence subsequent food intake.

The complex nature of the regulation of food intake is not surprising considering the varied inputs involved. This chapter reviews the physiological factors. A brief discussion on the central mechanisms regulating mammalian feeding is followed by an overview of the gastrointestinal control of food intake, which is the major focus of the research conducted in this thesis.

2.2 CENTRAL MECHANISMS CONTROLLING FOOD INTAKE

Although many of the sites involved in the central regulation of feeding behaviour have now been identified, largely through studies in rodent models, the spatial relationships between these sites, their orexigenic and anorexigenic products, and how their function as neurotransmitters between the multiple regions have only recently been realised. In addition, understanding is necessary of the integration of inputs from peripheral organs such as the gut and adipose tissue stores, and from plasma nutrients (Kalra *et al.*, 1999; Thomas *et al.*, 1992).

2.2.1 Neuroanatomical sites

A small number of discrete nuclei in the basal hypothalamus are associated with neural mechanisms affecting appetite. By employing techniques such as hypothalamic lesions or surgical transections of neural pathways in animal models, the ventromedial nucleus (VMN), dorsomedial nucleus (DMN), paraventricular nucleus (PVN), and lateral hypothalamus (LH) have been recognised as containing neural mechanisms affecting ingestive behaviour (Bernardis and Bellinger, 1996; Bray, 1984; Hetherington and Ranson, 1942; Luiten *et al.*, 1987; Sclafani, 1971). Studies in rodents demonstrate that the stimulation of the lateral hypothalamus results in overeating, while stimulation of the ventromedial hypothalamus causes aphagia. Ablation of these regions of the brain produces the opposite effects, i.e. destruction of the lateral hypothalamus results in under eating and of the ventromedial hypothalamus causes hyperphagia. The severity of symptoms depends on the precise location and size of the lesion (Hamilton and Brobeck, 1966); to a certain degree, the greater the damage the greater the effect on food intake. Recent research also implicates the arcuate nucleus (ARC), the perifornical hypothalamus (PFH) and the suprachiasmatic nucleus (SCN) as additional sites involved in the control of ingestive behaviour (Kalra *et al.*, 1999) (Figure 2.1).

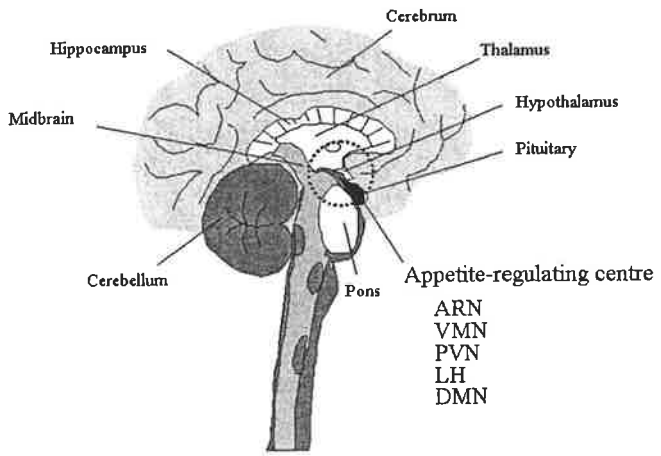


Figure 2.1 Diagrammatic representation of some of the central sites involved in the regulation of appetite (Kalra *et al.*, 1999). ARN, arcuate nucleus; VMN, ventromedial nucleus; PVN, paraventricular nucleus; LH, lateral hypothalamus; DMN, dorsomedial nucleus.

2.2.2 Chemical mediators

A large number of compounds, many discovered only recently, have been implicated in the central regulation of feeding (Table 2.1). These are released from central and peripheral sites and act both locally and at sites distal to their production. Orexic or anorexic effects are mediated by compounds including nitric oxide (NO) (Chapter 5 and Section 2.2.2.1), neuropeptide Y (NPY) (Section 2.2.2.2), and serotonin (Section 2.2.2.3). Many of these mediators, including the gastrointestinal peptides (Section 2.3.5), act at peripheral as well as central sites, and have direct effects on the gastrointestinal tract itself (Section 2.3, (McHugh *et al.*, 1975; Morley, 1987). The following discussion focuses on the involvement of endogenous NO in the central regulation of feeding, (also see Chapter 5) and some of the neurotransmitters with which it is proposed to interact. Studies investigating the effects of NO on food intake and gastrointestinal system function are described in Chapters 11-13 of this thesis.

2.2.2.1 Nitric oxide

Nitric oxide (NO) is a neuronal messenger originally identified in endothelial cells (Furchgott and Zawadzki, 1980), but also present in neural tissue and macrophgages (Bredt and Snyder, 1992; Moncada *et al.*, 1991). In the CNS, NO synthases (NOS), the enzymes responsible for NO production, are present within the cerebellum (Vincent and Kimura, 1992) and the hypothalamo-neurohypophyseal system of the rat (in the supraoptic and paraventricular nuclei) (Nelson *et al.*, 1997). Both brain regions are associated with regulation of ingestive behaviour. Nitric oxide synthase has also been shown to co-localise centrally with orexic hormones, including neuropeptide Y in the cerebral cortex and corpus striatum and oxytocin in the hypothalamus (Nelson *et al.*, 1997). Central NO has been implicated in many diverse biological processes, including memory formation (Shibuki and Okada, 1991), olfaction (Breer and Shepherd, 1993) and nociception (Moore *et al.*, 1991). Works from several research groups now also suggest the involvement of NO in the modulation of mammalian ingestive behaviour (Choi *et al.*, 1994; Morley and Flood, 1991; Morley and Flood, 1992; Morley and Flood, 1994; Squadrito *et al.*, 1993).

There is evidence that NO exerts stimulatory effects on feeding through central mechanisms. Intracerebral (ICV) administration of the non-specific NO synthase antagonist nitro-L-arginine (L-NO Arg) to rats reduces food intake, whereas the same dose administered peripherally has no effect on feeding (Squadrito *et al.*, 1993). In addition, food deprivation increases levels of brain NO synthase activity (Squadrito *et al.*, 1994).

Table 2.1 Endogenous compounds implicated in the central regulation of mammalian feeding behaviour. Adapted from (Hope, 2000; MacIntosh, 2001a)

	Stimulatory effect on food intake	Inhibitory effect on food intake
Pancreatic hormones		Amylin Insulin Insulin-like growth factor-1 Pancreatic glucagon
Monoamines	α-adrenergic regulators (eg. noradrenaline) Histamine	β-adrenergic regulators (eg. adrenaline) Dopamine Serotonin
Gastrointestinal peptides	Ghrelin Motilin	Bombesin Cholecystokinin Gastrin releasing peptide Glucagon-like peptide-1 Somatostatin
Gonadal hormones	Progesterone Testosterone	Estrogen
Adipose tissue signals		Leptin Tumor necrosis factor-α
Miscellaneous neurotransmitters	γ-aminobutyric acid Nitric oxide	N-methyl-D-aspartate
Miscellaneous peptides and hormones	β-endorphin / dynorphin Cytokines Galanin Growth hormone releasing hormone Melanin-concentrating hormone Neuropeptide Y Orexins Oxytocin Peptide YY Thyroid hormone Urocortin	α-melanocortin stimulating hormone Calcitonin Calcitonin gene related peptide Cocaine and amphetamine-regulated transcript Corticotrophin releasing factor Melanocortin and agouti-related protein Neurotensin Oxytocin (acute) Thyrotrophin-releasing hormone

One potential path responsible for the influence of nitric oxide on the central regulation of feeding behaviour involves the hormone serotonin (5-hydroxytryptamine [5-HT]) (Blundell, 1992). Administration of metergoline, a non-specific 5-HT receptor antagonist to rats before they received L-NO Arg (a non-specific NOS antagonist), attenuated the anorectic effect of L-NO Arg. In contrast, ritanserin a non-specific 5-HT_2 receptor antagonist had no effect, suggesting that the suppression of feeding by L-NO Arg may be mediated via 5-HT_1 receptors (Squadrito *et al.*, 1994). L-NO Arg administration in that study was also associated with increased levels of brain serotonin (Squadrito *et al.*, 1994), suggesting that NO normally inhibits serotonin synthesis, thus enhancing food intake. This provides strong evidence for the role of a common NO-serotonin pathway in the control of feeding.

Peripherally administered NOS inhibitors also counteract the stimulatory effects on feeding of centrally administered neuropeptide Y (Morley and Flood, 1992), morphine (Calignano *et al.*, 1993), substance P (Mancuso *et al.*, 1994) and clonidine (Choi *et al.*, 1995). These findings imply that any role for NO in the central regulation of feeding behaviour involves a number of mediators released secondary to NO production. Alternatively NO production may be stimulated by these substances.

2.2.2.2 Neuropeptide Y

Neuropeptide Y (NPY), a polypeptide originating predominantly from the sympatho-adrenomedullary nervous system, causes vasoconstriction and is probably involved in blood pressure regulation (Kokot and Ficek, 1999b). In addition, evidence largely from rodent studies, indicates that NPY is a potent orexigenic agent. It is thought to play a role in the hypothalamic regulation of eating behaviour through the activation of specific, central NPY receptors (Kokot and Ficek, 1999b).

When injected into the rodent brain, NPY increases feeding (Morley, 1987), apparently by pathways that interact with NO (Morley and Flood, 1992) (Section 2.2.2.1), leptin (Niimi *et al.*, 2001), opioids (Britton and Southerland, 2001) and amylin (Morris and Nguyen, 2001); increasing doses of amylin produce a dose-dependent inhibition of NPY-induced feeding (Morris and Nguyen, 2001). Brain NPY concentrations are not elevated, despite the reduced food intake. In rodents, stimulation of the NPY-ergic arcuate - paraventricular nucleus (ARC-PVN) pathway by exercise, fasting or energy loss increases food intake, via

a pathway inhibited by leptin (Kokot and Ficek, 1999a). Neuropeptide Y may therefore act to regulate feeding behaviour over longer periods than just between single meals.

2.2.2.3 Serotonin

Serotonin, a neurotransmitter derived from dietary tryptophan, is present in the highest concentrations in the enterochromaffin cells of the wall of the stomach and small intestine, in platelets and in the midbrain (Facer *et al.*, 1979; Maley *et al.*, 1990). In addition, serotinergic systems are directly linked to various central appetite-regulating sites; eg. lateral hypothalamic glucose-sensitive neurons receive direct modulation by serotonin (Angel, 1990).

The actions of serotonin are numerous and in the CNS, include the regulation of sleep, mood, stereotyped behaviour, pain perception, vomiting and feeding control. Considerable evidence supports a role for serotonin in the regulation of mammalian feeding behaviour. Studies usually using compounds that facilitate serotonin synaptic activity, such as fenfluramine and its derivatives, demonstrate that direct or indirect activation of serotonin receptors suppresses food intake (Blundell, 1984). Consistent with this, serotonin blockade or inhibition of serotonin metabolism increases feeding (Blundell, 1984). Serotonin-containing neurons may also have a role as amino acid 'sensors'. In support of this, there is a relationship between the tryptophan-large neutral amino acid (T-LNAA) ratio in the plasma and the proportion of carbohydrate to protein in the diet. High carbohydrate diets raise the plasma T-LNAA ratio, which increases brain tryptophan levels, leading to increased synthesis and release of serotonin (Blundell, 1992). This mechanism putatively signals dietary composition to the brain. At least in the rat, serotonin appears to be involved in the selection of specific macronutrients, namely protein and carbohydrate (Ashley and Anderson, 1975; Mok *et al.*, 2000).

2.3 PERIPHERAL MECHANISMS

Peripheral systems involved in the regulation of feeding differ markedly with respect to their origin and precise roles. Much of the information on the peripheral systems that act to influence feeding is derived directly from studies in humans. The mouth is the first part of the gastrointestinal tract to be exposed to food and taste plays an important primary role in determining not only food selection, but also energy intake (Rolls *et al.*, 1988). The upper gastrointestinal tract, including the mouth and stomach, probably has a major role in

the short-term regulation of feeding, and is therefore responsible for the generation of signals eliciting satiation (Read *et al.*, 1994). The rest of the gastrointestinal tract may regulate between-meal satiety. On the other hand, fat depots, the sizes of which are signaled by their circulating protein product leptin, appear to be involved in the regulation of long term feeding patterns by interacting with metabolic pathways (Campfield and Smith, 1998).

Peripheral signals are relayed to central feeding centres via a variety of pathways (Schwartz, 2000). The intrinsic and extrinsic innervations of the upper gastrointestinal tract, together with the extrinsic neural fields in the CNS, and their respective target neurons in the CNS, comprise the neural gut-brain axis. The humoral gut-brain axis describes the afferent signal produced by gut contact with ingested nutrients (Read *et al.*, 1994), that travel from the upper gastrointestinal tract to the CNS to mediate ingestive behaviour. Gut-brain peptides released into the blood stream following nutrient ingestion, provide an alternate afferent signalling pathway to sites in the CNS (Morley, 1987). The focus of this section is on the motor functions and secretions, including the gut peptides cholecystokinin, glucagon-like peptide-1, gastric inhibitory peptide and insulin, of the upper gastrointestinal tract. In addition, both the local and central interactions of these processes on mammalian feeding behaviour will be discussed. All of the studies described in this thesis have evaluated the roles of the gastrointestinal system in the regulation of food intake. (Figure 2.2)

2.3.1 Common gastrointestinal tract anatomy

The human diet is extraordinarily varied, so the gastrointestinal tract needs to be capable of digesting a large variety of compounds to their simplest, most absorbable state. The gut is comprised of a long hollow smooth muscle tube with the primary function of digesting and absorbing food. These processes are performed through both its mechanical activity and chemical secretion. In addition, the gastrointestinal tract is also a major endocrine system, under the control of its own integrated neural network, the enteric nervous system (ENS), which is partly responsible for signalling the intake of specific macronutrients to brain centres.

All of the activity of the gastrointestinal tract (except for the mouth, throat, upper oesophagus and anus) is involuntary and controlled by the ENS with modulation from the

sympathetic and parasympathetic nervous systems. The ENS consists of a large number of sensory, integrative and motor neurons, as well as a number of networks of ganglia. There

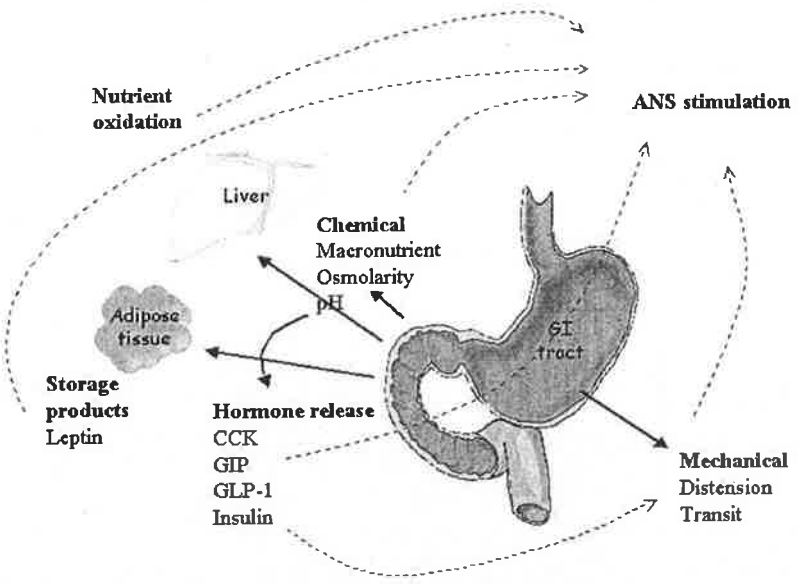


Figure 2.2 Overview of some of the major peripheral factors controlling appetite (Morley, 1990; Read *et al.*, 1994). ANS, autonomic nervous system; GI, gastrointestinal; CCK, cholecystokinin; GIP, gastric inhibitory peptide; GLP-1 glucagon-like peptide-1.

are two principle intramural plexuses in the tract; (i) the myenteric plexus, which lies between the outer, longitudinal and the middle, circular muscle layers and (ii) the submucous plexus, which is on the luminal side of the circular muscle layer. These plexuses are interconnected and their ganglion cells receive preganglionic parasympathetic fibres from the vagus, which are mostly excitatory. Incoming sympathetic fibres are largely postganglionic and these, in addition to innervating blood vessels, smooth muscle and some glandular cells directly, may end in the plexus (Hasler, 1995; Meyer, 1987).

Parasympathetic innervation is primarily by the vagus nerve. Vagal cell bodies in the brain stem give rise to the vagus nerve that passes to and innervates the oesophagus, then passes through the diaphragm to innervate the stomach, small intestine and the ascending colon. In addition to efferent fibres, 90% of the vagus nerve consists of sensory (afferent) fibres. Sympathetic innervation arises from the thoracolumbar portion of the spinal cord and branches from this region arrive at the sympathetic ganglia, synapsing with postganglionic nerve cells, whose fibres end at the intramural plexus (Hasler, 1995; Meyer, 1987).

Table 2.2 Neurotransmitter effects on stomach and small intestinal smooth muscle. Adapted from (Hasler, 1995; Meyer, 1987).

Neurotransmitter	Effect on smooth muscle
Acetycholine	Stimulatory
Angiotensin	Stimulatory
Cholecystokinin	Stimulatory
Dopamine	Inhibitory
Enkephalin	Inhibitory
Gastrin	Stimulatory
Histamine	Stimulatory
Motilin	Stimulatory
Nitric oxide	Inhibitory
Noradrenaline	Inhibitory
Serotonin	Stimulatory
Somatostatin	Inhibitory
Vasointestinal peptide	Inhibitory

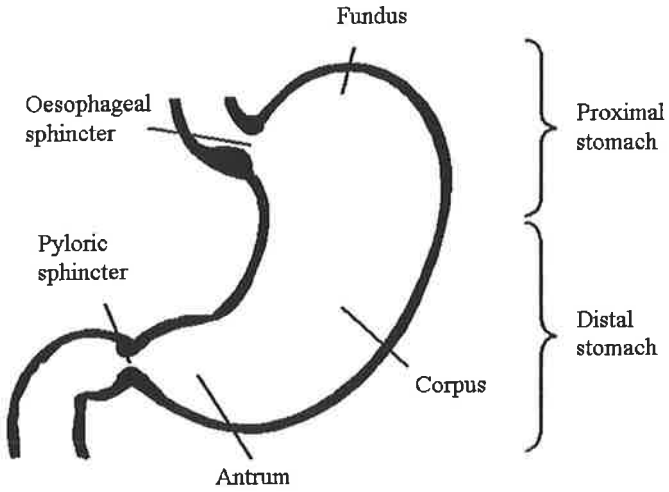
2.3.2 Stomach

The stomach is a J-shaped organ delimited by the lower oesophageal sphincter above, and the pylorus below. It comprises three anatomical regions, the fundus, corpus and antrum (Figure 2.3). Functionally, it is divided into a proximal compartment that acts as a reservoir for receiving and storing swallowed food, and a distal compartment comprising the antrum and the pylorus. Together with the proximal duodenum this is frequently viewed as a single 'antropyloroduodenal (APD) unit' and is largely responsible for the empting of chyme into the duodenum (Hasler, 1995; Meyer, 1987).

The mechanical function of the stomach is determined by the underlying electrical activity of the gastric myocytes. Gastrointestinal smooth muscle cells maintain a negative transmembrane electrical potential through ion pumps (Meyer, 1987). This membrane potential fluctuates rhythmically, with depolarisation occurring for 3 - 10 seconds when the potential difference reaches its maximum. Because the myocytes are in an electrical syncytium the area with the most frequent depolarization determines the frequency of the rest of the stomach. The gastric pacemaker, located on the greater curvature of the stomach, is responsible for generating this electrical activity. The frequency of this

electrical control activity is 3 cycles per minute in the stomach. The interstitial cells of Cajal transmit this rhythm distally at a rate of between 0.5 and 4 cm / s.

Figure 2.3 Basic anatomy of the stomach (Hasler, 1995; Meyer, 1987)



The electrical activity is responsible for two types of contractions in the gastrointestinal tract. A sudden spike potential generated in smooth muscle in the depolarised state, is always associated with muscle contraction and is responsible for phasic contractions. Tonic contractions, on the other hand, occur in the absence of spikes and are related directly to the size of the potential difference across the smooth muscle membrane (Meyer, 1987).

2.3.2.1 Gastric motor function

In the absence of food in the digestive tract the stomach exhibits a characteristic fasting motor pattern. Normal fasting motility consists of 3 phases, with an approximate cycle length of 100 min. Phase I (motor quiescence) and phase II (irregular contractions) are variable in duration, and phase III (regular high amplitude contractions at a maximum frequency and force of 3 per minute) occurs at the end of a cycle for ~ 7 - 10 min (Wingate, 1981). Subsequently the contractions fade and the stomach is once again quiescent (Phase I).

Two motor responses occur in the proximal stomach, consistent with its function as a food reservoir, allowing for the rapid expansion of gastric volume in response to eating. The first, termed receptive relaxation, is initiated by the action of swallowing; it lasts for about

20 seconds and is associated with a drop in stomach pressure (Meyer, 1987). To curb a large increase in pressure, and therefore prevent gastric contents from entering the small intestine too quickly, stretch sensors in the stomach are activated, and via a vago-vagal reflex proximal stomach smooth muscle relaxation occurs. This is termed adaptive relaxation and together with receptive relaxation, these reflexes ensure that the proximal stomach accommodates the increased intragastric volume with little or no increase in intragastric pressure (Malagelada, 1981).

During gastric emptying of a meal, proximal stomach tone increases, and together with pyloric and duodenal feedback this favours the delivery of partly digested foods from the distal stomach into the duodenum. Gastric motor function is mediated via a number of neural pathways, whereas hormonal mechanisms appear to be less important. For instance, fundic relaxation, at least in response to a carbohydrate meal, is not mediated by cholecystokinin (CCK). A carbohydrate-rich meal eaten at the same time as exogenous CCK administration does not induce fundic relaxation, whereas a fat-rich meal, which induces endogenous CCK secretion, does, despite similar plasma CCK levels (van der Schaar *et al.*, 2001). Serotonin (a neurotransmitter implicated in the central control of appetite and food intake) on the other-hand, probably plays a role in the regulation of meal-induced relaxation of the gastric fundus, via the 5-HT_1 receptor (Vingerhagen *et al.*, 2001). Furthermore, proximal gastric tone also seems to be regulated by cholinergic stimuli, which increase pressure, and non-adrenergic, non-cholinergic, possibly nitrergic (Gilja *et al.*, 1997; Tonini *et al.*, 2001) stimuli that induce relaxation. The involvement of NO mechanisms in the regulation of nutrient-induced inhibition of antral contractions is evaluated in the study described in Chapter 14.

2.3.2.2 Gastric distension

Gastric distension has been implicated in the generation of early post-prandial symptoms contributing to satiation and satiety. Gastric distension-induced sensations arise partly from activation of mechanoreceptors within the wall of the stomach (Feinle *et al.*, 1996) that signal the presence of food and enhance sensations of fullness and satiation, whilst decreasing sensations such as hunger and desire to eat (MacIntosh, 2001a). The effects of gastric distension on feeding are consistent with these sensory responses. For example distension of the stomach, independent of the presence of nutrients, inhibits feeding in animals (Grossman, 1949) and suppresses food intake in lean and obese human subjects (Geliebter *et al.*, 1988). When gastric factors are considered in isolation from duodenal

factors, gastric volume rather than nutrient content appears to be responsible for the inhibition of food intake. No greater suppression of subsequent food intake is observed after nutrient infusion than after saline infusion in the same volumes into pylorus-occluded rat stomachs (Degen and Phillips, 1996). This suggests that the contribution of stomach distension to reduced food intake, as well as the generation of post-prandial sensation, may be largely due to mechano- rather than chemoreceptor activation.

Local and central neural pathways are probably responsible for providing the feedback signalling the type and quantity of stomach contents. In rats, distension of the stomach produces changes in the firing rates in both the lateral and ventromedial hypothalami. The precise contribution of these central mechanisms is difficult to interpret though, as both increases and decreases in firing rate are observed (Maddison and Horrell, 1979). Changes in plasma glucose concentration (Hebbard *et al.*, 1996a; Hebbard *et al.*, 1996b) and in satiety hormone release (Melton *et al.*, 1992) probably also contribute to the sensation of stomach distension, perhaps via the vagus nerve (Troncon *et al.*, 1995).

2.3.2.3 Gastric emptying

The presence of fat, carbohydrate and protein in the upper small intestine, either in their pure state (Brener *et al.*, 1983; Heddle *et al.*, 1988a; Heddle *et al.*, 1988b; Shi *et al.*, 1997; Treacy *et al.*, 1990), or as part of a mixed nutrient meal (Hunt, 1980), delays gastric emptying. Feedback from small intestinal mechanisms contributes to this effect (Section 2.3.4). In addition, increases in plasma glucose concentrations are partly responsible for the small intestinal nutrient-induced delay in gastric emptying, independent of any local nutrient effects in the small intestine itself. For example, MacGregor *et al.*, (1976) reported an inverse relationship between the rate of gastric emptying of nutrient-containing meals and the blood glucose concentration after intravenous glucose administration. Acute hyperglycemia, during glucose clamping in patients with diabetes mellitus slowed emptying of nutrient-containing liquid and solid meals (Fraser *et al.*, 1990). Under hyperglycemic conditions (~ 15 mmol/l), gastric emptying of solid and liquid meals is slower than under euglycaemic conditions (Fraser *et al.*, 1990) and even increases in the blood glucose concentrations within the normal range (4 – 8 mmol/L) also slow gastric emptying (Schvarcz *et al.*, 1993). The contribution of gastric emptying to the regulation of appetite and food intake is probably indirect and related to stomach distension and antropyloroduodenal motor function.

2.3.3 Pylorus

The pylorus is a short (~2 cm) region connecting the antrum to the duodenal bulb. Its primary function is that of a sieve, to regulate outflow of intraluminal contents from the stomach (Hasler, 1995). Although its pacesetter potential is the same as that of the stomach (Hasler, 1995), the pylorus is subject to neurohormonal control distinct from the adjacent antrum and duodenum and has more neurons containing vasointestinal peptide, substance P, enkephalin, neuropeptide Y and galanin than either the antrum or the duodenum (Hasler, 1995). Accumulating evidence supports the theory that the pylorus acts to regulate gastric outflow, in response to small intestinal feedback. The precise coordination of the series of events involved is complex, and incompletely understood.

The role of the pylorus during the interdigestive period is unclear. It does not appear to act as a tonic sphincter, as holding open the pylorus with an intraluminal stent does not change gastric emptying rates (Crider and Thomas, 1937). The development of the 'pyloric sleeve' incorporated into the manometric assemblies used to measure intraluminal gut pressures (Heddle *et al.*, 1988a), has enabled precise measures of pyloric contractile activity. These have revealed that the fed state is characterized by increased basal pyloric tone and the onset of isolated pyloric waves (IPPW) (Section 2.3.4).

2.3.3.1 Pulsatile transpyloric flow

Transpyloric flow is tightly regulated and optimized to ensure adequate grinding and mixing of stomach contents, as well as efficient absorption of nutrients along the length of the small intestine. Animal (Anvari *et al.*, 1995) and human (King *et al.*, 1984) studies demonstrate that transpyloric flow is principally pulsatile, and includes episodes of flow from the duodenum back into the stomach. These antegrade (forward) and retrograde (backward) flow pulses depend on the complex organisation of antropyloroduodenal (APD) motor unit activity and are influenced by gastric, pyloric and duodenal factors. To date, studies that have evaluated the effects of intraduodenal nutrients on appetite and / or APD motor unit activity have used continuous intraduodenal nutrient infusions.

The relationship between antroduodenal motility and transpyloric fluid movement in humans was first recognized using real-time ultrasound. This demonstrated that both forward and backward movements occur during the course of normal emptying, but that the majority of the flow pulses were in discrete 2 - 5 second episodes (King *et al.*, 1984), rather than as continuous flow. Sonographic and fluoroscopic techniques have now

established that pulsatile flow accounts for between 71% and 80% of all transpyloric fluid movement (King *et al.*, 1984; Malbert and Ruckebusch, 1991).

The mechanical events that result in flow are controversial. Until recently, gastric emptying of liquids was believed to occur in response to the pumping action of antral peristaltic contractions (Brener *et al.*, 1983; Cooke and Clark, 1976), or as a steady flow (induced by a pressure gradient maintained and regulated by proximal stomach tone) (Strunz and Grossman, 1978) interrupted by intermittent closure of the pylorus (Kelly, 1980). A steadily advancing peristaltic contraction or a constant intragastric pressure cannot however, explain the observed intermittent pattern of gastric emptying. There are currently two theories on the generation of the intermittent nature of flow. Studies in pigs (Treacy *et al.*, 1994) and dogs (Malbert and Ruckebusch, 1991) suggest that antral pumping temporarily ceases, concurrent with stimulation of IPPWs. A second hypothesis proposes that intermittent increases in gastric pressure (Anvari *et al.*, 1995), concurrent with decreases in phasic resistance in the distal stomach underlie the intermittent flow activity. Clearly, there is an integration of motor mechanisms involving the proximal and distal stomach, the pyloric sphincter itself, and most likely the proximal small intestine. The precise nature of this interaction warrants further investigation.

The consequences of pulsatile transpyloric flow on the cascade of events that follows gastric emptying; the distribution of nutrients along the small intestine, absorption of nutrients, stimulation of satiety hormones and resulting satiety, are unknown. Some are investigated in the study described in Chapter 10.

2.3.4 Small intestine

The small intestine is a tube ~ 5 m in length and consisting most proximally of the duodenum of about 25 cm, the jejunum (~ 2 m long) and most distally the ileum (~ 3 m long). In the duodenum and the terminal ileum, electrical activity cycles at about 12 and 8 - 9 times per minute, respectively. Food absorption occurs principally in the duodenum and the jejunum and the mucosal cells in this region are highly sensitive to luminal contents. Both gastric (Section 2.3.2) and small intestinal factors independently influence the postprandial control of feeding. In addition, they interact to produce strong and persistent feedback, signalling satiety and affecting subsequent food intake.

The contact of partly digested food with gastrointestinal endocrine cells and enteric neurons modulates gastric emptying and stimulates gastrointestinal symptoms and hormone release, all of which signal satiety. The organisation of antropyloroduodenal (APD) motility contributes to the regulation of gastric emptying, whereas the release of gastrointestinal hormones produces satiety that persists well after food has emptied from the proximal into the distal small intestine. These interactions are influenced by the specific contents of the lumen, particularly by the type of nutrient as discussed in Chapter 4.

2.3.4.1 Satiating effects of intraduodenal nutrients

Nutrients infused directly into the small intestine of humans or experimental animals dose-dependently decrease energy intake at a subsequent meal or sham feeding (Castiglione *et al.*, 1998). In lean and obese humans, the decrease in food intake is preceded by decreases in hunger and desire to eat, and increases in fullness (Chapman *et al.*, 1999). A comparison of the satiating effects of intraduodenal and intravenous glucose in doses that produce equivalent increases in blood glucose concentrations demonstrates that intraduodenal delivery of nutrients is significantly more satiating (Lavin *et al.*, 1996), illustrating the importance of the interactions of duodenal nutrients with small intestinal receptors in the regulation of appetite.

A significant component of the appetite response to intraduodenal nutrients is mediated by their effect on gastrointestinal sensations, including those that are pleasant such as fullness and satiation, and those that are unpleasant, such as bloating and nausea. Intraduodenal lipid (Chapman *et al.*, 1999; Cook *et al.*, 1997) and carbohydrate (Rayner *et al.*, 2000) evoke sensations of fullness and decrease hunger and desire to eat. Little is known about the time course of the development of gastrointestinal symptoms. However, it is likely that a greater nutrient interaction with small intestinal receptors leads to more intense sensation. The return of hunger appears to be related directly to a decline in the exposure of the small intestine to nutrient stimuli (Sepple and Read, 1989). Generation of sensations by intraduodenal nutrients depends on the products of nutrient digestion. For example, the intensity of the post-prandial symptoms and the decrease of appetite suppression produced by combined gastric distension and intraduodenal lipid depends on the breakdown of triglycerides to free fatty acid. Lipase inhibition using Orlistat significantly decreases the intensity of the nausea and bloating (Feinle *et al.*, 1999b). Tolerance for intraduodenal lipid shows large inter-individual variation and is also dependent on dietary habits of

subjects. Independent of their effects on other regions of the gastrointestinal tract, nutrients in the small intestine affect satiety by stimulating both mechanical and chemical feedback and these effects are detailed below.

2.3.4.2 Duodenal distension

The first consequence of intraduodenal nutrient infusion is duodenal distension. Volumes of air greater than 10 ml, administered via duodenal balloon distension, produce antropyloroduodenal motor unit responses similar to those produced by intraduodenal nutrients, i.e. increases in pyloric motility and decreases in both antral and duodenal motility (Edelbroek *et al.*, 1994; Heddle *et al.*, 1988a). The finding that feedback inhibition of gastric emptying by duodenal balloon distension is less than to a comparable volume nutrient infusion (Edelbroek *et al.*, 1994) however, suggests that other non-mechanical factors must contribute. Consistent with this, an increase in blood glucose enhances the pyloric motor response to duodenal balloon distension (Lingenfelser *et al.*, 1999), indicating that nutrients themselves modify antropyloroduodenal motor unit activity, and potentially post-prandial satiety signals.

2.3.4.3 Antropyloroduodenal motility

The marked delay in gastric emptying that occurs in response to duodenal nutrients (Azpiroz and Malagelada, 1985; Heddle *et al.*, 1988a; Lin *et al.*, 1989; Lin *et al.*, 1990) is a result of a decrease in gastric propulsive forces. This is caused by suppression of antral pressure waves (Heddle *et al.*, 1988a) and a reduction in proximal gastric tone, (Azpiroz and Malagelada, 1985) together with an increased resistance to transpyloric flow as a result of stimulation of basal pyloric pressure and phasic pressure waves localized to the pylorus (isolated pyloric pressure waves (IPPWs)) (Heddle *et al.*, 1988a; King *et al.*, 1984; Malbert and Mathis, 1994). Increases in basal pyloric pressure and isolated pyloric pressure waves are associated with cessation of transpyloric flow suggesting that the stimulation of pyloric motility may be the most important of these mechanisms (Horowitz *et al.*, 1994).

Feedback from duodenal receptors is important in regulating distal gastric motility. Both intraduodenal glucose and lipid suppress antral and stimulate pyloric motility although the response to lipids is delayed by 10-15 min (Heddle *et al.*, 1988a) compared to that of glucose (Edelbroek *et al.*, 1992). This difference probably reflects the time taken for conversion of lipid to free fatty acids that interact with duodenal chemoreceptors. The pyloric motor response to intraduodenal protein is less well characterized. In cats,

duodenal infusion of mixed amino acid solutions increase the amplitude of pyloric contractions (Reynolds *et al.*, 1985). This response is specific for L-tryptophan, as neither intraduodenal infusion of L-phenylalanine nor L-glycine influence pyloric activity (Reynolds *et al.*, 1985). In humans intraduodenal infusions of L-tryptophan delay gastric emptying (Carney *et al.*, 1994) increase phasic and tonic motility (Edelbroek *et al.*, 1994). Changes in APD motor unit activity are not only caused by intraduodenal nutrients. Non-nutrient hyperosmolar solutions also delay gastric emptying (Lin *et al.*, 1993), although the extent to which they do so is less. These studies demonstrate how different nutrients add to the complexity of co-coordinating antropyloroduodenal motility.

Alterations in the absorptive and / or structural characteristics of the small intestine may modify the motor response to intraduodenal lipid. For example, the IPPW response to continuous infusions of lipid for greater than one hour is attenuated when compared to the initial response. The frequency of IPPWs decreases from a maximum of ~ 2.4 / min to ~ 1.4 / min and there is a reduction in amplitude, and homogeneity of IPPWs, with those occurring towards the end of the infusion becoming poorly defined and irregular in frequency (Fraser *et al.*, 1993). Similarly, phasic and tonic pyloric pressure wave increases are not sustained throughout a continuous 90 min intraduodenal dextrose infusion, although, the suppression of antral contractions persists (Edelbroek *et al.*, 1992). This may indicate that different mechanisms are responsible for controlling monosaccharide-induced feedback to different regions of the stomach. An intraduodenal triglyceride infusion started immediately after the end of that dextrose infusion re-stimulated both phasic and tonic pyloric pressure waves (Edelbroek *et al.*, 1992), which suggests that the adaptive changes in motor responses and therefore small intestinal chemosensors, are nutrient specific. The APD motor responses to intraduodenal lipid delivered continuously and in a pulsatile fashion are evaluated in Chapter 10.

2.3.4.4 Region and length of intestinal contact

Although infusions of lipid and / or carbohydrate into the duodenum, jejunum or the ileum (Castiglione *et al.*, 1998; Chapman *et al.*, 1999; Cook *et al.*, 1997; Welch *et al.*, 1985) all suppress appetite and subsequent food intake, the degree of suppression depends on the region and length of the intestine exposed to the nutrients.

Studies in humans, have shown that while overall food intake was not significantly different between infusions, infusion of fat into the jejunum results in a greater decrease in

sensations of hunger and rate of food ingestion than infusion of an identical amount of fat into the terminal ileum (Welch *et al.*, 1988). These findings support the probability that nutrients in different parts of the small intestine may suppress food intake by different mechanisms and probably reflect the differences in receptor populations present in isolated regions of the small intestine (Grundy and Scratched, 1989), or the different absorptive capacities of the small intestinal regions; the ileum absorbs fat at one half the rate of the jejunum (Wu *et al.*, 1975).

The length of the small intestine exposed to nutrients, also affects the efficiency with which these nutrients suppress appetite and food intake. In humans, Welch *et al.*, (1988) demonstrated that infusions of a corn oil emulsion into the jejunum significantly reduced hunger before a meal and reduced the rate of meal ingestion, whereas more distal ileal infusion did not influence either parameter. Consistent with this, studies in dogs (Lin *et al.*, 1989; Lin *et al.*, 1990) have shown greater suppression of feeding after infusions of nutrient into greater lengths of intestine. These responses are probably due to the greater recruitment of receptors. As has been customary when assessing the effects of small intestinal nutrients on feedback processes affecting appetite and APD motility, the infusions administered in the studies in this thesis (Chapters 10 and 14) were delivered into the duodenum.

2.3.5 Gastrointestinal peptides

Hormones released from gastrointestinal cells mediate many of the consequences (such as the suppression of appetite and food intake) arising from contact of nutrients with the small intestinal mucosa. Many processes depend on the vagus nerve in order to produce their satiating effects (Table 2.3). The gastrointestinal peptides discussed below are those were measured in the studies described in Chapters 7, 10 and 11.

2.3.5.1 Cholecystokinin

Cholecystokinin (CCK) is a peptide produced in the small intestine and released into the circulation, in response to luminal nutrients, particularly fat and protein (Peikin, 1989). It is localised within mucosal I-cells of the duodenum and jejunum, in concentrations of approximately 15-50 pmol per gram of tissue, and is also found in enteric nerves, mucosa, smooth muscle cells of the lower gastrointestinal tract, and in ascending fibres of vagus nerve. Cholecystokinin circulates in a number of forms including CCK-4, CCK-8 (which contains all of the biological activity) and CCK-33. In the central nervous system CCK-8S

(sulphated CCK-8) acts as a neurotransmitter after release from nerve terminals. In humans, circulating levels increase from fasting 1 ± 0.2 pmol/l to approximately 5 – 10 pmol/l after a meal.

Table 2.3 Gastrointestinal and pancreatic hormones that modulate food intake after peripheral administration. ? indicates unknown. Adapted from Morley, (1990).

	Effect on food intake	Dependence on vagal nerve
Pancreatic hormones		
Glucagon	Decrease	Yes
Insulin	Increase	No
Amylin	Decrease	No
Gastrointestinal peptides		
Bombesin	Decrease	Partially
Cholecystokinin	Decrease	Yes
Gastrin-releasing peptide	Decrease	?
Ghrelin	Increase	?
Motilin	Increase	?
Satietin	Decrease	?
Somatostatin	Decrease or no effect	Yes
Thyrotropin-releasing hormone	Decrease	Yes

Two CCK receptor subtypes have been identified; CCK-A and CCK-B. CCK-A receptors are found mainly in the periphery, including pancreatic acinar cells, the gall bladder, the smooth muscle of the pylorus (Smith *et al.*, 1984) and in vagal afferent nerve fibres (Moran *et al.*, 1987). They are also found in some areas of the CNS, notably the nucleus tractus solitarius, area postrema, dorsal medial hypothalamus, interpendular nucleus and habenulum. CCK-B receptors are found mainly in the CNS, including in vagal afferents, the cerebral cortex, the olfactory bulb and hypothalamus. Both peripheral and central CCK-A receptors are identical to gastrin receptors, and CCK-B receptors exhibit a high degree of homology to gastrin receptors.

Oral ingestion, and intragastric, and intraduodenal administration of fat and protein stimulate the release of CCK in mammals (Drewe *et al.*, 1992). Intravenous fat infusions are not associated with increases in plasma CCK concentrations, indicating the need for a direct contact effect of fat on the gastrointestinal system to release CCK (deBoer *et al.*, 1992). Tetrahydrolipstatin (a gastrointestinal lipase inhibitor) suppresses fat-induced

increases in plasma CCK concentrations by 77% (Hildebrand *et al.*, 1998) and in patients with pancreatic exocrine insufficiency (i.e. lipase deficiency) duodenal perfusion of oleic acid as free fatty acid, but not as triglycerides, generates a two-fold increase in CCK release (Guimbaud *et al.*, 1997). In rats, Pluronic L-81 (which inhibits chylomicron formation) but not Pluronic L-63 (a chemically similar surfactant that has no effect on chylomicron formation) blocks lipid-induced CCK release (Raybould *et al.*, 1998). Thus, the release of CCK after a fatty meal is dependent on (i) hydrolysis of fat to free fatty acids and (ii) chylomicron formation. Whether the mode of lipid delivery into the duodenum influences the release of CCK is unknown, and this has been evaluated in the study reported in Chapter 10.

Central and / or peripheral CCK administration suppresses food intake in a variety of non-human species, including rats (Gibbs *et al.*, 1976), dogs (Pappas *et al.*, 1985) and rhesus monkeys (Gibbs *et al.*, 1976). Physiological doses of CCK appear to be responsible for the observed affects (Figlewicz *et al.*, 1989). The satiating effect of CCK may be centrally mediated, as intraventricular CCK delays gastric emptying as well as reduces meal size, whereas similar doses of intravenous CCK delay gastric emptying but do not affect meal size (Figlewicz *et al.*, 1989). In healthy young (Liddle *et al.*, 1986; Pi *et al.*, 1982) and older (MacIntosh *et al.*, 2001b) humans and in obese individuals (Pi *et al.*, 1982), intravenous infusions of CCK-8, in doses that increase circulating CCK concentrations into the normal post-prandial range (Lieverse *et al.*, 1995), decrease food intake. There is no difference in the satiating effect of CCK between lean and obese subjects (Lieverse *et al.*, 1993) but there is evidence that the satiating effect may be greater in old than young adults (MacIntosh *et al.*, 2001b). There is evidence that cholecystokinin administration reduces intake of fatty, rather than carbohydrate- or protein-rich foods (Lieverse *et al.*, 1995), suggesting a nutrient-specific action of CCK on food intake.

There remains some uncertainty as to whether the satiating effects of CCK are physiological or pharmacological. Some studies suggest that CCK reduces food intake by inducing malaise (Moore and Deutsch, 1985), whilst direct comparisons of the effects of CCK and lithium chloride (LiCl) (a known aversive agent) in rats, argue against an aversive effect of exogenous CCK by showing that the patterns of suppression of food intake differ between these two agents. There are few reports of non-specific effects of exogenous CCK on food intake in humans. Studies in which CCK has been administered systemically have not reported any significant effects on sensations of nausea (MacIntosh

et al., 2001b). The induction of sleepiness produced by a high- but not low-fat meal (Wells *et al.*, 1997b) may be mediated by CCK acting through receptors other than CCK-A, as loxiglumide increased ratings of fatigue and sleepiness after a fatty meal (Wells *et al.*, 1997a). In humans at least, it is unlikely that the satiating effect of CCK is a result of aversive effects induced following its administration.

Studies demonstrating that CCK antagonists enhance food intake have strengthened the hypothesis that endogenous CCK acts as a satiety factor in mammals. Systemic administration of devazepide (CCK antagonist) increases test meal size in animals (Cheng *et al.*, 1993; Covasa and Forbes, 1994; Ebenezer and Baldwin, 1995; Ebenezer *et al.*, 1990; Hewson *et al.*, 1988; Moran *et al.*, 1993; Silver *et al.*, 1989). The results from human studies however, are less convincing. Loxiglumide is one of the few CCK-A receptor antagonists available for human use (Feinle *et al.*, 1999a). The effects of a 643 kJ appetizer on hunger and fullness in humans were antagonized by loxiglumide (Gutzwiller *et al.*, 2000) and consistent with this, loxiglumide has recently been shown to increase short-term food intake in humans (D'Amato, 2001). On the other-hand, in a relatively small number of free feeding individuals who received oral loxiglumide three times daily for 3 days, there was little effect on daily food intake or test meal intake (French *et al.*, 1994). The acute reduction of feeding by CCK may therefore not be sustained and this implies that CCK is satiety factor that acts acutely rather than over long periods.

The CCK-A receptor antagonist loxiglumide abolishes the suppression of food intake produced by intraduodenal fat infusion (Lieverse *et al.*, 1995), indicating that fat acts through CCK-A receptors to stimulate satiety. A synergistic effect of gastric and small intestinal CCK mechanisms appears important, as oral pre-loads alone have no effect on CCK release, while co-administration with intraduodenal fat leads to CCK-dependent reduction in energy intake, greater than that induced by intraduodenal fat alone (Matzinger *et al.*, 1999). There is convincing evidence from studies in humans that CCK suppresses feeding by augmenting fullness and reducing hunger after a meal. In this context, CCK probably amplifies neural activity from the distended stomach. Feinle *et al.*, (1996) demonstrated that during gastric distension, CCK release by intraduodenal lipid-induced is involved in the induction of meal-like sensations of fullness. Cholecystokinin-A receptors probably mediate this response, as intravenous loxiglumide attenuated the effect. Therefore, endogenous CCK probably regulates food intake by directly affecting gastric sensation.

2.3.5.2 Glucagon-like peptide-1

Glucagon-like peptide-1 (GLP-1) is a member of the glucagon class of peptides. It is an insulinotropic peptide hormone secreted from the endocrine cells in the gut mucosa in response to meal ingestion. In addition to being processed in the periphery, where it acts as an incretin (stimulating insulin secretion) and inhibits upper gastrointestinal motility ('ileal brake'), it is also secreted by cells in the central nervous system where it is thought to have a role as a satiety hormone.

Populations of α-cells in the pancreas, and L-cells in the distal ileum and colon process GLP-1 from proglucagon. The major source of circulating GLP-1 is the intestinal L-cell (Turton *et al.*, 1996). In the body, GLP-1 is present in a number of forms, the biologically active of which is GLP-1 (7-36). Glucagon-like peptide-1 immunoreactive nerve fibres and terminals are widely distributed in the brain with the highest density in the hypothalamus, thalamus, and septal regions, and the lowest concentrations in the cortex and hindbrain (Kieffer and Habener, 1999). Glucagon-like peptide-1 receptors have been identified in the brain, lungs, pancreatic islets, stomach, hypothalamus, heart, intestine, and kidney (Kieffer and Habener, 1999). This receptor is highly specific for GLP-1 and does not bind other peptides of the glucagon superfamily. Co-localisation of GLP-1 receptors and glucose sensing-related proteins in hypothalamic neurons suggests a role for GLP-1 in the hypothalamic regulation of macronutrient selection (Navarro *et al.*, 1996). Glucagon-like peptide-1 (7-36), but not (7-16) or (7-33) suppresses food intake in the neonatal chick, suggesting that the C-terminal amino acids of GLP-1 play an important role in its bioactivity in the CNS (Furuse *et al.*, 1998). Although the bioactivity of the molecule does not depend on an intact N-terminal, this region does determine its efficacy. When the N-terminal histidine is replaced with tyrosine, there is an 11–13-fold decrease in suppressive effect of GLP-1 on food intake (Bungo *et al.*, 1999).

Glucagon-like peptide-1 is released into the circulation after a meal (Kieffer and Habener, 1999; Kreymann *et al.*, 1987) in response to direct contact of nutrients with the gastrointestinal lumen. Oral and intraduodenal nutrients stimulate a rise in plasma GLP-1 from a basal concentration of 0.4 - 1.4 pmol to a maximal post-prandial concentration of 10 - 12 pmol. Release of GLP-1 after oral glucose is biphasic, peaking at ~ 30 - 60 min post-glucose ingestion. Hormonal and neural inputs probably control the early release, while direct nutrient contact with the L-cells may mediate later secretion (Kieffer and

Habener, 1999). The release of GLP-1 from the isolated perfused ileum requires sodium (Herrmann *et al.*, 1995), implicating the sodium / glucose transporter in this effect. Consistent with this, other sugars that utilise this co-transporter for absorption (eg. galactose) also stimulate GLP-1 release (Kieffer and Habener, 1999). Oral amino acid challenges also induce rapid rises in plasma GLP-1 concentrations, while fat (in the form of corn oil) induces strong, long-lasting (> 120 min) increases in GLP-1. The chain-length and degree of saturation of the fatty acids affect the ability of fats to stimulate GLP-1 secretion. Monounsaturated long-chain fatty acids (= C16) are more effective than short-chain or medium-chain, polyunsaturated or saturated fatty acids (Kieffer and Habener, 1999). In addition, GLP-1 release is strongly influenced by the physical characteristics of a meal; significantly more GLP-1 is released after a liquid meal, than after a solid meal otherwise identical in composition (Brynes *et al.*, 1998). Comparative GLP-1 release after isoenergetic glucose and fructose loads in diabetic and non-diabetics have been evaluated in Chapter 11.

One of the actions of GLP-1 on the hypothalamus is to reduce food intake (Bungo *et al.*, 1999; Davis *et al*, 1998; Gutzwiller *et al.*, 1999), and GLP-1 antagonises well-known orexigenic hormones such as NPY (Furuse *et al.*, 1997). The administration of GLP-1 into the CNS of rats profoundly decreases food consumption (Hwa *et al.*, 1998), while administration of the GLP-1 receptor antagonist exendin (9-39) blocks this inhibitory effect of GLP-1 and increases feeding when given alone. Intraperitoneal GLP-1 is ineffective in reducing food intake in rats (Turton *et al.*, 1996), and there is some debate as to whether the diminished food intake responses to intracerebroventricular administration are due to satiety or food aversion (Thiele *et al.*, 1997) although mice lacking functional GLP-1 receptors display normal feeding behaviour (Scrocchi and Drucker, 1998), this may be explained by the great redundancy present in feeding control with multiple compensatory mechanisms present.

In healthy young adult humans intravenous GLP-1 (50 pmol/kg/h) enhances satiety and fullness after a fixed energy breakfast and reduces food intake at an *ad libitum* lunch by 12% compared with saline (Flint *et al.*, 1998). Graded intravenous infusions (0, 0.375, 0.75 and 1.5 pmol/kg/min) reduce food intake (maximum inhibition 35% versus the saline control infusion) in a dose-dependent manner (Gutzwiller *et al.*, 1999). Intravenous GLP-1 infusions also enhance satiety and fullness and reduce energy intake in people with type 2 diabetes (Gutzwiller *et al.*, 1999). Infusions of GLP-1 for 2, 6, 8 and 48 h all reduce food

intake (Kieffer and Habener, 1999) in man. As no adverse events, or undesirable symptoms have been reported with these infusions, this is likely to be a true satiety effect. It remains unclear however, whether GLP-1 is a physiological as well as a pharmacological satiety hormone.

Chronic studies, thus far limited to rats, indicate that GLP-1 may have some therapeutic potential in the treatment of food intake-related disorders, such as obesity. Six days of daily intracerebroventricular GLP-1 administration reduced food intake and body weight in schedule-fed wild-type and genetically obese rats, whereas the administration of the GLP-1 antagonist exendin (9-39) increased both food intake and body weight (Meeran *et al.*, 1999). After the same period of chronic daily intracerebroventricular GLP-1 dosing of genetically obese rats, significant reductions in both cumulative food intake and body weight were observed (Davis *et al.*, 1998). Chronic daily administration of exendin decreased daily food intake and blocked weight gain in obese rats (Rodriquez-de *et al.*, 2000). In lean rats however, the reduction in food intake during daily GLP-1 was diminished after 2 days, and disappeared after 3 days (Donahey *et al.*, 1998). This apparent adaptation of satiety pathways to chronic GLP-1 dosing may limit the usefulness of GLP-1 in the treatment of obesity. Further studies are required to evaluate metabolic responses to chronic GLP-1 administration in humans.

Glucagon-like peptide-1-induced suppression of food intake involves both mechanical and chemical mechanisms. Intravenous GLP-1 infusions delay gastric emptying in both lean (Long *et al.*, 1999) and obese (Naslund *et al.*, 1998) subjects, which may suppress food intake by prolonging sensations of fullness. In lean individuals, this delay in gastric emptying by GLP-1 was achieved using doses that produced double the usual post-prandial plasma GLP-1 concentrations (Long *et al.*, 1999). The biological relevance of this finding is therefore difficult to assess. Consistent with GLP-1 being a determinant of gastric emptying however, Wishart *et al.*, (1998) found that the early rise in GLP-1 following a 75 g oral glucose load was significantly and inversely related to the rate of gastric emptying. Vagal afferents probably mediate the inhibitory action of GLP-1 on gastric motor function (Imeryuz *et al.*, 1997).

Glucagon-like peptide-1-mediated satiety signals initiated by nutrient-based stomach distension are apparently due to the nutrients, and not the mechanical distension they induce (Kieffer and Habener, 1999). Glucagon-like peptide-1 may interact with several

chemical mediators to reduce sensations of appetite and subsequent food intake. Irrespective of the duration of fasting, GLP-1 strongly inhibited food intake as rapidly as 10 min after central administration. Central NPY enhanced food intake, but co-injection of GLP-1 decreased food intake in a dose-dependent manner (Furuse *et al.*, 1997).

The metabolic benefits of stimulating GLP-1 release using non-pharmacological agents, such as monosaccharides is discussed in Chapter 4 and evaluated in Chapter 11.

2.3.5.3 Gastric-inhibitory peptide

Gastric-inhibitory peptide (GIP) is a 42-amino acid peptide that inhibits gastric acid secretion and stimulates pancreatic insulin release in the presence of glucose (Morgan, 1996) (Chapter 4). It is derived from the amino acid residue peptide preproGIP, whose proteolytic cleavage gives rise to GIP and N- and C-terminal peptides of 22- and 59-amino acids, respectively (Morgan, 1996). In humans, fats (Falko *et al.*, 1975) and carbohydrates (Rayner *et al.*, 2000) potently stimulate GIP secretion. There is some suggestion that release of GIP after monosaccharides may be different as glucose, but not fructose, stimulates GIP release (Rayner *et al.*, 2000). Amino acids are weak stimulants of GIP (O'Dorisio *et al.*, 1976). Gastric-inhibitory peptide-receptor expression is present in the pituitary gland, but not in the rat hypothalamus. Gastric-inhibitory peptide-receptor mRNA has also been detected in the rat adrenal cortex (McIntosh *et al.*, 1996).

To date, there are no data in support of GIP acting as a satiety factor in either humans or animals. When administered to rats, GIP was without effect on food intake (Garlicki *et al.*, 1990). There is evidence however, that GIP may be involved in the stimulation of other peptides known to affect food intake. For example, *in vitro*, exogenous GIP administration produces a dose-dependent rise in PYY concentration, with a maximal response at 800% above basal levels (Plaisancie *et al.*, 1995). Ileal transposition and jejunoileal bypass significantly reduced the post-prandial GIP response (Canbeyli and Koopmans, 1984), demonstrating that at least in rats, the distal small intestine is necessary for meal-stimulated GIP release.

2.3.5.4 Insulin

Insulin is a peptide hormone produced by the pancreatic β-cells in response to stimulation by glucose. Its primary role is one of glucose homeostasis and in this capacity it regulates

blood glucose concentrations. The role of insulin in the regulation of mammalian food intake is controversial. Administration of intravenous glucose is associated with decreased food intake, hyperglycemia and hyperinsulinemia (Van Itallie, 1990; Woods *et al.*, 1984). Chronic central insulin administration in the rat (Brief and Davis, 1984) and peripheral insulin administration in the baboon (Woods *et al.*, 1984) also suppress food intake.

It is unclear whether physiological hyperinsulinemia itself is responsible for the reduction in food intake, because in the absence of altered blood glucose concentrations, hyperinsulinemia does not appear to influence satiety (Chapman *et al.*, 1998). Consistent with this finding, glucose and insulin administration infused in such a manner as to mimic the 'normal' insulin and glucose levels seen shortly before a meal, did not affect meal size or duration in healthy, normal-weight humans (Woo *et al.*, 1984). Mayer's glucostatic theory of food intake regulation however, proposed that changes in the body's carbohydrate stores, rather than changes in blood glucose concentrations are important determinants of energy intake as it relates to the steady state body composition, rather than specific mealtime energy intake (Mayer, 1991; Mayer, 1996). Consistent with blood glucose concentrations affecting food intake, hyperinsulinemia, unrelated to changes in plasma glucose concentration, has been reported to increase hunger, heighten the palatability of sucrose, and increase food intake (Rodin *et al.*, 1985). Peripherally administered long-acting insulin stimulates food intake and weight gain in rodents, however, when infused intracerebroventricularly insulin decreases food intake and body weight (Larue and Le, 1984). It is likely that the peripheral effects of insulin on food intake and appetite differ from those mediated centrally.

2.4 CONCLUSION

This chapter has reviewed topics related to the regulation of mammalian, specifically human, appetite and food intake. Central mechanisms have been discussed briefly, with the specific focus on nitrergic pathways (Chapter 5). The preceding discussion has also examined the importance of the timing of intraduodenal nutrient delivery and subsequent effects on antropyloroduodenal motor function (Chapter 9), and the involvement of cholecystokinin (Chapter 9), glucagon-like peptide-1 (Chapters 4 and 10), gastric inhibitory peptide (Chapters 4 and 10) and insulin (Chapters 4 and 10) in the regulation of normal feeding behaviour. The hypothesis arising from the review of appetite physiology presented in this chapter, and not dealt with elsewhere in the introductory chapters is that:

Pulsatile intraduodenal triglyceride infusions will suppress appetite and food intake to a greater extent than continuous intraduodenal triglyceride infusions.

This has been evaluated in the study described in Chapter 10.

Chapter 3

DIETARY MACRONUTRIENTS AND THEIR COMPARATIVE EFFECTS ON APPETITE AND FOOD INTAKE

3.1 INTRODUCTION

Eating triggers characteristic feedback responses that increase satiety (Read *et al.*, 1994). These responses depend on both the type of food consumed i.e. the specific macronutrient composition (Rolls, 1986; Rolls and Hammer, 1995), and the timing of food consumption i.e. the frequency of meals (Speechly and Buffenstein, 1999a; Speechly *et al.*, 1999b). The physical and chemical properties of foods are largely responsible for their effects on satiety. Macronutrients differ in their ability to suppress appetite and food intake and it has been proposed that there is a satiety hierarchy. Evidence from studies on food intake regulation in both humans and animals support a greater satiating efficiency of protein than either carbohydrate or fat (Poppitt *et al.*, 1998; Porrini *et al.*, 1995; Stubbs *et al.*, 1996; Teff *et al.*, 1989). The data from studies evaluating the comparative satiating efficiencies of carbohydrate and fat are controversial. There is some indication, however, that differences in the duration of satiety induced by these nutrients may provide an explanation for these conflicting data. Only a limited number of studies have evaluated the comparative timing of appetite suppression by the three dietary macronutrients and these are discussed below. A better understanding of the satiating effects of dietary macronutrients would allow the design of diets to maximise satiety in weight loss regimens, which could in turn promote greater compliance by individuals on these diets.

The literature is divided on the issue of beneficial eating patterns. Snacking is often regarded as predisposing to weight gain and obesity, with the required daily energy intake optimally obtained by adhering to a strict 3-meal per day eating pattern. Such a plan is therefore often prescribed in weight reduction programmes. Conversely, chronic dietary intervention and population studies have demonstrated a number of metabolic advantages to spreading the daily nutrient load over more than 3 meals. The direct effects of meal frequency on food intake are unknown.

To address these issues studies were performed that aimed to determine:

1) The effects of protein, carbohydrate and fat ingestion on satiety, when the time interval to subsequent food intake is fixed (Chapter 7) or when the time interval is not fixed and food intake is spontaneous, and (Chapter 8).

2) The effect of meal frequency on *ad libitum* food intake (Chapter 9).

3.2 MACRONUTRIENT SATIATING IIEIRARCHY

Carbohydrate, fat and protein differ in their ability to suppress hunger and subsequent food intake. The specificity of macronutrient-induced satiety has been evaluated using a variety of methods. Generally, *ad libitum* food intake is quantified at a predetermined time interval following ingestion of pre-loads manipulated with respect to macronutrient composition. Alternatively, a chronic dietary intervention is performed, where foods high in a particular macronutrient are consumed *ad libitum* / to satiety and food intake is assessed using diet diaries. The ease with which short-term studies can be performed probably explains why these dominate the literature. A major disadvantage of such studies, however, is their inability to assess downstream effects such as those that are influenced by macronutrient utilisation. Furthermore, results are very sensitive to the time interval between the pre-load and the test meal, which is usually fixed. Despite these limitations data from acute (within day) interventions supports a hierarchy of food intake suppression, where protein is the most and fat the least satiating macronutrient (Poppitt *et al.*, 1998)(Porrini *et al.*, 1995; Stubbs *et al.*, 1996; Teff *et al.*, 1989).

3.2.1 Dietary interventions and pre-loading studies

A number of studies have evaluated the comparative effects of macronutrients on satiety and subsequent food intake, however, only few have assessed the effects of protein, carbohydrate and fat in the same study, whilst also controlling for the different taste and textural properties of these macronutrients. Furthermore, manipulation of macronutrient ratios in previous studies has often been extreme with meal compositions outside the physiological range and well beyond the changes recommended for weight loss. In some studies appetite and feeding responses to pure nutrients have been assessed. Studies assessing food intake responses to less severe dietary interventions would contribute to an understanding of the actual biological relevance of dietary manipulations. Such studies should ideally use pre-loads with characteristics similar to actual whole foods.

A number of studies evaluating responses to readily available food items have shown that protein-rich foods produce greater satiety than low protein foods (Poppitt *et al.*, 1998)(Porrini *et al.*, 1995; Rolls *et al.*, 1988b) whereas foods high in carbohydrate, particularly simple sugars, may stimulate appetite and hunger (Geiselman and Novin, 1982; Rodin *et al.*, 1985). Rolls *et al.*, (1988) reported that isoenergetic pre-loads comprising actual food items, high in fat, carbohydrate (sweet or non-sweet), protein or a mixture of nutrients, had differing effects on food intake at a buffet meal 2 h later. Fullness was greatest following ingestion of both the high protein and the high starch, compared with the high fat, high sucrose and mixed nutrient pre-loads. Consistent with this, the weight and the energy content of the food consumed at the subsequent self-selection lunch were almost 40% lower after the high protein than after the high fat pre-loads (Rolls *et al.*, 1988b). It is possible however, that the different satiating effect of these pre-loads may have been influenced by preconceptions about the foods items offered. For example, the high protein and high starch pre-loads were chicken and pasta, respectively, traditionally considered 'main meal' type foods. The high fat and high sucrose pre-loads were chocolate and Turkish delight, traditionally considered 'snack' type foods and possibly therefore perceived as less satiating. In another study that also evaluated satiety responses 2 hours after 'main meal' type foods high in carbohydrate (pasta) or protein (meatballs), food intake following high protein pre-load the was less than after the high carbohydrate pre-load (Porrini *et al.*, 1995). Food intake reflected differences in sensations of fullness and satiety that were also significantly less after the pasta pre-load (Porrini *et al.*, 1995). These studies suggest that irrespective of perception of food items, foods with high protein content are more satiating than foods with high carbohydrate content, but do not exclude the possibility that some of this difference is due to different perceptions of protein- and carbohydrate-containing foods. By and large, laboratory studies utilizing pure nutrient infusions in rats support this satiating hierarchy as both intragastric (Geliebter, 1979) and intravenous (Walls and Koopmans, 1992) protein administration is more satiating than either fat or carbohydrate.

3.2.2 Time course of effects

The timing of subsequent food intake following previous nutrient ingestion is probably important in determining the relative satiating effects of different macronutrients. In previous studies where the time interval between pre-load and test meal ingestion has been fixed and is less than 60 min, isoenergetic pre-loads of carbohydrate, fat and protein were

similar in satiating efficiency (Geliebter, 1979). After longer intervals, however, significant differences have been reported (Porrini *et al.*, 1995; Stubbs *et al.*, 1996; Teff *et al.*, 1989).

The temporal effects of fat and carbohydrate on suppression of food intake were assessed in a series of studies by Blundell and collegues (Blundell *et al.*, 1993) in which the interval between the pre-load and test meal ingestion was varied. Breakfasts of orange juice, scones and fruit yoghurt, (1.82 MJ) (normal) or the same supplemented to 3.36 MJ with fat (polyunsaturated margarine and dairy cream) or carbohydrate (sucrose, maltodextrin and glucose) were consumed by healthy male subjects who then ate lunch *ad libitum* 4 hours later (Blundell *et al.*, 1993). In all meals the protein content was constant. In an initial study where sensation only was assessed, in the 4 hours following ingestion, carbohydrate supplemented breakfasts suppressed hunger, desire to eat and prospective consumption, and increased ratings of fullness, whereas fat supplemented breakfasts did not affect these sensations. In subsequent studies with similar protocols but designed to also assess feeding responses, food intake at a lunch presented 90 minutes after breakfast was suppressed by the carbohydrate-supplemented, but not the fat-supplemented breakfast. In contrast food intake at lunch presented 270 minutes after both breakfasts was unchanged. These results support the hypothesis that carbohydrate is more satiating than fat but also suggest the presence of characteristic time intervals for satiety induced by different macronutrients. Rolls *et al.*, (1991) measured food intake at meals 30, 90 and 180 min after isoenergetic high fat or high carbohydrate yoghurt pre-loads and found that the macronutrient content of the pre-loads did not differentially affect food intake at test-meals offered, regardless of the time lag between the pre-load and the test lunch. Food intake 90 min intake after the high carbohydrate yoghurt was less than after the high fat yoghurt, once again demonstrating that carbohydrate is more satiating than fat, but also that specific and different time intervals may exists during which satiety following the macronutrients is maximal. The sensory properties of foods probably played a significant role in establishing food intake responses to macronutrients. In this case the subtle differences between the macronutrients might best be examined using pre-loads of pure nutrients instead of foods varying in macronutrient composition.

In the above studies the time between the pre-load and the test meal varied between experimental days. However, this interval was fixed on each specific day and subjects had

no control over when they ate. Whether intake at a test meal would change after different pre-loads when the time interval between the pre-load and the test meal is not fixed, i.e. when subjects can spontaneously select from a range of foods and eat at will is unknown. This has important implications not only for total food intake, but also the accuracy of compensation between two subsequent meals i.e. the amount by which food intake at the test meal is reduced in response to pre-load ingestion. It is well recognised that as the time between the pre-load and the test lunch is increased, the degree of compensation diminishes (Rolls *et al.*, 1991).

In a recent study, Marmonier *et al.*, (2000) assessed the timing and quantity of food intake following consumption of isoenergetic (~ 1 MJ) high protein, high fat and high carbohydrate afternoon snacks in the satiated state. The high protein snack, comprising cooked chicken breast and a low-fat dressing, delayed dinner requests by 60 min, compared to the control, whereas the delays induced by high fat (cream cheese and toasted bread) and high carbohydrate (rye bread with raisins) snacks were shorter (25 and 34 min respectively) (Marmonier *et al.*, 2000). In this acute study total daily food intake was similar across treatment conditions, but in the longer term small differences in timing to further food intake accumulate and could potentially contribute to a decreased number of eating episodes and overall reduction in food intake. Whether protein suppresses food intake by prolonging the duration of appetite suppression, decreasing eating frequency, suppressing overall food intake or a combination of these is yet to be evaluated in a study where hedonic factors and sensory properties of the foods are masked. (Chapter 8)

3.2.3 Implications for weight loss

A greater intake of fat in the diet has been proposed as a mechanism whereby over-consumption of energy leads to obesity. The greater energy density of fat (over twice the energy per gram of protein and carbohydrate) is one possible reason for fat over-consumption (Rolls, 2000), while its failure to elicit satiety is another mechanism that has been extensively studied (Rolls and Hammer, 1995). Dietary recommendations therefore usually support reduced fat diets in weight loss regimes (Garg *et al.*, 1992; Heilbronn *et al.*, 1999).

To be successful a weight loss diet should result in suppression of appetite and food intake, which is maintained, and unaccompanied by adaptive responses. In this context, there is

support for protein producing a suppressive effect on appetite even over prolonged periods. de Castro *et al.*, (1987), assessed macronutrient-specific feedback effects on spontaneous food intake by way of diet diaries in free-living individuals. Protein in the stomach, but not fat, or carbohydrate had a large negative influence on the amount of food ingested at the next meal, implying that healthy individuals who consume higher proportions of dietary protein may eat less food overall. Skov *et al.*, (1999) have recently compared the metabolic benefits of replacing fat in the diet with protein or carbohydrate. Sixty-five overweight or obese subjects were placed on high protein (25%) or high carbohydrate (58%) diets for 6 months. The benefits of a high protein diet on weight loss were present by 3 months (high protein 7.5 kg v high carbohydrate 5 kg, $P < 0.05$), and persisted for the duration of the trial (high protein 8.7 kg v high carbohydrate 5 kg, $P < 0.001$). The high protein diet was also associated with greater loss of total body and intra-abdominal fat, and did not adversely affect plasma free fatty acids. It is unclear whether the weight loss was due to high protein foods being consumed in smaller proportions, as a result of their greater satiety, or whether some metabolic effect of high protein consumption contributed to the observed weight loss. This is an area that warrants further investigation.

3.3 CHARACTERISTICS OF FOODS AFFECTING SATIETY

3.3.1 Sensory properties of macronutrients

The sensory properties of foods, especially taste, smell and texture help to determine food preferences and eating habits, and also affect the satiating efficiency of specific macronutrients (Rolls and Shide, 1992; Warwick *et al.*, 1993). Taste receptor cells in the oral cavity respond to basic tastes such as salty, sour, sweet and bitter, the taste of protein or 'umami' and astringency (Mattes, 1999). High-fat foods are more palatable than low-fat foods and may induce active overconsumption, for example, in rats, high fat diets are preferred over high carbohydrate diets (Warwick and Weingarten, 1994).

Learned and apparently innate preferences exist for proteins, carbohydrates and fats alike, and some of these have been linked to characteristic chemical stimuli. The insulin release following ingestion of high carbohydrate foods may be responsible for an acquired desire to eat foods with sweet tastes, whereas specific amino acid recognition following protein ingestion may assist the recognition of protein in foods (Stubbs, 1999). A preference for dietary fat has been suggested to be closely related to its distinct taste and texture. Specific

fat receptors have been detected in the mouth and differential modulation of potassium channels by fatty acids has been proposed as one possible mechanism for oral sensation of fat (Gilbertson *et al.*, 1997). The effects of palatability on feeding behaviour have not been studied in this thesis but the sensory properties of the oral pre-loads used were consistent to be held constant in the studies conducted in Chapters 7, 8 and 11 to avoid this confounding variable.

3.3.2 Volume and weight

The precise contribution of volumes or weights of food ingested to the regulation of appetite and food intake are unknown. Volume and weight probably influence early satiety directly by an effect of volume on gastric distension (Read *et al.*, 1994; Rolls *et al.*, 1998). Studies in healthy subjects demonstrate the importance of weight and volume of food in the regulation of food intake. When low and high energy, isoenergetic tomato soup pre-loads were consumed by 24 females, hunger sensations and stomach fullness were similar. In addition, despite these differences in the energy density of the pre-loads, there were no differences in the weights of the food eaten at the first or the second courses of the test lunch (Rolls *et al.*, 1988a). As meal volume was held constant whilst energy density was altered, these data suggest that the amount rather than the energy content affects food intake. In a comprehensive study on 37 healthy subjects, the effect of meal volume on food intake, independent of energy density, were assessed. Yoghurt pre-loads differing in both energy (1.26, 2.51 MJ) and volume (250, 500 and 750 g) were administered. Increases in both the energy content and volume of pre-loads decreased food intake in a dose-dependent fashion. In addition, there was a greater correlation between weight and energy content of the pre-loads, revealing a greater effect of the weight of the pre-load on subsequent food intake, than between the energy content of the pre-load and test meal intake (de Graaf and Hulshof, 1996). Although this would suggest that volume might be a more important factor than energy content in determining short-term feeding response to a meal, this effect on food intake is potentially a result of differences in gastric emptying rates of the pre-loads.

A further study evaluated the appetite-suppressive effects of isoenergetic (2088 kJ) milk-based drinks of varying volumes (300, 450 and 600 ml). Pre-loads were followed 30 min later by a self-selection lunch and > 4 h later by dinner (Rolls *et al.*, 1998). This experimental design allowed both the suppression of food intake at lunch and the

compensation at dinner to be assessed. Pre-load volumes of 300 and 450 ml suppressed energy intake at lunch by 18% and 14% respectively compared to the control (no drink) day. Compensation was better after the 600 ml than after the 300 ml pre-load (in the latter, subjects overate, relative to the control day). These results confirm that the volume of food consumed is an important determinant of satiety.

Within the physiological range, the effect of meal volume on subsequent food intake is probably due to an effect of greater stimulation of gastric mechanoreceptors, rather than slowing of gastric emptying, or induction of discomfort (Geliebter *et al.*, 1988). It was therefore necessary in the studies reported in chapters 7 – 11, that the pre-load volumes were well tolerated and representative of 'normal' meals.

3.3.3 Energy density

The energy density of foods (kJ/g [kcal/g]) also plays a role in the control of food intake. It has been suggested that over consumption of fat is largely attributable to its high energy density (~ 2.2 times more energy per gram than either protein or carbohydrate) (Rolls *et al.*, 1999). To some extent the controversies surrounding different satiating efficiencies of macronutrients are due to differences in experimental design. Many evaluations of the effect of energy density on food intake have altered the fat content (and consequently the macronutrient ratios) of the foods in order to manipulate their energy density. A large body of literature now describes the food intake responses to covert manipulations of energy density.

When equally attractive diets differing in fat content (2.9 kJ/g v 6.3 kJ/g) and thus energy density were consumed to satiety, by non obese and obese subjects over a 5 day period, satiety was reached on the low energy density diet at a mean daily energy intake one half that of the high energy density diet. This indicated that higher energy density foods are less satiating than lower energy density foods (Duncan *et al.*, 1983). In support of this finding, several studies have subsequently demonstrated that the energy intake increased as the proportion of fat in the diet increased (Lissner and Heitmann, 1995; Stubbs *et al.*, 1995a; Stubbs *et al.*, 1995b). For example, subjects fed diets varying in both energy density (4.8, 5.6 and 7.0 kJ/g) and fat content (20, 40 and 60%) for 14 days, consumed a constant weight of food across dietary treatments, which resulted in the highest energy consumption on the high fat diet, and lowest energy consumption on the low fat diet (Stubbs *et al.*, 1995b)

The independent effects of energy density on food intake have been evaluated by manipulating the energy density of foods whilst holding macronutrient content and palatability constant. In a study by *Rolls et al.*, (1989) entrees manipulated with respect to energy density (5.23 kJ/g and 7.32 kJ/g) as well as fat content (25%, 35% and 45%), were consumed by lean and obese women. Although women ate less in the low than in the high energy density condition, hunger and fullness were similar following the high and low fat entree intake, demonstrating that the amount of food consumed was related more closely to energy density than fat content in that study. In a shorter study normal weight women were tested for 2 days. Lunch, dinner and evening snacks were eaten *ad libitum* from a selection of foods varying in energy density (low, med or high) but not palatability. This study design ensured that the majority of the daily food intake was derived from these manipulated entrees. By weight, similar amounts of food were consumed in all three groups, resulting in greater energy intake in the high energy density group, than in the medium energy density group (18% more than medium energy density), and in the low energy density group (10% less than the medium energy density group) (Bell *et al.*, 1998). Subject ratings of hunger or fullness did not differ between the groups. These studies show that energy density plays an important role, independent of either fat content or palatability, on the control of food intake. In the study described in Chapters 7 and 8 the volume, weight and energy density of the pre-loads consumed was controlled in order to accurately assess macronutrient-specific effects of foods on subsequent food intake.

3.3.4 Frequency of feeding

The effect of the frequency of meals on eating behaviour has been studied in recent years. A number of investigators have studied the 'gorging' versus the 'nibbling' patterns of eating (Bellisle *et al.*, 1997; Verboeket-van *et al.*, 1993). Although the benefits of more frequent eating are yet to be established, chronic dietary intervention and population studies have demonstrated a lower incidence of overweight and obesity, a cholesterol-lowering effect, and a favourable impact on glycemic control when meal frequency is increased (Bellisle *et al.*, 1997; Fabry *et al.*, 1964; Metzner *et al.*, 1977). Establishing whether metabolic processes are dependent on timing of nutrient ingestion may provide further understanding of the effects and potential benefits of different eating patterns.

Fabry and collegues, (1964) provided evidence of an inverse relationship between meal frequency and body weight in humans. In a study of 379 Czech men aged 60 - 64 years,

the proportion of overweight subjects and the amount of body fat, as assessed by mean skinfold thicknesses, were inversely related to the meal frequency (3 meals or less v 3-4 meals, $P < 0.02$; 3 meals or less v 5+ meals, $P < 0.01$). Work by the same group in school-aged (6 – 16 y) children supported these initial findings (Wurtman *et al.*, 1988). Children attending three schools followed 3, 5 or 7 meals per day *ad libitum* eating frequency regimens. After one year, body weight, height and four skin-fold measurements were recorded. The average daily energy intake was not different between the schools. For the older (10 - 16 y), but not the younger group of children, increases in weight relative to height (indicative of increases in body weight) were greater in the 3 meal, than in either the 5 or 7 meal schools. Changes in triceps, subscapular and abdominal skin-folds reflected these changes. Metzner *et al.*, (1977) reported findings from a long-term epidemiological study performed on a community of about 10,000 individuals in the USA (Tecumseh Community Health Study). Twenty-four hour dietary recall was used to assess meal frequency in men (n = 948) and women (n = 1080) aged 35-49 y. The definition of a meal was based on energy intake and time between eating episodes. While the specific hypothesis tested was slightly different (i.e. adiposity, rather than changes in body weight), the results appeared to follow the same trends. Adiposity index (i.e. a combination of four different skin-fold measures and BMI) was inversely related to meal frequency, for meal frequencies between 2 and 6 per day, in both men and women. Similar reports by others have confirmed these findings in a variety of age ranges, demonstrating significant inverse relationships between meal frequency and percentage body fat and BMI (Bellisle *et al.*, 1997) and waist:hip ratios (Edelstein *et al.*, 1992).

Consistent with these findings, it has previously been observed that obese individuals often skip breakfast (Huenemann *et al.*, 1966) and tend to eat most of their food in one meal (Huenemann, 1972). Kulesza, (1982) also found that obese subjects consumed fewer meals per day than the non-obese subjects. However, the results of these studies have been criticised because of the well-recognised problem of dietary under-reporting, especially by over-weight individuals. Additional, well-controlled, studies are necessary to determine whether meal frequencies affect *ad libitum* food intake.

3.3.4.1 Effect of eating frequency on energy utilisation

A possible explanation for the effects of differences in meal frequency on body weight is an increase in diet-induced thermogenesis. However, there is little evidence to support this from short-term studies (4-48 h) and the underlying mechanism remains unclear.

Several studies have now assessed the influence of meal frequency on weight loss during intentional energy restriction and most have found no significant effect of meal pattern on weight reduction. For example, in a strictly controlled study by Durrant *et al.*, (1982) in which fourteen obese women were housed in a metabolic ward for 3 weeks, the consumption of 3.4 MJ/day in either one or five meals produced an equivalent reduction in weight. These studies employed the same general experimental protocol. A low meal frequency eating pattern, where the recommended daily energy intake for weight loss was consumed entirely in one or two meals per day was compared to a high meal frequency pattern where the daily energy intake was consumed in three to six meals per day. The major shortcoming with this study design in determining the benefits of one meal frequency regime over another on food intake is the lack of an *ad libitum* food intake option; subjects were instructed to consume a fixed amount of food per day. As it is now believed that low and high meal frequencies produce similar energy expenditure responses (see above), it is not surprising that isoenergtic diets consumed under different eating patterns produce equal weight loss.

3.3.4.2 Effect of eating frequency on food intake

The most likely mechanism by which increased meal frequency can reduce body weight therefore seems to be a reduction in chronic food intake. No long-term studies have evaluated the effects of meal frequency on *ad libitum* food intake. For example, instructing subjects to eat, nine times per day versus three times per day would provide information on the satiating efficiency of the different eating patterns. In a recent study Speechly and co-workers, (1999b) evaluated the acute effects of feeding frequency on the relationship between perceived hunger and subsequent food intake and appetite in obese men (n = 7). Subjects consumed isoenergetic pre-loads (4.1 MJ]) 33% average daily energy requirement) orally, in either a single meal or divided evenly over 5 meals given hourly and then ate *ad libitum* from a test meal 1 h after the last pre-load. Despite similar ratings of hunger, when the subjects consumed a single meal, food intake was 27% greater than when the meal was divided. A key question remaining to be addressed in relation to the acute

appetite responses to increased meal frequency is whether covert manipulations of meal frequency affect *ad libitum* food intake in healthy adults.

3.4 CONCLUSION

This chapter has reviewed issues related to the satiating efficiencies of carbohydrate, fat and protein. It appears that there is a physiological macronutrient satiating hierarchy where protein is more satiating than carbohydrate, which is more satiating than fat. A comprehensive study, controlling for hedonic and sensory factors, whilst orally administering pre-loads high in fat, carbohydrate and protein is necessary to define how each of the macronutrients fit into their place in the satiating hierarchy. The discrepancies highlighted between previous studies however, appear to be related to differences in experimental design. In particular, the duration of satiety appears to differ between macronutrients. Very few studies have explored the how these variations affect appetite suppression and spontaneous food intake.

In addition, the work reviewed above has highlighted the metabolic benefits (decreased body weight in people with obesity attempting to lose weight), of increasing the frequency at which meals are taken. The available literature points towards a decreased body weight being a result of a reduction of total food intake, as enhanced meal frequency has little effect on energy utilisation, although this is yet to be evaluated formally.

The studies described in later chapters of this thesis test the following hypotheses arising from this review:

1) High protein yoghurt pre-loads will suppress hunger and desire to eat, enhance fullness and satiety, and decrease food intake to a greater extent than isoenergetic pre-loads high in carbohydrate or high in fat (Chapter 7).
2) High protein yoghurt pre-loads will delay spontaneous food requests longer than isoenergetic pre-loads high in carbohydrate or high in fat (Chapter 8).
3) More frequent pre-loads will suppress hunger and desire to eat, enhance fullness and satiety, and decrease total food intake to a greater extent than isoenergetic meals administered (Chapter 9).

Chapter 4

EFFECTS OF MONOSACCHRIDES ON GLYCEMIC CONTROL AND APPETITE REGULATION IN HEALHTY INDIVIDUALS AND IN PATIENTS WITH NON-INSULIN DEPENDENT DIABETES MELLITUS

4.1 INTRODUCTION

Carbohydrates are classified as monosaccharides, disaccharides, and polysaccharides. This chapter is concerned with dietary monosaccharides. The monosaccharides of significant importance in nutrition are glucose, fructose, and galactose. Fructose is a naturally occurring monosaccharide present in fresh fruits and honey (Hardinge *et al.*, 1965), and has been promoted as an alternative sweetener for patients with non-insulin dependent diabetes mellitus (NIDDM) (Anderson *et al.*, 1989; Koivisto, 1978; Osei and Bossetti, 1989). The primary aim of diabetes treatment is to reduce blood glucose concentrations, and in the case of many people with type 2 diabetes, who are overweight, to reduce body weight. The basis of the recommendation to consume fructose in the diabetic diet is the smaller post-prandial rise in blood glucose produced by fructose, compared to isoenergetic sucrose (glucose:fructose disaccharide) or glucose. The glycemic index (GI) is the area under the postprandial blood glucose curve for a food expressed as a percentage of the area after the consumption of a reference food, usually glucose (Thorburn *et al.*, 1986). The GI of fructose is, at 20, considerably lower than other commonly consumed sugars including sucrose (GI: 60) and lactose (GI: 90) (Thorburn *et al.*, 1986). In addition, there is evidence that fructose may produce a greater insulin response in diabetics than in non-diabetics (Nuttall *et al.*, 1992a), although this has not been directly evaluated. Finally, while there is conflicting evidence on the relative satiating efficiencies of fructose and glucose, some data suggest that fructose has a greater satiating efficiency than isoenergetic glucose (Rodin, 1990; Rodin, 1991; Rodin *et al.*, 1988; Spitzer and Rodin, 1987). This finding remains to be confirmed (Kong *et al.*, 1999), but if true, would by itself support the use of fructose as a substitute for glucose in the diet of overweight patients with diabetes mellitus where obesity is common and a major contributor to insulin resistance.

This chapter reviews dietary intervention studies that have evaluated the potential benefits of including fructose in the diabetic diet, as well as acute studies assessing the comparative blood glucose, insulin, and appetite responses to different monosaccharides in diabetics and non-diabetics. The following discussion raises the question in patients with type 2 diabetes mellitus and non-diabetic controls of whether oral glucose and fructose pre-loads have different effects on:

1) Post-prandial blood glucose concentration,
2) Stimulation of insulin, glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP) release and
3) Appetite and food intake.

These issues are evaluated in Chapter 11.

4.2 FRUCTOSE IN THE DIABETIC DIET

A number of studies have assessed the glycemic, insulin and triglyceride responses to chronic fructose administration in type 2 diabetes, and these data are summarised in Table 4.1. There was no effect on or suppression of fasting plasma glucose and HbA_{1c} (a reflection of chronic blood glucose control). Furthermore, only 1 in 8 studies showed an increase in plasma triglyceride concentrations. It is possible that these outcomes are a result of differences in experimental conditions, such as the duration of the intervention, the background diet, the degree of metabolic control of the subjects or the type of carbohydrate replaced by fructose in the diet. Furthermore, the parameters used to assess glycemic control have differed markedly. In addition, the patients to whom this type of dietary intervention is most relevant are those with type 2 diabetes who are controlling their diabetes by dietary modification alone, or who are on hypoglycaemic medications that do not alter insulin secretion (eg. metformin).

4.2.1 Glycemic control

A small number of studies have reported significantly lower fasting blood glucose concentrations after high-fructose diets in patients with type 2 diabetes mellitus (Osei and Bossetti, 1989; Osei *et al.*, 1987). In general though, fasting blood glucose concentration does not seem to be markedly altered by the consumption of high-fructose diets (Bantle *et al.*, 1986; Crapo *et al.*, 1986; Thorburn *et al.*, 1990). For example, when patients with type 2 diabetes consumed fructose (as 20% of their daily energy intake) instead of either sucrose

(23%) or starch (55%) for 8 days, fasting plasma glucose was reduced by only 6.8% after fructose consumption (Bantle *et al.*, 1986), a reduction that was not significant. Similarly, fasting blood glucose concentration was no lower after 14 days fructose supplementation compared with at baseline (prior to fructose feeding) (Crapo *et al.*, 1986).

In contrast to the marginal effects on fasting blood glucose concentrations, a high-fructose diet does appear to have beneficial effects on post-prandial glycemic control (Bantle *et al.*, 1986; Crapo *et al.*, 1986). This is important as post-prandial hyperglycemia contributes to overall poor glucose control and is being increasingly recognised as a possibly independent risk factor for the cardiovascular and other complications of diabetes. In a study by Crapo *et al.*, (1986) patients with type 2 diabetes consumed fructose (in place of sucrose) for 14 days. Peak serum glucose responses to a 50 g oral glucose load were 15% lower after this fructose feeding than at baseline. It is interesting that after this time, serum glucose responses to a 50 g oral fructose pre-load remained unchanged but significantly lower than to isocaloric oral glucose. Thus, there did not appear to be any detrimental adaptation to continued fructose ingestion (Crapo *et al.*, 1986). Interventional studies of between one and six months duration indicate improvements in post-prandial hyperglycemia and HbA_{1c} by the incorporation of fructose into the diabetic diet. It is therefore of interest and importance to understand the mechanisms that produce this favourable response in diabetics

4.3 MONOSACCHARIDE ABSORPTION

Nutrient transport processes in the small intestine adapt to physiological, pharmacological, and environmental requirements, and monosaccharide transport is no exception. For example, small intestinal transport of glucose is altered in diabetes mellitus. Glucose and other monosaccharides are transported into and across cells by a family of transport proteins known as the GLUT proteins (GLUT 1-7). Those directly related to glucose and fructose uptake in the gastrointestinal system are described below.

4.3.1 Monosaccharide transporters

GLUT2 is a facilitative glucose transporter localised to the basolateral membrane of liver, pancreatic β-cells, small intestine, and kidney. It has a low affinity (high Km) for glucose (Thorens *et al.*, 1990; Thorens *et al.*, 1988) and transport of glucose into hepatocytes

Table 4.1 Chronic fructose intake by patients with type 2 diabetes

Subjects	Added fructose	Duration	Substitute in control diet	FBG	HbA_{1c}	TG	Reference
n = 9	60 g/d	12 w	Sucrose mainly	9% ↓ (P< 0.05)	↓	↔	(Osei *et al.*, 1987)
n = 13	60 g/d	6 m	Complex CHO mainly	12% ↓ (P< 0.02)	↓	↔	(Osei and Bossetti, 1989)
n = 7	80-115 g/d	14 d	Sucrose	↔	-	↔ or ↑	(Crapo *et al.*, 1986)
n = 12	21% daily energy	8 d	Sucrose (23%) Starch (55%)	↔	-	↔	(Bantle *et al.*, 1986)
n = 10	45-65 g/d	4 w	Complex CHO	↔	↓	↔	(Koivisto and Yki, 1993)
n = 8	30 g/d	9 w	Starch	↔	↔	↑	(Grigoresco *et al.*, 1988)
n = 10	25% daily CHO	4 w	Low-fructose diet	↔	-	↔	(McAteer *et al.*, 1987)
n = 11	50-60 g/d	24 w	Starch	↔	↔	↔	(Anderson *et al.*, 1989)
n = 5	76-124 g/d	3 m	Sucrose	↔	↔	↔	(Thorburn *et al.*, 1990)
n = 5	13% daily energy as fructose	3 m	Sucrose	-	-	↔	(Thorburn *et al.*, 1989)

↑ significant increase, ↓ significant decrease, ↔ no significant change, - variable not assessed; FBG, fasting blood glucose concentration; HbA_{1C}, glycosylated haemoglobin; TG, fasting plasma triglycerides.

and pancreatic β-cells by GLUT2 is not rate limiting, increasing linearly as glucose concentrations increase. GLUT2 exhibits a higher Km for glucose than the other isoforms. This is thought to be of benefit to hepatic gluconeogenesis, glucose sensing activity of β-cells, and transepithelial transport of glucose in the kidney and small intestine (Gould and Holman, 1993). GLUT2 may be responsible for the efflux of fructose from the basolateral

surface of absorptive epithelial cells of the small intestine (Thorens *et al.*, 1990) and is likely to be the sole hepatic fructose transporter.

GLUT5 is an intestinal hexose transporter, localised to the apical brush boarder on the luminal side of epithelial cells (Burant *et al.*, 1992; Burant *et al.*, 1992) and is also expressed in muscle, brain and adipose tissues (Kayano *et al.*, 1990). It is a high affinity fructose transporter, whose mRNA and protein levels are regulated by fructose intake (Inukai *et al.*, 1993), which exhibits poor ability to transport glucose.

4.3.2 Glucose transport

Glucose is absorbed in the small intestine either by (i) traversing the enterocyte (trans-cellular) or (ii) entering between the enterocytes (para-cellular). Trans-cellular transport can occur against a chemical gradient (greater glucose concentrations within the enterocyte) where it is coupled with sodium absorption, with energy supplied by the sodium / potassium ATPase pump on the basolateral membrane (Hediger *et al.*, 1987). Trans-cellular glucose transport occurs via the sodium-glucose cotransporter (SGLT1) (Wright *et al.*, 1991). Other sugars including D-glucose, 3-*O*-methyl-glucoside, D-galactose and α-methyl glucoside are also transported by SGLT1(Wright *et al.*, 1991). Passage of glucose out of the cell into the blood, is by diffusion, facilitated by a sodium-independent transporter. Para-cellular transport of glucose takes place through the tight junctions that exist between enterocytes (Philpott *et al.*, 1992).

The dietary history or pathological state of the organism also has an important effect on glucose transport. An increase in the amount of dietary carbohydrate increases glucose absorption rate, probably by increasing the total number of glucose carriers (Thorens *et al.*, 1988). Glucose absorption is also increased in streptozocin-induced diabetic rats with a 5- to 7-fold increase in 3-*O*-methyl-D-glucopyranose absorption, rather than an increase in carrier affinity (Fedorak *et al.*, 1987; Fedorak *et al.*, 1989). In this model, transport capacity is increased at the onset of the disease and remains high (Fedorak *et al.*, 1987). Whether glucose transport is similarly affected in human diabetes is unknown.

4.3.3 Fructose transport

Absorption of fructose across the small intestine is less well characterised. It probably occurs via an oxygen-dependent transport system at a slower rate than glucose (Rumessen

and Gudmand, 1986). When given by gavage to rats, fructose is absorbed more slowly than glucose (59% vs 91% absorbed in 120 min), but faster than passively transported sugars such as sorbitol (Niewoehner, 1986). Studies in humans indicate that fructose transport, unlike glucose, is neither active nor sodium-dependent (Levin, 1984). The monosaccharide transporter GLUT5 was identified as a fructose transporter located on the brush border membrane of human and rodent enterocytes (Burant *et al.*, 1992; Rand *et al.*, 1993). Transport across the basolateral membrane is probably via GLUT2. Fructose absorption occurs in direct proportion to its concentration and appears to be facilitated by the presence of glucose (Truswell *et al.*, 1988), which suggests the presence of a glucose-dependent fructose transport mechanism. Consistent with the promotion of fructose absorption by hyperglycemia GLUT5 gene expression (Lenzen *et al.*, 1996) and protein production (Burant *et al.*, 1994; Corpe *et al.*, 1996) is increased in rats with streptozotocin-induced diabetes increases. These studies imply that fructose absorption may be increased in type 2 diabetes, where blood glucose concentrations are increased. The post-absorptive consequences of enhanced fructose absorption by diabetics may have important implications in diabetes treatment.

4.4 EFFECTS OF MONOSACCHARIDES ON APPETITE

4.4.1 Comparative satiating efficiencies of monosaccharides

Controversy surrounds the efficiencies with which isoenergetic oral pre-loads of glucose and fructose suppress appetite and food intake at a subsequent test meal. Studies in healthy human subjects report equal suppression by glucose and fructose (Guss *et al.*, 1994; Kong *et al.*, 1999), or greater suppression by fructose (Rodin, 1990; Rodin, 1991; Rodin *et al.*, 1988; Spitzer and Rodin, 1987). Studies carried out in the same laboratory as the studies in this thesis have demonstrated an equal suppression of hunger and total food intake 3 h after 75 g oral pre-loads of these monosaccharides in healthy young adult males (Kong *et al.*, 1999). Similarly, Guss and collegues (1994) found no difference between the effects of 50 g glucose and fructose pre-loads on appetite ratings and food intake 135 min later in non-obese and obese women. Other studies, albeit all performed by the same group, have shown greater suppression of food intake by fructose than glucose. Rodin *et al.*, (1991) studied food intake in healthy subjects 135 min after the consumption of a 'pudding' pre-load where fructose or glucose (50 g each) was the sole source of CHO in a protein, fat and CHO mixture. When the pre-load contained 50 g fructose only, subjects consumed less

energy at the subsequent test meal 2.25 h later than when 50 g glucose or 50 g fructose plus starch 15 g was ingested. In another study which included non-obese and obese subjects, Rodin *et al.*, (1988) reported that 50 g fructose produced a lower intake of fat (grams and calories) at a test meal than isoenergetic glucose pre-loads. The pre-load consisted of a 500 ml drink of fructose, glucose, or aspartame in lemon flavouring, and was consumed 38 min before the buffet lunch. Surprisingly, in one of these studies this group demonstrated enhanced food intake after glucose pre-loads. Here, 50 g glucose or fructose in 500 ml water was ingested 2.25 h before food was eaten *ad libitum* from a buffet meal (Spitzer and Rodin, 1987). It is possible that the differences between the relative satiating efficiencies of the monosaccharides glucose and fructose in these studies is accounted for by differences in the total volume of pre-load ingested (300 ml in the study of Kong *et al.*, (1999) v 500 ml in the studies of Rodin and collegues). This appears unlikely, however as Guss *et al.*, (1994) who found no difference in satiating efficiency between glucose and fructose also used a pre-load volume of 500 ml. Further studies are necessary to clarify these discrepancies.

Possible discrepancies between the satiating efficiencies of glucose and fructose, may relate to different gastric emptying rates of these monosaccharides. In monkeys, glucose empties into the small intestine in a linear fashion, which is concentration-dependent. In contrast, fructose empties more rapidly than glucose and in an exponential fashion (Moran and McHugh, 1981). In humans, emptying of glucose is also slower than fructose; both sugars, empty more slowly than water (Gluc v Fruc: t1/2 = 93.61 v 65.45 min) (Guss *et al.*, 1994). Rayner *et al.*, (2000) however, found that intraduodenal fructose pre-loads suppressed food intake more than isoenergetic intraduodenal glucose, indicating that gastric emptying rates probably do not play a large role in affecting the satiating efficiency of these monosaccharides.

There is some suggestion that differences in the chemical structures of monosaccharides may influence their satiating capacity. In rats, for example, intestinal satiety was only achieved by sugars with affinity for the glucose transporter, such as D-xylose and galactose, but not by fructose (Meyer *et al.*, 1998). Similarly, glucose, but not fructose or lipid, significantly reduces 2-deoxy-D-glucose-induced feeding in rats (Singer and Ritter, 1994). Together these results suggest that the satiating effect of monosaccharides may be mediated by receptors that are responsive to glucose or sugars similar in structure to

glucose. There is no information as to the effect of diabetes on the concentrations of these receptors.

4.4.2 Peptide hormones

Nutrient-induced release of peptides from the gastrointestinal tract, contributes to the sensation of satiety. Carbohydrate ingestion stimulates the release of glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP). While administration of GLP-1 suppresses food intake, its role as a satiety hormone remains controversial (Chapter 2.3.5.2). In contrast, there is no evidence to suggest that GIP acts independently as a satiety hormone (Chapter 2.3.5.3), although at concentrations within the physiological range it stimulates the release of GLP-1 (Kieffer and Habener, 1999).

4.4.2.1 Glucagon-like peptide 1

Oral glucose ingestion results in a greater increase in plasma GLP-1 concentration than isoenergetic ingestion of fructose (Kong *et al.*, 1999). As with their satiating efficiencies, the ability of monosaccharides to stimulate the release of GLP-1 probably depends on their structure, although the data on this are conflicting. Perfusion of canine ileal loops with D-glucose, but not D-fructose, increased plasma concentrations of GLP-1 (Shima *et al.*, 1990). Sugars with similar structures stimulated GLP-1 release and it was suggested that this was due to recognition by glucose sensors. In contrast, in the rat, elevated GLP-1 concentrations in the mesenteric vein were detected when both D-glucose and D-fructose solutions were used to perfuse the ileum (Ritzel *et al.*, 1997). If GLP-1 is an endogenous satiety hormone, differential effects of the two monosaccharides on GLP-1 secretion could produce differences in satiating effects.

4.4.2.2 Gastric inhibitory peptide

Gastric inhibitory peptide (GIP) (also termed glucose-dependent insulinotropic peptide) is an intestinal hormone that both inhibits gastric acid production, and stimulates insulin secretion. At physiological concentrations, GIP stimulates GLP-1 synthesis and secretion, by direct activation of a protein kinase A and indirectly through the nervous system (either vagal or myoenteric) rather than by a direct effect on the L cell (Kieffer and Habener, 1999).

Glucose may act directly on the GIP-secreting K cells to stimulate GIP release. In healthy humans, fructose has a modest stimulatory effect on plasma GIP concentrations (Crapo *et al.*, 1980), which is probably less than that of glucose. In dogs the GIP response to fructose is much less than that to glucose (Williams *et al.*, 1981) but there are no data on the relative effects of glucose and fructose on GIP release in humans.

There are few data on the potential role of GIP as a satiety hormone (Chapter 2.3). It is also unclear whether GIP release has a role in appetite suppression following glucose or fructose ingestion (Chapter 11).

4.5 METABOLIC RESPONSES TO MONOSACCHARIDE INGESTION

4.5.1 Blood glucose

Ingestion of 75 g glucose produces rapid rises in blood glucose concentration from a mean of about 4.8 mmol/L to 8.5 mmol/L in healthy fasting adults, with a peak 30 minutes after ingestion (Hilton *et al.*, 2002; Kong *et al.*, 1999). The ingestion of fructose produces a much flatter curve, with lower maximal responses of 5.8 mmol/L, also occurring after 30 min (Kong *et al.*, 1999). The fructose-induced increase in plasma glucose is due to the conversion of two thirds of ingested fructose by the liver to glucose, which either accumulates as glycogen or is released into the systemic circulation. The release of insulin and the incretins is also different after glucose and fructose ingestion, influencing the glycemic state as discussed below.

4.5.2 Peptide hormones

The stimulation of insulin secretion by oral glucose ingestion occurs both via a direct interaction of glucose with pancreatic β-cells and by the stimulation of the incretins, GLP-1 and GIP (Nauck *et al.*, 1986). In keeping with its effect on plasma glucose, fructose has a modest effect on plasma insulin concentration, with levels 25% of those seen after ingestion of a comparable amount of glucose (Nuttall *et al.*, 1992b).

The biphasic insulin response to glucose ingestion, with peaks in excess of 40 uU/ml between 30 and 120 minutes post-glucose ingestion is not observed after fructose. Instead, plasma insulin concentrations after fructose consumption increase to a maximum of about 15 uU/ml. Stimulation of insulin secretion by oral fructose is also highly variable,

depending on glycemic state. Dietary fructose either does not stimulate or produces only minor rises in insulin secretion in non-diabetic humans (Dunnigan and Ford, 1975; Reiser *et al.*, 1987), whereas in patients with type 2 diabetes it clearly stimulates insulin secretion (Lawrence *et al.*, 1980; Nuttall *et al.*, 1992a). Consistent with this, oral fructose is a potent insulinogen in non-diabetic humans who become hyperglycaemic either due to prior oral (Dunnigan and Ford, 1975) or intravenous glucose administration, (Reiser *et al.*, 1987). This observation has important implications for the administration of fructose to diabetics.

The insulin response to oral fructose and isoenergetic glucose has been evaluated in people with type 2 diabetes (Nuttall *et al.*, 1992b) and without diabetes (Kong *et al.*, 1999) in separate studies. Although these responses have not been directly compared in the same study, the insulin response to fructose appears to be ~ 30% greater in diabetics than in non-diabetics. Nuttall *et al.*, (1992) who evaluated the glycemic, insulin and C-peptide responses to 50 g fructose and glucose in otherwise healthy type 2 diabetics, reported a 150-fold stimulation of insulin from baseline by fructose and 300-fold stimulation by glucose. Our group have demonstrated a smaller fructose-induced rise in plasma insulin in non-diabetics, in response to 75 g fructose (Kong *et al.*, 1999).

There are a number of possible mechanisms for a greater fructose-induced insulin response in type 2 diabetics than in non-diabetics. Although in the non-diabetic state metabolites of fructose (such as d-glyceraldehyde) have the capacity to stimulate insulin secretion (Jain *et al.*, 1975), this appears unlikely as in streptozotocin-(Morin *et al.*, 1997) and genetically-(Molina *et al.*, 1984) diabetic rats, insulin responses to d-glyceraldehyde are diminished compared with non-diabetic animals. Similarly, although a specific receptor site on the β-cell has been proposed (Lawrence *et al.*, 1980), this has not been identified. It is also possible that a yet unidentified incretin could account for this response. Alternatively, hyperglycemia which enhances insulin secretion in response to fructose may underlie the effect. However, it is unclear if patients with type 2 diabetes have the same heightened response. If so, then a further glucose-dependent mechanism may be responsible. It is unknown whether type 2 diabetics would exhibit the same heightened response to fructose if their blood glucose concentration were the same as those of people without diabetes. If so, then it would suggest that fructose may exert its effect on insulin release by a mechanism independent of elevated blood glucose concentrations, perhaps one that is specific to the pathogenesis of diabetes.

Greater fructose- than glucose-induced insulin responses have been observed after intravenous administration (Lawrence *et al.*, 1980), arguing against a role for incretins in the regulation of fructose-induced insulin release. Insulin release following intravenous fructose in patients with type 2 diabetes is slow and follows a biphasic pattern (MacDonald *et al.*, 2000). When fructose was infused intravenously into diabetics and euglycemic non-diabetics the insulin response was greater in diabetic than in non-diabetic subjects. Both the peak insulin levels were higher and the mean total insulin output in patients with type 2 diabetes were almost double, those of the controls although this was not statistically significant (Lawrence *et al.*, 1980). When fructose was infused intravenously after priming non-diabetics with glucose, the insulin responses in diabetic and non-diabetic subjects were similar (Lawrence *et al.*, 1980). This suggests that a potential effect of fructose, to promote differential insulin secretion in patients with and without type 2 diabetes could be related to post-absorptive factors other than hormone release. A formal evaluation of insulin and incretin concentrations following oral glucose and fructose ingestion in these two groups of subjects is required.

4.6 CONCLUSION

This chapter has discussed topics related to the use of fructose as a treatment for type 2 diabetes. After acute oral ingestion of a single fructose meal, patients with type 2 diabetes have a markedly flattened blood glucose curve and possibly a greater rise in plasma insulin than do non-diabetics. The mechanisms responsible for this have been discussed, and include the release of incretin hormones GLP-1 and GIP. In addition, the satiating efficiency of fructose has been discussed, as has the contribution of GLP-1 to mediating this effect. The study described in Chapter 11 relates to this work, and tests the hypothesis that in patients with type 2 diabetes fructose ingestion is associated with a:

1) Smaller rise in post-prandial blood glucose concentration,
2) Greater rise in plasma insulin than non-diabetics and
3) Greater suppression of food intake

when compared to oral glucose.

Chapter 5

THE ROLE OF NITRIC OXIDE MECHANISMS IN THE REGULATION OF MAMMALIAN FEEDING BEHAVIOUR

5.1 INTRODUCTION

A variety of mechanisms interact to influence food intake. These include nutrient supply to the brain, the release of gastrointestinal hormones and neurotransmitters, gastrointestinal transit of ingested food, metabolic products and the state of nutrient stores (Campfield and Smith, 1998; Morley, 1987; Morley, 1990; Read *et al.*, 1994). It has become apparent that a complex circuitry exists, in which a variety of excitatory and inhibitory neurotransmitters act to integrate the inputs from these mechanisms. The focus of much appetite research has been to determine how these processes are integrated. The inhibitory neurotransmitter, nitric oxide (NO) has emerged as a key potential mediator.

Endogenous NO has been suggested to modulate mammalian appetite, possibly by acting centrally as a neurotransmitter in feeding-related pathways and peripherally by regulating gastrointestinal motility. The potential development of new therapies to treat appetite-related disorders, such as obesity, makes these findings especially interesting. This chapter summarizes the literature regarding nitric oxide in the regulation of mammalian feeding behaviour and possible involvement in the regulation of human appetite.

Studies described later in this thesis were designed to determine the role of nitrergic mechanisms in the regulation of human appetite, feeding behaviour and upper gut motility (Chapter 12 and 13).

5.2 NITRIC OXIDE PHYSIOLOGY

5.2.1 Endogenous NO production

Nitric oxide (NO) is a slightly water-soluble gas produced by mammalian tissues and first identified in the cardiovascular system (Furchgott and Zawadzki, 1980). It exists in three forms: the nitrosium ion (NO^+), the neutral free radical ($NO^\bullet$) and the nitroxyl ion (NO^-)

(Stamler *et al.*, 1992). While the redox state depends on the tissue in which it is produced, its biochemical properties are largely those of a free radical. The differing actions of NO may be explained by the existence of different redox states.

Synthesis of NO depends on the availability of its precursor L-arginine. In humans, this is either obtained from the diet or synthesised endogenously. L-arginine is converted to NO and L-citrulline by the NADPH-dependent enzyme NO synthase (NOS) (Moncada *et al.*, 1991) of which three isoforms have been identified; endothelial, neuronal and macrophage. In humans, the genes for these isoforms reside on chromosomes 7,12 and 17 respectively (Marsden *et al.*, 1993). Endothelial and neuronal NOS are known as constitutive NOS (Moncada *et al.*, 1991), as they are present under physiological conditions. Constitutive (calcium-dependent) NOS is released in response to binding of endogenous mediators, such as acetylcholine or bradykinin, to cell surface receptors, or mechanical forces such as the pulsatile flow of blood through arteries, which increase intracellular calcium concentrations and promote calcium / calmodulin complex formation. Macrophage, or inducible NOS, is activated directly by exposure of immune cells to immune reaction mediators such as cytokines or endotoxins (Moncada and Higgs, 1993; Moncada *et al.*, 1991). Inducible NOS will not be discussed, as its function is principally in pathological states such as septic shock. The focus of this chapter and the studies described in chapters 12-14 are on the role of nitrergic mechanisms involved in normal feeding behaviour.

L-arginine analogues, such as NG-monomethyl-L-arginine (L-NMMA) and NG-nitro-L-arginine methyl ester (L-NAME) inhibit NO formation. While the specificity and selectivity, of these analogues is highly variable (Moncada *et al.*, 1991) their use to indirectly inhibit NO formation has provided much information on the physiological interactions involving nitrergic mechanisms. The development of pharmacological agents that inhibit specific isoforms of NOS and animals with targeted disruption of genes encoding specific isoforms of NOS are currently being used to clarify more precisely, the roles of each different NOS isoform.

5.2.2 Nitric oxide synthase localisation

Direct measurement of NO is particularly difficult due to the extremely small quantities produced and its instability. Immunohistochemical techniques utilising pure brain antisera have been developed and used to localise neuronal and endothelial NOS, however this

antisera does not recognise macrophage NOS (Snyder and Bredt, 1991). Molecular cloning techniques have enabled NOS mRNA to be localised by *in situ* hybridisation techniques (Snyder and Bredt, 1991). A close parallel between immunoreactivity and NOS catalytic activity in different brain regions has confirmed the specificity of these immunohistochemical techniques.

In the CNS the highest density of neuronal NOS has been observed within the cerebellum. In particular neuronal NOS and neuronal mRNA occurs most prominently within glutaminergic granule cells and GABAergic basket cells (Bredt and Snyder, 1992; Moncada *et al.*, 1991; Snyder and Bredt, 1991). High concentrations of neuronal NOS have also been identified within the olfactory bulb, particularly within the accessory olfactory bulb. Within the cerebral cortex and hippocampus, the pedunculopontine tegmental nucleus, superior and inferior colliculi, islands of Callejae, caudata-putamen and dentate gyrus of the hippocampus contain high concentrations of neuronal NOS (Bredt and Snyder, 1992; Moncada *et al.*, 1991).

While neuronal NOS does not consistently colocalize with a single neurotransmitter, in the cerebral cortex and corpus striatum NOS neurons are colocalized with somatostatin and neuropeptide Y (Bredt and Snyder, 1992; Snyder and Bredt, 1991), and within the hypothalamus some neuronal NOS neurons stain positive for oxytocin and vasopressin (Bredt and Snyder, 1992; Snyder and Bredt, 1991). Throughout the brain, and largely throughout the peripheral nervous system, NOS and NAPDH-diaphorase localisations are identical.

5.2.3 Cellular targets of NO

Recent studies have demonstrated the ability of NO to act as a signalling molecule both at intra- and extra-cellular sites. Three modes of NO signalling exist: (i) locally to stimulate cGMP within the cell in which it was formed, (ii) as a neurotransmitter by diffusing to neighbouring cells where it activates cGMP, and (iii) in a feedback loop where it diffuses from the external environment back to the cell of origin to activate guanylyl cyclase (Bruhwyler *et al.*, 1993; Bult *et al.*, 1990; Snyder and Bredt, 1991).

A known intracellular target for NO is the soluble guanylyl cyclase (sGC) enzyme. The heme moiety of sGC is sensitive to NO, which when bound is capable of inducing a

conformational change as the nitrosyl-heme complex itself activates sGC to convert GTP to cGMP (Bruhwyler *et al.*, 1993; Bult *et al.*, 1990; Snyder and Bredt, 1991). In the smooth muscle, these events result in protein phosphorylation or ion-channel modulation and ultimately in tissue relaxation. Studies have identified roles for cGMP-operated ion channels, cGMP-dependent protein kinases and phosphodiesterases in the CNS.

In addition to promoting cGMP formation NO species interact with a variety of other cellular targets, the details of which are less well characterised. The free radical form of NO targets all heme containing proteins such that not only does it modify its own synthesis through heme containing NOS, but also interacts with cyclooxygenases 1 and 2, cytochrome P450, haemoglobin and myoglobin. The nitroxide ion specifically acts upon intracellular proteins containing iron-sulphur clusters, but is also capable of protein nitration, and reactions with DNA, while in its oxidised form, NO is capable of nitrosylating a variety of proteins including G proteins, adenylyl cyclase and protein kinase C, where it may indirectly influence insulin secretion (Nelson *et al.*, 1997). It is through nitrosylation of the NMDA receptor that NO has been suggested to modulate glutamatergic neurotransmission in the CNS.

5.2.4 Physiological functions of NO

It is not surprising, that NO has diverse functions given its multiple intracellular sites of action and its widespread production. In the cardiovascular system, endogenous NO, regulates vasodilator tone essential for the regulation of blood flow and pressure (Rees *et al.*, 1989). In the brain it is involved in memory formation (Shibuki and Okada, 1991), vision (Venturini *et al.*, 1991), olfaction (Breer and Shepherd, 1993) and nociception (Moore *et al.*, 1991) while in the peripheral nervous system NO contributes to relaxation of the airways (Belvisi *et al.*, 1992) and adaptive relaxation of the stomach to accommodate food (Desai *et al.*, 1991). Data from several independent research groups now also suggest an involvement of NO in the modulation of mammalian ingestive behaviour.

5.3 NITRIC OXIDE AND FOOD INTAKE

5.3.1 Nitric oxide antagonists on food intake

There is increasing evidence that endogenous NO synthesis influences mammalian feeding behaviour. Morley and Flood, (1991) first demonstrated that peripheral administration of a

single dose of the NOS inhibitor N-arginine to 24 h food-deprived mice is associated with dose-responsive suppression of food intake. An acute anorectic effect of NOS inhibition has since been demonstrated in rats (Squadrito *et al.*, 1993) and chickens (Choi *et al.*, 1994). Five days of administration of L-NAME to normal mice suppressed food intake and reduced body weight for the entire treatment period (Morley and Flood, 1992). Decreased food intake during L-arginine analogue administration probably represents a specific effect of suppressed production of NO. Consistent with this, high doses of L-arginine (1000 mg / kg) promote feeding in mice (Morley and Flood, 1991). Furthermore, L-arginine administration prevents the effects on feeding of NOS inhibition. This effect of L-arginine analogues is stereo-isomer specific as D-arginine does not attenuate the effects of NO synthase inhibition (Morley and Flood, 1991).

5.3.2 Nitric oxide synthase inhibition in genetically obese animals

Studies with genetically obese rodents indicate that obesity is associated with an increased sensitivity to the food intake-suppressant actions of NOS inhibition. Squadrito *et al.*, (1993) reported that lean rats became tolerant to the anorectic effect of repeated L-NO Arg administration during a week of treatment at low doses (100 mg / kg / day), while the suppression of food intake and subsequent weight loss were maintained for 24 days in genetically obese (FA/FA) rats. In that study, obese rats ate more at baseline than lean rats (~26 vs 20 g / 24 h) and it is interesting that the different effects of L-NO Arg in the two groups resulted in the lean and obese rats having equivalent food intake (~22 g / 24 h) during L-NO Arg treatment. Morley and Flood, (1994) have demonstrated similar effects in lean compared with obese (ob/ob) mice. These findings are intriguing because they suggest that obesity may be caused by or associated with increased NO tone. Consistent with this, levels of NOS and NOS mRNA are enhanced in genetically obese mice (Morley *et al.*, 1995). Nevertheless, while these studies suggest that genetically obese animals are particularly sensitive to the satiating effect of NOS inhibition, it is not known whether animals obese for other reasons (eg. overfeeding) are also more sensitive.

5.3.3 Effects of food intake on NO production

Chronic food deprivation studies in rats demonstrate decreases in the concentrations of feeding-related chemical messengers thyrotropin-releasing hormone (TRH), CRH, galanin and neuropeptide Y in the paraventricular nucleus (PVN) (Ueta *et al.*, 1995). Similarly, 48

h food-, but not water-deprivation significantly decreases NOS gene expression and the prevalence of NOS transcripts in the rat PVN and supraoptic nucleus (Ueta *et al.*, 1995). However, measurement of the conversion of L- [^{3}H] arginine to L- [^{3}H] citrulline in the diencephelon, indirectly reveals that NOS activity is actually enhanced in rats fasting overnight, compared with normally fed rats. These apparently conflicting findings may reflect compensatory mechanisms on the part of the NOS enzyme, such that activity is enhanced in order to offset the decrease in its production, or may be a direct result of the use of different techniques to characterise NO formation.

5.3.4 Non-specific effects of NOS antagonists

Whether the suppression of feeding associated with administration of L-arginine analogues in animal studies is a specific effect of NOS inhibition or relates to other effects of NO inhibition is uncertain. For example, no study has measured blood pressure to determine whether the appetite suppression produced by L-arginine analogues is a non-specific effect of hypertension, rather than a specific effect of NOS inhibition. However, this seems unlikely, as spontaneously hypertensive Zucker rats are reportedly hyperphagic, compared with their genetically normal counterparts (Wexler and McMurtry, 1981).

L-arginine analogues may suppress feeding, at least in part, by inducing nausea, drowsiness or some other non-specific aversive or behavioural effect. There are conflicting reports on the effects of NO on conditioned taste aversion. Prendergast *et al.*, (1997) suggested that L-NAME produces malaise, while the data of Hui and Chan, (1995) indicate that L-NAME reduces food intake via a mechanism independent of aversion. Nor do NOS antagonists appear to influence patterns of exploratory behaviour or preferences for favourable sensory cues (Hui and Chan, 1995). Additional studies in this area are necessary in order to clarify whether NO is responsible for regulating feeding behaviour physiologically. The study reported in Chapter 13 was designed to assess non-specific effects of NOS antagonists on mammalian feeding by evaluating food intake responses to varying doses of L-NMMA and L-NAME.

5.4 MECHANISMS REGULATING NOS INHIBITION-INDUCED ANOREXIA

5.4.1 Central mechanisms

Nitric oxide may act indirectly via central pathways and via interactions with other neurotransmitters to modulate feeding behaviour. In the central nervous system, the highest densities of nNOS are present within the cerebellum (Snyder and Bredt, 1991), but nNOS positive neurons are also present in the hypothalamo-neurohypophyseal system of the rat (in the supraoptic and paraventricular nuclei (Snyder and Bredt, 1991)) a region of the brain, which is associated with regulation of ingestive behaviour. Evidence that NO exerts effects on feeding partly or predominantly through central mechanisms is provided by the finding that intracerebral administration of L-NO Arg to rats reduces food intake, whereas the same dose administered peripherally has no effect on feeding (Squadrito *et al.*, 1993). Consistent with this food deprivation increases levels of brain NOS activity (Squadrito *et al.*, 1994). The potential neurotransmitters mediating and / or interacting with central nitrergic food intake regulating pathways are discussed in Chapter 2.

5.4.2 Peripheral mechanisms

Enteric excitatory and inhibitory neurons act together to innervate gastrointestinal smooth muscle and a balance between both mechanisms controls gastrointestinal motility. Nitric oxide is an inhibitory neurotransmitter released by motor neurons and producing smooth muscle relaxation (Snyder and Bredt, 1991). The gastrointestinal tract is heavily innervated by non-adrenergic, non-cholinergic (NANC) nerves (Meyer, 1987), which provide a rich source of inhibitory innervation. While the identity of the neurotransmitter at these neurons remained elusive for many years, studies now indicate that NO or a related nitroso compound is likely to be the major inhibitor mediator. Given the prominence of NO as an inhibitory neurotransmitter throughout the gastrointestinal tract, it is not surprising that a role for the L-arginine-NO pathway has been described in relation to oesophageal peristaltic contractions (Anand and Paterson, 1994; Knudsen *et al.*, 1994), reflex relaxation of the stomach to accommodate food (Desai *et al.*, 1991), the modulation of gastric motility and gall bladder emptying (Fiorucci *et al.*, 1995), coordination of the migrating motor complex (Russo *et al.*, 1999) and relaxation of the internal anal sphincter (O'Kelly *et al.*, 1993). The involvement of nitrergic mechanisms in the regulation of gastric accommodation and gastric emptying is discussed below.

5.4.2.1 Gastric accommodation / sensation

There are two major reservoir functions of the stomach that determine the handling of ingested foods. These are receptive and adaptive relaxation (Chapter 2). The ability of an individual to accurately accommodate for changes in gastric volume influences post-prandial symptoms such as fullness and nausea, and has significant bearing on subsequent food intake. This is especially important under pathological conditions, such as functional dyspepsia, where symptoms of early satiety and fullness, abnormal bloating, nausea and pain are reported after consumption of a standard meal. While the mechanisms responsible for receptive and adaptive relaxation are different, NO-dependent neural pathways are involved in both reflexes. Thus, nitrergic mechanisms appear to be responsible for the normal functioning of these reflexes and when abnormal may contribute to the pathology of abnormal accommodation.

Work on the guinea-pig fundus has revealed that adaptive relaxation is NO-dependent (Desai *et al.*, 1991). Incubation of the guinea-pig stomach with the NOS inhibitor L-NMMA or L-NAME abolished pressure-induced adaptive relaxation, an effect that was partially reversed by L- but not D-arginine. In support of a NO-mediated pathway, incubation of the stomach with methylene blue (an inhibitor of soluble guanylate cyclase to which NO binds intracellularly) inhibited adaptive relaxation of the stomach. Two pathways have been proposed, a local reflex arc where afferent fibres sense changes in intraluminal pressure, and a second pathway which is dependent on stimulation by a ganglionic nicotinic receptor agonist. Consistent with the involvement of nitrergic pathways, sublingual glyceryl trinitrate (GTN) (NO donor) improves the accommodation to a mixed liquid-solid meal in patients with functional dyspepsia (Gilja *et al.*, 1997).

5.4.2.1 Gastric emptying and antropyloroduodenal motility

Although a direct association between delayed gastric emptying and decreased feeding has not been established, it is possible that suppression of food intake may occur due to the prolongation of gastric distension. While delayed gastric emptying prolongs gastric distension it also retards the movement of food into the small intestine, and thus both delays the onset, and prolongs the duration of small intestine satiation effects. The precise mechanism by which nitrergic modulation of gastrointestinal motility affects feeding remains uncertain, although there is increasing evidence from studies in animals, as well as humans, that NO serves as a neurotransmitter involved in the regulation of gastric emptying.

Studies in animals suggest that endogenous NO accelerates gastric emptying. Consistent with this, acute inhibition of NOS delays gastric emptying in rats (Plourde *et al.*, 1994) and increases phasic pyloric activity in ferrets (Lingenfelser *et al.*, 1997). In contrast, available evidence suggests that endogenous NO delays, rather than accelerates gastric emptying in humans. For example, the NO donor GTN prolonged gastric emptying in humans (Konturek *et al.*, 1995). Further, systemic L-NMMA administration enhanced gastric emptying in healthy human subjects (Konturek *et al.*, 1999). These studies demonstrated that whilst nitrergic mechanisms are involved in the regulation of mammalian gastric emptying, the precise roles may vary depending on species.

While some of the conflicting findings relating NO modulation of gastric emptying may be explained by citing species differences or the use of different antagonists, the role of NO may be more complicated than previously thought. In a study in dogs Orihata and Sarna, (1994) showed that gastric emptying of a solid meal was slowed by inhibition of NOS using intravenous L-NAME but also (although to a lesser extent) by intravenous L-arginine. Their data showed that L-NAME slowed linear phase gastric emptying whereas L-arginine inhibited gastric emptying by partially inhibiting gastropyloroduodenal contractions (Orihata and Sarna, 1994). This suggests that nitrergic mechanisms play a role in the regulation of gastric emptying via the coordination of fundic, antral, pyloric and duodenal motor function. Whether this directly affects food intake is unknown.

Studies in humans indicate that NO may also play an important role in the modulation of feedback from intraduodenal nutrients regulating gastric emptying. However, it appears that NO exerts its effect in a nutrient specific manner, though, as stimulation of phasic and tonic pyloric motility by intraduodenal lipid (Sun *et al.*, 1996) are attenuated by the NO donor, nitroglycerin. To date, there are no reported studies of the role of endogenous NO on antropyloroduodenal responses to intraduodenal nutrients (Chapter 14).

5.5 CONCLUSION

While the precise role of NO in the regulation of feeding behaviour is yet to be defined, it is not be surprising that it has an important contribution, as it is central to so many other biological responses. Studies performed by different groups in several animal species have

shown that both acute and sub-chronic NOS inhibition suppresses food intake, suggesting that NO has a stimulatory effect on mammalian appetite. Whether this effect is mediated centrally or peripherally is unknown, but it is probable that both components act synergistically. The mechanism(s) of action of NO remains largely unexplored.

The studies described later in this thesis relate to the role of nitrergic mechanisms in the regulation of mammalian feeding behaviour, and evaluate whether NO

1) Stimulates appetite and food intake in humans (Chapter 12)
2) Is involved in regulating post-prandial gastroduodenal motility in humans (Chapter 13).

Chapter 6

COMMON METHODOLOGIES

6.1 INTRODUCTION

The methods presented in this chapter are techniques common to the human studies described in Chapters 8 - 13 of this thesis. These techniques have all been used previously, validated and are well accepted approaches to assess feeding behaviour and antropyloroduodenal motility (Chapman *et al.*, 1999; Cook *et al.*, 1997; Heddle *et al.*, 1989; Heddle *et al.*, 1988a; Heddle *et al.*, 1988b; Lavin *et al.*, 1996; Lavin *et al.*, 1998; MacIntosh *et al.*, 1999; Sepple and Read, 1989). Where a new technique has been established, this will be described in detail in the relevant chapter (Chapter 10).

6.2 SUBJECTS

Healthy young subjects (18 - 40 y) were recruited via the placement of advertisments and information sheets on notice boards located within the local hospital and university communities. Where necessary, advertisements were also placed in the local newspaper. Prior to enrolment, subjects were screened to exclude previous or current gastrointestinal illness, cardiovascular disease, diabetes, epilepsy, the use of medications that could affect gastrointestinal function or appetite, a current alcohol intake of > 20 g per day or use of illicit drugs. Subjects were also required to complete a 'Three-Factor Eating Questionnaire' of Stunkard and Messick, (1985) to measure dietary restraint. In addition, subjects participating in the studies detailed in Chapters 8 and 9 also completed the 'Eating Attitudes Test' to detect symptoms of eating disorder (Garner and Garfinkel, 1979), and the 'Zung Self-Rating Depression Scale' (Zung, 1986), to detect depression.

Subjects with type II diabetes mellitus were recruited according to the description in Chapter 11.

A brief dietary history was obtained. Subjects were classed as vegetarians or omnivores, and by way of prompts of 'balanced', 'high fat', 'high protein' and 'high carbohydrate',

were asked to describe the types of foods they would usually eat. In addition subjects were asked whether they participated in any regular physical activity. Subjects were instructed to maintain their 'normal' lifestyle whilst participating in the study. For studies involving the administration of drugs (Chapters 12 and 13), plasma concentrations of liver enzymes were screened prior to enrolment and following completion of the study. Abnormal results precluded subjects from enrolment in the study to decrease the likelihood of experiencing adverse effects from drug administration.

Signed, informed consent was obtained from each subject prior to participation in each study. All subjects understood they were free to discontinue studies at any time. All subjects were offered an honorarium for their participation.

6.3 ETHICS APPROVAL

All protocols were approved by the Royal Adelaide Hospital Research Ethics Committee. Where appropriate (i.e. for the studies conducted in Chapters 12 and 13 using NG-nitro-L-arginine methyl ester (L-NAME) and NG-monomethyl-L-arginine (L-NMMA) the Royal Adelaide Hospital Investigational Drug Sub-Committee and Therapeutic Goods Administration (TGA) of Australia (Drug Safety and Evaluation Branch) approvals were sought, prior to the recruitment of subjects.

6.4 STUDY ENVIRONMENT

Feeding behavior, especially sensations associated with appetite, is extremely subjective and can therefore be influenced, often to a large extent, by external factors including social interaction, time of the day and temperature (Blundell and Stubbs, 1997). It is therefore necessary, that these variables are as constant as possible, both on the study day itself, and across multiple study days.

All studies presented in this thesis were conducted in the clinical research study rooms of the Department of Medicine and the Gastrointestinal Investigation Unit at the Royal Adelaide Hospital. Subjects remained in these rooms for the duration of the study, except to use the toilet. Rooms were isolated from the external environment; air temperature was maintained constant at 24°C by reverse-cycle air conditioning and subjects were not

permitted to open windows. There was no opportunity for contact with individuals other than investigators. Subjects were permitted to read (but not about food or related topics), listen to the radio or pre-recorded music or to study. Studies were performed lying (in the case of all studies involving manometry recording) (Chapter 10 and 13), supine with the upper body elevated 30° above the horizontal, (in the case of studies where blood pressure was an important outcome measure) (Chapter 12), or subjects were free to move about (Chapters 7 - 9 and 11). Where timing of food intake was an important outcome measure (Chapter 8), subjects were not permitted to wear a watch or listen to the radio. Subjects were seated during meals.

6.5 DRUG PREPARATION AND ADMINISTRATION

NG-monomethyl-L-arginine (L-NMMA) and NG-nitro-L-arginine methylester (L-NAME) were prepared according to similar protocols. Doses were based on subject weight, which was taken upon arrival, each study day. Pre-prepared frozen aliquots of stock solution were thawed on the morning of the study and the required dose of drug was diluted using sterile saline (0.9%). Both drugs were administered intravenously. Control infusions were sterile saline (0.9%), administered at the same rate as drug infusions.

6.6 PRE-LOAD PREPARATION AND ADMINISTRATION

6.6.1 Yoghurt pre-loads

The aim of a well-designed pre-loading technique is to mask all sensory aspects of food intake by ensuring that the sight, smell, taste and texture of the foods consumed are kept similar. Pure nutrient pre-loads are not representative of normal meals and usually empty from the stomach more rapidly than solid meals, as they are often liquids (Chapman *et al.*, 1999; de Graaf *et al.*, 1992). In addition, they are often administered beyond the oral cavity (Cook *et al.*, 1997). Yoghurt modified with respect to macronutrient composition make excellent pre-loads (Mudge, 2000; Rolls *et al.*, 1991).

Yoghurt pre-loads were prepared within the research kitchen using commercially available ingredients. High carbohydrate (HC), high fat (HF), high protein (HP), and high fat/protein (HF/P) pre-loads, each contained fruit flavoured yoghurt (Yoplait, National Foods Limited, Morwell, Vic, Aust.) as the base ingredient. Cornflour (White Wings Foods, Smithfield,

SA, Aust.) and glucose powder (Glucodin energy powder, Boots Healthcare, North Ryde, NSW, Aust.) were used as the carbohydrate source. Canola oil (Meadow Lea Foods Ltd., Mascot, NSW, Aust.) and cream were used as the fat source. Casec powder (Powder CAS.E.M.C, protein supplement, Mead Johnson, Evansville, IN, USA) or ProMod (Abbott Laboratories, Columbus, Ohio, USA) and egg whites were used to supply protein. Pre-loads were designed to be indistinguishable from each other by sight, smell, texture or taste. These variables were assessed in the study on macronutrient pre-loads, and results are reported in Chapter 8. Strawberry topping (Schweppes Cottee's, Liverpool, NSW, Aust.) and methyl cellulose (Ace Chemical Company, Camden Park, SA, Aust.) were added to the yoghurts for colour and texture respectively. The specific compositions of pre-loads are detailed in Chapters 7 and 8. Yoghurts were freshly prepared prior to the commencement of the study.

6.6.2 Triglyceride emulsion

Commercially available Intralipid® 10 % (Kabi Pharmacia Ltd., Milton Keynes, UK.) containing 10 % triglycerides as fractionated soy bean oil (50 g/500 ml), 1.2 g fractionated egg phosophlipids and 2.25 g glyceryl anhydrous, was used as the nutrient infusion in the studies described in Chapters 10 and 14. Intralipid® was delivered intraduodenally at rates specified in the relevant chapters.

6.7 TECHNIQUIES

6.7.1 Assessment of feeding behavior

6.7.1.1 Three-factor eating restraint questionnaire

All of the studies presented in this thesis were designed to evaluate the effects of a specific intervention on appetite sensations and food intake in healthy individuals. It was therefore necessary to identify and exclude individuals possessing or with pre-dispositions to 'abnormal' eating behaviour, in particular those whose eating behavior would not change even in the presence of an intervention which would alter the eating behavior of an 'average' person. The questionnaire of Stunkard and Messick, (1985) is currently the most widely used method of screening for 'abnormal' eating behaviour. It was devised to measure three factors related to human feeding habits: 1) cognitive restraint of eating, 2) dis-inhibition and 3) hunger. The factor relevant to the studies reported in this thesis is 'restrained eating', which was described by Herman and Mack, (1975) as the tendency of

individuals to restrict their food intake in order to control their body weight. In their work, Herman and Mack, (1975) assessed dietary restraint by predicting food intake in response to a) food pre-loads, b) alcohol ingestion and c) dysphoric emotions.

The reproducibility of assessing these three factors, using a questionnaire administered to *a priori* assigned 'free-eaters' (n = 45) and 'dieters' (n = 53) was evaluated by Stunkard and Messick, (1985). The reported test-retest reliability co-efficient (a measure of the capacity to provide the same measurement on different occasions) of the questionnaires was high for each of the three factors; 0.92 for restraint, 0.91 for dis-inhibition and 0.85 for hunger respectively, where greater than 0.8 was considered acceptable. Among the 'dieters' and 'free-eaters', the reliability of restraint measures was marginally different (0.79 and 0.92 respectively). There was a correlation between restrained eater's current weight (correlation co-efficient 0.2, $P < 0.01$), with heavier people having higher restraint scores. In addition, the sex of the respondent's was also related to dietary restraint (correlation co-efficient 0.31, $P < 0.01$); women scored significantly higher than men. Consistent with the original reproducibility study, a follow-up assessment using the Stunkard and Messick (1985) questionnaire in 17 subjects (including 3 overweight) produced a one-month test-retest reliability of 0.93 for factor I (Ganley, 1985, personal communication with (Stunkard and Messick, 1985)).

The Stunkard and Messick, (1985) questionnaire revealed a final mean score ± S.E.M.M for factor 1, 'dietary restraint' among 'dieters' of 14.3 ± 3.6 whilst among 'free-eaters' the score was significantly lower at 6.0 ± 5.5. In all studies reported in this thesis, dietary restraint prior to enrolment of subjects was measured using the Stunkard and Messick, (1985) questionnaire, a score of < 10 for 'dietary restraint' (i.e. outside the range observed in 'dieters') used as a cut off to participate. This cut-off was chosen as it has been used in other studies to recruit unrestrained eaters (Rolls *et al.*, 1988; Rolls *et al.*, 1991); at half way between the mean 'free-eater' and the more restrained 'dieters' in the validation study above, it permits exclusion of the most restrained eaters.

6.7.1.2 Diet diaries

Several methods are available to evaluate the food habits and energy intake of an individual, including weighed records, 24 h recall and food frequency questionnaires (FFQ) with diet records being the most rigorous and widely accepted method (Bathalon *et al.*,

2000; Bingham *et al.*, 1994; Jorgensen *et al.*, 1992; Willett *et al.*, 1985). For the purposes of the work conducted in this thesis, a 3 day diet diary (Appendix 1) was considered to provide adequate information on the baseline diets of healthy, unrestrained, motivated subjects. The use of 24 h recalls (Beaton *et al.*, 1979), 1 day records (Sempos *et al.*, 1985), 2 day records (Hartman *et al.*, 1990) and 3 day records (Hunt *et al.*, 1983) have all been used previously.

There are limitations associated with the use of diet diaries to collect data on an individuals' feeding behaviour. This is especially true in unhealthy, or unmotivated populations. For example, it is well known that body weight is positively correlated with under-reporting of energy intake in weighed diet records (Schoeller, 1990). Dietary restraint (Section 6.7.5.1) also potentially affects reporting accuracy in subjects completing diet diaries (Bingham *et al.*, 1994). In the study described in Chapter 9, 3 day diet diaries were used to establish subject baseline food intake.

6.7.1.3 Visual analogue scale

Sensations of appetite including hunger, fullness, satiety and desire to eat are influenced by individual physiology and psychology, as well as by external cues such as temperature, time and prior food intake. They are therefore extremely subjective and difficult to quantify. The most common form of assessment are visual analogue scales (VAS), which usually consist of horizontal lines (of a specified length) with words describing the two extremes of a sensation anchored at either end. Subjects make a vertical mark along the line corresponding to the strength with which they are experiencing the particular sensation at a given time. The VAS used in this thesis were in the form of a questionnaire with the opposites of a particular sensation written at either end of a 10 cm horizontal line; for example hungry v not hungry and full v empty (Cook *et al.*, 1997; Sepple and Read, 1989) (Appendix 2). Subjects were instructed to place a vertical mark at the appropriate place on each line to indicate the strength of the nominated symptom. The pre-treatment values were averaged to provide a baseline. Sensations associated with appetite were then quantified (mm) as a change from baseline.

The reproducibility, power and validity of VAS in the assessment of appetite sensations after a single test meal were recently reported by Flint *et al.*, (2000). Fifty-five healthy male subjects were tested on 2 occasions, separated by 1-3 weeks. Under identical

experimental conditions, subjects consumed a 2MJ breakfast and completed VAS questionnaires rating satiety, hunger, fullness and prospective consumption every 30 min for the following 4.5 hours, at which time foods from a buffet meal were eaten *ad libitum*. Scores of appetite-related symptoms were similar on both test days with the co-efficients of variation between 9 and 24 %. Similarly, Raben *et al.*, (1995) found that when the same 9 subjects received identical test meals, under the same standardized conditions, on 2 different days, the co-efficients of variation again ranged from 9.6 % (for hunger) to 24.9 % (for fullness). Power calculations in the study of Flint *et al.*, (2000) revealed that a sample size of 16 was sufficient to detect baseline differences of 10 mm (out of 100 mm) in hunger and fullness, with a 20 % chance of a type 2 statistical error. Double this number were required to detect similar differences at the peak / nadir responses.

A number of groups have demonstrated the validity of appetite ratings. In an overt trial, Flint *et al.*, (2000) correlated *ad libitum* food intake, at a buffet lunch 4.5 h after breakfast, with VAS scores. Perhaps not surprisingly, correlation co-efficients immediately before lunch (i.e. 4.5 h post breakfast) were higher than at baseline (i.e. fasting) (eg. for hunger 0.5 v 0.32, $P < 0.001$). The relationships between ratings of hunger and fullness, and food intake at a subsequent test meal were also recently assessed (in two overt and one covert trial) in our department. In seventy-four healthy subjects (18-85 y) energy intake was directly related to the mean hunger before subjects consumed food from a buffet meal ($F = 0.37$, $P < 0.0001$), and inversely related to fullness ($r = -0.28$, $P < 0.0001$) ratings (MacIntosh, 2001). Although in both studies, the relationships were relatively weak, these data indicate that in general the more hungry and less full the subjects feel immediately before a meal, the more energy they consume. Together these studies demonstrate that VAS has some validity as a measure of symptoms associated with food intake. They were therefore used in all of the clinical studies reported in this thesis.

6.7.1.4 Food intake

The end-point of feeding behaviour is energy intake. Therefore, the major end-point of all the clinical studies presented in this thesis was an assessment of food intake. The most accurate and effective way of obtaining such a measure of energy intake is to quantify food ingested by subjects at an *ad libitum* eating episode, or test meal. A standard buffet meal was employed for this purpose (Lavin *et al.*, 1996; Lavin *et al.*, 1998) (Appendix 3) and this was presented to the subjects either at a fixed time interval after the study intervention

(Chapters 7, 9-11, 13 and 14) or when requested by the subject (Chapter 8). The buffet meal contained the same standard food items for each of the clinical experiments presented. Specific details of the buffet meals are in Appendix 3 and also the relevant chapters.

Test meals were always presented as a cold buffet lunch. The quantity of food was prepared in excess of what the subject would normally be expected to eat (Lavin *et al.*, 1996). Subjects were asked to eat until they were comfortably full. The total energy content of the food offered was approximately 10 MJ. All food items were weighed (to the nearest 0.1 g) before and after the meal. Energy consumption (kJ) and macronutrient intake (% of total) were calculated from the amount of food consumed during the buffet meal, using the DIET/4 food composition computer programme (Xyris Software, Highgate Hill, Qld) (Lavin *et al.*, 1996; Lavin *et al.*, 1998) or Food Works Nutritional Software (Version 2, Xyris Software [Aust] Pty Ltd).

6.7.2 Blood sampling

Cannulae for blood sampling were inserted into a forearm vein, under local anaesthetic (Lignocaine HCl, 1%) (Delta West, Technology Park, WA, Aust.). Subjects were allowed to rest for a recovery time of at least 5 minutes before starting the study. Blood samples (10 ml) were drawn at time points specified in the relevant chapters. Blood samples were immediately placed into tubes containing dipotassium EDTA and the protease inhibitor Trasylol (480 ul / 10 ml whole blood) (Bayer, Leverkeusen, FRG, Germany). Plasma was isolated within 2 hours of blood sample collection by centrifugation at 3200 rpm for 12 min, at 4°C and stored at either -20°C or -70°C until required for assaying.

6.7.3 Haemodynamic variables

In Chapter 13 the heart rate was measured by cardiac auscultation using a stethoscope. Blood pressures (BP) were measured, in the right arm, using a mercury sphygmomanometer (Accoson, London, England). Systolic pressure was recorded as the appearance of the first and diastolic pressure as the fourth Korotkov sounds. Mean arterial blood pressure (mmHg) was calculated as diastolic BP + 1/3 (systolic BP- diastolic BP). In blood pressure and heart rate were measured using a Dinamap™ vital signs monitor 8100 (Critikon, Sydney, Aust.) (Chapters 12 and 13).

6.7.4 Biochemical variables

Blood glucose and the concentrations of plasma hormones associated with feeding behaviour were quantified for individual studies as described below.

6.7.4.1 Blood glucose

Glucose concentration (mmol/L) of whole blood was measured at the bedside with a Medisense II glucometer (Medisense Inc, Bedford, MA, USA) utilising the glucose oxidase method (Kong *et al.*, 1999). The accuracy of this method has been confirmed previously using the hexokinase technique (Horowitz *et al.*, 1991).

6.7.4.2 Plasma insulin

Plasma insulin concentration was measured in the Institute of Medical and Veterinary Science Laboratory using the Abbott IMx Microparticle Enzyme Immunoassay (Abbott Laboratories, Diagnostic Division, Dainabot, Tokyo, Japan). The sensitivity of the assay (concentration at 2 SD from the zero standard) was 1.0 uU/ml. The intra-assay coefficients of variation were 4% at 8.3 uU/ml, 2.9% at 40.4 uU/ml, and 2.5% at 121.7 uU/ml. The inter-assay coefficients of variation were 4.5% at 8.3 uU/ml, 3.4% at 40.4 uU/ml, and 3.6% at 121.7 uU/ml.

6.7.4.3 Plasma glucagon-like peptide-1 (GLP-1)

Plasma GLP-1 (7-36) concentration was measured, after ethanol extraction of plasma samples by a radioimmunoassay method (Kong *et al.*, 1999; Wishart *et al.*, 1998). The antibody, provided by Professor SR Bloom (Hammersmith Hospital, London), had been raised in a rabbit immunized with GLP-1 (7-36) conjugated to bovine serum albumin (BSA) by carbodiimide. The antibody had 100% cross-reactivity with synthetic entire GLP-1 (7-36) (Penninsula Laboratories, CA, USA), but does not cross react with GLP-1 (7-37), glucagon, GIP, or other gut or pancreatic peptides and has been demonstrated by chromatography to measure intact GLP-1 (7-36) amide. The minimum detectable limit for the assay was ~ 2 fmol/L and the interassay coefficient of variation was 18%.

6.7.4.4 Plasma gastric inhibitory peptide (GIP)

Plasma GIP concentration was measured, after ethanol extraction of plasma samples by a radioimmunoassay method (Wishart *et al.*, 1992). Commercially available antiserum was used (Penninsula Laboratories, CA, USA). Bound fragments were separated from free

fragments using the double-antibody technique. The minimum detectable limit for the assay was ~ 15 pmol/L and the interassay coefficient of variation was 15%.

6.7.4.5 Plasma cholecystokinin (CCK)

Plasma CCK concentration was measured in the laboratory of Professor A Shulkes (Department of Surgery, University of Melbourne) after ethanol extraction of plasma using a radioimmunoassay. Antiserum 92128 (generous donation of Jens Rehfeld, University Hospital, Copenhagen) and ^{125}I-Bolton-Hunter-CCK-8 label (Amersham Laboritories, Buckinghamshire, England) were used as described previously (Zavros and Shulkes, 1997). The antiserum is specific for CCK amide with negligible cross-reactivity to gastrin amide or gly- extended forms of gastrin and CCK. The intra- and inter-assay coefficients of variation were 7% and < 14% respectively.

6.7.5 Manometry

The motor function of the antropyloroduodenal region is described in detail elsewhere (Chapter 2). The use of perfusion manometry to measure intraluminal pressures provides accurate and optimal assessment of antral, pyloric and duodenal pressures. Transducers linked to a manometric catheter allow the concurrent recording of luminal pressures at multiple points along the gastrointestinal tract. Over even short distances intraluminal pressures exhibit significant variation, therefore pressure sensors are spaced close together (1 - 1.5 cm apart). In addition, the pylorus has a narrow contractile zone. Optimal measurement of antropyloroduodenal motility is therefore performed using a catheter incorporating both side holes and a sleeve sensor. The advantages of this technique are that it allows not only assessment of pressure wave frequency, but also permits characterization of pressure waves including their amplitude and descriptions of propagation (aborally or orally) (Horowitz and Dent, 1991).

The position of the catheter can be independently monitored continuously using transmucosal potential difference (TMPD) (Heddle *et al.*, 1988a). The transmucosal potential difference (stomach vs duodenum) is measured via the side holes located at the oral and anal margins of the sleeve sensor (i.e. the most distal antral side hole and the most proximal duodenal side hole). The channels corresponding to these side holes are perfused independently from each other and from the other pressure recording channels. Potassium chloride (Crown Scientific, SA, Aust.) filled agar (Critchley Electrical Products, NSW,

Aust.) bridges connect each of these side holes, via Y-connectors, to calomel half-cells (Ionode, QLD, Aust.) and together with a common reference electrode (a sterile plastic saline-filled 21 G cannula placed subcutaneously on the left forearm, also connected to a calomel half-cell), an electric circuit enables the continuous measurement of TMPD across the stomach and small intestine. The secretions of the stomach (primarily hydrochloric acid) and the proximal duodenum (bicarbonate ions) produce electrically negative and electrically neutral charges, respectively, when compared to a reference point (the forearm). Using TMPD recordings, measured via voltage transducers connected to these two channels, the precise position of the tube can be monitored.

All studies involving the collection of manometry data were conducted using a 4 mm (outer diameter) silicone rubber manometric assembly (Dent-Sleeve, SA, Aust.). The assembly consisted of sixteen channels (0.1 mm diameter) for recording pressure, surrounding a central feeding channel (0.4 mm diameter) through which the intraduodenal infusions were introduced.

After an overnight fast (except for water) the manometric assembly was inserted into the stomach via an anaesthetised nostril (Lignocaine 5%, PeadPharm, Aust.). The tip of the tube was allowed to pass into the duodenum. This took between 30 and 180 min. A 21 G saline-filled reference electrode was inserted subcutaneously into the subjects' forearm to measure transmucosal potential difference (TMPD), so that the position of the manometric assembly could be monitored continuously (Heddle *et al.*, 1989). A 16-lumen sleeve/side hole manometric assembly, which included a 4.5 cm-long pyloric sleeve sensor, was used to measure antropyloroduodenal pressures. Two side-holes were located along the sleeve ± 0.75 cm from its centre. Side holes at each end of the sleeve sensor recorded intraluminal pressure and TMPD concurrently (Heddle *et al.*, 1988a). Pressures were also recorded from one antral and three duodenal side holes. All side holes were positioned at 1.5 cm intervals. A 1 mm infusion port was located 15 cm distal to the mid-point of the sleeve sensor. All side holes were perfused at a rate of 0.15 ml/min; the TMPD side holes with degassed, 0.9 % saline, and the remaining seven channels with degassed, distilled water (Sun *et al.*, 1996).

Antropyloric pressures were recorded using a computer-based recording system (Macintosh 6200-75, Apple Computer, Cupertino, California, USA), running Acqknowledge Software

(Version 3.2, BIOPAC Systems, Santa Barbara, California, USA). Manometric data were only analysed when the TMPD criteria indicated that the sleeve was positioned correctly across the pylorus (Heddle *et al.*, 1988a). The parameters assessed were: isolated pyloric pressure waves (IPPWs), basal pyloric pressure, antral pressure waves and duodenal pressure waves. An IPPW was defined as a pressure wave >10mmHg in amplitude recorded by the sleeve sensor with or without a concurrent pressure wave in one side hole along the sleeve and without an antral or duodenal pressure wave with an onset within 5 sec of the onset of the IPPW (Cook *et al.*, 1997; Heddle *et al.*, 1988a; King *et al.*, 1984). Basal pyloric pressure was measured by subtracting the mean basal pressure, (excluding phasic pressures), in an antral side hole 2.25 cm proximal to the mid-point on the sleeve from the mean basal pressure recorded by the sleeve (Edelbroek *et al.*, 1992; Sun *et al.*, 1996). This was calculated each minute, and the sum of 30 min blocks was determined. The number of antral and duodenal pressure waves >10 mmHg was counted for each 30 min of the study (Heddle *et al.*, 1988a). To determine antral and duodenal pressure wave amplitudes, pressure waves >10 mmHg on the most distal antral channel and on the most proximal duodenal channel, respectively, were averaged in 30 min periods. (Appendix 4)

6.8 STATISTICAL ANALYSES

All data are expressed as mean ± S.E.M. Study sample sizes were determined based on previous studies performed in the same laboratory (Lavin *et al.*, 1996; Lavin *et al.*, 1998). The major outcome measures of plasma gastrointestinal hormone concentrations, appetite sensations, and food intake were the same as in the studies reported in this thesis. The techniques used to evaluate these variables were also the same. These methodologies allowed detection of differences in the various parameters assessed, using sample sizes between 8 – 20 subjects, as were used in the studies reported here. Pre-treatment (baseline) ratings of appetite and antropyloroduodenal pressures on the different study days in each protocol were assessed using a Student's paired *t*-test or repeated-measures one-way analysis of variance (ANOVA). All appetite ratings are expressed as changes from baseline, defined as the mean of all pre-treatment values. Appetite ratings, antropyloroduodenal pressures, blood pressure, pulse rate, blood concentrations of hormones and glucose throughout the studies were analysed using repeated measures two-way ANOVA (with time and treatment being the two factors). Post-hoc analyses were performed when the initial comparison was found to be significant. A Student's paired *t*-

test, one-way ANOVA (with treatment as a factor) with repeated measures or two-way ANOVA (with treatment and patient group as the two factors) were used to compare the means for energy intake and macronutrient composition. The Sigma Stat (Jandel Scientific, Australia) or the SuperANOVA Version 1.11 (Abacus Concepts Inc. Berkley, California) software packages were used to perform analyses in all of the studies. A P value of < 0.05 was considered significant.

Chapter 7

THE EFFECTS OF INCREASED DIETARY PROTEIN ON SATIETY ARE ATTENUATED BY FAT

7.1 SUMMARY

The effects of isocaloric 2.5MJ yoghurt preloads differing in macronutrient composition on sensations of appetite and food intake at a subsequent meal were compared in 7 healthy subjects, (4 male; 3 female), each of whom, visited the research unit on five separate occasions. They consumed one of four yoghurt preloads (high carbohydrate (HC), high fat (HF), high protein (HP), or high fat / protein (HF / P) or no preload (N)), in a double blind, randomised crossover design. Hunger, fullness, and desire to eat, plasma glucose, insulin, and free fatty acid concentrations were measured before, immediately after, and at 15 min intervals for 180 min, following which a buffet meal was offered to assess food intake. Hunger decreased and fullness increased after the HC and HP, but not HF and HF / P compared to control preloads ($P < 0.05$). The plasma insulin to glucose ratio was greatest after the HP and least after the HF preload ($P < 0.05$). Plasma free fatty acids decreased after the HC and HP preloads ($P < 0.05$) but increased after 60 min in proportion to the amount of fat, in response to the HF and HF / P preloads. The effect of preloads with increased protein content to increase satiety and to induce a relative suppression of energy intake at the subsequent meal was partially abolished by increasing the fat content.

7.2 INTRODUCTION

Obesity is an increasingly prevalent disease (WHO, 1997) associated with substantial morbidity and mortality (WHO, 1997). The cause of the 'obesity epidemic' probably relates to the constant and readily available supply of high fat, energy dense foods, in an environment where energy expenditure is easily minimised. Evidence from cross-sectional, population, and observational studies implicates dietary fat, independent of overall energy intake, in the development of obesity (Rolls and Shide, 1992; WHO, 1997). An inability to adjust the oxidation of fat to match the dietary intake of fat has been demonstrated in obese-prone, obese, post-obese and normal individuals (Flatt, 1996). The

most common dietary approach for the treatment of obesity is a low energy, low-fat, high-carbohydrate diet. Diets with increased protein or an increase of both protein and fat at the expense of carbohydrate are, however, becoming increasingly popular. An increase in the ratio of protein to carbohydrate has recently been shown to result in greater weight loss in obese women as compared to a standard low-fat, high-carbohydrate diet (Skov *et al.*, 1999), but there is little evidence to support the use of very low carbohydrate diets.

Although protein has been shown in both animals (Walls and Koopmans, 1992) and humans (Porrini *et al.*, 1995; Stubbs *et al.*, 1996; Teff *et al.*, 1989) to be more satiating than either carbohydrate or fat, the data are derived predominantly from single nutrient studies. There is a lack of information relating to the effect of mixed macronutrient preloads on subsequent food intake. Since the majority of studies have involved an alteration in the ratio of carbohydrate to fat (Rolls and Hammer, 1995) with fixed amounts of protein, there is relatively little information available on the effect of variable combinations of protein and fat.

In obese women, hypoenergetic diets, with high carbohydrate content have been shown to decrease insulin sensitivity and increase fasting free fatty acids (FFA), whereas those with an increase in the dietary protein to carbohydrate ratio have been shown to improve insulin sensitivity (Piatti *et al.*, 1994). Both insulin and non-insulin-mediated glucose utilisation improve in normal subjects when plasma FFA concentrations are reduced (Piatti *et al.*, 1991).

The aim of this study was to determine the effect of preloads of varying macronutrient composition on plasma glucose, insulin, and FFA concentrations, and appetite sensations over a 180 min period followed by food intake at a subsequent meal in healthy, non-obese male and females.

7.3 METHOD

7.3.1 Subjects

Seven subjects (4 male and 3 female) were recruited according to Chapter 6.2. All subjects were told that the primary objective of the study was to determine the effects of macronutrient compositions of food on metabolic rate, macronutrient-oxidation and plasma

glucose and insulin. Subjects were not informed that appetite sensations and subsequent food intake were the primary outcome measures. Subjects were informed that a range of sensations and emotions would be monitored. Although the subjects did not sign consent knowing that the nature of the trial would be covert, The Royal Adelaide Human Ethics committee approved this form of 'blinding' the subjects from the true primary outcome measure being assessed. The subjects were fully informed post-trial.

7.3.2 Protocol

Subjects attended the Clinical Research Centre, Department of Medicine, Royal Adelaide Hospital at 0830 or 0900 h (consistent for each subject) on 5 separate occasions, separated by at least 48 h, after a 10 h overnight fast. The order of the preloads was randomised, and apart from the control day, both the subject and primary investigator were unaware of the preload condition. Female subjects attended the clinic during the follicular stage of their menstrual cycle (days 1-14). For 24 hours prior to each study visit, subjects recorded their food intake (baseline diet) and physical activity in diaries, and were instructed not to alter these before each study.

Upon arrival subjects lay down and an intravenous cannula was placed in a forearm vein for blood collection. After 30 minutes rest a visual analogue scale questionnaire (VAS) was completed and the first blood sample was collected (t = -15 min). Subjects then received one of four macronutrient preloads (Table 7.1), which were designed to have similar taste, consistency, and approximate energy, but varied in macronutrient content. This was determined by informal assessment by four Department of Medicine research staff blinded to the nature of the pre-loads. On the control day they consumed no preload. Subjects were instructed to consume the entire preload within 15 min, which was followed by another VAS and collection of a blood sample (t = 0 min). Subjects then returned to the ventilated hood for a further 180 min. Throughout this time a VAS was completed every 15 min, with blood collection every 15 min until 60 min and then every 30 min until 180 min. At the completion of the metabolic measurements, subjects were offered a cold buffet-style meal (Table 7.2) and instructed to eat until comfortably full. Thirty minutes after commencing the meal, a final VAS was completed, a blood sample was collected and the cannula was removed.

At the conclusion of all five visits, subjects answered questions relating to the study. They were asked to write down if they felt any different when comparing the preloads (hungry, full), after eating the pre-loads, later in the day and the following day. Finally they recorded the order in which they thought they consumed the preloads.

7.3.3 Macronutrient preloads and buffet meal

See Chapter 6.6.1 for yoghurt pre-load preparation. The specific composition of each preload is shown in Table 7.1. The tastes of the pre-loads were not formally assessed in this study (see Chapter 8).

The buffet meal contained a variety of foods and drinks that varied in macronutrient composition (Appendix 3) and was available for *ad libitum* consumption (Lavin *et al.*, 1996; Lavin *et al.*, 1998). Food intake was measured as previously described in Chapter 6.7.1.

7.3.4 Appetite sensations

Visual analogue scales (VAS) were used to measure sensations of appetite; hunger, fullness and desire to eat (Flint *et al.*, 2000; Sepple and Read, 1989) (Chapter 6.7.1.3).

7.3.5 Biochemical variables

Assays for blood glucose and plasma insulin are described in Chapter 6.7.4.

7.3.6 Statistical analyses

All data are expressed as mean ± S.E.M. Statistical analysis was performed using SPSS for Windows (Version 10, SPSS Inc., Chicago). Baseline measurements (t = -15 min) on each study day were compared using one-way ANOVA. All subsequent data were analysed by two-way repeated measures ANOVA with post-hoc Bonferroni's corrections. Results were considered significant at $P < 0.05$.

7.4 RESULTS

7.4.1 Subjects, baseline diets and blinding of preloads

The four males (age 26.0 ± 2.6, range 20-32 years; BMI 23.5 ± 0.6, range 22.1-24.9 kg/m^2) and three females (age 21.7 ± 0.9, range 20-23; BMI 21.0 ± 1.0, range 19.0-22.1 kg/m^2) completed the study without incident. Two of the volunteers were vegetarian (1 male; 1 female). The male subject consumed the vegetarian buffet meal but the female subject consumed the normal buffet meal on each occasion. In all subjects energy and macronutrient intakes were similar in the 24 hours preceding each study day (baseline diet); mean (± S.E.M.) intakes of energy, fat, carbohydrate and protein were 8731.7 ± 929 kJ, 34.7 ± 2.5%, 43.4 ± 3.2%, and 19.6 ± 2% respectively.

On each occasion, preloads were consumed in less than 15 min and the majority of subjects were unable to discern any differences between them. Only one subject correctly guessed the preload being consumed on each study visit. Questionnaires completed at the end of the study (prior to unblinding) revealed that all subjects felt fuller after the HC, HP, and HF/P preloads as compared to N or HF, and 4 subjects reported that they were hungrier after the HF preload as compared to any of the other preloads. Three subjects recorded that they were hungrier later in the day after the HF and HF/P preloads.

7.4.2 Appetite sensations

The changes in the sensations of appetite are shown in Figure 7.1. At baseline (t = -15 min) there were no significant differences between each study day for hunger, fullness or desire to eat. All subsequent data are expressed as change from baseline. There was a significant effect of preload ($P < 0.001$) and a significant interaction between preload and time ($P < 0.001$) for the sensation of hunger. Immediately after the consumption of the HC, HP, and HF/P preloads (t = 0 min) by ANOVA followed by Bonferroni's there was a significant decrease in hunger compared to N ($P < 0.05$), whereas after the HF preload the small decrease in hunger did not reach statistical significance. Thereafter, hunger remained significantly less after the HC ($P < 0.01$), HP ($P < 0.01$), and HF/P preloads ($P < 0.05$) (with no significant difference between them) as compared to N, when a small progressive increase in hunger occurred. After the HF preload, hunger was not significantly different from N.

There was a significant effect of preload on the sensation of fullness ($P < 0.01$) and a significant interaction between preload and time ($P < 0.001$). Fullness increased

immediately after all the preloads, as compared to N ($P < 0.05$ by ANOVA followed by Bonferrroni's), and remained greater over the 3 h after the HC ($P < 0.05$) and HP ($P < 0.05$) preloads, but not after the HF or HF/P preloads where fullness was similar to N ($P > 0.05$). There was an effect of preload ($P < 0.05$) and an interaction between preload and time ($P < 0.001$) on desire to eat, but no post hoc tests were significant.

There were no significant relationship between baseline diet and the sensations of appetite in response to any of the macronutrient preloads.

7.4.3 Energy and macronutrient intakes

None of the subjects ate all the food offered at the buffet meal, nor did they eat for the full 30 min time allotment. Energy intakes after the HC, HF, HP, and HF/P preloads decreased by 9.6 ± 7.9%, 4.2 ± 8.4%, 19.3 ± 2.8%, and 7.3 ± 5.1%, respectively, as compared to N, although this was not statistically significant by ANOVA ($P = 0.07$) and on post-hoc testing only the decrease in energy intake after the HP preload was significantly different to the control day ($P > 0.05$) (Figure 7.2). There were no significant differences in macronutrient composition of the food consumed at the buffet meal (% fat, % carbohydrate, % protein) between the control and any of the preloads, or between any of the preloads. There was also no significant relationship between baseline diet and the amount of energy consumed after each of the macronutrient preloads.

7.4.4 Plasma glucose and insulin

Fasting plasma glucose and insulin concentrations were similar at baseline (t = -15 min) on each study day. There was an effect of preload ($P < 0.01$) and a significant interaction between preload and time ($P < 0.001$) on plasma glucose concentrations over the 180 min. After the HF/P preload, plasma glucose concentrations decreased transiently before returning to baseline (Figure 7.3) and were significantly higher in response to the HC preload than the other treatments ($P < 0.05$). There was an effect of preload ($P < 0.01$) and an interaction between preload and time ($P < 0.001$) on plasma insulin concentrations over the 180 min. Plasma insulin concentrations increased from baseline after all of the preloads (Figure 7.3), whereas it remained unchanged after N. After the HP ($P < 0.05$) and HF/P ($P < 0.05$) preloads, plasma insulin concentration was significantly greater over the 3 hours as compared to N; it remained unchanged after HF and HF/P. The insulin to glucose ratio was greatest after the HP and least after the HF preloads ($P < 0.05$). Thirty minutes

after the commencement of the buffet meal (t = 210 min) there was no significant differences in plasma glucose and insulin concentrations between any of the study days.

7.4.5 Plasma Free Fatty Acids

Plasma FFA concentrations were similar at baseline on each study day (Figure 7.4). There was a significant effect of preload ($P < 0.001$) and a significant interaction between preload and time ($P < 0.001$). Plasma FFA concentrations decreased and reached a nadir at 60 min after the consumption of each preload, followed by an increase in FFA concentration over the remaining 120 min after the HF and HF/P preloads, while plasma FFA concentrations remained low after the HC and HP preloads. Post-buffet meal (t = 210 min), plasma FFA concentrations decreased following HF/P and N, remained low on the HP and HC days as compared with N ($P < 0.05$), and did not change further on the HF day.

7.5 DISCUSSION

These data demonstrate that when the protein content of a meal is high, energy intake is reduced at a subsequent meal, whilst in contrast, when the carbohydrate or the fat content of a meal are high, energy intake at a subsequent meal is unchanged. The data also show that a high fat content may counteract the satiating effect of a high protein meal. Of the two pre-loads with 15% protein, the 'meal' with carbohydrate/fat ratio of 2.4 (HC) suppressed food intake more than that with a ratio of 1.125 (HF) [9.6% v 4.2%], while of the pre-loads with 30% protein, that with the carbohydrate/fat ratio of 1.8 (HP) suppressed food intake more than that with a ratio of 0.75 (HF/P) [19.3% v 7.3%]. This is not, however, an independent effect of protein since when combined with an increase in the ratio of fat to carbohydrate the benefits of additional protein on appetite were reduced. Similarly the insulin to glucose ratio, which occurred after the high protein preload, were abolished when the fat content was increased concomitantly at the expense of carbohydrate. These data suggest that the observed effects were not simply an effect of increased dietary protein but relate also to the ratio of carbohydrate to fat. Furthermore both the HF and HF / P preload resulted in higher plasma FFA concentrations than either of the other preloads.

Although there were small differences in the energy content of the four diets, the HC and HF contained similar amounts of energy (2545 and 2510 kJ respectively) and the energy contents of the HP and HF/P diets were well matched (2821 and 2877 kJ respectively),

there were no significant relationships between the energy content of the diets and any of the outcome measures. The energy densities of these pre-loads were not different (5.8 v 5.9 v 5.9 v 6.0 kJ / g for HC v HF v HP v HF/P respectively). It appears unlikely that these differences confound the conclusions drawn from the data.

The relatively low intakes of subjects (established from diet diary records) were surprising. To remain in energy balance it would be expected that healthy adult males would be required to consume approximately 12.5 kJ. It is likely that these subjects under-reported their total energy intake.

Many previous studies have compared the effects of preloads varying in fat and carbohydrate content on sensations of appetite or energy intake (Porrini *et al.*, 1995; Stubbs *et al.*, 1996; Teff *et al.*, 1989). Marmonier *et al.*, (2000) found that the consumption of a HF snack resulted in a shorter latency to a dinner request than after the consumption of an isoenergetic HC snack. Consistent with this, we found that the HF preload suppressed energy intake at a subsequent meal less than the HC preload, although this was not statistically different. Few studies however, have evaluated the effect of HP preloads on sensations of appetite and subsequent food intake in both male and female subjects. The present data demonstrate a strong trend ($P = 0.07$) towards a suppression of food intake as a result of a greater protein content in the pre-load (compared with those high in fat or carbohydrate). Although the decreased hunger, and desire to eat and increased fullness after the HP preload, did not reach statistical significance the small sample size ($n = 7$) and possible gender differences may have contributed to a type 2 error. This implies that if high protein diets are to be used in weight loss diets, they may be of benefit by reducing food intake.

Plasma glucose levels increased in proportion to the carbohydrate content of the preload, whereas the plasma insulin levels increased to a similar extent after the HC and HP preloads, presumably because dietary protein markedly potentiates the insulin response to co-ingested carbohydrate (McCarty, 2000). Therefore the substitution of protein for carbohydrate may be of particular metabolic benefit by achieving a lower blood glucose level for a given level of insulin. This may be advantageous for individuals with impaired glucose tolerance or type 2 diabetes mellitus, particularly as postprandial hyperglycaemia is known to be a major determinant of diabetic complications. An increase in the fat content,

in addition to protein in the preload, at the expense of carbohydrate, resulted in even lower plasma glucose levels without a concomitant increase in insulin. This observation is consistent with the study of Golay *et al.*, (1996) showing that an increase in monounsaturated fat at the expense of carbohydrate lowered plasma glucose levels. In this study, however, after an initial decrease, plasma FFA concentrations increased in proportion to the fat content of the preload, an effect which may limit any potential metabolic benefit of additional fat.

Diets that are low in fat, with carbohydrate replacement by protein, have been shown to result in increased weight loss, suggesting that the short-term benefits of increased dietary protein may be sustained over a longer period and have significant benefits for the dietary management of obesity. In contrast, a further reduction in dietary carbohydrate and substitution by fat may attenuate the short-term benefits of increased dietary protein and has unfavourable metabolic effects, suggesting that this is a sub-optimal approach for the long term dietary management of obesity. Further studies will need to be conducted to establish whether this effect is reproducible not only in lean, but also in obese individuals. The mechanism underlying the effects of protein in this study, together with the role of gender differences, and whether the subjects are lean, obese prone, obese and post-obese individuals also remains to be determined.

Acknowlegement: The work reported in this chapter was submitted as part of an Honours degree by J Mudge (University of Adelaide, 2000)

Table 7.1 Ingredients and macronutrient content of yoghurt preloads[1]

	Yoghurt preload			
	HC[2]	HF[2]	HP[2]	HF/P[2]
Ingredients (g)				
Yoghurt, light fruit of the forest	300	300	300	300
Strawberry flavoured topping	16	16	16	16
Canola oil	15	22	17	24
Cornflour	26	16	25	-
Thickened cream	-	10	-	12
Glucose powder	18	-	-	-
Casec powder	-	-	28	28
Raw egg white (number)	2	2	3	3
Energy from Fat (%)	25	40	25	40
Energy from Carbohydrate (%)	60	45	45	30
Energy from Protein (%)	15	15	30	30
Carbohydrate/Fat ratio	2.4	1.125	1.8	0.75
Total weight (g)	437	426	479	473
Total Energy (kcal)	608	599	674	687
(KJ)	2545	2510	2821	2877

[1]All amounts are for 1 serving. Glucose powder: Glucodin energy powder, Smithfield, Australia; Casec powder: protein supplement, MeadJohnson, Evansville, USA.

[2]HC = High Carbohydrate, HF = High Fat, HP = High Protein, HF / P = High Fat / Protein

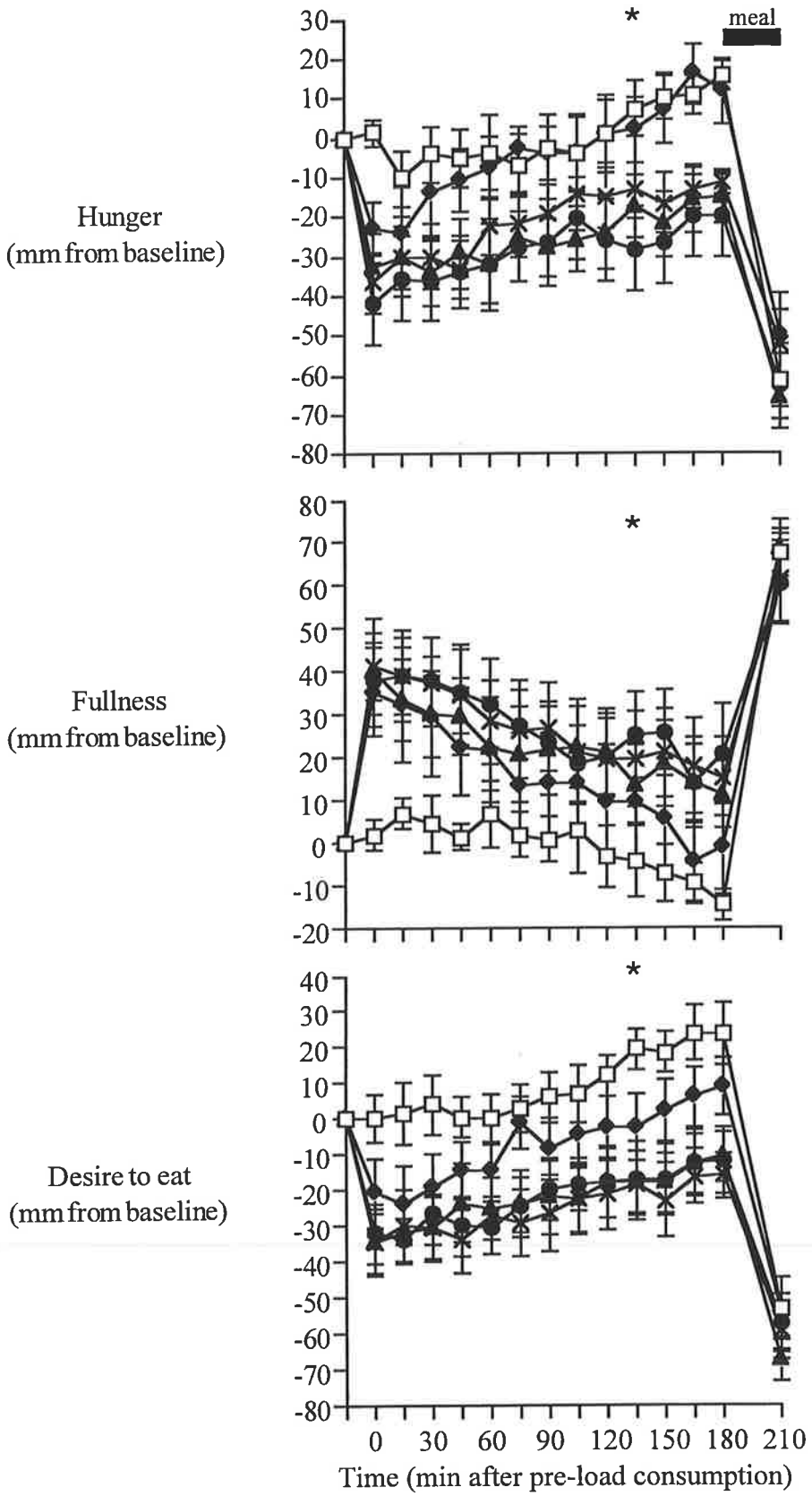


Figure 7.1 Hunger, fullness and desire to eat following ingestion of pre-loads HF (◆), HP (●) HC (▲), HP / F (✕) or N (□) by healthy adults (n = 7). Values are mean ± SEM. * $P < 0.05$ for whole curve.

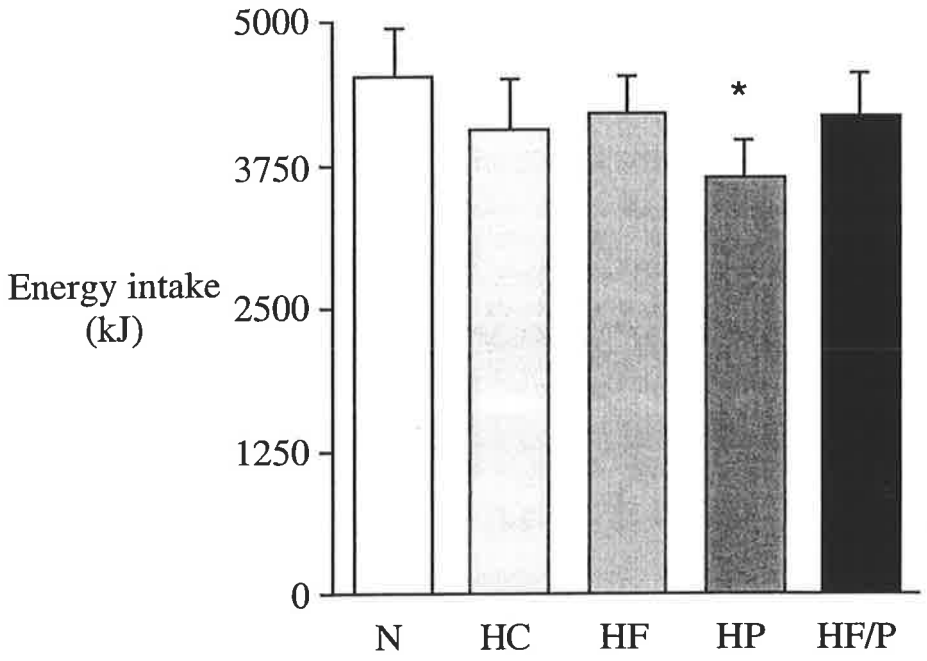


Figure 7.2 *Ad libitum* energy intake at a buffet meal following ingestion of yoghurt pre-loads high in fat (HF), protein (HP), carbohydrate (HC), protein and fat (HP / F) or no pre-load (N) by healthy adults (n = 7). Values are mean ± SEM. * $P < 0.05$ HP v N by post-hoc analysis.

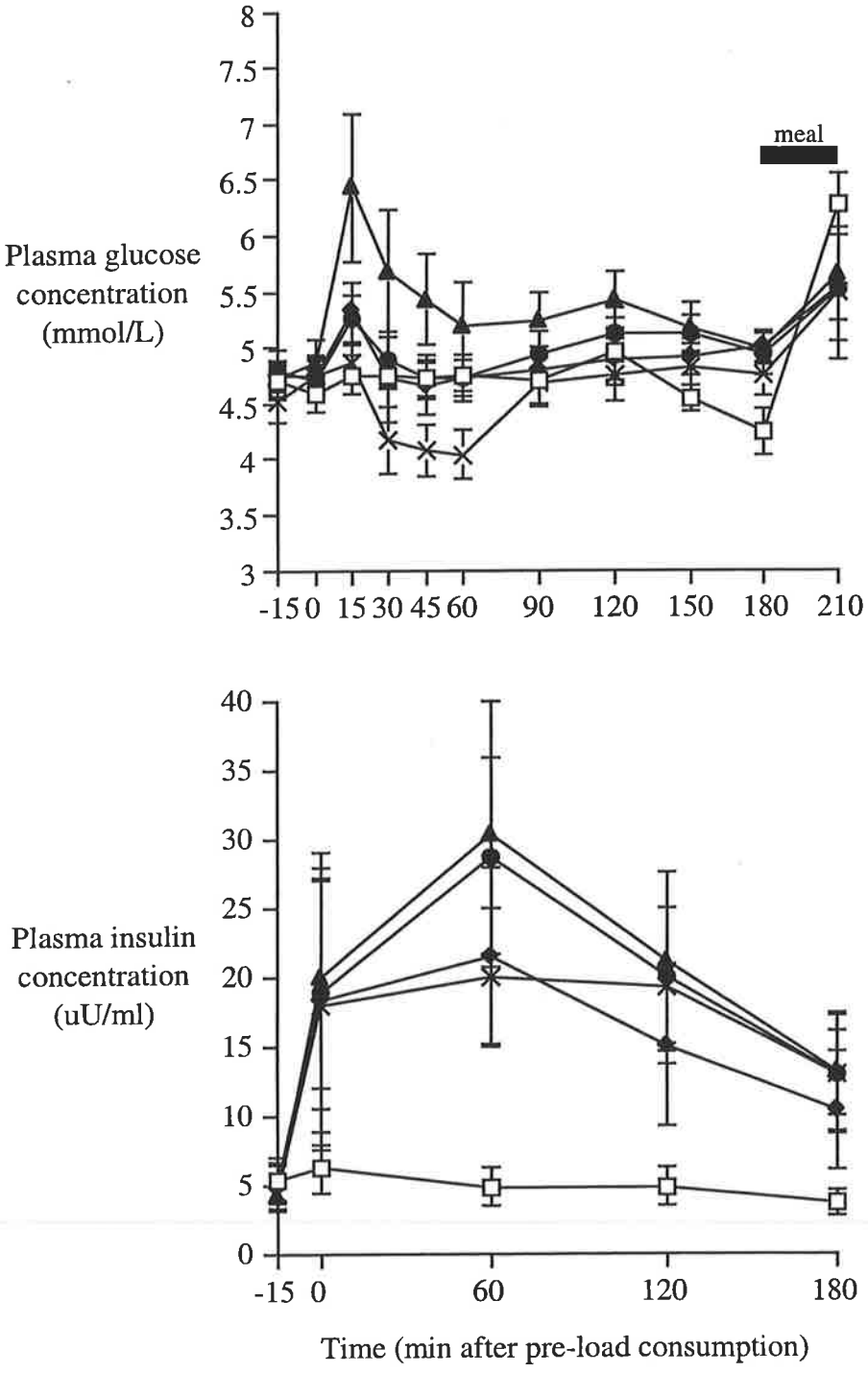


Figure 7.3 Plasma glucose and plasma insulin concentrations following ingestion of yoghurt pre-loads HF (◆), HP (●), HC (▲), HP / F (✕) or N (□) by healthy adults (n = 7). Values are mean ± SEM.

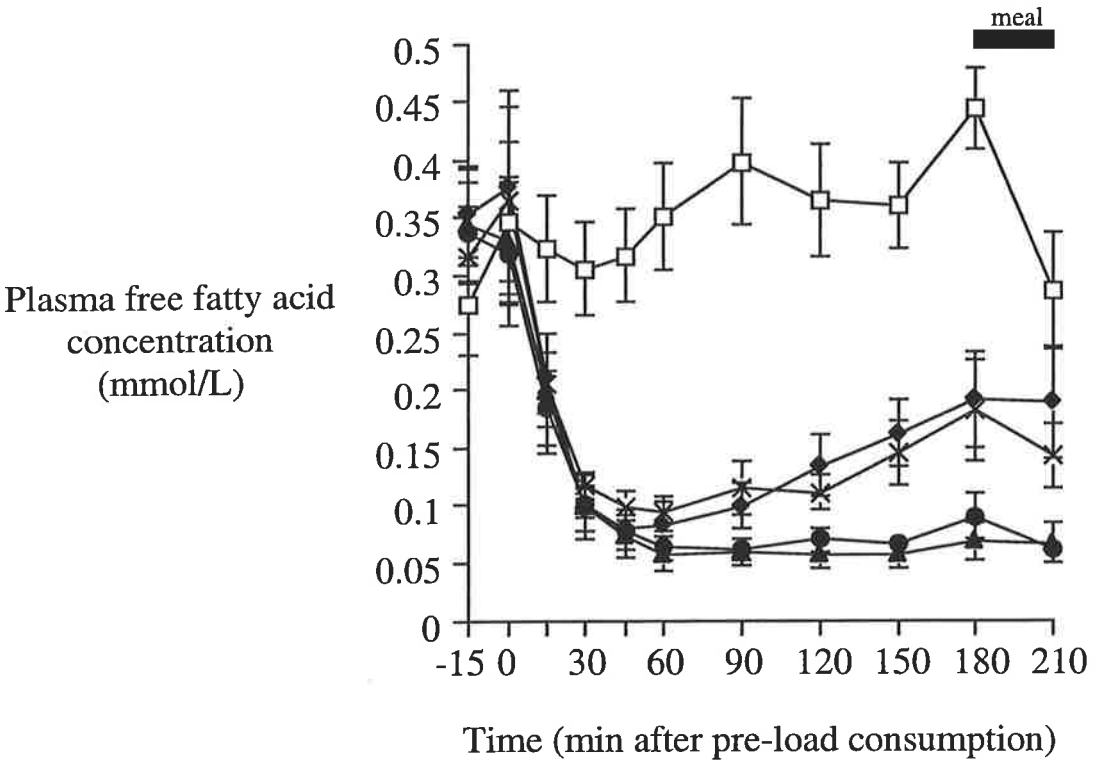


Figure 7.4 Plasma free fatty acid concentrations following ingestion of yoghurt pre-loads HF (◆), HP (●), HC (▲), HP / F (✕) or N (□) by healthy adults (n =7). Values are mean ± SEM.

Chapter 8

HIGH PROTEIN, HIGH CARBOHYDRATE AND HIGH FAT YOGHURT PRE-LOADS AND THEIR EFFECT ON SUBSEQUENT SPONTANEOUS FOOD INTAKE IN HEALTHY SUBJECTS

8.1 SUMMARY

Pre-loads high in protein, as compared to carbohydrate and fat, produce greater satiety and reduce food intake after a fixed time interval. To determine the effect of macronutrient composition on spontaneous eating behaviour, 16 fasted, healthy, non-obese men blinded to the true purpose of the study, consumed on four separate occasions isoenergetic ~3000 kJ yoghurt pre-loads of equivalent weight (~ 510 g) high in fat (40%) [HF], carbohydrate (60%) [HC] or protein (29%) [HP], and no pre-load [N], in a randomized, single blind fashion. Subjects ate at will from a selection of food items for the remainder of the day (7 h). The time of food requests, and quantity and types of food eaten were recorded. The taste, pleasantness, texture, creaminess and colour of the pre-loads were similar. Subsequent food intake was suppressed 29% (HP), 20% (HF) and 17% (HC) by the 3 pre-loads ($P < 0.05$ for HP and HF v N). Compensation after consumption of HP was greater than after HF and HC; total daily food intake was significantly greater after the HF and HC (but not the HP) pre-loads than after no pre-load. Suppression of subsequent food intake by the nutrient pre-load was due to a combination of reduced meal size and frequency, with no difference between the effects of the different pre-loads. The high protein pre-load was the most satiating in subjects free to choose when and how much they ate.

8.2 INTRODUCTION

Excess energy intake, particularly in the form of palatable, energy-dense fat, facilitates weight gain (Bray and Popkin, 1998). Most weight loss diets therefore recommend a reduction in energy and fat intake, and usually advocate replacement of fat by carbohydrate (Garg *et al.*, 1992; Heilbronn *et al.*, 1999). There is some evidence that protein is more satiating than either fat or carbohydrate (Teff *et al.*, 1989). High protein diets are being increasingly recommended for the treatment of both insulin resistance (Heilbronn *et al.*, 1999) and obesity (Skov *et al.*, 1999). This is despite the fact that protein only comprises

about 15-20% of the average western diet (National Task Force on the Prevention and Treatment of Obesity, 2000), and there are difficulties in increasing protein intake without simultaneously increasing fat intake.

The evidence that protein is the most satiating macronutrient is largely derived from studies in rats (Walls and Koopmans, 1992) and humans (Stubbs *et al.*, 1996; Geliebter *et al.*, 1988; Geliebter, 1979; Porrini *et al.*, 1995; Teff *et al.*, 1989), in which the time interval between the nutrient pre-load and the subsequent "free" consumption of a test meal is fixed. These studies usually involve administration of pure macronutrient preloads, or preloads containing concentrations of a particular macronutrient far exceeding those in normal diets. These study designs do not, therefore, reflect free-living conditions, where foods are of mixed nutrient composition and the timing of food intake has the potential to be highly variable. No studies have evaluated whether protein remains more satiating than the other macronutrients when people can eat freely.

The aim of this study was to determine the relative satiating effects of protein, fat and carbohydrate, and the temporal characteristics of these effects. Equi-energetic, mixed macronutrient oral preloads containing high-physiological concentrations of each of these macronutrients were administered to healthy men, after which they were able to eat freely with respect to time and quantity (spontaneously) for the next seven hours.

8.3 METHODS

8.3.1 Subjects

Sixteen healthy men aged 18-27 y, with body mass indices 19.0-25.1 kg / m^2 were recruited according to Chapter 6.2. Subjects were blinded to the purpose of the study, believing that the study was designed to test the effect of consuming different macronutrients on mood.

8.3.2 Protocol

Subjects attended the Clinical Research Centre, Department of Medicine, Royal Adelaide Hospital for approximately 8 hours on four occasions, separated by at least 48 hours. Subjects were instructed not to undertake any vigorous exercise or drink alcohol in the 24 hours before each study. Subjects arrived at approximately 0830 after an overnight fast, except for water from 2000, and rested quietly for 5 minutes before two baseline visual

analogue scale (VAS) were administered (t = -15 and 0 min) (Chapter 6.7.1). Additional sensations including anxiety, energy level, happiness, efficiency, friendliness, how settled subjects felt, and interest, were assessed. Subjects then ingested one of three isoenergetic yoghurt preloads of equivalent weight (high fat (HF), high protein (HP) or high carbohydrate (HC)) over 15 minutes, or no yoghurt (No pre-load control (N)) in random order. Except on the no pre-load (N) day subjects were unaware of treatment order. Immediately following yoghurt ingestion subjects completed a VAS questionnaire, rating the pre-load for taste, pleasantness, texture, creaminess, colour and sweetness. For the remainder of the day subjects were encouraged to act as they 'normally' would, within the confines of the research environment. Visual analogue questionnaires were administered at 15 min intervals for the duration of the study.

During the rest of the study day (i.e. until t = 435 min) subjects were required to inform the investigator when they were hungry enough to eat. Food could be requested at any time from 30 minutes after the yoghurt ingestion, and subjects were allowed to eat as often as they wished. The time, quantity and macronutrient composition of each eating episode was recorded. At the end of the fourth visit, subjects were asked to record the order in which they thought the preloads were consumed.

8.3.3 Yoghurt pre-loads

The yoghurt pre-loads were prepared to be similar by sight, smell, texture and taste (Chapter 6.6.1) and all contained ~3000 kJ, in similar amounts (~510 g). The yoghurt pre-loads were prepared in advance and consumed within 5 minutes of removing them from 4°C storage. Yoghurt pre-load composition is shown in Table 8.1.

8.3.4 Buffet meal

A cold, buffet style, self-selection meal varying in fat, carbohydrate and protein content (Chapter 6.7.1.4) was made available to the subjects throughout the day. Subjects viewed photos of each food item on their first visit, to ensure that they were familiarized with the selection. Foods were always available in excess of what the subject would normally be expected to eat (Lavin *et al.*, 1996).

8.3.5 Statistical Analyses

Perceptions of the taste, pleasantness, texture, creaminess and colour of the yoghurt pre-loads were analysed using a one-way analysis of variance (ANOVA) with repeated measures (yoghurt pre-load type as a factor). The energy intake subsequent to yoghurt ingestion, total daily energy intake including the yoghurt pre-load, energy intake per eating episode, frequency of eating episodes, timing to the first eating episode, energy eaten at the first eating episode and the energy intake at eating episodes subsequent to the first eating episode (n = 5 only) were analysed using a one-way analysis of variance (ANOVA) with repeated measures (treatment condition as a factor). When a significant interaction was observed, contrasts were used to test hypotheses of interest, enabling specific paired comparisons between two treatments. Daily macronutrient intake and intake at the first eating episode were analysed using a one-way analysis of variance (ANOVA) with repeated measures (treatment condition as a factor). Sensations including nausea, bloating, satiety, fullness, hunger, prospective consumption and desire to eat were also analysed using a one-way analysis of variance (ANOVA) with repeated measures (treatment condition as a factor). SuperANOVA Version 1.11 (Abacus Concepts Inc. Berkley, California, USA) software was used to perform these analyses. A P value of <0.05 was considered statistically significant. All data are expressed as means ± S.E.M.

8.4 RESULTS

8.4.1 Yoghurt pre-loads

All subjects completed the studies and there were no adverse events reported. Fifteen of the 16 subjects ingested the yoghurt within the required 15 min time period, the remaining subject ate the yoghurt pre-loads over 20 min.

Questionnaires completed immediately following the yoghurt ingestion revealed that the sensory properties of the yoghurts were similar. Taste (35 ± 4 v 32 ± 3 v 35 ± 4 mm; HP v HC v HF), pleasantness (40 ± 4 v 34 ± 4 v 33 ± 4 mm; HP v HC v HF), texture (40 ± 6 v 36 ± 6 v 40 ± 6 mm; HP v HC v HF) and colour (40 ± 6 v 56 ± 6 v 56 ± 5 mm; HP v HC v HF) were not statistically different between pre-loads ($P > 0.05$ for each parameter). Only one subject correctly identified the pre-load order.

8.4.2 Food intake

One subject ate no food following HP. His time to first food intake on that day has been designated 7 hours (the end of the study day) and his energy intake and macronutrient content of meals on all study days have been omitted from analysis (n = 15).

Compared with the no yoghurt day, energy intake following the yoghurt pre-loads was suppressed 29 ± 10%, 20 ± 9% and 17 ± 6% by HP, HF and HC respectively (Fig 8.1a: effect of treatment $P = 0.01$). Suppression by HP and HF yoghurts was statistically significant (HP vs N, $P < 0.01$, HF vs N, $P < 0.05$), but suppression by HC was not ($P = 0.053$). Because the reduction in energy intake after the 3 yoghurts was not enough to compensate for the energy content of the yoghurts (3000 kJ), total energy intake (pre-load plus intake for the rest of the day) was greater on all three yoghurt days than on the no yoghurt day (Fig 8.1b). The daily intake was significantly greater on the HF and HC than N day ($P < 0.05$ v N), but not on the HP day, indicating a greater suppressive effect of the HP than HF and HC on food intake during the seven hours after the pre-load.

8.4.3 Timing and size of eating episodes

The reduction in energy intake after the three yoghurt pre-loads was due to the combination of a non-significant reduction in the amount of energy consumed per eating episode after the HP and HF yoghurts (Fig 8.2a, $P > 0.05$), and a non-significant reduction in the number of eating episodes after all three yoghurts (HP v HF v HC v N; 1.8 ± 0.2, 2.0 ± 0.2, 1.8 ± 0.2, 2.1 ± 0.2; Fig 8.2b, $P > 0.05$). The latter was largely due to a marked delay in the first spontaneous eating episode after all three yoghurts (HP v HF v HC v N; 3.1 ± 0.4 v 2.8 ± 0.3 v 3.1 ± 0.3 v 1.3 ± 0.3 h, $F = 8.4$, $P < 0.001$), with no effect on the amount of energy consumed at that first episode (HP v HF v HC v N; 3369 ± 405 v 3286 ± 451 v 3720 ± 451 v 3201 ± 305 kJ, $P > 0.05$). There was however a slight reduction in the amount of energy consumed at each eating episode after the first voluntary intake on the yoghurt days compared to the no yoghurt day; (HP v HF v HC v N; 2888 ± 560 v 3741 ± 526 v 4146 ± 368 v 4552 ± 994 kJ, $P > 0.05$) (n = 5), although the fact that only 5 subjects had more than one voluntary eating episode on all 4 study days prevented proper statistical analysis of this result. There was no significant difference between any of the three yoghurt pre-loads in their effects on the timing and size of voluntary eating episodes.

8.4.4 Macronutrient intake

Ingestion of yoghurt high in a particular macronutrient did not differentially alter subsequent total daily macronutrient intake (Table 8.2a). Protein intake at the first meal following yoghurt pre-loads high in protein however, was increased relative to protein intake following high fat yoghurts and no yoghurt (Table 8.2b).

8.4.5 Appetite sensations

Yoghurt ingestion did not induce nausea or bloating (Table 8.3). Irrespective of macronutrient composition, satiety and fullness, increased after yoghurt ingestion (Table 8.3). Hunger, prospective consumption and desire to eat all decreased after yoghurt ingestion (Table 8.3). There were no effects of the pre-loads on other sensations including anxiety, energy level, happiness, efficiency, friendliness, how settled subjects felt, and interest ($P > 0.05$). There were no differences between the effects of any of the three pre-loads on any sensation.

8.5 DISCUSSION

The major finding of this study was that, in subjects who were unrestricted in both timing and quantity (free to eat spontaneously) of eating episodes, pre-loads high in fat, carbohydrate or protein exerted similar effects on subsequent food intake. This is in contrast to the findings of Chapter 7, where, when meal times were fixed, pre-loads high in protein (but not high in carbohydrate or fat) suppressed food intake at a subsequent meal. The compensatory reduction in food intake after the high protein pre-load was such that the total energy intake for the high protein day (including the pre-load itself) was not significantly greater than when no pre-load at all was consumed. In contrast, the total energy intake on both the high fat and high carbohydrate days was significantly greater than on the no pre-load day.

Studies in both humans (Porrini *et al.*, 1995; Poppitt *et al.*, 1998; Stubbs *et al.*, 1996) and animals (Burton *et al.*, 1997; Geliebter, 1979; Walls and Koopmans, 1992) in which the time interval between the pre-load and the test meal was fixed (and is greater than 60 min), have demonstrated that protein has a greater satiating efficiency than either fat or carbohydrate. These studies did not, however, determine the relative satiating effects of the macronutrients when the timing of food was under voluntary control. In normal life, although the timing of meals is determined to some extent by habit and social factors, it

remains largely under voluntary control. This study demonstrates that no difference in the satiating efficiencies of macronutrients exists under spontaneous eating conditions. This finding suggests that under free-eating conditions, as occurs in real-life, energy density and / or energy load may be the more important factors in determining meal times, and total amount of calories consumed.

Only one previous study has compared the satiating effects of different macronutrients in freely eating humans. In that study, Marmonier *et al.*, (2000) found that healthy men delayed asking for their test dinner significantly longer after a high protein pre-load than after isoenergetic high carbohydrate and high fat pre-loads (186 v 161 v 151 min; $P < 0.02$ protein v fat and carbohydrate). In this study, however, the delay to first meal request did not differ after the three nutrient pre-loads. Also in contrast to the present study, Marmonier *et al.*, (2000) did not show a difference between the amounts of food eaten at the test meal on the three pre-load days, with total daily energy intake significantly greater on all three pre-load days than on the no pre-load day.

The differences in findings between the current study and that of Marmonier *et al.*, (2000) may relate to study design. The subjects had fasted for about thirteen hours before the pre-load in this study, whereas those of Marmonier *et al.*, (2000) had eaten a lunch containing approximately 3200 kJ four hours before the pre-load, and would have had some food still in the stomach at the time of pre-load ingestion. In the study by Marmonier *et al.*, (2000) the high protein pre-load had a greater volume than the high fat and high carbohydrate pre-loads (198 g compared to 65 g for high fat and 100 g for high carbohydrate), and is therefore likely to have produced more gastric distension, at least initially. Differences in the perceptions of the types of foods eaten i.e. chicken breast (high protein) considered a 'main meal' type food item, with rye bread with raisins (high carbohydrate) and cream cheese (high fat) considered 'snack' type food items may also have contributed to prolonged satiety following high protein pre-loads. In addition, the high protein pre-load was very high in protein (77% compared with 29% in this study), with similarly very high levels of carbohydrate (84%) and fat (58%) in the other two pre-loads. This study avoided these potentially confounding factors by the use of three indistinguishable, isoenergetic pre-loads, of equivalent weight and volume containing concentrations of the three macronutrients high in the normal range or not much above it, and by allowing subjects to eat on more than one occasion following the pre-load. The latter was of particular importance, as it was only after the first voluntary meal that the greater satiating effect of

protein was observed. This suggests that the greater satiating effects of protein are slow in onset, or depend for their initiation on some interaction with subsequently ingested food.

This study design allowed examination of the way in which the different pre-loads suppressed subsequent food intake – by reducing the size of meals, the frequency of meals, or both. It had been hypothesized that protein would exert its enhanced satiating effect, at least in part, by delaying voluntary food intake longer than fat or carbohydrate. This prediction was based on the results of Marmonier *et al.*, (2000) and the earlier study in our laboratory (Chapter 7). In these studies hunger ratings and elevated fullness ratings returned to baseline more slowly after isoenergetic protein than fat and carbohydrate pre-loads. In the current study this was not the case, and all three pre-loads delayed the time to first spontaneous food intake equally. However, both fat and protein pre-loads reduced subsequent meal size, whereas the carbohydrate pre-load had no effect on this parameter The effects of the pre-loads on meal size did not differ significantly from each other. Overall there was no evidence for a difference between macronutrients in their suppressive effects on subsequent food intake. The reduction in meal size and frequency was proportional to their overall suppressive effect.

It has previously been demonstrated that the increased satiating effect of protein may have implications for the design of weight loss diets (Skov *et al.*, 1999). This is based on the findings that demonstrate the efficacy of modifying dietary macronutrient ratios in weight loss regimes; high protein foods eaten *ad libitum* decreased food intake in obese subjects on low fat diets, i.e. the total energy intake on high protein diets was lower than on high carbohydrate diets (Skov *et al.*, 1999). This effect occurred within three months of starting these diets and persisted for the duration of the six-month study (Skov *et al.*, 1999). However, there was no information on whether the decrease in food intake was due to a decrease in the amount of food eaten, a reduction in the total number of meals, or both. In addition to reducing food intake, this substitution of protein for carbohydrate also promoted weight loss. The thermic effect of feeding is higher in the 24 h following ingestion of high protein / high carbohydrate than high fat pre-loads (Westerterp *et al.*, 1999), suggesting that weight loss in obese people on high protein diets may be due to increased energy expenditure as well as reduced food intake. Thus, whilst in the acute setting, high protein pre-loads have little effect on spontaneous food intake, in the chronic situation; a diet high in protein appears to produce reductions in overall energy intake.

Although, in the present study subjects ate a greater percentage of protein at their first voluntary meal on the protein pre-load day than the other days, a mean of 17% of the total post pre-load nutrient ingestion was protein, 35% fat and 45% carbohydrate, and these percentages did not differ after the different pre-loads. This is consistent with previous studies (Johnson and Vickers, 1993; Marmonier *et al.*, 2000; Rolls *et al.*, 1991), showing no effect of pre-loads differing in the macronutrient composition of subsequent voluntarily chosen food.

A limitation of this study is the small subject numbers. Food intake was suppressed 819 kJ more after the high protein than high carbohydrate pre-load, a difference equal to 12% of the total daily energy intake on the control day. This difference would, if sustained in a weight loss diet for obesity, be likely to have substantial beneficial effects. Nevertheless, due to the variability in human feeding behaviour, even within a relatively homogenous group such as the young men in this study, the differences between the effects of the three nutrient pre-loads did not achieve statistical significance. Comparison of the effects of the three pre-loads with those on the no pre-load day, however, strongly supports a greater satiating effect of protein than the other macronutrients. Another limitation of the study was the lack of a control treatment that blinded subjects to what they were receiving on that day. However, very low energy yoghurts similar in sensory properties were, in practise, very difficult to achieve.

In summary, this study demonstrates that in spontaneously feeding subjects the ingestion of protein in physiological quantities is as satiating as both fat and carbohydrate. Sensory properties of high protein foods may play a role in producing the greater satiating effect that has been noted of high protein foods in previous studies.

Table 8.1 Specific ingredients and macronutrient content of yoghurt preloads high in protein (HP), fat (HF) and carbohydrate (HC).

	HC[1]	HF[1]	HP[1]
Ingredients (g)			
Yoghurt, light fruit of the forest	350	350	350
Strawberry flavoured topping	19	19	19
Canola oil	18	26	14
Cornflour	30	19	20
Thickened cream	-	12	9
Glucose powder	21	-	-
Casec powder	-	-	29
Raw egg white	73	73	73
Energy from fat (%)	24	40	24
Energy from carbohydrate (%)	60	42	44
Energy from protein (%)	14	14	29
Total weight (g)	511	499	514
Total energy (kJ)	2984	2934	2951

[1]All amounts are for 1 serving.

Table 8.2 Specific macronutrient intake following intake of yoghurt high in protein (HP), fat (HF), carbohydrate (HC) and following no pre-load.

	Pre-load condition				
	HP	**HF**	**HC**	**No pre-load**	***P* value**
a. *Ad libitum* daily food intake					
Protein (% of total intake)	17 ± 2	17 ± 1	17 ± 1	17 ± 1	0.9
Fat (% of total intake)	34 ± 3	37 ± 2	34 ± 2	36 ± 2	0.8
Carbohydrate (% of total intake)	41 ± 3	45 ± 2	47 ± 2	45 ± 2	0.2
b. First eating episode					
Protein (% of total intake)	19 ± 2	15 ± 1[a]	16 ± 1	16 ± 1[a]	0.049
Fat (% of total intake)	34 ± 2	36 ± 3	36 ± 2	30 ± 3	0.1
Carbohydrate (% of total intake)	45 ± 3	47 ± 3	42 ± 3	51 ± 3	0.06

Values are mean ± S.E.M, (n = 15). [a] indicates significant versus HP yoghurt.

Table 8.3 Appetite sensations 30 min following intake of yoghurt high in protein (HP), fat (HF), carbohydrate (HC) and following no pre-load.

	Pre-load condition				
	HP	**HF**	**HC**	**No pre-load**	***P* value**
Sensations					
Fullness	29 [a]	37 [a]	37 [a]	-3	< 0.001
Prospective consumption	-29 [a]	-15 [a]	-26 [a]	-8	< 0.001
Hunger	-34 [a]	-22 [a]	-37 [a]	0	< 0.01
Satiation	37 [a]	29 [a]	25 [a]	0	< 0.001
Desire to eat	-36 [a]	-27 [a]	-38 [a]	-4	< 0.001
Bloating	8	12	20	5	0.1
Nausea	0	1	3	3	0.9

Values are mean sensation scores relative to baseline (mm) (n = 16). [a] indicates significant versus No pre-load control condition.

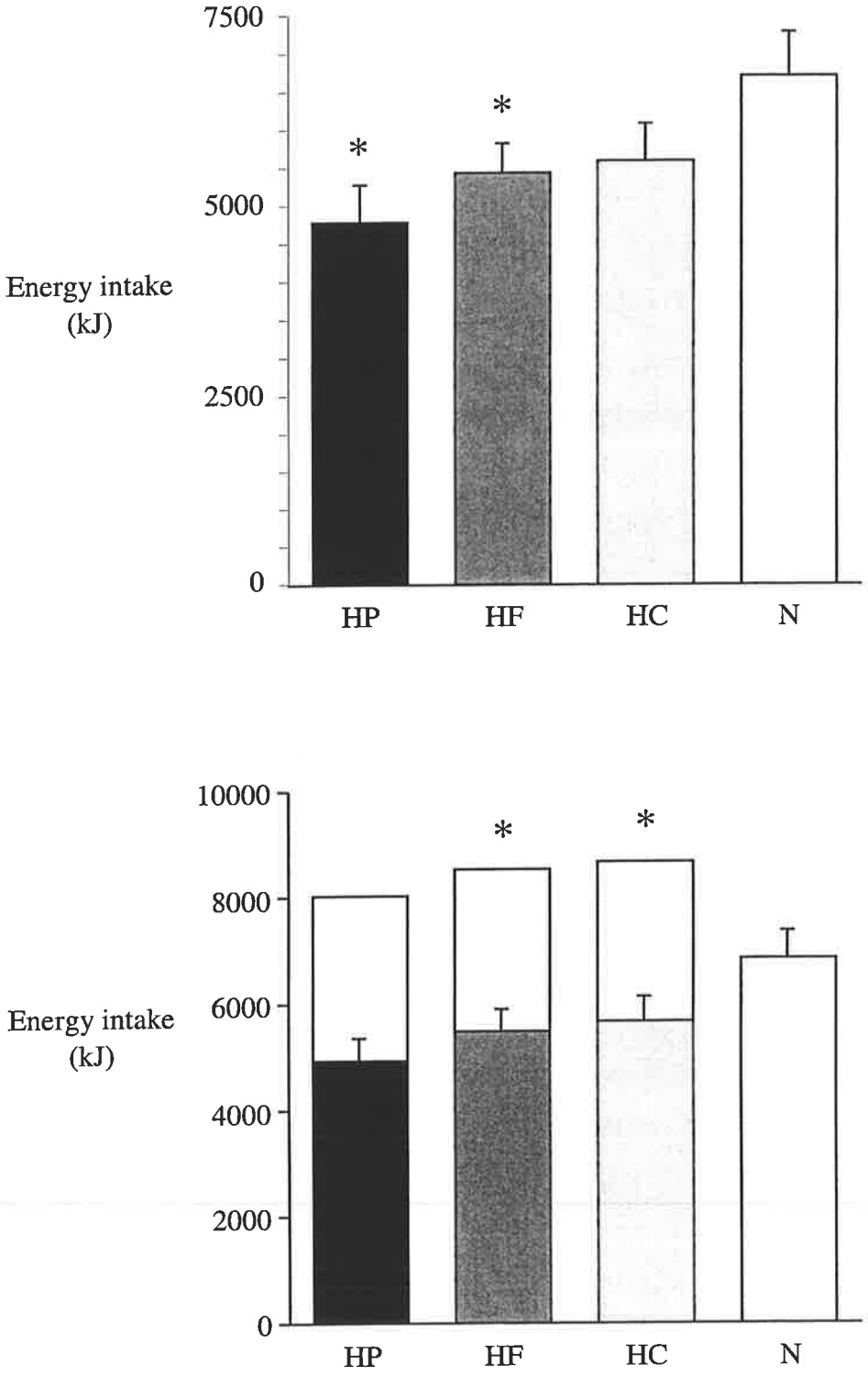


Figure 8.1 *Ad libitum* energy intake following ingestion of yoghurt pre-loads high in protein (HP), fat (HF), carbohydrate (HC) and no yoghurt (N) and total daily energy intake including yoghurt pre-loads (hatched bars indicate pre-load) (n = 16). Values are mean ± SEM. * $P < 0.05$ v N.

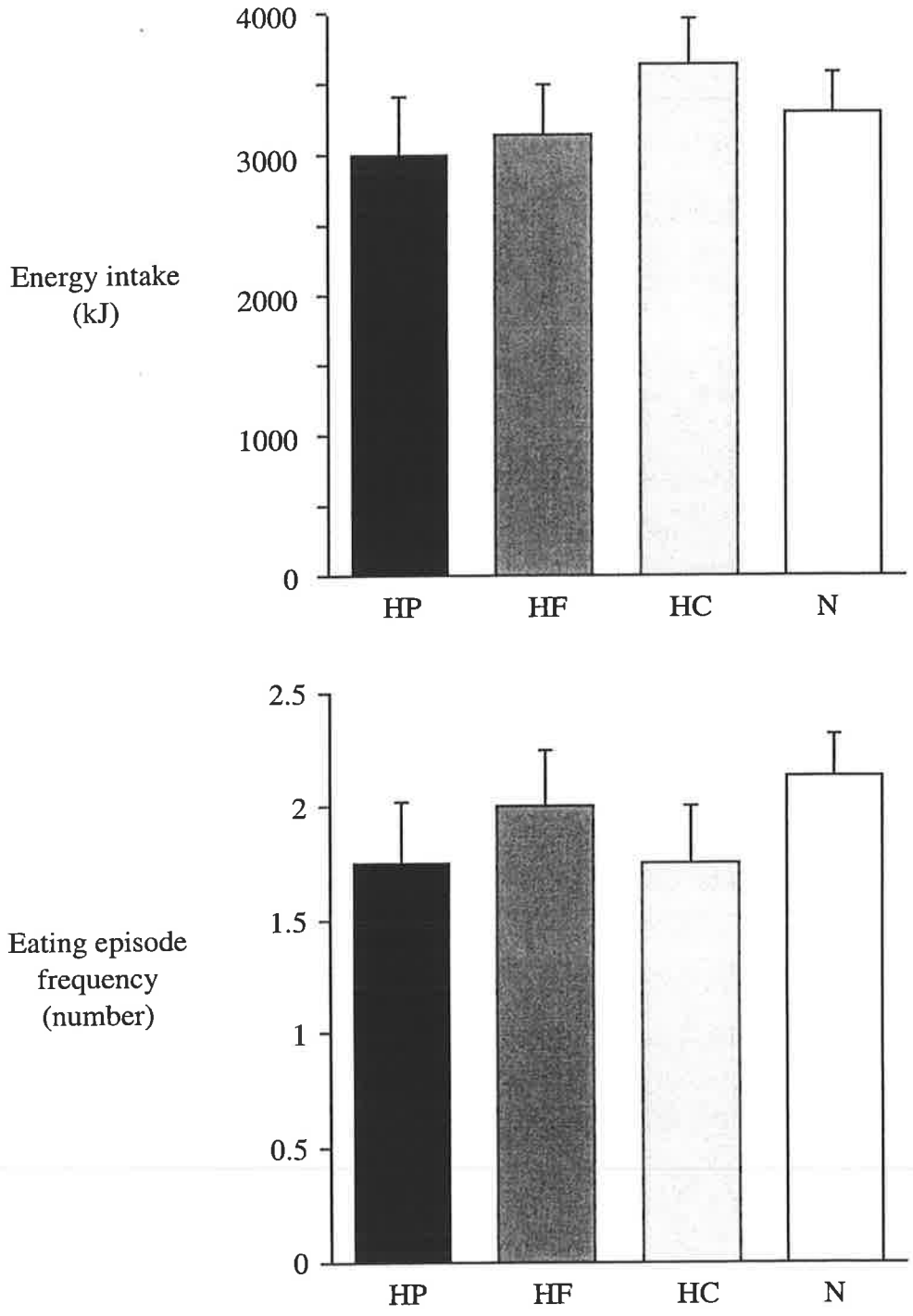


Figure 8.2 *Ad libitum* energy intake at each eating episode and total number of eating episodes following ingestion of yoghurt pre-loads high in protein (HP), fat (HF), carbohydrate (HC) and no yoghurt (N) (n = 16). Values are mean ± SEM. No significant differences between pre-loads.

Chapter 9

EFFECT OF MEAL FREQUENCY ON FOOD INTAKE

9.1 SUMMARY

To evaluate the benefits of increasing meal frequency on food intake responses, 12 healthy males were studied. On five different mornings, subjects received mixed nutrient equivolumetric, isoenergetic, intragastric or oral pre-loads, differing in frequencies of administration. Blood glucose was quantified, and hunger, fullness and nausea were assessed at regular intervals throughout the rest of the day. Energy and macronutrient intakes at an *ad libitum* lunch (t = 270 min) and dinner (t = 540 min) were quantified. Meal frequency had no significant effect on blood glucose concentrations, hunger, desire to eat, fullness and satiation or on *ad libitum* food intake. These findings do not support the promotion of increased meal frequency as a means to reduce food intake.

9.2 INTRODUCTION

Treatments for overweight and obesity are numerous, with many dietary interventions focusing on reducing the total daily energy intake of an individual (Lissner and Heitmann, 1995), or on decreasing the fat:carbohydrate ratios of food consumed by obese people (Heilbronn *et al.*, 1999). Attention has also turned to evaluating the potential benefits of specific eating patterns to prevent the onset of obesity, as well as treating individuals with the disease (Bellisle *et al.*, 1997). Numerous studies have demonstrated an inverse relationship between the number of meals consumed per day and the general state of health. Acute studies favouring more frequent meals have shown improved carbohydrate metabolism, enhanced lipid metabolism, greater insulin sensitivity, reduced adiposity and improved weight control (Edelstein *et al.*, 1992; Jenkins *et al.*, 1994). As a consequence more frequent meals have been prescribed both for the management of obesity, and diabetes and cardiovascular disease also.

Early epidemiological studies on the relationships between enhanced meal frequency and body weight have methodological limitations, most notably due to under-reporting by

obese individuals. The results of more recent work tend to refute suggestions that a greater meal frequency promotes decreased body weight (Crawley and Summerbell, 1997; Summerbell *et al.*, 1995; Summerbell *et al.*, 1996). In terms of energy expenditure, there is no evidence to suggest that either short-term (diet-induced thermogenesis) or longer-term (total energy expenditure) (Bellisle *et al.*, 1997) energy expenditure is enhanced when smaller meals are eaten more frequently. In addition, studies on the effect of meal frequency on weight loss in obese individuals suggest that meal pattern has no impact on weight loss during intentional energy restriction (Bellisle *et al.*, 1997).

Until very recently the effects of varied meal frequencies on subsequent food intake had not been evaluated. Speechley *et al.*, (1999 a,b) demonstrated an inverse relationship between the number of meals and the amount of food consumed at a subsequent meal in both lean (Speechly and Buffenstein, 1999a) and obese (Speechly *et al.*, 1999b) males, suggesting a better 'intuitive' assessment of appetite and energy intake with more frequent, smaller meals, than with one large meal. Alternatively, the administration of multiple small meals may slow the absorption of nutrients, and provide limited stimulation of gastrointestinal hormone release. If an exclusively psychological mechanism is responsible for this effect then subjects blinded to the number of meals consumed would be likely to eat equal amounts of food when offered lunch from a self-selection buffet and eat less when the same nutrients are administered orally. The aim of the present study was to examine the effect of intragastric and oral pre-loads of varying meal frequencies on *ad libitum* food intake in healthy, non-obese males.

9.3 METHODS

9.3.1 Subjects

Studies were performed in twelve healthy male volunteers who were recruited as described in Chapter 6.2.

9.3.2 Protocol

Subjects were studied on five occasions each separated by at least five days. Prior to their participation in the study, subjects completed a 3-day diet diary. Subjects were instructed not to undertake vigorous exercise or consume alcohol for at least 24 hours before each study day. Subjects arrived at the Department of Medicine at 0800, following an overnight

fast. A nasogastric catheter (10 French) was introduced into the stomach via an anaesthetised nostril and under local anaesthetic a cannula was placed in a forearm vein for blood sampling (Cook *et al.*, 1997). The intravenous line was kept patent using a continuous low-rate saline (0.9%) infusion. Subjects rested quietly for 5 min before the commencement of the study, which involved a 15 min baseline period followed 4.5 h later by a four and a half hour pre-load intervention period, a 30 min lunch test meal, followed by a 30 min dinner test meal.

9.3.3 Pre- loading schedule

The pre-loading schedule is detailed in Table 9.1. Pre-loads were either given as a bolus (i.e. in a single administration) or in multiple administrations (either intragastrically or orally). When a bolus was administered, it was all of the energy content administered in the first administration. This was either in a third of the total volume (i.e. 300 ml) or in the majority of the volume (i.e. 600 ml). When multiple administrations were infused into the stomach or ingested in one third the total volume. The control was a no energy saline pre-load. The oral pre-load, the fifth arm of the study in which only 9 of the 12 volunteers participated, was carried out after the completion of the other four treatments and therefore was not in random order. At this visit volunteers were informed that the pre-load was a flavoured drink, which may or may not contain energy (i.e. that half of the subjects would receive no nutrient and that half of the subjects would receive a nutrient-containing pre-load). The control pre-load was flavoured with low-joule orange cordial (Cottee's, Australia).

At t = 200 min (at the end of the infusion of the final pre-load), the nasogastric tube was removed. Subjects were required to remain in a semi-recumbent position until lunch time, after which they were free to lie on a bed, sit or stand, and could move about within the study room.

At 15 minute intervals until the commencement of the lunch test meal, and immediately after the lunch meal venous blood samples were drawn for the determination of blood glucose concentration.

9.3.4 Pre-load composition

The pre-load was a mixed nutrient, liquid meal 'Deliver' (MeadJohnson, Australia) (8.36 kJ/ml (8.36 kJ/ml): CHO; 40%, PROT; 15%, FAT; 45%). This commercially available liquid meal was modified by the addition of glucose (247 g glucose/L 'Deliver') in order to increase its energy density (to 12.54 kJ/ml (12.5 kJ/ml): CHO; 59%, PROT; 10%, FAT; 31%). The pre-load was designed such that ~33% average daily energy requirement was administered to each subject (~3800 kJ total), calculated on a group basis for healthy adult males. This meal was administered intragastrically or taken orally. Isotonic saline (0.9%), adjusted for viscosity with methylcellulose, was used as the control pre-load and to match volumes on the multiple pre-load treatment days. Pre-loads were administered in randomised, single-blind fashion.

9.3.5 Test meals

The presentation of the lunch test meal is described elsewhere (Chapter 6.7.1).

The dinner test meal was prepared in excess of what subjects would normally be expected to consume. At the screening visit subjects were required to select one of three possible dinner meals: lasagna, or chicken or fish that was prepared with mixed vegetables. Subjects were also offered bread rolls, margarine, cheese and crackers, jelly, ice cream, tinned fruit, iced coffee and orange juice.

The total energy content of this meal was ~8400 kJ, and once again was consumed *ad libitum*; subjects were encouraged to request additional food if they desired. The vegetarian subject was offered vegetarian cannelloni. Appetite and food intake were assessed as described in Chapter 6.7.1.

9.3.6 Statistics

All baseline values were an average of t = -15 and 0 min values and analysed using a one-way (treatment) ANOVA. Blood glucose concentration and sensations associated with appetite were analysed using a two-way (time x treatment) ANOVA with repeated measures. Area under the blood glucose concentration curve was calculated using the trapezoidal rule and analysed using a one-way (treatment) ANOVA with repeated measures. Total food intake and macronutrient composition of the food consumed was analysed using a one-way (treatment) ANOVA with repeated measures. Diet diary food intakes were analysed using a one-way (treatment) ANOVA with repeated measures.

Analyses were performed using the SuperANOVA Version 1.11 (Abacus Concepts Inc. California) software package. A P value of < 0.05 was considered statistically significant. All data are expressed as means ± S.E.M.

9.4 RESULTS

9.4.1 Blood glucose

Baseline (pre-treatment) blood glucose concentrations were similar on all treatment days ($P > 0.05$). Blood glucose concentrations fell between breakfast and lunch ($P < 0.0001$ for time) irrespective of the meal frequency. Blood glucose concentrations increased after all nutrient-containing pre-loads ($P < 0.05$ v No pre-load [N]) and on the multiple [M] and oral [O] pre-load days this increase was greater after the first than after subsequent pre-loads ($P < 0.0001$). The areas under the blood glucose concentration curves were higher after pre-loads containing nutrients (single uncontrolled [SU] v single controlled [SC] v M v O; 245 ± 9.8 v 229 ± 13.1 v 241 ± 8.4 v 239 ± 8.2 mmol/L/h) than after the non-nutrient containing pre-load (208 ± 5.3 mmol/L/h), although this was not statistically different and there were no differences between the pre-loads containing nutrients ($P > 0.05$ for treatment). The peak blood glucose concentrations following the pre-loads were SU v SC v M v O v N; 8.1 ± 0.4 v 8.0 ± 0.5 v 8.1 ± 0.3 v 6.0 ± 1.1 v 5.7 ± 0.1 mmol/L, ($P > 0.05$). There was a time x treatment ($P < 0.0001$) interaction such that blood glucose concentrations were higher after higher meal frequencies (M and O) than after low meal frequencies (SC and SU). (Figure 9.1)

9.4.2 Appetite sensations

Baseline ratings of hunger, fullness, desire to eat and satiety were similar on all treatment days ($P > 0.05$ for treatment for each sensation). Overall increases in hunger ($P < 0.0001$ for t = 15 - 270 min) and desire to eat ($P < 0.0001$ for t = 15 - 270 min), and decreases in fullness $P < 0.0001$ for t = 15-270 min) and satiation ($P < 0.0001$ for t = 15 - 270 min) occurred between breakfast and lunch. Satiation and fullness were lower immediately before the lunch meal than they were when the study commenced ($P < 0.0001$ for time). Ingestion of both the lunch and the dinner meals increased fullness ($P > 0.05$) and satiation ($P > 0.05$) and decreased hunger ($P > 0.05$) and desire to eat ($P > 0.05$). Subjects reported

greater feelings of fullness and lesser feelings of hunger following lunch consumed after any of the pre-loads, compared with lunch following no pre-load ($P < 0.0001$ for time 270 - 300 min for each sensation). Consumption of the dinner meal decreased hunger and desire to eat, and increased satiation and fullness to the same extent, irrespective of the treatment received at breakfast ($P < 0.0001$ for t = 270-300 min for each sensation).

Satiation, fullness and desire to eat all changed in response to specific treatments. Hunger was lower after O than after N ($P < 0.001$ for interaction between time and treatment [t = 105, 225 min]) and desire to eat was smaller after SU, M and O pre-loads than after N ($P < 0.001$ for treatment). Satiation was greater after SC ($P > 0.0001$ for treatment), than either N or SU, and greater after M than SU, and fullness was greater after O ($P > 0.0001$ for treatment) than after N, SU, or SC.

9.4.3 Food intake

Neither the frequency nor the nature of the pre-load administration affected the amount (energy content or weight) of food ingested at the lunch meal (Table 9.2). None of the pre-loads significantly suppressed subsequent food intake compared to no pre-load at all. There was a trend for oral ingestion of pre-loads to decrease the percentage of energy consumed as fat (by 22% compared with no pre-load) ($P = 0.09$), and increase the percentage of energy consumed as protein (by 13% compared with no pre-load) ($P = 0.08$), although these differences were not statistically significant. There was no effect of pre-load administration frequency on energy or macronutrient ratios consumed at the dinner meal (Table 9.3) or when lunch and dinner were combined (Table 9.4).

9.5 DISCUSSION

The primary aim of this study was to assess the appetite and food intake responses to oral and intragastric mixed nutrient, mixed frequency meals. Surprisingly the data show that whilst appetite sensations (hunger and desire to eat) were lower and satiety-related sensations (fullness and satiation) higher after oral, than after both nutrient and non-nutrient intragastric pre-loads, food intake was similar at all frequencies and modes of pre-load administration. In addition, multiple meals (intragastric or oral) produced multiple rises in blood glucose concentrations, whereas single meals produced rises followed by

gradual declines in blood glucose concentrations. There was however, no difference between the pre-loads on glucose AUC's across the 270 min before lunch.

The finding that pre-loads of 3762 kJ did not suppress food intake at a buffet meal 4.5 h later was unexpected. It is particularly surprising that the oral pre-loads did not suppress food intake. Cecil *et al.*, (1999) have reported that oral pre-loads are more effective at reducing energy intake from the test meal than the same pre-loads administered intragastrically (~15% greater suppression following orally than intragastrically administered pre-loads), and the suppression of appetite and stimulation of fullness after the oral pre-load was greater than after intragastric infusion in the present study. This could be due to a number of factors. The energy content and weight of the pre-loads may have been insufficient to trigger feedback to signal satiety; this appears unlikely as energy contents as low as 2090 kJ in 300 ml (Rolls *et al.*, 1998) and 1254 kJ in 250, 500 and 750 g (de Graaf and Hulshof, 1996) have previously suppressed food intake at subsequent test meals in healthy adults. A greater energy content (4180 kJ) was tested in preliminary nasogastric infusion studies (n = 2) to this study and subjects either reported severe nausea during or vomited immediately following the infusion. The energy content of the pre-loads was therefore reduced, but still within the previously reported range to suppress food intake.

It is possible that the time interval between the pre-loads and the test meal in this study were outside the window of satiety for these pre-loads. In previous studies test meals have been offered between 0.5 and 2 h of the pre-load (Cecil *et al.*, 1999; Rolls *et al.*, 1998).

It is also possible that this response was specific to these subjects (none of whom had participated in previously in studies by our group). These subjects were all young males studying at the local universities. A larger proportion (than in other studies) (33%) were foreign exchange students, and it is possible that a combination of differences in foods and culture have altered their responses to cues that would usually suppress feeding.

It seems most likely that the lack of suppression of food intake following the oral pre-loads may have been due to an order effect. The oral pre-load treatment days were the fifth time that the subjects had been exposed to these test meals and their food choice may have been a result of familiarity rather than hunger. The oral condition was added to the study only

after no difference was detected between the effects of the different intragastric pre-load conditions. Ideally, to directly study this hypothesis, the study would need to be repeated with all five treatments randomised.

More frequent meals have been suggested to improve blood glucose control (Ellis, 1934). When meals were ingested at three regular intervals in place of one in the present study, overall blood glucose concentrations were not reduced; total areas under the curve were similar for multiple and single meals, and there were no differences between the peak blood glucose responses. The peak blood glucose concentrations were no higher after a single meal (containing the entire pre-load energy content), than after one multiple pre-load (1/3 total energy and glucose content). In both situations, concentrations peaked 30 min after the first pre-load and decreased thereafter until the next meal. After single pre-loads there were no further increases in blood glucose concentrations, whereas the glucose concentrations rose after each of the multiple (intragastric and oral) pre-loads. These subsequent peaks were not as great as after the first pre-load (probably as a result of enhanced insulin release due to priming of the pancreas by previous glucose ingestion). The administration of a mixed-nutrient meal probably contributed to the smaller than expected rise in blood glucose concentration after the single pre-loads. For example, the ingestion of fat with a meal slows gastric emptying (Cunningham and Read, 1989). It is also possible that these responses were specific to this study population of healthy non-diabetic adults. The benefits on blood glucose of a feeding regimen with more frequent meals may only be observed in a population such as type 2 diabetics, in whom blood glucose concentrations are not as tightly regulated.

In the present study overall appetite was decreased in response to the nutrient-containing pre-loads. Sensations associated with food intake (desire to eat and hunger) were decreased and sensations associated with satiety (satiation and fullness) were increased when nutrients were consumed. There were however, no transient changes in these parameters, as one might expect if the gastrointestinal tract recognised each pre-load as a separate meal. Gastric emptying rates regulate the flow of nutrients into the small intestine (Horowitz and Dent, 1991), and it is possible that multiple and single pre-load ingestion produced the same overall pattern of nutrient flow from the distal stomach into the duodenum. Presumably the signals (eg. satiety hormones) arising from the small intestine

would not be different from each other, and this would explain similarities in appetite sensations between treatments.

The studies of Speechly *et al.*, (1999 a,b) showed that increasing the frequency of meals produced suppression of food intake in both lean and obese individuals. In their studies however, subjects were unblinded to their treatment, and in addition, no control (or no pre-load) treatment day was incorporated into the study. It is possible that these two factors have biased their results in the direction that lead to their suggestion that meal frequency is inversely related to energy intake. Ideally, a study would have subjects blinded to their treatment, as was the aim of this study.

Although in the present study the subjects were blinded to the infusions there was a lack of a suppression of food intake by any of the pre-loads. This prevented this study providing new information on the effects of meal frequency on food intake. Further studies would need to, using a dose-response protocol, establish a dose of pre-load sufficient to suppress food intake over a specified time interval in preliminary studies. Following this, an adequate dose could be used to test for potential differences between meal frequencies in suppression of *ad libitum* food intake.

Table 9.1 Energy and volume distribution of pre-loads during administration of single uncontrolled (i.e. volume was different at each time point) (SU), single controlled (i.e. volume was same at each time point) (SC), multiple (M), oral (O), no pre-load (N). [ ] indicates the time (min) over which nutrient containing and non-nutrient containing pre-loads were administered.

Pre-load	Intragastic				Oral
	Bolus (SC)	Bolus (SU)	Multiple	No pre-load	
t=0-20 min					
Volume (ml)	300	600	300	300	300
Energy (kJ)	3762	3762	1254	1254	1254
Infusion rate (kJ / min) [min]	188	188	188 [0-6.6] 0 [6.7- 20]	0	188 [0-6.6] 0 [6.7- 20]
Infusion rate (ml / min)	15	30	15	15	15
t=90-110 min					
Volume (ml)	300	200	300	300	300
Energy (kJ)	0	0	1254	1254	1254
Infusion rate (kJ / min) [min]	188	0	188 [90-96.6] 0 [96.7-110]	0	188 [90-96.6] 0 [96.7-110]
Infusion rate (ml / min)	15	10	15	15	15
t=180-200 min					
Volume (ml)	300	100	300	300	300
Energy (kJ)	0	0	1254	1254	1254
Infusion rate (kJ / min) [min]	188	0	188[180-86.6] 0 [186.7-200]	0	188[180-86.6] 0 [186.7-200]
Infusion rate (ml / min)	15	5	15	15	15
Total volume (ml)	900	900	900	900	900
Total energy content (kJ)	3762	3762	3762	0	3762

Table 9.2 Energy and macronutrient intake at lunch following administration of pre-loads differing in meal frequency. Single uncontrolled (SU), single controlled (SC), multiple (M), oral (O), no pre-load (N). Values are mean ± S.E.M. No significant difference between treatments.

Pre-load	Energy (g)	Energy (kJ)	Protein (%)	CHO (%)	Fat (%)
SU	1058 ± 99	5709 ± 451	17	52	30
SC	1137 ± 88	6061 ± 426	15	55	28
M	1104 ± 96	6035 ± 509	16	54	28
O	1059 ± 99	5822 ± 530	15	58	24
N	1031 ± 86	5797 ± 418	16	51	31

Table 9.3 Energy and macronutrient intake at dinner following administration of pre-loads differing in meal frequency. Single uncontrolled (SU), single controlled (SC), multiple (M), oral (O), no pre-load (N). Values are mean ± S.E.M. No significant difference between treatments.

Pre-load	Energy (g)	Energy (kJ)	Protein (%)	CHO (%)	Fat (%)
SU	992 ± 96	4752 ± 497	21	52	25
SC	1072 ± 64	5434 ± 384	21	52	25
M	1031 ± 108	6073 ± 493	20	54	23
O	968 ± 75	5024 ± 284	20	53	24
N	1089 ± 70	5538 ± 280	20	52	25

Table 9.4 Total daily energy and macronutrient intake (excluding pre-loads) following administration of pre-loads differing in meal frequency. Single uncontrolled (SU), single controlled (SC), multiple (M), oral (O), no pre-load (N). Values are mean ± S.EM. No significant difference between treatments.

Pre-load	Energy (g)	Energy (kJ)	Protein (%)	CHO (%)	Fat (%)
SU	2050 ± 181	10466 ± 865	19	52	27
SC	2209 ± 136	11499 ± 752	18	53	27
M	2135 ± 150	12113 ± 912	18	53	28
O	1530 ± 290	7900 ± 1454	17	56	24
N	2120 ± 132	11361 ± 614	19	51	28

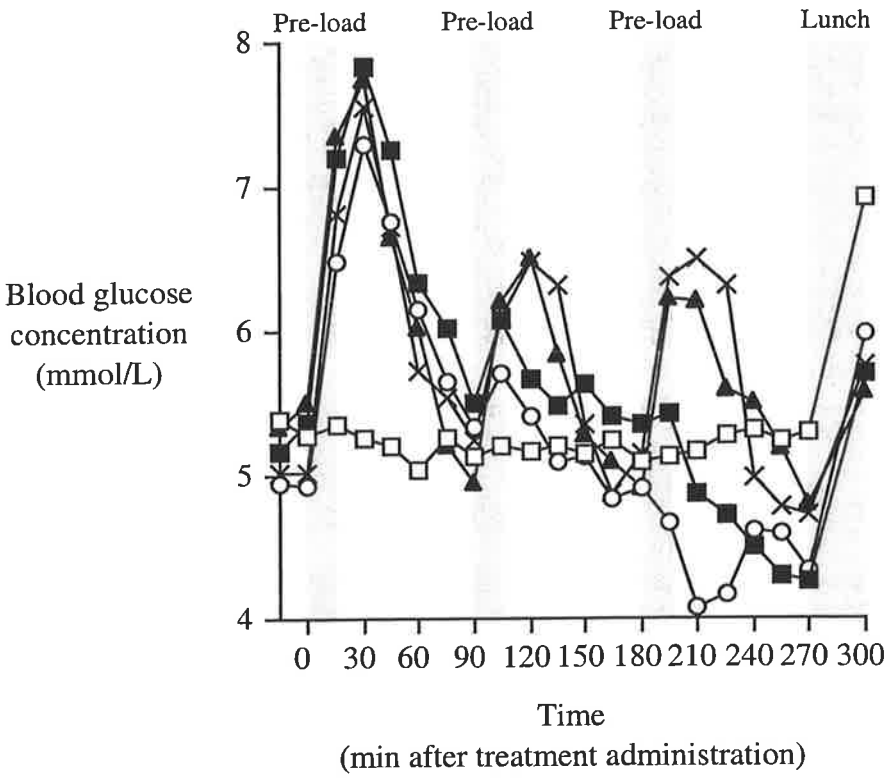


Figure 9.1 Blood glucose concentrations following mixed nutrients pre-loads differing in frequency of administration: Single uncontrolled volume (■), single controlled volume (○), multiple (▲), oral (×) and no pre-load (□). Values are means. There was no significant difference between treatments.

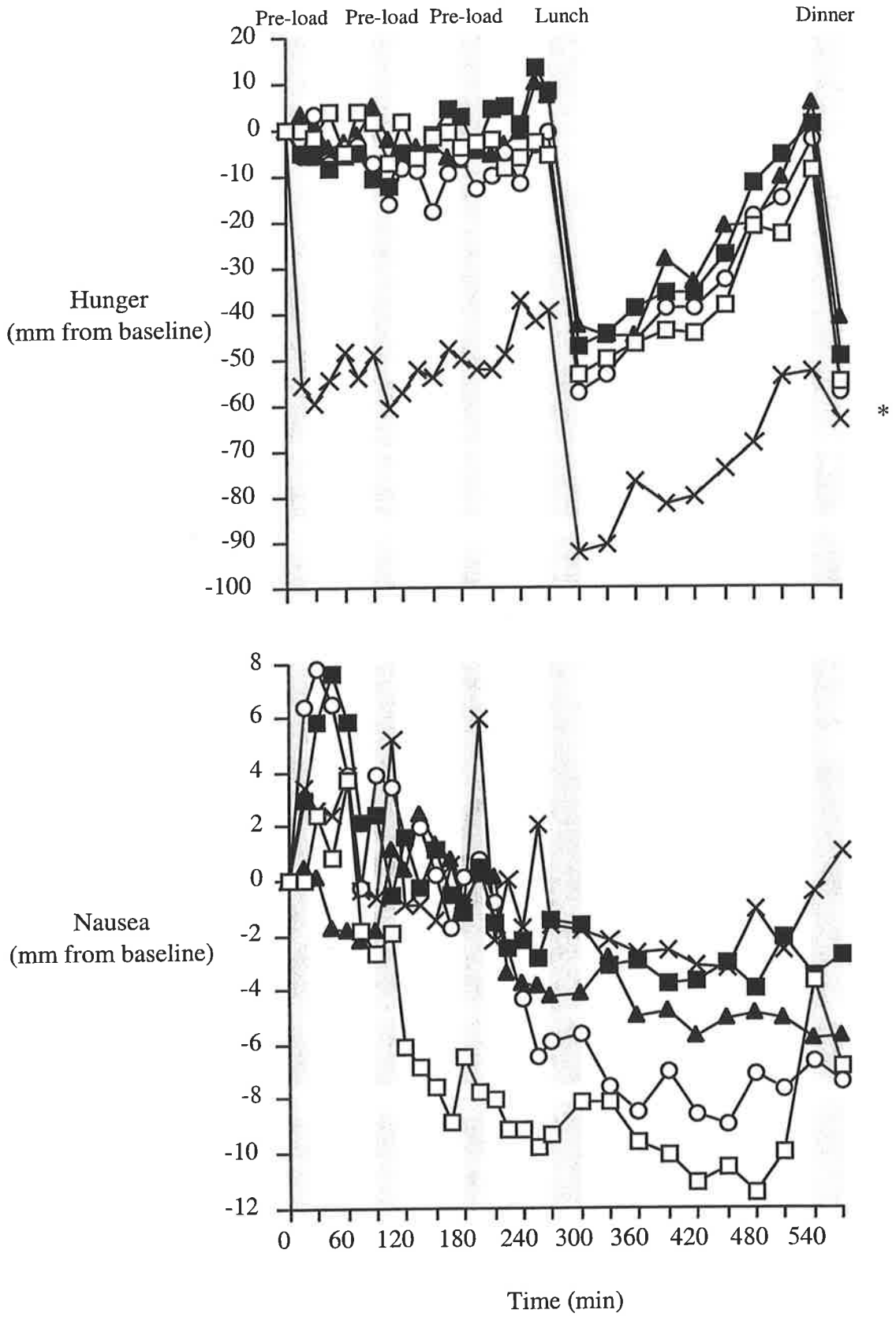


Figure 9.2 Changes in VAS scores for hunger and nausea following mixed nutrient pre-loads differing in frequency of administration: Single uncontrolled volume (■), single controlled volume (○), multiple (▲), oral (⊗) and no pre-load (□). Values are means. * $P < 0.05$ for entire curve, oral v all other treatments.

Chapter 10

ANTROPYLORODUODENAL, CHOLECYSTOKININ AND FEEDING RESPONSES TO PULSATILE AND NON-PULSATILE INTRADUODENAL LIPID INFUSION

10.1 SUMMARY

Gastric emptying occurs as pulses, rather than steady transpyloric flow, which are modulated by small intestinal feedback. The contribution of this pulsatile nature of gastric emptying to small intestinal feedback mechanisms modulating antropyloroduodenal motility and appetite is unknown. To investigate this the infusions were given on separate days, in single-blind fashion. Eight healthy male volunteers (18-34 y) received intraduodenal 10% Intralipid (8.36 kJ/min), either continuously [CONT], or in a pulsatile manner [PULS] (5 sec on / 15 sec off) or 0.9% saline [SAL] administered continuously, at a rate of 1.8 ml/min for 3 h. During each infusion subjective ratings of appetite were assessed and antropyloroduodenal pressures recorded. Plasma cholecystokinin was measured from blood collected at regular intervals throughout the infusion. At the end of each infusion the manometric assembly was removed, subjects were offered a buffet meal and the energy and macronutrient content of the food consumed measured. Both lipid infusions stimulated isolated pyloric pressure waves (IPPWs) ($P < 0.001$) and basal pyloric pressure ($P < 0.01$) and suppressed antral ($P < 0.05$) and duodenal ($P < 0.05$) pressure waves when compared to saline; there was no difference between antropyloroduodenal pressures during continuous and pulsatile lipid on antropyloroduodenal pressures. Infusions of lipid significantly increased plasma CCK concentrations ($P < 0.05$) compared with saline, but were not different between the two modes of lipid delivery ($P > 0.05$, continuous v pulsatile lipid). Both intraduodenal lipid infusions decreased hunger ($P < 0.05$), increased fullness ($P < 0.05$) and reduced energy intake ($P < 0.05$) when compared with saline. There was no difference between continuous and pulsatile lipid. We conclude that at the infusion rate of ~8.36 kJ/min the acute effects of intraduodenal lipid on

antropyloroduodenal pressures, plasma CCK concentration and appetite are not modified by a pulsatile mode of lipid delivery into the duodenum.

10.2 INTRODUCTION

The interaction between nutrients and mucosal chemoreceptors in the small intestine plays a major role in the regulation of both gastric emptying (Heddle *et al.*, 1989; Heddle *et al.*, 1988a; Heddle *et al.*, 1988b; Tougas *et al.*, 1992) and appetite (Cook *et al.*, 1997; Lavin *et al.*, 1996; Read *et al.*, 1994). Exposure of the small intestine to nutrients slows gastric emptying via a decrease in propulsive forces (Azpiroz and Malagelada, 1985; Heddle *et al.*, 1988a), together with an increased resistance to transpyloric flow (Heddle *et al.*, 1988a; King *et al.*, 1984; Malbert and Mathis, 1994). Stimulation of pyloric motility may be the most important of these mechanisms; increases in basal pyloric pressure and isolated pyloric pressure waves are associated with cessation of transpyloric flow (Tougas *et al.*, 1992).

Intraduodenal infusions of triglyceride reduce appetite and subsequent food intake (Castiglione *et al.*, 1998; Chapman *et al.*, 1999; Cook *et al.*, 1997; Lavin *et al.*, 1996) and stimulate cholecystokinin (CCK) secretion (Lilja *et al.*, 1984). Cholecystokinin is believed to play a major role in the regulation of gastroduodenal motor activity. Intravenous CCK administration stimulates pyloric motility in humans (Fraser *et al.*, 1993) and the CCK-A receptor antagonist, loxiglumide, restores tonic and phasic gastric activity during lipid infusions (Feinle *et al.*, 1996), thus directly implicating CCK as a mediator of lipid-induced gastric contractile activity. In addition, considerable evidence supports the role of CCK as a satiety hormone in humans (Geary *et al.*, 1992; Lieverse *et al.*, 1995).

A limitation of previously reported studies, which have evaluated the effects of small intestinal lipid infusion on gastric motility and appetite, is the mode of lipid delivery into the intestine. Lipid has been continuously infused at a constant rate into the duodenum whereas in both animals (Anvari *et al.*, 1995; Carlson *et al.*, 1966; Malbert and Mathis, 1994; Malbert and Ruckebusch, 1991) and humans (Hausken *et al.*, 1998; King *et al.*, 1984) transpyloric flow is predominantly pulsatile rather than continuous.

The slowing of gastric emptying and suppression of appetite by intraduodenal lipid infusion of the small intestinal receptors is rapid, and commences in the pre-absorptive phase (Greenberg, 1998). Mucosal chemoreceptors have an adaptation half time of 3 to 10 sec, so that after 30 sec of continuous nutrient exposure the firing rate of these receptors is dramatically reduced (Grundy and Scratched, 1989). A pulsatile delivery flow of nutrients into the duodenum, with each flow pulse lasting for about 5 sec, as has been described in humans (Malbert and Mathis, 1994; Malbert and Ruckebusch, 1991), may result in more sustained chemoreceptor activity than continuous infusion. This may produce more pronounced satiety signals and greater suppression of subsequent food intake than continuous lipid infusion, but to date has not been studied. It is also unknown whether the mode of lipid delivery into the duodenum influences the release of CCK. Therefore, the aims of the current study were to evaluate the effects of pulsatile intraduodenal lipid infusion on antropyloroduodenal pressures, CCK release and appetite in healthy subjects when compared to continuous infusion.

10.3 METHODS

10.3.1 Subjects

Studies were performed in eight healthy male volunteers (18-32 y) and they were recruited according to Chapter 6.2.

10.3.2 Protocol

Each subject was studied on three occasions separated by at least seven days. Subjects were instructed not to undertake vigorous exercise or consume alcohol for at least 24 hours before each study day. At 0900, after an overnight fast (except for water), a 4 mm silicone rubber manometric assembly (Dent-Sleeve, Australia) was inserted into the stomach via an anaesthetised nostril (Lignocaine 5%, PeadPharm, Australia). The tip of the tube was allowed to pass spontaneously into the duodenum (time taken 30 - 180 min) as assessed by TMPD (Chapter 6.7.5). Under local anaesthetic an intravenous cannula was inserted into a forearm vein, for venous blood sampling at t = -15, 0, 10, 15 min and every 15 min thereafter until lunch (i.e. t = 180 min) and again after lunch at t = 210 and 240 min. During each study subjects were allowed to read (but not about food- or eating-related subjects) or listen to the radio.

At time t = 0 min, during antral phase I, (within 10 min of the end of phase III of the migrating motor complex (Chapter 2.3.2)), an intraduodenal (ID) infusion was commenced via an infusion port, located approximately 15 cm distal to the pylorus, and continued for 180 minutes. Infusions were either continuous 10% Intralipid (Kabi Pharmacia) at a rate of 1.8 ml/min [CONT], pulsatile 10% Intralipid at a rate of 1.8 ml/min, 5 sec on (i.e. 7.2 ml/min for 5 sec) /15 sec off [PULS], or continuous 0.9% saline [SAL] at a rate of 1.8 ml/min. Treatments were administered in a randomised, single blind fashion. A Gemini PC-2 volumetric infusion pump (IMED, CA, USA), controlled via purpose-designed software (courtesy of Professor Charles Malbert) was used to deliver all ID infusions. The program was compiled in Labview 3.0.1 (National Instruments) running on a Macintosh 6200-75. Treatments were administered in randomised, single blind fashion. The rate of energy delivery of lipid was 8.36 kJ/min. Subjective ratings of appetite were obtained at t = -15, 0, 10, 20 and 30 min and every 15 min thereafter, for 4 h (Sepple and Read, 1989). Antropyloric pressures were recorded continuously during the ID infusions.

Each infusion was continued for 180 minutes, at which time the infusion was ceased and the manometric assembly removed. The subjects were then offered a cold buffet meal, containing in excess of what they would normally be expected to eat, and invited to eat as much as they wanted in the following 30 minutes and / or until they were comfortably full. The rate of ingestion and the total amount and macronutrient composition of food consumed were quantified (Lavin *et al.*, 1996; Lavin *et al.*, 1998). After consumption of the meal subjects remained in the laboratory for a further 30 minutes. Subjects remained supine for the entire study, except during the meal, when they were seated.

10.3.3 Measurement of antropyloroduodenal pressures

Antropylorodudoenal pressures were assessed as described in Chapter 6.7.5.

10.3.4 Quantification of plasma cholecystokinin

Plasma CCK was assessed as described in Chapter 6.7.4.5.

10.3.5 Assessment of appetite and food intake

Assessment of appetite and food intake are described elsewhere (Chapter 6.7.1).

10.3.6 Statistical Analyses

All data are expressed as mean ± SEM. Pre-treatment ratings of appetite and antropyloroduodenal pressures were assessed using a repeated-measures one-way analysis of variance (ANOVA). Appetite ratings, plasma CCK concentrations and antropyloroduodenal pressures and antral and duodenal pressure wave amplitudes throughout the study were analysed using two-way ANOVA (with time and treatment being the two factors) with repeated measures, using the Sigma Stat software package (Jandel Scientific, Australia). Post-hoc analyses were performed using the Student-Newman-Keuls procedure. A one-way ANOVA (with treatment as a factor) with repeated measures was used to compare the means for caloric intake and macronutrient composition. A P value of < 0.05 was considered significant.

10.4 RESULTS

The studies were well tolerated by the subjects, with no adverse effects reported.

10.4.1 Antropyloroduodenal pressures

Antropyloroduodenal pressures were evaluable in 7 of the 8 volunteers (in one volunteer the manometric catheter was positioned incorrectly for the majority of the study). In these 7 subjects TMPD measurements indicated that the sleeve sensor was positioned correctly across the pylorus for 5114 min of a total recording time of 5355 min (95%).

10.4.1.1 Isolated pyloric pressure waves and basal pyloric pressure (Figures 10.1 and 10.2)

There were no differences in the pre-treatment frequency of isolated pyloric pressure waves (IPPWs) between SAL, CONT and PULS ($P > 0.05$). CONT and PULS infusions both increased the number of IPPWs by approximately eight-fold ($P < 0.05$) whereas SAL had no effect on IPPW frequency ($P > 0.05$). There was no difference in IPPW frequency between CONT and PULS ($P > 0.05$); IPPW frequency was greater during both CONT and PULS when compared with SAL ($P < 0.001$).

There was no difference in pre-treatment basal pyloric pressure between the three treatments ($P > 0.05$). Saline had no effect on basal pyloric pressure. Both CONT and PULS increased basal pyloric pressure when compared with SAL ($P < 0.01$). This increase

persisted in all studies ($P > 0.05$). There was no difference in basal pyloric pressure between CONT and PULS ($P > 0.05$)

10.4.1.2 Antral pressure waves (Figures 10.1 and 10.2)

Pre-treatment antral pressure wave frequency was not significantly different between the three treatment groups ($P > 0.05$). Antral pressure waves were detected throughout SAL infusions ($P < 0.05$) and their frequency increased from 1.1 ± 1 per 30 min interval to a maximum of 15 ± 8.1 per 30 min interval. In contrast, antral pressure waves remained suppressed and virtually abolished throughout intraduodenal lipid infusions ($P < 0.05$) and there was no difference between CONT and PULS with respect to the number of antral pressure waves ($P > 0.05$).

10.4.1.3 Duodenal pressure waves (Figures 10.1 and 10.2)

Pre-treatment duodenal pressure wave frequency was similar in all treatment groups ($P > 0.05$). Duodenal pressure waves were suppressed by both CONT and PULS when compared with SAL ($P < 0.05$ for treatment from t = 0-120 min). Duodenal pressure waves persisted throughout SAL ($P > 0.05$). There was no difference between CONT and PULS in the number of duodenal pressure waves ($P > 0.05$).

10.4.2 Plasma cholecystokinin

Pre-treatment plasma CCK concentrations were not significantly different between treatment days ($P > 0.05$). Plasma CCK concentrations were greater after intraduodenal infusions of lipid than after infusions of saline ($P < 0.0001$). Plasma CCK increased within 10 minutes of commencing the infusion and there was no significant difference in the magnitude between the continuous and pulsatile lipid-induced rise in plasma CCK concentrations ($P > 0.05$). When calculated as a mean of the individual peak responses there were no differences between the peak plasma CCK concentrations in response to continuous and intermittent lipid (CONT 17.6 ± 4.1 v PULS: 12.1 ± 3.2 pmol/L; $P > 0.05$, values are means of individual peak responses) or the time at which they occurred (CONT 59.4 ± 15.1 v PULS: 60.0 ± 20.8 min; $P > 0.05$, values are means of individual time to peak responses) (Figure 10.3)

10.4.3 Appetite and food intake (Figure 4)

Pre-treatment ratings of hunger, fullness and nausea did not differ significantly between the three treatment days. Scores for hunger were less ($P < 0.05$) and fullness greater ($P < 0.05$) during CONT and PULS infusion compared to SAL; without any difference between CONT and PULS. Consumption of the meal increased fullness ($P < 0.0001$) and decreased hunger ($P < 0.0001$) irrespective of intraduodenal infusion. Overall nausea ratings were not affected by type or mode of intraduodenal infusion ($P > 0.05$). (Figure 10.4)
No subject consumed all the food offered, nor ate for the entire 30 minutes. Eating time was similar in all three treatment groups (SAL v CONT v PULS, 18.6 ± 2.4 v 18.3 ± 3.8 v 16.8 ± 2.5 min; $P > 0.05$) (Table 10.1). Energy and food intake was suppressed by both CONT ($P < 0.05$) and PULS ($P < 0.05$) (12% and 15 % respectively compared with SAL) (Table 10.1). There were no significant differences in proportions of fat, carbohydrate or protein intakes after SAL, CONT and PULS infusions ($P < 0.01$) (Table 10.1). There were no differences between CONT and PULS in total energy intake, individual macronutrient consumption or total eating time ($P > 0.05$) (Table 10.1).

Post-prandial sensations of hunger were less after both CONT and PULS compared to SAL ($P < 0.01$), but there was no significant difference between CONT and PULS ($P > 0.05$). There was no significant difference between SAL, CONT and PULS with respect to post-prandial fullness ($P > 0.05$) or nausea ($P > 0.05$).

10.5 DISCUSSION

This study has evaluated for the first time the influence of pulsatile, compared with continuous intraduodenal lipid infusion on small intestinal feedback responses affecting antropyloroduodenal activity, cholecystokinin (CCK) release and appetite. Continuous lipid infusions have been used in many previous studies of feeding behaviour and gastrointestinal motility. Pulsatile and continuous intraduodenal lipid infusions did not differ in their acute effects on i) the antropyloroduodenal pressures, associated with the cessation of transpyloric flow (Tougas *et al.*, 1992) and the suppression of gastric emptying (Heddle *et al.*, 1988a), ii) plasma CCK concentration and iii) subjective ratings of appetite and food intake. These data suggest that mechanical and chemical responses to intraduodenal lipid are independent of the temporal mode of intraduodenal lipid delivery.

The present data support previous work on the antropyloroduodenal motor, CCK and appetite responses to intraduodenal lipid (Chapman *et al.*, 1999; Cook *et al.*, 1997; Fraser *et al.*, 1992). The absence of any detectable differences between the effects on feeding, motility and hormone release between pulsatile and continuous lipid infusions suggest that the absence of pulsatile delivery of intraduodenal lipid did not affect the outcomes of previous studies examining these parameters (Cook *et al.*, 1997; Heddle *et al.*, 1989; Heddle *et al.*, 1988a; Holloway *et al.*, 1997; MacIntosh *et al.*, 1999).

The rate of lipid delivery to the small intestine in this study (8.36 kJ / min) was to provide a comparison with previous studies conducted by our group and others, utilising this lipid infusion technique (Cook *et al.*, 1997; Edelbroek *et al.*, 1992). Thus in the current study, these are believed to approximate the 'normal' rate and characteristics of gastric emptying in humans (Brener *et al.*, 1983; Hunt, 1983). The precise rate of gastric emptying of lipids is unclear, although rates of fat emptying into the duodenum as low as 4.18 kJ / min and high as 13.5 kJ / min have been reported (Meyer *et al.*, 1996). Whether these responses would be observed at lower or higher rates of lipid infusion is unknown.

The suppression of gastric emptying by small intestinal lipid (and other nutrients) is influenced by the length of the intestine (and therefore number of chemoreceptors) exposed to the nutrient (Lin *et al.*, 1989; Lin *et al.*, 1990). Although continuous and intermittent infusion might be expected to produce different degrees of spread of lipid along the small intestine, thus generating different feedback effects on antropyloroduodenal pressures and the rate of gastric emptying, our observations suggest this is not the case. It has been proposed that a constant infusion results in a steady concentration of nutrient at the level of the duodenal chemoreceptors (Muller and Blum, 1984). However, villus contractions and / or duodenal secretions may influence the concentration of lipid in the immediate vicinity of the chemoreceptors. The current data suggest that even under conditions of continuous intraduodenal lipid infusions, intermittent chemical stimulation is maintained, and chemoreceptors continue to fire or alternately, that intermittent infusion in this study results in continuous occupation of receptors. The finding that plasma CCK concentrations are independent of the temporal mode of lipid delivery may also suggest that timing of lipid delivery to the duodenum has little effect on concentrations at the mucosal level. A possible confounder is the potential for CCK to activate neural pathways via local paracrine pathways, not reflected by changes in blood concentrations. Co-administration of

a CCK-A antagonist with the continuous or pulsatile lipid may enable the paracrine effects of endogenous CCK to be examined.

These data are consistent with previous reports that infusions of lipid into the duodenum, jejunum or the ileum reduce voluntary food intake at a subsequent meal (Chapman *et al.*, 1999; Cook *et al.*, 1997; Lavin *et al.*, 1996; Miller *et al.*, 1981; Welch *et al.*, 1985). Although no studies have evaluated the effects of duodenal distension on appetite or food intake in mammalian species, there is evidence of synergy between mechanically- and chemically-induced feedback mechanisms on sensations associated with appetite; the effects of duodenal distension on perceptions of appetite sensations are enhanced by hyperglycemia (Lingenfelser *et al.*, 1999). Furthermore, sensations of hunger are reduced after duodenal and jejunal but not ileal lipid infusions (Welch *et al.*, 1985), suggesting either that the proximal small intestine exerts a greater control over sensations associated with appetite (but not overall satiety) than the distal small intestine, that the length of the small intestine exposed to the lipid is proportional to its satiating efficiency, or both. Results from a recent study provide evidence for greater suppression of food intake following exposure of a greater length of the small intestine to lipid. Infusion of disaccharides or oleate into increasing lengths of the small intestine of rats produced dose-responsive inhibition of food intake at all sites tested (Meyer *et al.*, 1998), establishing that suppression of food intake, like gastric emptying, is dependent on the length of the small intestine exposed to nutrients. These observations suggest that after pulsatile and continuous infusions, the lengths of small intestine exposed to lipid are comparable.

The characteristics of the lipid pulses represent an approximation of normal human gastric emptying, based on studies showing pulses occurring approximately 3.8 times per min, in discrete ~ 5 sec periods (Hausken *et al.*, 1998). Although the delivery of nutrient was into the second part of the duodenum, the chemoreceptor populations in the proximal duodenum (in particular in the first 5 cm) are unlikely to contain fat-responsive receptors; as intraduodenal acids, but not sodium oleate, or hypertonic glucose slow gastric emptying in dogs (Cooke and Clark, 1976). Therefore infusions of lipid more proximal than 12-15 cm distal to the pylorus (Cook *et al.*, 1997; Fraser *et al.*, 1992) are unlikely to have produced different antropyloroduodenal motor responses. However, the distribution of mechanoreceptors may be region specific (Azpiroz and Malagelada, 1990). It is thus unlikely that the region of the duodenum into which the Intralipid was infused played a

large part in the observed lack of a difference between the two modes of infusion; proximal and distal duodenal distensions do not appear to have different effects on the number or amplitude of antral waves, or on the basal pyloric pressure (Edelbroek *et al.*, 1994). Duodenal balloon distension stimulates IPPWs and basal pyloric pressure and inhibits antral pressure waves (Edelbroek *et al.*, 1994; Lingenfelser *et al.*, 1999) and there is evidence for synergy in the effects of small intestinal nutrient and mechanical stimuli (Edelbroek *et al.*, 1994). There are, however, no data on the effects of pulsatile delivery of non-nutrient liquids stimulating mechanisms that slow gastric emptying. Saline does not stimulate IPPWs or CCK release and it is for this reason that a pulsatile saline arm was not included. Furthermore, whereas chemoreceptors respond exclusively to chemical stimuli, so-called 'polymodal' receptors, which respond to both distension and contraction of the duodenum, are also modified by chemical stimuli (Mei and Lucchini, 1992). This also suggests that chemical (i.e. nutrient) and hormonal stimuli play the dominant role in providing feedback from the small intestine.

In summary, the antropyloroduodenal motor, CCK and appetite responses to the infusion of ~ 8.36 kJ / min intraduodenal lipid infusion are apparently independent of the temporal mode of delivery into the duodenum. It is unknown whether this also applies for infusions of other nutrients or mixed nutrients. We speculate that the previously observed pulsatility of gastric emptying is probably a consequence of gastric pumping mechanisms, rather than a requirement for normal, nutrient-induced small intestinal signalling.

Table 10.1 Weight, energy content, macronutrient content (percentage of the total energy intake) and total eating time for a buffet meal consumed by healthy subjects after intraduodenal infusion of continuous lipid [CONT], pulsatile lipid [PULS] (both 8.36 kJ/min) and saline [SAL] (n = 7).

	SAL	CONT	PULS
Total weight (g)	1120 ± 124	957 ± 122*	947 ± 126*
Energy intake (kJ)	5337 ± 622	4397 ± 639*	4773 ± 865*
CHO (% of total)	57.4	58.2	55.3
PROT (% of total)	24.0	23.8	23.7
FAT (% of total)	18.6	18.1	21.9
Eating time (min)	18.6 ± 2.4	18.3 ± 3.8	16.8 ± 2.5

Values are means ± SEM. * $P < 0.05$ SAL v. CONT and SAL v PULS, by one-way repeated measures ANOVA.

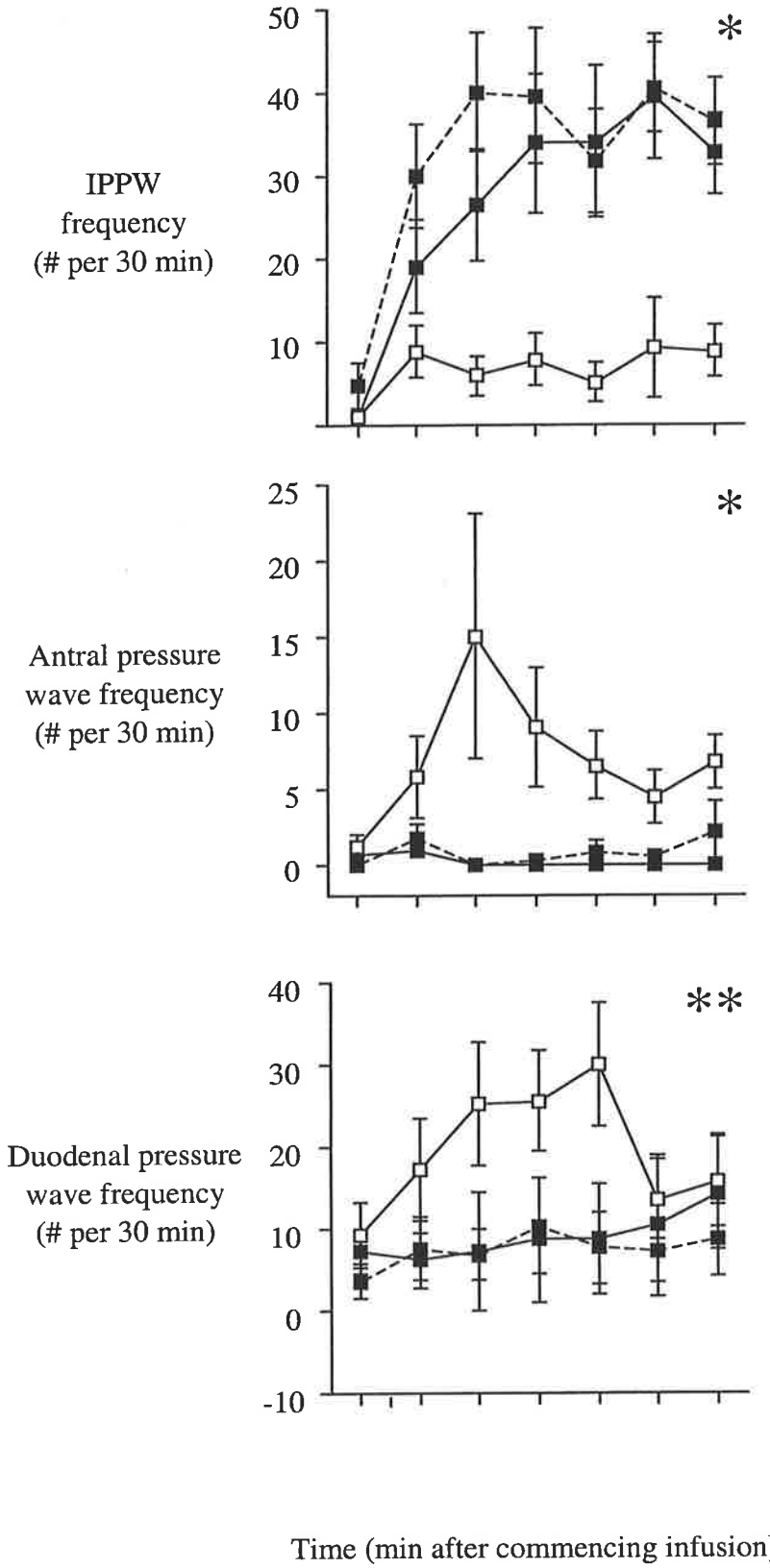


Figure 10.1 Isolated pyloric (IPPW), antral and duodenal pressure wave frequency during intraduodenal continuous lipid (—■—), pulsatile lipid (- ■ -) and continuous saline (—□—) (n = 8). Values are mean ± SEM. * $P < 0.05$ both lipid infusions v saline (t = 0 - 180 min), ** $P < 0.01$, both lipid infusions v saline (t = 0 - 180 min).

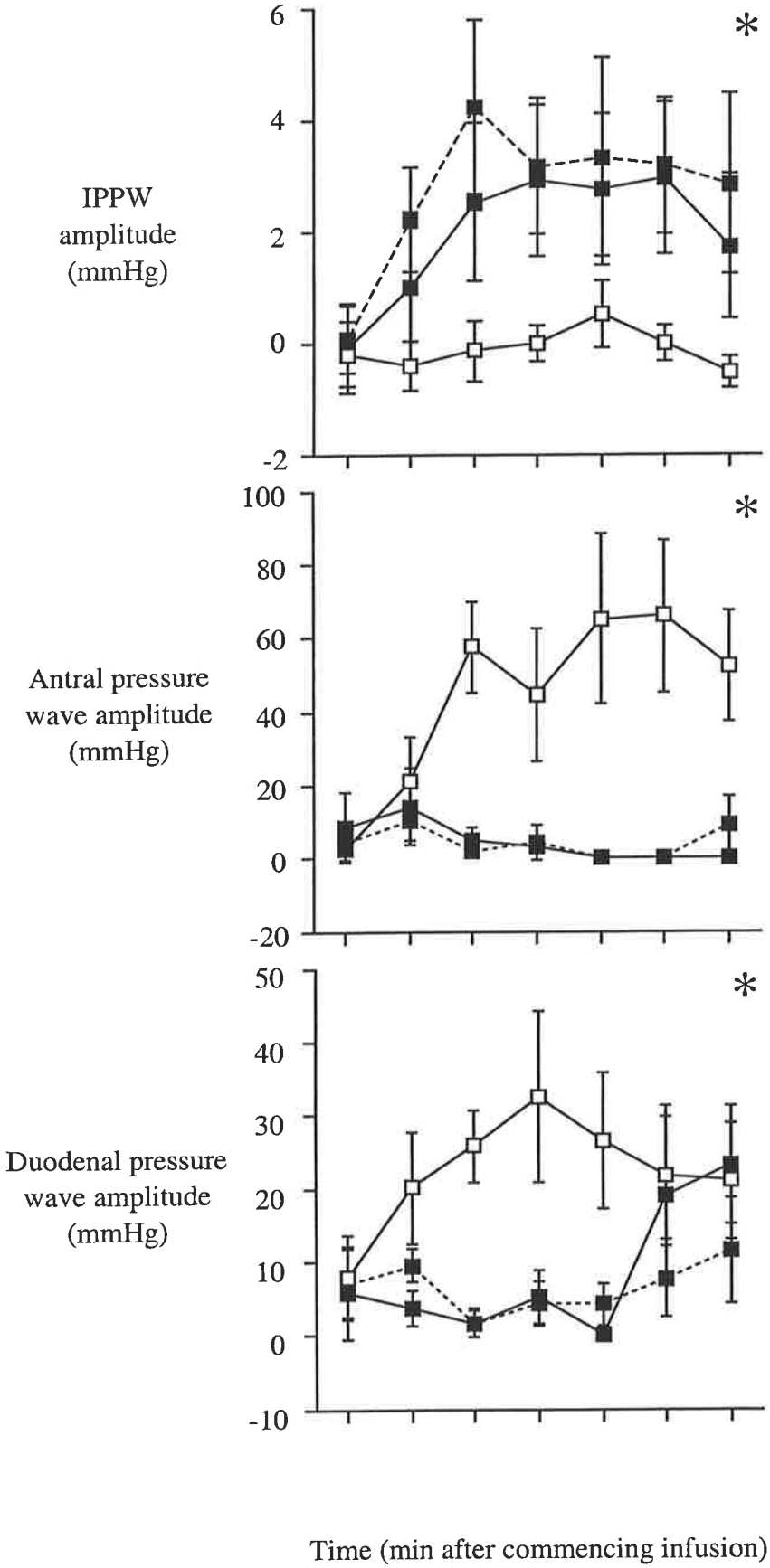


Figure 10.2 Isolated pyloric (IPPW), antral and duodenal pressure wave amplitudes during intraduodenal continuous lipid (—■—), pulsatile lipid (- ■ -) and continuous saline (—□—) (n = 8). Values are mean ± SEM. * $P < 0.05$ both lipid infusions v saline (t = 0 - 180 min).

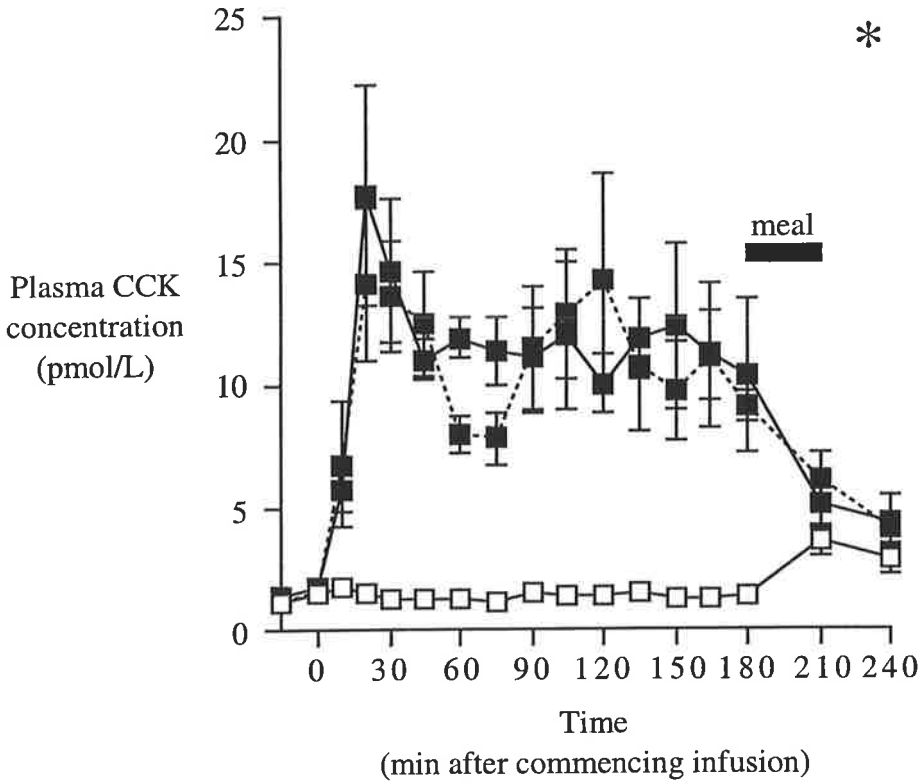


Figure 10.3 Plasma cholecystokinin (CCK) concentrations during intraduodenal continuous lipid (—■—), pulsatile lipid-(■ -) and continuous saline (—□—) (n = 8). Values are mean ± SEM. * $P < 0.05$ both lipid infusions v saline (t = 0 - 180 min).

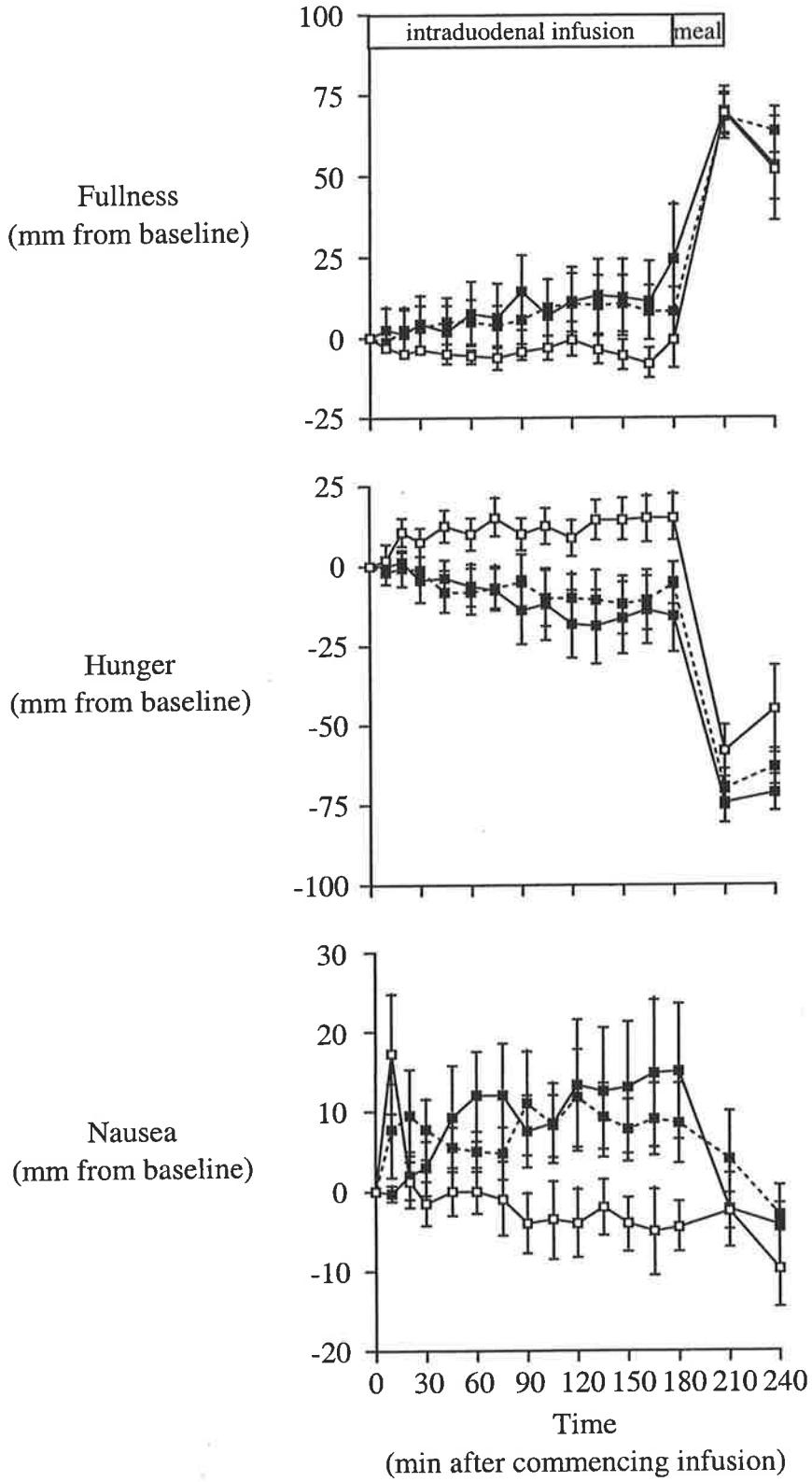


Figure 10.4 Fullness, hunger and nausea during intraduodenal continuous lipid (—■—), pulsatile lipid (- ■ -) and continuous saline (—□—) (n = 8). Values are mean + SEM. * $P < 0.05$ both lipid infusions v saline (t = 0 - 180 min).

Chapter 11

GLYCEMIC, HORMONE AND APPETITE RESPONSES TO MONOSACCHARIDE INGESTION IN PATIENTS WITH TYPE 2 DIABETES

11.1 SUMMARY

The aim of treatment of patients with type 2 diabetes, who are often also overweight, is to reduce fasting and post-prandial blood glucose concentration, and body weight. To investigate the relative effects of fructose and glucose on blood glucose, plasma insulin and incretin (glucagon-like peptide-1 and gastric inhibitory peptide) concentrations, and acute food intake, 11 (7M: 4F) patients with diet-controlled type 2 diabetes [NIDDM] (44-69 y; BMI 27-38 kg/m^2) and 10 age-, and BMI-matched (6M: 4F) non-diabetic, control subjects with varying degrees of glucose tolerance [NON], were studied on 3 days. In random order, subjects drank isoenergetic pre-loads of glucose (75 g) [GLUC], fructose (75 g) [FRUCT] or vehicle (300 ml water with non-caloric flavoring) [VEH] 3 h before an *ad libitum* buffet lunch. Mean glucose concentrations were lower after FRUCT than GLUC in both NIDDM (FRUCT v GLUC: 6.2 ± 0.5 v 8.2 ± 0.9 mmol/L, $P < 0.001$) and NON (FRUCT v GLUC: 6.0 ± 0.2 v 7.2 ± 0.3 mmol/L, $P<0.05$). Mean insulin concentrations were ~50% higher after FRUCT in diabetics than in non-diabetics (NIDDM v NON: 22.5 ± 4.9 v 15.1 ± 1.3 μU/ml; $P < 0.0001$). Fructose-induced GLP-1 concentrations were not different between diabetics and non-diabetics ($P > 0.05$). Glucose but not FRUC, increased GIP concentrations that were not different between diabetics and non-diabetics ($P > 0.05$). Food intake was suppressed 14% by GLUC ($P < 0.05$ v CONT) and 9% by FRUC ($P < 0.05$ v CONT), with no difference between the amount of food consumed after GLUC and FRUC treatment in either diabetics or non-diabetics ($P > 0.05$). The data indicate that fructose produces a lower post-prandial blood glucose response than isoenergetic glucose and demonstrated that i) fructose produces greater insulin release in diabetics than non-diabetics, which is not due to greater incretin release and ii) fructose and glucose have equivalent short-term satiating efficiency in both diabetics and non-diabetics. On the basis

of improved glycemic control, but not satiating efficiency, fructose may be useful as a replacement for glucose in the diet of obese patients with diabetes.

11.2 INTRODUCTION

Glucose ingestion promotes insulin secretion by a direct action on the pancreatic β-cells and by stimulating the release of incretin hormones such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP). Incretin release accounts for over 50% of the rise in insulin after ingestion of a glucose load in healthy individuals (Nauck *et al.*, 1986). Fructose ingestion also induces insulin secretion, but to a lesser extent glucose. While no study has directly compared the effect of fructose ingestion on plasma insulin in adults, with and without non-insulin dependent diabetes mellitus (NIDDM), the results of separate studies (Kong *et al.*, 1999; Nuttall *et al.*, 1992) suggest that oral fructose is a more potent insulin secretagogue in diabetics. This may be due to greater fructose-induced release of incretins in patients with diabetes. In non-diabetics, fructose stimulates GLP-1 secretion in non-diabetics, but to a smaller degree glucose (Kong *et al.*, 1999), and has no effect on GIP concentrations (Rayner *et al.*, 2000). In NIDDM, GIP is secreted in response to glucose, but has almost no insulinotropic activity, whereas the extent of GLP-1 release in people with NIDDM after glucose ingestion is unclear; studies have reported both enhanced and decreased secretion compared to non-diabetics (Nauck *et al.*, 1993; Orskov *et al.*, 1991). The relative effects of fructose on insulin, GLP-1 amide and GIP release in patients with and without NIDDM have not been evaluated.

The comparative effects of glucose and fructose on appetite remain controversial. In healthy people, results of several (Rodin, 1990; Rodin, 1991; Spitzer and Rodin, 1987), but not all (Guss *et al.*, 1994; Kong *et al.*, 1999) studies indicate a greater suppression of short-term food intake by oral fructose than isoenergetic glucose. The relative satiating effects of these monosaccharides have not been examined in people with diabetes. Intravenous GLP-1 reduces food intake in healthy humans without diabetes (Gutzwiller *et al.*, 1999b). It is not yet clear if this is a physiological or pharmacological effect, but GLP-1 may be an endogenous satiety factor (Gutzwiller *et al.*, 1999a), in which case its secretion may account for some of the reduction in food intake following glucose and fructose ingestion.

This study was conducted to determine the relative acute effects of oral glucose and fructose on appetite and food intake, and plasma insulin and incretin concentrations in people with and without NIDDM.

11.3 METHODS

11.3.1 Subjects

Eleven patients with early (< 4 years since diagnosis), well controlled, type 2 diabetes mellitus [NIDDM] and 11 non-diabetic subjects with varying degrees of impaired glucose tolerance [NON] were recruited from the Royal Adelaide Hospital diabetes clinic and by advertisement. One of the NON subjects was subsequently excluded as he was diabetic in response to 75g oral glucose tolerance test (Subject characteristics are detailed in Table 11.1). The diabetic patients were all treated with diet alone i.e. none was taking oral hypoglycaemic agents or insulin. Non-diabetic subjects had a screening visit fasting venous whole blood glucose concentration of < 6.1 mmol/L, and HbA_{1c} < 6% (Alberti and Zimmet, 1998). All subjects were non-smokers, and unrestrained eaters (see Chapter 6.2). Subjects with significant gastrointestinal symptoms, disease or surgery, intake of >20 g of alcohol/day on a daily basis and current use of medications that might affect glycaemic control, gastrointestinal motor function or appetite were excluded. The Royal Adelaide Hospital Human Ethics Committee approved the study protocol, and written, informed consent was obtained from each subject prior to enrolment.

11.3.2 Protocol

Each subject was studied on three occasions, separated by at least five days. Subjects refrained from vigorous exercise and alcohol intake for 24 h before each study. Subjects attended the study centre at 0830 following an overnight fast, except for water. On arrival, a blood sampling cannula was inserted into a forearm vein. Subjects remained either seated or lying on a bed during all 3 studies and could, apart from during the meal, read (but not about food-related topics) or listen to the radio.

Fifteen minutes after intravenous cannulation (t = 0 min), subjects received, in randomised order and single-blind fashion, a non-caloric lemon flavoring (Green's Foods, Glendenning, NSW, Australia) (60 ml) in water (240 ml) (i) alone (vehicle) [VEH], (ii) plus anhydrous glucose (75 g) [GLUC] or (iii) plus fructose (75 g) [FRUCT], which was

consumed in two minutes. Three hours later subjects were offered a buffet meal and asked to eat until comfortably full or 30 min had elapsed. Subjects were monitored for 0.5 h post-prandially. Venous blood samples (10 ml) were taken at t = -15, 0, 5, 10, 15, 30, 45, 60, 75, 90, 120, 150 and 180 min for measurement of glucose, glucagon-like peptide-1 (GLP-1), gastric inhibitory peptide (GIP) and insulin and also at t = 210 and 240 min for measurement of glucose and insulin. Visual analogue scale questionnaires (VAS) (Sepple and Read, 1989) were administered at 15 minute intervals, starting at t = -15 min except during the meal.

11.3.3 Biochemical measurements

Blood samples were collected on ice into EDTA tubes containing a protease inhibitor (Trasylol; Bayer, Leverkeusen, FRG). Plasma was obtained by centrifugation at 4°C at 3200 rpm for 12 min, and stored at -20°C until assayed. Blood glucose, plasma insulin, GLP-1 and GIP were quantified as indicated in Section 6.7.4

11.3.4 Assessment of appetite

Appetite was assessed using linear visual analogue scales (VAS) (Section 6.7.1.3). Food intake was quantified as described in Section 6.7.1.4.

11.3.5 Statistical analysis

Baseline data were analysed using two-way analysis of variance (ANOVA) with repeated measures, with treatment and patient group as factors. Hunger, fullness and nausea, blood glucose and plasma insulin, GLP-1 and GIP concentrations were initially analysed using a three-way ANOVA with repeated measures with patient group and treatment and time as factors. When a significant interaction was observed, contrasts were used to test hypotheses of interest, enabling paired comparisons between the studies. Fructose-induced insulin GLP-1 and GIP release were assessed using two-way ANOVA with repeated measures with patient group and time as factors. Relationships between baseline and fructose-induced rises in blood glucose and plasma insulin were assessed using Pearson's correlations. Food intake was compared using a two-way ANOVA with repeated measures with treatment and patient group as factors. Subject characteristics were compared with Student's unpaired t-tests. SuperANOVA Version 1.11 (Abacus Concepts Inc. Berkley, California) software was used to perform these analyses. A P value of <0.05 was considered statistically significant. All data are expressed as means ± S.E.M.

11.4 RESULTS

One subject experienced a severe headache prior to commencing the buffet meal on the fructose treatment day; all food intake data for this subject were omitted from the food intake analysis. All other subjects tolerated studies well; with no untoward side effects reported.

11.4.1 Biochemical measurements

11.4.1.1 Blood glucose

All diabetics met World Health Organisation and American Diabetic Association criteria for the diagnosis of diabetes, with a screening fasting blood glucose concentration ≥ 6.1 mmol/L (Alberti and Zimmet, 1998). (Diagnostic values for venous whole blood (≥ 6.1 mmol/L) are lower than those for venous plasma (≥ 7.0 mmol/L) and the same as capillary whole blood (Alberti and Zimmet, 1998). On the basis of their responses to the 75 g oral glucose load, and fasting whole blood glucose concentrations 7 of the NON subjects had impaired glucose tolerance, 2 had impaired fasting and 1 normal glucose tolerance.

Fasting (pre-treatment) blood glucose concentrations were greater in diabetics than in NON (6.9 ± 0.2 v 5.6 ± 0.1mmol/L, $P < 0.05$) with no difference between the 3 treatment days for either subject group ($P > 0.05$).

Glucose concentrations were higher in diabetics, than in NON, following both GLUC (mean: 10.5 ± 0.1 v 7.3 ± 0.3 mmol/L; $P < 0.001$) and FRUCT (mean: 7.3 ± 0.02 v 5.9 ± 0.2 mmol/L; $P < 0.001$). The rise in blood glucose concentration after GLUC, but not FRUCT, was greater than after VEH in all subjects ($P < 0.001$). At the time of the meal ingestion, blood glucose concentrations were higher in diabetics than in NON (6.8 ± 0.6 v 5.1 ± 0.2 mmol/L, $P < 0.01$), and these were higher after GLUC than FRUCT (NIDDM 8.2 ± 0.9 v 6.2 ± 0.5 mmol/L; NON 7.3 ± 0.3 v 6.0 ± 0.2 $P < 0.05$ for both). Blood glucose concentrations after lunch were lower after GLUC ($P < 0.001$) and FRUCT ($P < 0.001$) than VEH, with no difference between the monosaccharides (NIDDM; G v F: 7.8 ± 0.8 v 7.6 ± 0.5 mmol/L, $P > 0.05$, NON; G v F: 5.8 ± 0.3 v 6.7 ± 0.3 mmol/L, $P > 0.05$) in all subjects. (Figure 11.1)

11.4.1.2 Plasma insulin

Baseline plasma insulin concentrations were similar in diabetics and NON (11.8 ± 1.2 v 8.6 ± 0.6 μU/ml; P = 0.2) with no differences between the treatments in either subject group. Mean insulin concentrations were higher after GLUC, than VEH, in diabetics (36.7 ± 2.6 v 22.5 ± 1.9 μU/ml; P < 0.001) and NON (47.2 ± 3.6 v 15 ± 0.7 μU/ml; P < 0.001), and higher after GLUC than FRUCT in diabetics (36.7 ± 2.6 v 22.5 ± 1.9 μU/ml; P < 0.01) and NON (G v F: 45.2 ± 1.2 v 14.9 ± 0.2 μU/ml; P < 0.0001). Insulin concentrations were higher after FRUCT than VEH in all subjects; this was significant in diabetics (P < 0.01). There was an interaction between time and patient group effect such that an hour after pre-load ingestion, the insulin response to FRUCT was greater in diabetics than NON (P < 0.0001). The mean insulin concentration between FRUCT ingestion and lunch (5 - 180 min) was ~ 50% higher in the subjects with diabetes than in NON (22.5 ± 4.9 v 15.1 ± 1.3 μU/ml). Fructose-induced increases in plasma insulin concentrations from baseline were also greater in diabetics than in NON (P < 0.05 time x patient group). Peak plasma insulin concentration responses were delayed in diabetics compared with in NON, regardless of the monosaccharide ingested (72.4 ± 7.4 v 51.5 ± 8.0 min; P < 0.01). Fructose-induced insulin release remained higher in diabetics than in NON until lunch (16.0 ± 3.2 v 8.0 ± 0.8 μU/ml, P < 0.05). Post-lunch insulin concentrations on the fructose day did not differ between diabetics and NON (51.6 ± 10.2 v 53.8 ± 9.2 μU/ml, P > 0.05) and there was no difference between peak post-lunch insulin concentrations after the three pre-loads in diabetics (P > 0.05).

Fructose-induced increases in plasma insulin concentrations were not correlated with baseline blood glucose concentrations in the whole subject group (r = -0.07, P > 0.05), or in either NON (r = -0.48, P > 0.05) or diabetics (r = -0.24, P > 0.05) when they were assessed separately. Rises in insulin after fructose ingestion were not correlated with rises in blood glucose after fructose ingestion in diabetics (r = -0.37, P > 0.05) or when all subjects were assessed together (r = -0.1, P > 0.05), but there was a positive correlation in NON (r = 0.6), which was almost significant (P = 0.07). (Figure 11.2)

11.4.1.3 Plasma GLP-1

Baseline GLP-1 concentrations were slightly, but not significantly higher, in diabetics (11.1 ± 1.8 pmol/L) than NON (7.9 ± 1.2 pmol/L), (P = 0.12), with no difference between 3 treatment days in either subject group (P > 0.05). Overall GLP-1 concentrations rose after

both GLUC and FRUCT compared with VEH ($P < 0.001$), with no difference following GLUC and FRUCT ($P > 0.05$). There was a non-significant increase in plasma GLP-1 concentrations following FRUCT relative to VEH in diabetics and in NON ($P > 0.05$). Fructose-induced GLP-1 concentrations were no different between diabetics and NON (14.7 ± 0.6 v 10.2 ± 0.6 pmol/L; $P > 0.05$). Glucagon-like peptide-1 concentrations were marginally greater after GLUC than FRUCT in NON (12.2 ± 0.5 v 10.2 ± 0.5 pmol/L; $P > 0.05$), but not in diabetics (13.9 ± 0.6 v 14.1 ± 0.6 pmol/L; $P > 0.05$). At the time of the ingestion of the meal, plasma GLP-1 concentrations were similar in diabetics and NON (11.1 ± 1.6 v 9.7 ± 1.4 pmol/L, $P > 0.05$) and not different after different treatments in either subject group ($P > 0.05$). (Figure 11.3)

11.4.1.4 Plasma GIP

Baseline GIP concentrations were similar in diabetics (43.6 ± 13.3 pmol/L) and NON (32.6 ± 6.8 pmol/L) ($P = 0.4$), with no difference between the treatments in either subject group ($P > 0.05$). In all subjects, GIP concentrations increased following GLUC ($P < 0.0001$), compared with VEH ($P < 0.05$) and FRUCT ($P < 0.05$) ingestion. Plasma GIP responses to FRUCT were similar in diabetics and NON ($P > 0.05$). (Figure 11.4)

11.4.2 Appetite and food intake

11.4.2.1 Sensations of appetite

Baseline hunger, fullness and nausea ($P > 0.05$) did not differ between the three treatment days for either diabetics or NON.

Fullness ($P < 0.0001$) and nausea ($P < 0.001$) decreased and hunger increased ($P < 0.0001$) between pre-load and lunch ingestion. Both monosaccharides increased fullness (G v V, $P < 0.01$; F v V, $P < 0.05$) and decreased hunger (G v V and F v V, $P < 0.05$) and neither had an effect on nausea ($P > 0.05$). Nausea was higher after FRUCT, than GLUC and VEH, more so in NON than non-diabetic subjects, although this was not statistically significant ($P = 0.07$). There were no differences in hunger or fullness perceptions between GLUC and FRUCT in either subject group ($P > 0.05$ for NIDDM and NON). Lunch intake increased fullness ($P < 0.0001$) and decreased hunger ($P < 0.0001$) similarly in all subjects regardless of the pre-load type (no effect of treatment). (Figure 11.5)

11.4.2.2 Food intake

No subject consumed all the food offered, but one subject ate for the full 30 minutes on all of the three treatment days. Energy intake was suppressed approximately 11% (~ 493 kJ) compared to VEH, by 75 g monosaccharide ingestion ($P < 0.05$), with no difference between the suppressive effect of GLUC and FRUCT (G v F v V: 3891 ± 405 v 3933 ± 330 v 4485 ± 418 kJ, $P > 0.05$). This suppression represents ~ 40% of the energy content of the pre-loads. Diabetics (V: 3991 ± 589 v G: 3670 ± 568 v F: 3544 ± 568 kJ) ate less than NON (V: 4978 ± 581 v G: 443 ± 426 v F: 4317 ± 338 kJ) on every study day and about 19% less overall; this difference was not significant ($P > 0.05$). Total daily energy intake including the pre-loads was similar in diabetics (G: 4924 ± 572 v F: 4798 ± 572 kJ) and NON (G: 5367 ± 614 v F: 5571 ± 3427 kJ) ($P > 0.05$). There was no patient group x treatment interaction ($P > 0.05$) and no differences in the macronutrient composition of the foods eaten on different days ($P > 0.05$) or eaten by diabetics and NON ($P > 0.05$). (Figure 11.6)

11.5 DISCUSSION

This study has confirmed that fructose ingestion produces smaller increases in blood glucose concentrations than glucose ingestion in humans both with, and without diabetes. In addition, the data provide evidence that fructose ingestion produces a greater insulinogenic response in diabetics than in non-diabetics, and that this is not due to greater stimulation of glucagon-like peptide 1 (GLP-1) or gastric inhibitory peptide (GIP). Consistent with previous finding that oral fructose and glucose were equally satiating in lean, young adults without diabetes (Kong *et al.*, 1999), the current study has found them to be equally satiating in older, overweight people with and without type 2 diabetes.

The finding of lower blood glucose concentrations after fructose than glucose ingestion is consistent with the results of previous studies in people with diabetes (Gannon *et al.*, 1998; Nuttall *et al.*, 1992) and obese (Reiser *et al.*, 1987) and non-obese (Kong *et al.*, 1999) people without diabetes, showing smaller increases in blood glucose concentration after ingestion of the fructose than glucose or the glucose-containing disaccharides sucrose and lactose (Thorburn *et al.*, 1986). The small rise in circulating glucose concentrations after fructose ingestion is probably the result of hepatic conversion of fructose to glucose (Chandramouli *et al.*, 1993; Uusitupa, 1994). Lowering blood glucose concentrations in people with diabetes reduces the microvascular and possibly macrovascular complications

of this disease (Hanefeld and Temelkova, 1997). Therefore substitution of fructose for glucose in the diet of diabetics may represent a way of lowering average blood glucose concentrations and possibly reducing hyperglycemia-induced diabetic complications. Several studies that have examined the chronic effect of including fructose in the diet of diabetics, either as a supplement (Anderson *et al.*, 1989; Osei and Bossetti, 1989; Osei *et al.*, 1987; Thorburn *et al.*, 1989), or instead of other sugars (Thorburn *et al.*, 1990), demonstrate either no change or improved glycemic control after 3-6 months. There are, however, suggestions that chronic fructose ingestion may increase circulating triglyceride and / or cholesterol concentrations (Crapo *et al.*, 1986). While addition of fructose to the diabetic diet for its glucose lowering effects is a practical treatment option, it cannot be assumed that this will reduce metabolic complications. Studies to determine if this is so have not been performed.

This study demonstrates for the first time in this study that plasma insulin concentrations are higher after fructose ingestion in diabetic subjects than in age- and weight-matched non-diabetic subjects. Fructose is therefore apparently a more potent insulin secretagogue in type 2 diabetics than in non-diabetics. It is not surprising to find a significant degree of impaired glucose tolerance in an older and overweight 'healthy' population. This highlights the contribution of increasing body weight to the development of insulin resistance, impaired glucose tolerance and eventually diabetes mellitus. As a consequence of the variable glucose tolerance in the NON group, the variances between insulin responses to monosaccharide ingestion in diabetics and non-diabetics were large, and often overlapped. It is likely that in a lean non-diabetic population free of impaired glucose tolerance, the differences between diabetics and non-diabetics would have been even more marked.

The hypothesis was that a greater insulin response to fructose in diabetics than non-diabetics would be a result of higher fructose-induced GLP-1 concentrations. This was not the case. Although absolute GLP-1-amide concentrations were higher after fructose in diabetics than in non-diabetics, this was likely due to higher basal GLP-1 concentrations in diabetics. As previously reported (Toft *et al.*, 2001) the increase in GLP-1 after fructose was similar in the two subject groups (from ~ 8 to 12 pmol/L in the non-diabetics and from ~11 to 16 pmol/L in diabetics). As the assay measures both active and inactive GLP-1, the exact proportions of each are unknown and cannot exclude the possibility that the ratio of

active:inactive GLP-1 is different in diabetics to non-diabetics. Similarly, it is difficult to be sure that the degradation of GLP-1 following monosaccharide ingestion will be similar for each sugar. There was a greater variability in these levels in the diabetics than non-diabetics, and the sample size was relatively small, so a type 2 statistical error cannot be excluded. Nonetheless the lack of an enhanced GLP-1 response to fructose in people with NIDDM is consistent with a recent report by Toft-Neilson *et al.*, (2001) that the GLP-1 response to a mixed meal has less in NIDDM than NON. While increased GLP-1 release seems unlikely to be a cause of the enhanced insulin release in diabetics, increased sensitivity to the insulinotropic actions of GLP-1 does remain a possibility. Baseline GIP concentrations were higher (although not significantly) in diabetics, possibly as a consequence of the glucose-dependent nature of GIP release (Morgan, 1996). There was no increase in plasma GLP-1 concentration after fructose ingestion in either NIDDM or NON as previously reported by Ebert and Creutzfeldt, (1987).

Fructose-induced insulin release is known to be glucose-dependent, and may be enhanced in diabetics by the hyperglycemia characterizing this condition. *In vitro* pancreas and isolated islet preparation studies show that fructose is incapable of stimulating insulin in the complete absence of glucose (Ashcroft *et al.*, 1972; Coore and Randle, 1964; Hager *et al.*, 1972) and insulin release is greater after intravenous fructose during hyper- than during euglycemia in non-diabetics (Dunnigan and Ford, 1975). Fructose has a weak insulinogenic action during euglycaemia in people without diabetes, but elevation of the blood glucose concentration even slightly (eg. from 5.5 to 6.4 mmol/L), substantially increases the stimulatory effect of fructose on insulin release (Reiser *et al.*, 1987). Baseline blood glucose levels were similarly elevated in the diabetics compared to the non-diabetics in this study (6.9 v 5.8 mmol/L). In addition to enhancing the stimulatory effects of fructose on the β-cell, hyperglycaemia may indirectly enhance the insulinotropic effects of fructose by increasing the insulinotropic actions of GLP-1. Glucagon-like peptide-1 acts on the pancreatic β-cell to stimulate insulin secretion and this insulinotropic effects is enhanced by hyperglycaemia (Goke *et al.*, 1993). Although hyperglycaemia may provide an explanation for the fructose-induced insulin hypersecretion in diabetics, in the present study, the lack of a significant correlation in diabetic subjects between the increase in plasma insulin concentration following fructose ingestion and either fasting blood glucose concentration or the rise in glucose concentrations after ingestion of the pre-load does not support this. Nevertheless, the substantial variances in insulin concentrations in the

diabetic subjects and the relatively small subject numbers mean that this possibility can not be excluded and further studies will be needed to directly explore this possibility.

The relative effects of monosaccharides on food intake are controversial. Three previous studies from the same center (Rodin, 1991; Rodin *et al.*, 1988; Spitzer and Rodin, 1987) have demonstrated oral fructose to be more satiating than isoenergetic glucose in subjects without diabetes, whereas three other studies including this one have shown no difference (Guss *et al.*, 1994; Kong *et al.*, 1999). The discrepancy may be related to differences in study design. Studies in which fructose has been more satiating than glucose have tended to use higher volume pre-loads (500ml v 300ml in our study (Spitzer and Rodin, 1987)) and have shorter time-periods between pre-load and test meal ingestion. In this study higher concentrations of sugar were used in the pre-load (than did Rodin *et al.*, (1987, 1988, 1991) (75 g in 300 ml v 50 g in 500 ml)). The higher concentration may result in small intestinal receptors signaling satiety in response to different monosaccharides become saturated, producing similar satiating effects of the two sugars.

Fructose may have suppressed appetite and food intake in part by making the subjects nauseous. This seems unlikely. The fructose solution in this study was sweeter than the glucose solution, and nausea ratings were somewhat higher in the first sixty minutes after fructose ingestion than glucose ingestion in both diabetics and non-diabetics. Nevertheless, this difference was not significant, and any nauseating effect of fructose, as indicated by these ratings, had resolved by the time of meal ingestion.

This was an acute study that involved the ingestion of each monosaccharide in isolation. It did not investigate the possibility that fructose and glucose have different effects on satiety or blood glucose when ingested chronically from naturally occurring sources (eg. fructose in fruit). Nevertheless, the findings do not support a use for fructose in the diet of people with type 2 diabetes as a means of suppressing food intake and reducing body weight. Furthermore, diabetic patients were studied soon after diagnosis (all < 4 years) on diet treatment alone. It is unclear whether fructose will have the same effect in patients who have had diabetes for longer and / or those who are taking hypoglycaemic medication. Further studies will be needed to examine the reduced glycemic response to dietary fructose, particularly its possible ability to suppress post-prandial hyperglycaemia, and effects on long-term diabetic complications.

In summary, in patients with type 2 diabetes and in non-diabetic controls, fructose ingestion causes a smaller increase in blood glucose concentration than isoenergetic oral glucose ingestion. In addition, oral fructose is associated with a greater rise in plasma insulin concentration in diabetics than in non-diabetics. However, there is no difference in satiation between fructose and glucose.

Table 11.1 Subject characteristics: gender, age, body mass index (BMI), body weight, body fat content, fasting blood glucose concentration, and glycated hemoglobin (HbA_{1c}) comparisons between non-diabetic (control) and type 2 diabetic (type 2) subjects.

	Control	Type 2	*P* value
Gender	7M, 4F	7M, 4F	-
Age (y)	54.7 (44 - 69)	55.7 (44 - 71)	0.77
Weight (kg)	84.8 (71 - 120.9)	87.9 (62 - 112.3)	0.64
BMI (kg/m^2)	30.9 (27 - 37.7)	30.0 (25.3 - 36.2)	0.67
Duration of known diabetes (mth)	-	18 (0.5 – 43)	-
Body fat (%)	37.7 (27.4 - 45.8)	33.9 (20 - 48.7)	0.39
Fasting blood glucose (mmol/L)[1](mean of three study days)	5.8 (5.2 - 6.0)	6.9 (5.5 - 8.8)	0.001 *
HbA_{1c} (%)[2]	5.2 (4.9 - 5.4)	5.8 (4.8 - 7.1)	0.07

Body fat was quantified using bioelectrical impedance (Lukaski *et al.*, 1985).

Values are mean (range) (except for Gender). Comparisons were performed using a Student's un-paired *t*-test. * indicates $P = 0.001$.

[1]Mean of the 3 study days

[2] HbA_{1c} (%) measurements were performed using the HPLC with cation exchange column method of Philcox *et al.*, (1992). Reference range for non-diabetics 4–6 %.

Diagnostic values for diabetes mellitus for venous whole blood are ≥ 6.1 mmol/L, for venous plasma and for capillary whole blood they are ≥ 7.0 mmol/L (Alberti and Zimmet, 1998). Eg. A patient with venous whole blood glucose of 6.5 mmol/L would correspond to venous plasma and or capillary whole blood glucose of 7.5 mmol/L. the approximate conversion of venous whole blood glucose concentration to venous plasma and or capillary whole blood glucose concentration is x1.154.

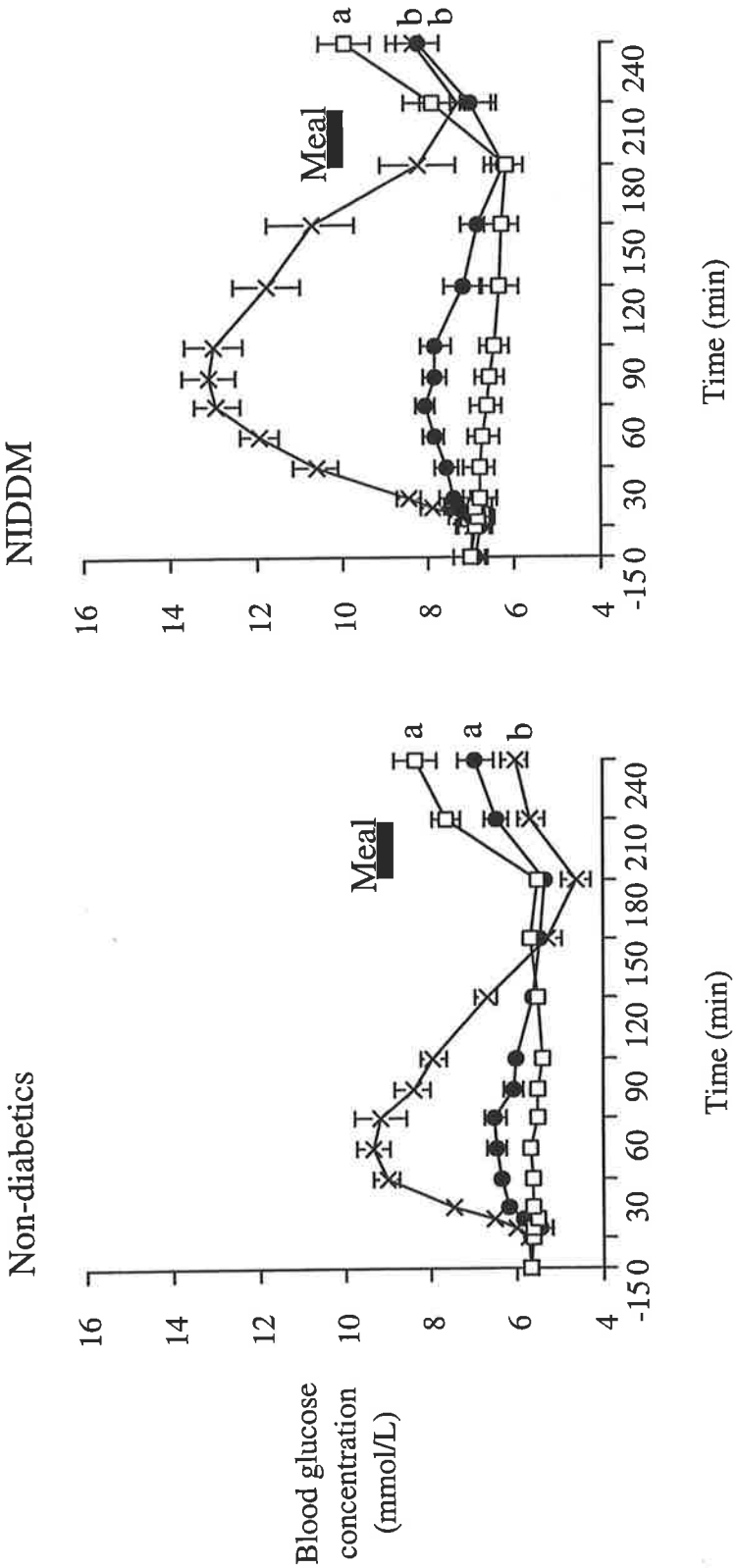


Figure 11.1 Blood glucose concentrations in non-diabetics (n = 11) and type 2 diabetics (n = 11) following glucose (75 g) (×), fructose (75 g) (●) or vehicle (□). Values are mean ± S.E.M. Different superscript letters indicate curves are significantly different ($P < 0.05$) from t = 0 - 180 min.

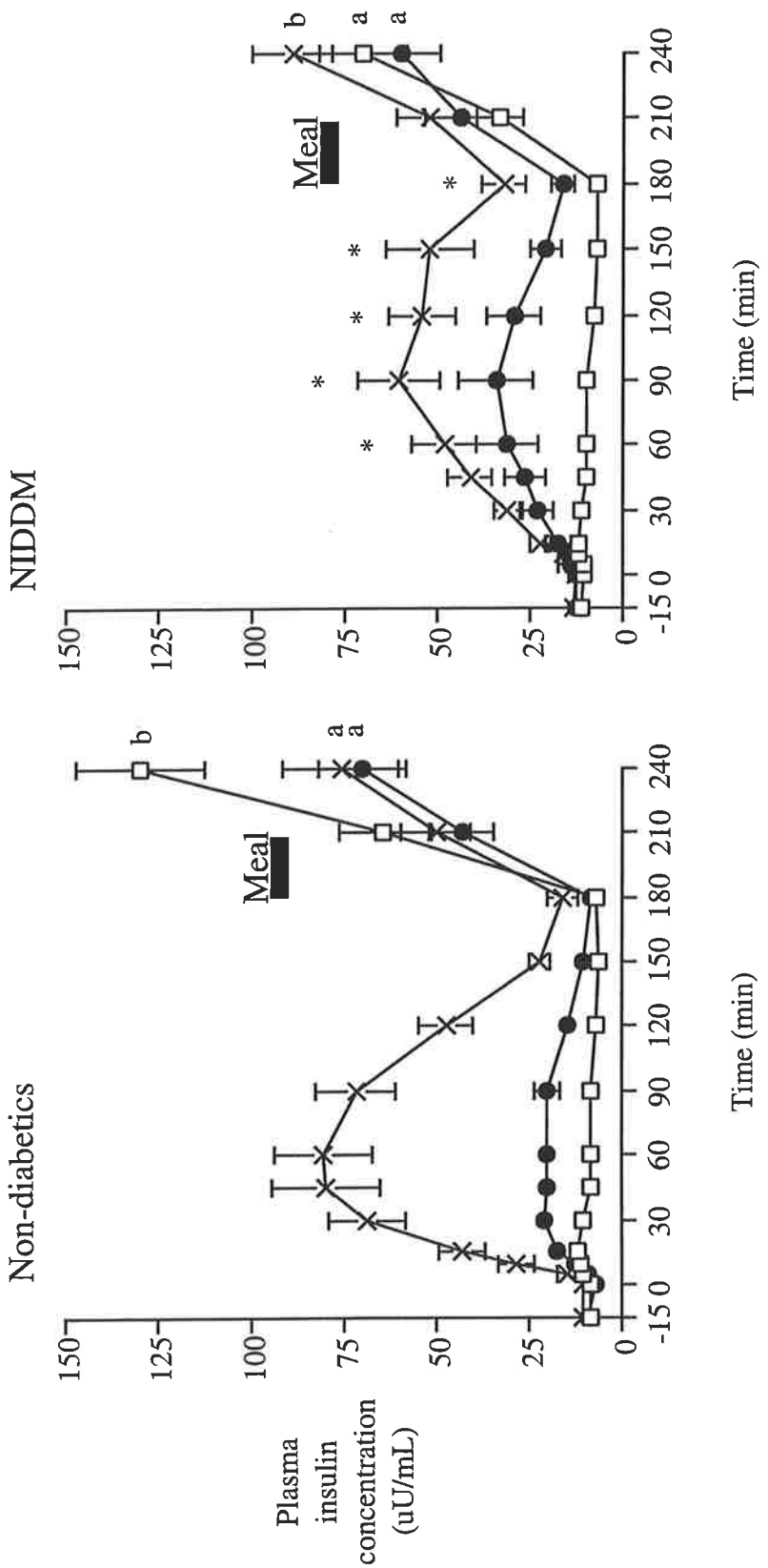


Figure 11.2 Plasma insulin concentrations in non-diabetics (n = 11) and type 2 diabetics (n = 11) following glucose (75 g) (✕), fructose (75 g) (●) or vehicle (□). Values are mean ± S.E.M. * $P < 0.05$ for fructose in type 2 diabetics v non-diabetics. Different superscript letters indicate curves are significantly different ($P < 0.05$) from t = 0 - 180 min.

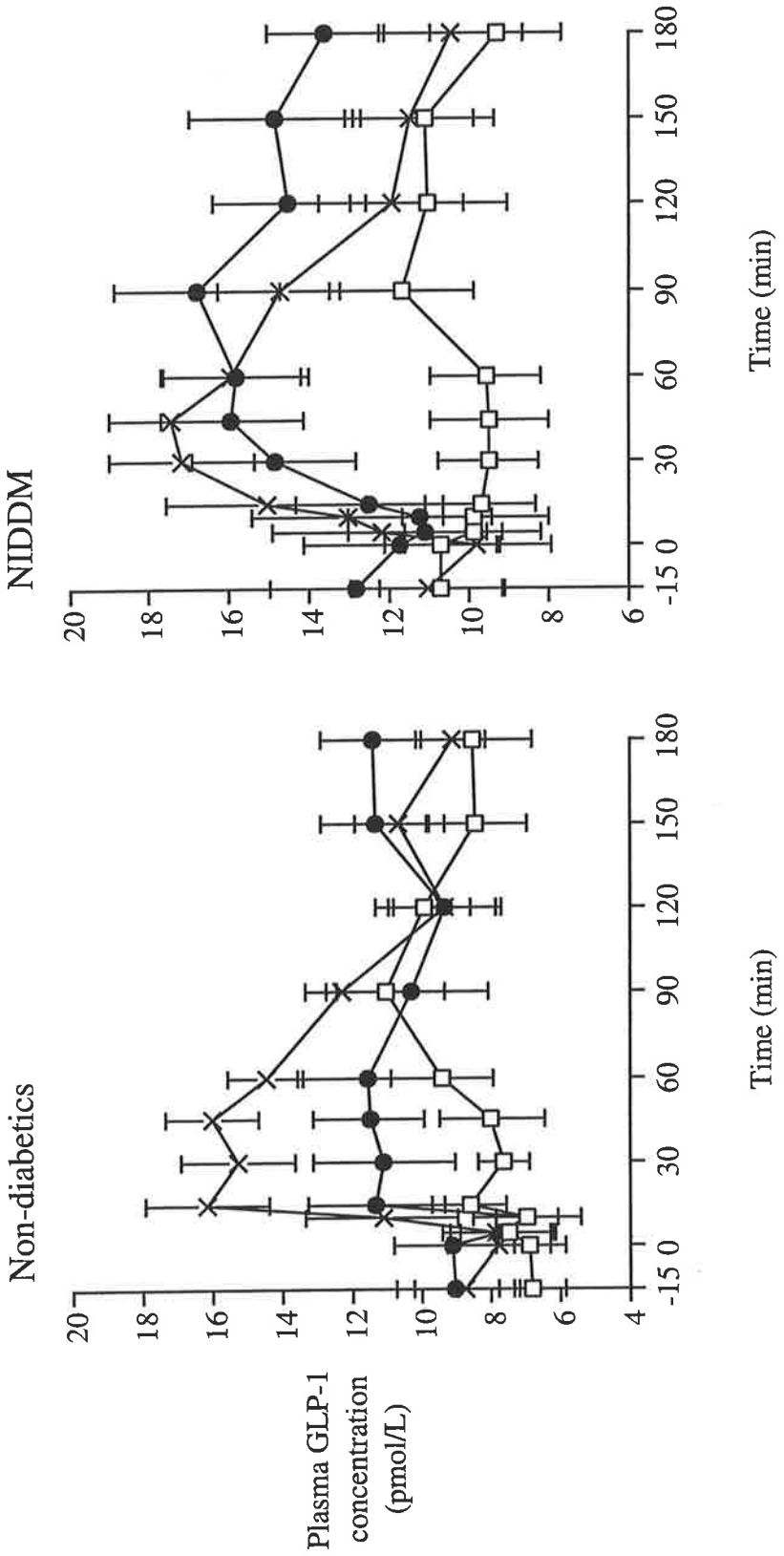


Figure 11.3 Plasma glucagon-like peptide-1 (GLP-1) concentrations in non-diabetics (n = 11) and type 2 diabetics (n = 11) following glucose (75 g) (×), fructose (75 g) (●) or vehicle (□). Values are mean ± S.E.M.

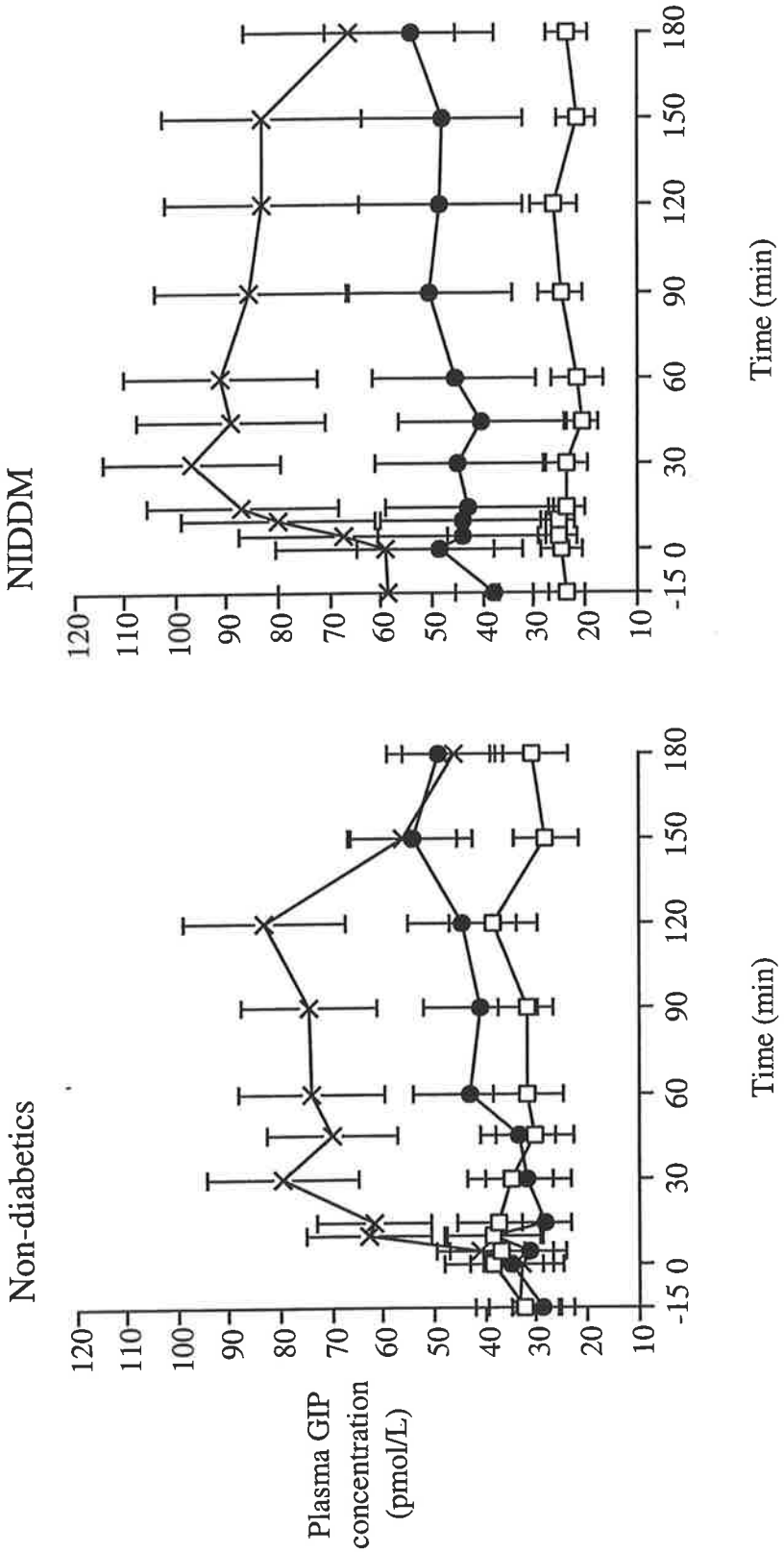


Figure 11.4 Plasma gastric inhibitory peptide (GIP) concentrations in non-diabetics (n = 11) and type 2 diabetics (n = 11) following glucose (75 g) (✕), fructose (75 g) (●) or vehicle (□). Values are mean ± S.E.M.

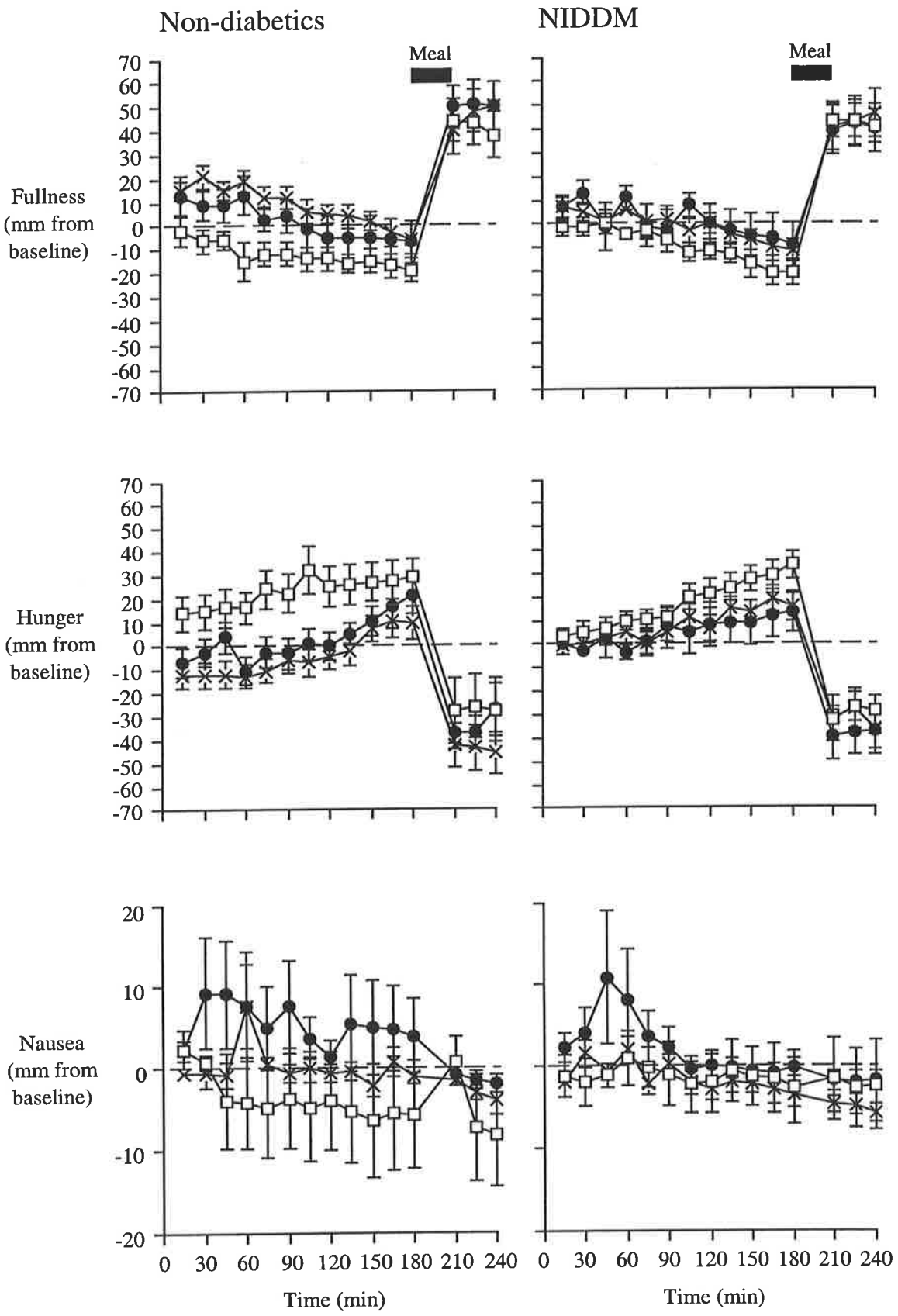


Figure 11.5 Differences in VAS scores for fullness, hunger and nausea in non-diabetics (n = 11) (left panels) and type 2 diabetics (n = 11) (right panels) following glucose (75 g) (×), fructose (75 g) (●) or vehicle (□). Values are mean ± S.E.M. No significant difference between treatments, or between diabetics and non-diabetics.

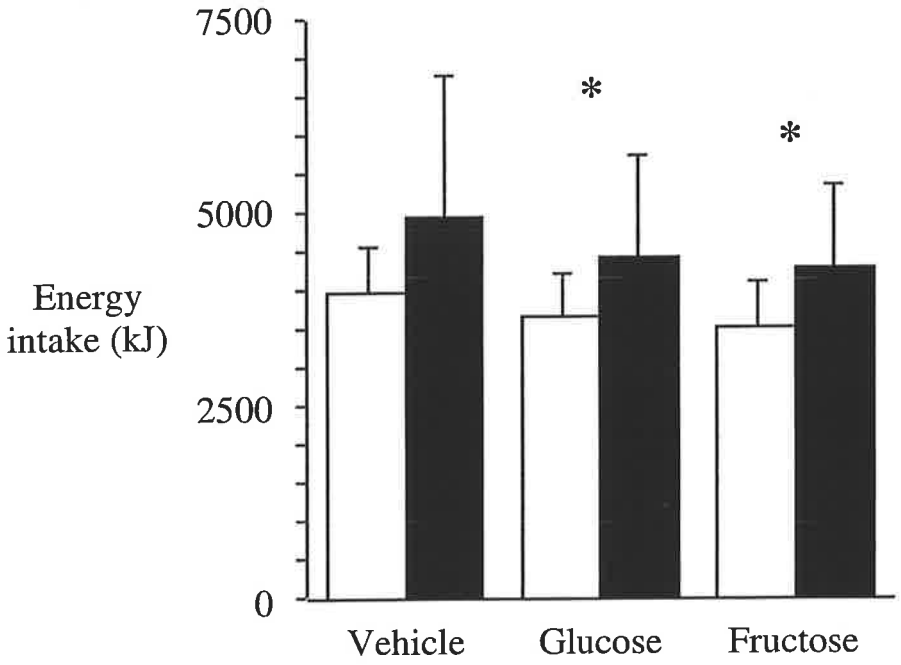


Figure 11.6 Energy intake at a buffet meal 3 hours after glucose (75 g), fructose (75 g) or vehicle in non-diabetics (n = 11) (solid columns) and type 2 diabetics (n = 11) (open columns). Values are mean ± SEM. * $P < 0.05$ v vehicle.

Chapter 12

EFFECT OF INTRAVENOUS ADMINISTRATION OF NITRIC OXIDE (NO) SYNTHASE INHIBITORS, L-NMMA AND L-NAME, ON SHORT-TERM APPETITE AND FOOD INTAKE IN HUMANS

12.1 SUMMARY

Animal studies suggest that nitric oxide (NO) may be a physiological regulator of appetite; NO synthase inhibition suppresses food intake in rats, mice and chickens. It is not known whether NO has any effect on appetite in humans. NG-nitro-L-monomethyl arginine (L-NMMA) and NG-nitro-L-arginine methyl ester (L-NAME), both competitive, non-selective inhibitors of NO synthase (NOS), were used in two separate experiments, to evaluate the role of NO in the short-term regulation of appetite in humans. In experiment I, 13 men (18 - 25 y) underwent paired studies, in randomised, double blind fashion. L-NMMA (4 mg/kg/h) or saline (0.9%) was infused intravenously at a rate of 40 ml/h for 1.5 hours. In experiment II, 8 men (18-26 y) underwent three randomised, double blind studies. L-NAME (75 μg/kg/h or 180 μg/kg/h) or saline (0.9%) was infused intravenously at a rate of 20 ml/h for 120 min. Both experiments were performed after an overnight fast. Hunger and fullness were measured using visual analogue scales; blood pressure and heart rate were monitored, and 30 minutes before the end of the infusion subjects were offered a cold buffet meal. Total energy intake and the macronutrient composition of the meal were determined. Both L-NMMA ($P = 0.052$) and L-NAME ($P < 0.05$; both doses) decreased heart rate, L-NMMA increased diastolic blood pressure ($P < 0.01$) and L-NAME increased systolic blood pressure ($P = 0.052$). Neither drug had any effect on energy intake or sensations of hunger or fullness. Despite having significant effects on cardiovascular function in the doses used, neither L-NMMA nor L-NAME, had any effect on feeding, suggesting that NO does not affect short-term appetite or food intake in humans.

12.2 INTRODUCTION

The results of animal studies suggest that NO may also be involved in the regulation of feeding behaviour (Chapter 12); (Morley, Flood, 1991)(Squadrito et al., 1993)(Choi et al., 1994) (See Chapter 5 for details). However, no studies have examined the effects of NO on appetite and food intake in humans.

As there are substantial differences in both the potencies and selectivity for different NOS isoforms by all L-arginine analogues (Fukuto, Chaudhuri, 1995) the effect of two analogues has been examined. In the present study the NOS inhibitors, L-NMMA and L-NAME, were administered to healthy young men to determine whether NO has a role in the short-term regulation of human appetite. It was hypothesised that acute NOS inhibition would suppress appetite and food intake.

12.3 METHODS

12.3.1 Subjects

Two separate studies were performed. In experiment I, L-NMMA was infused (n = 13), and in experiment II, L-NAME (n = 8). Healthy male volunteers (18 - 26 y) with body mass indices (BMI) between 20.3 - 26.1 kg/m^2 were studied (Section 6.2), and no subject participated in both studies.

12.3.2 Protocol

12.3.2.1 Both studies

Studies were separated by at least seven days. Subjects were instructed not to undertake vigorous exercise or consume alcohol for at least 24 hours before each study day. Subjects arrived at the test centre at 0830, having fasted (except for water) from 2100 the previous night. At this time intravenous (iv) cannulae were inserted into a vein in each forearm, one to receive infusions, the other from which blood samples were taken. Subjects then rested for 30 minutes until t = 0 min. All studies were performed in a quiet room. Subjects were allowed to read or listen to the radio, but not to read about food or eating related subjects. In both studies, if systolic blood pressure increased by $\geq$ 30 mm Hg from baseline or diastolic blood pressure increased $\geq$ 20 mm Hg from baseline, the infusion was to be terminated. If blood pressure did not return to pre-treatment levels within 30 min, an anginine tablet would be administered. All subjects were tested for possible adverse

reactions to anginine at their screening. Subjects were informed of this possible side effect. On their final study day all subjects were informally asked, by the primary investigator blinded to the treatment infusions, in what order they thought they had received the different treatments.

Effect of L-NMMA (Experiment I)

Subjects were studied on two separate days. An iv infusion of 0.9% saline (60 ml/h) was commenced at t = 0 min and continued until t = 150 min. Starting at t = 60 min, subjects received a 90 minute iv treatment infusion (40 ml/h) of either saline (0.9%), or L-NMMA (Clinalfa AG, Läufelfingen, Switzerland) (4 mg/kg/h) in saline (0.9%), in randomized, double blind order. At t = 120 min, 30 minutes before the end of the treatment infusion subjects were offered a cold buffet meal, prepared in excess of what they would normally be expected to eat, and invited to eat as much as they wanted in the following 30 minutes and / or until they were comfortably full. Subjects received the same meal on both days. The infusion ended at the conclusion of the 30 minute meal period (t = 150 min). Subjects then rested for a further 30 minutes to the end of the study. Except during mealtime, when they were seated, subjects remained in a supine position with the upper body elevated 30° above the horizontal, for the entire study.

Effect of L-NAME (Experiment II)

Subjects were studied on three separate days. An iv infusion of 0.9% saline (60 ml/hr) was commenced at t = 0 min and continued until t = 150 min. Starting at t = 30 min subjects received a 120 minute iv treatment infusion (20 ml/h) of either saline (0.9%) or L-NAME (Clinalfa AG, Läufelfingen, Switzerland) (75 or 180 μg/kg/h) in saline (0.9%), in randomized, double-blind fashion. At t = 120 min (30 minutes before the end of the treatment infusion) subjects were offered a cold buffet meal, prepared in excess of what they would normally be expected to eat, and encouraged to eat as much as they wanted in the following 40 minutes and/or until they were comfortably full. Subjects received the same meal on all three study days. The meal ended at t = 150 minute and the infusion at t = 160 min. Subjects then rested for a further 20 minutes till the end of the study. Except during meal time, when they were seated, subjects remained in a supine position with the upper body elevated 30° above the horizontal, for the entire study.

12.3.2.2 Measurements of sensations of appetite and food intake

Visual analogue scales (VAS) assessing hunger, fullness and nausea ratings (Chapter 6.7.1.3) were administered, every 15 minutes until from t = 0 - 120 min, and then at t = 150 and t = 180 min (Study I) or at t = 160, 165, 180 and 195 min (Study II). In study I, subjects were told that the primary aim of the study was to assess the effects of L-NMMA on sensations of appetite and on food intake. However, in study II, subjects were not informed of the major aim of the study, but were told that the study would assess the effects of L-NAME on cardiovascular function in the pre- and post-prandial states.

Food intake was quantified as described in Chapter 6.7.1.4. Vegetarian subjects were offered a boiled egg (n = 2; Study I) or additional vegetables: beetroot, carrot and three-bean mix (n = 1; Study II), in place of the meats.

12.3.2.3 Measurements of blood pressure and pulse rate

Haemodynamic variables were measured as described in Section 6.7.3.

12.3.3 Statistical Analyses

All data were analysed as described in Chapter 6.8. A two-tailed, Student's paired *t*-test was used to compare the means for energy intake and macronutrient composition in study I.

12.4 RESULTS

All treatments were well tolerated by all subjects and there were no adverse events. On direct questioning, subjects experienced no symptoms that allowed them to determine the NOS inhibitor they were receiving on a particular day and did not identify the treatment days more often than by chance alone.

12.4.1 Effects of L-NMMA and L-NAME on blood pressure and heart rate

Both L-NMMA and L-NAME increased blood pressure and decreased heart rate (Figure 12.1 and 12.2). There was a significant effect of time on blood pressure in both studies, such that mean arterial blood pressure during and after the meal was 3-5 mm Hg higher than at the beginning of the treatment infusion. Blood pressure did not increase to an extent where these infusions needed to be terminated in any subject. There was an effect of L-NMMA and L-NAME such that heart rate was lower than on saline infusion days.

12.4.1.1 L-NMMA

During the baseline saline infusions there were no significant differences in systolic (control v L-NMMA, 120.5 ± 1.7 v 122 ± 1.8 mmHg; P > 0.05), diastolic (control v L-NMMA, 82.2 ± 2 v 79.8 ± 2 mmHg; P > 0.05), or mean arterial (control v L-NMMA, 94.8 ± 1.8 v 93.9 ± 1.8 mmHg; P > 0.05), blood pressure between control and L-NMMA infusion days. While L-NMMA had no effect on the change in systolic blood pressure from baseline (P < 0.05) (Figure 12.1), L-NMMA increased diastolic blood pressure by 4.5% during treatment infusions (control v L-NMMA, 81.7 ± 1.3 v 85.4 ± 1.0 mmHg; P<0.01), an effect which was evident 30 minutes after commencing the treatment infusion (Figure 12.1). There was also a significant effect of L-NMMA on mean arterial blood pressure (P < 0.05); L-NMMA increased mean arterial blood pressure by 3.0 ± 0.7 mmHg, during treatment infusions, compared with the saline control.

During the baseline saline infusions, there were no significant differences in heart rate between control and treatment days (control v L-NMMA, 56.9 ± 2.0 v 56.4 ± 2.1 bpm; P = 0.6). While there was no effect of L-NMMA on heart rate (P > 0.05) (Figure 12.2), there was an interaction between time and treatment that just failed to reach statistical significance, such that the increase in heart rate that occurred at the end of both treatment infusions was not as great when L-NMMA was infused, compared with when saline was infused (saline v L-NMMA, 7.2 ± 1.8 v 4.4 ± 1.9 change in heart rate from baseline, mmHg; P = 0.052).

12.4.1.2 L-NAME

During baseline saline infusions there were no significant differences in systolic (control v low v high, 120.3 ± 3.1 v 122.8 ± 3.2 v 121.9 ± 3.1 mmHg; P>0.05) diastolic (control v low v high, 56.7 ± 3 v 59.7 ± 3.1 v 61.3 ± 3.1 mmHg; P > 0.05) or mean arterial (control v low v high, 82 ± 2.9 v 84.9 ± 2.8 v 86.8 ± 3.1 mmHg; P > 0.05) blood pressure between control, low dose L-NAME and high dose L-NAME infusion days. L-NAME increased systolic blood pressure during the infusion (control v low v high, 120.1 ± 1.4 v 121.2 ± 1.4 v 123.4 ± 1.3 mmHg; P = 0.052) (Figure 12.1), however this failed to reach statistical significance. L-NAME had no effect on diastolic blood pressure (P > 0.05) (Figure 12.1), or on mean arterial blood pressure (P > 0.05).

During the baseline saline infusions, there were no significant differences in heart rate between control and treatment days (control v low dose v high dose, 61.7 ± 2.4 v 59.0 ± 2.5 v 58.4 ± 2.4 bpm; P=0.3). Both doses of L-NAME decreased heart when rate throughout the treatment infusion compared with the saline control (control v low dose v high dose, 6.9 ± 1.4 v 1.4 ± 1.4 v 0.2 ± 1. 9 change in heart rate from baseline, mmHg; $P < 0.05$) (Figure 12.2).

12.4.2 Effect of L-NMMA and L-NAME on sensations of appetite and on food intake

Pre-treatment ratings of hunger ($P > 0.05$), fullness ($P > 0.05$) and nausea ($P > 0.05$) did not differ significantly between the two treatment days in experiment I, nor between the three treatment days in experiment II (hunger, $P > 0.05$; fullness, $P > 0.05$; nausea, $P > 0.05$). Consumption of the meal increased fullness (L-NMMA, $P < 0.0001$; L-NAME, $P < 0.0001$) and decreased hunger (L-NMMA, $P < 0.0001$; L-NAME, $P < 0.0001$). Nausea ratings were not significantly affected by the meal (L-NMMA, $P > 0.05$; L-NAME, $P > 0.05$). Neither L-NMMA nor L-NAME, at any dose studied, had any effect on ratings of hunger (L-NMMA, $P > 0.05$; L-NAME, $P > 0.05$), fullness (L-NMMA, $P > 0.05$; L-NAME, $P = 0.5$) or nausea (L-NMMA, $P > 0.05$; L-NAME, $P > 0.05$).

No subject consumed all the food offered, and only one subject ate for the entire 40 minutes (Experiment II). Energy intake was unaffected by both L-NMMA ($P > 0.05$) and L-NAME ($P > 0.05$) (Tables 12.1 and 12.2). Neither the L-NMMA infusion nor the L-NAME infusions were associated with any significant changes in macronutrient composition of the food consumed or duration of eating (Tables 12.1 and 12.2).

There was no effect of either L-NMMA on L-NAME on other parameters assessed by VAS including drowsiness, anxiety, tiredness, clear-headedness, sociability, happiness, predicted strength, efficiency, friendliness, satiety, thirst, desire to eat, and prospective food consumption.

12.5 DISCUSSION

Animal studies, in mice (Morley, Flood, 1991), rats (Squadrito et al., 1993), chickens (Choi et al., 1994) and marsupials (Chapter 12) have suggested that NO may have a role in the regulation of feeding behaviour. Administration of L-arginine analogues, which act as

competitive, apparently non-selective nitric oxide (NO) synthase inhibitors, to food deprived animals results in suppression of food intake. This study is the first to examine the role of NO in the regulation of human feeding. We have demonstrated that 1.5 and 2 hour intravenous infusions of the L-arginine analogues L-NMMA and L-NAME had no effect on appetite ratings or on food intake during the later part of the infusions. This finding occurred despite an effect of L-NMMA and L-NAME on the cardiovascular system consistent with peripheral NOS inhibition; viz suppression of heart rate and elevation of blood pressure. This data therefore indicate that NO is unlikely to be involved in the regulation of short-term human appetite in humans.

It is interesting that the macronutrient composition of the chosen buffet meals were considerably different between the two studies (eg 32% fat for study I, v 15% fat for study II). We have no specific explanation for this, as all subjects were healthy young men and the macronutrient composition of the buffet meals offered in the two studies did not differ. It is most likely that the different subjects studied (no subject participated in both studies) had different food choices, although this was not formally assessed using diet diaries. This reflects the large variation in inter-individual food choice, even within a theoretically homogenous group such as healthy, lean males.

The potencies and selectivity of L-arginine analogues differ markedly (Fukuto, Chaudhuri, 1995). There is evidence that in relation to vasoconstriction, L-NAME is at least ten times more potent than L-NMMA (Rees et al., 1990). We therefore studied the effects of two analogues. In view of this it is not surprising that comparable effects on blood pressure and heart rate were observed after both drugs, despite the fact that the dose of L-NAME administered was 17 - 40 times lower than that of L-NMMA. It has also been demonstrated that NG-methyl-L-arginine exhibits competitive inhibition of endothelial NOS and irreversible inhibition of macrophage NOS (Olken et al., 1991). Furthermore, NG-nitro-L-arginine is selective for brain compared to inducible NOS (Furfine et al., 1993). Both of the drugs studied had similar effects, neither drug had any effect on appetite, and both drugs produced comparable haemodynamic effects. The increase in blood pressure and heart rate (on the control day) was unexpected, but possibly resulted from the change in posture (from supine to sitting) and slight increase in activity while eating. The study however provides no evidence for a selective action of either L-NMMA or L-NAME on any NOS isoform.

The apparent discrepancy between suppression of food intake in animals by L-arginine analogue administration and the absence of such effect in humans may be due to species differences. Alternatively, it is possible that in the doses required to reduce food intake in animals these drugs produce a non-specific noxious effect leading to aversion, rather than a true suppressive effect on appetite. The dose of L-NAME required to produce anorexia in animals (10 mg/kg in mice, 50 mg/kg in rats (Morley, Flood, 1991; Squadrito et al., 1993) is higher than the dose in this study was without effect on appetite in humans. The issue of whether drugs produce appetite-suppressive effects secondary to the production of malaise is optimally addressed in animals using conditioned taste aversion tests, see Chapter 5.3.4 for detailed discussion.

The reasons for the observed lack of effect of L-arginine analogues on appetite and food intake in the current study may include inadequate duration of NOS blockade and / or inadequate dose of the L-arginine analogues. The first explanation seems unlikely, given that the infusions were administered for 90 and 120 minutes. Moreover, infusions had significant cardiovascular effects during this time, due to endothelial NOS inhibition (Coiro *et al.*, 1997); L-NMMA produces endothelium dependent vasoconstriction (Moncada *et al.*, 1991; Palmer *et al.*, 1988). Furthermore, L-arginine analogues have been shown to suppress food intake in animals within 30 minutes of a single intraperitoneal injection (Morley and Flood, 1991).

The doses of L-NMMA and L-NAME used were based on those shown to have effects on factors associated with appetite regulation, namely hormone secretion and gut motility, in previous human studies. Comparable doses of L-NAME have been shown to affect insulin and adrenocorticotrophic hormone (ACTH) secretion in healthy young men. A dose of 90 μg/kg iv over 60 mins reduces the stimulatory effect of L-arginine on insulin secretion (Coiro *et al.*, 1997), as well as producing a marginal increase in mean arterial blood pressure. A 40 μg/kg bolus plus a further 50 μg/kg for one hour enhanced the ACTH response to hypoglycemia (Volpi et al., 1996). The higher of the two L-NAME doses in our study is greater, both in total dose administered (360 μg/kg) and hourly rate (180 μg/kg/h) than in those studies, whereas the lower dose delivered a slightly lower hourly rate (75 μg/kg/h compared with 90 μg/kg/h) but somewhat greater total dose (150 μg/kg compared with 90 μg/kg). The dose of L-NMMA used in this study, 4 mg/kg/h, has been

shown to stimulate small intestinal fasting motility in normal subjects (Russo *et al.*, 1999). This dose of L-NMMA, when infused for 1h, increased the number of small intestinal migrating motor complexes. Furthermore, the doses of L-NMMA and L-NAME administered in the present studies were sufficient to exert cardiovascular effects, consistent with suppression of peripheral NOS activity. Therefore, although not definitely the case, it seems likely that the doses used were sufficient to influence appetite if nitric oxide has such an effect. While higher doses of these L-arginine analogues may theoretically have effects on appetite, peripheral administration of such doses would probably be accompanied by even greater effects on blood pressure and heart rate, which would very likely render these analogues unacceptable for clinical use.

Several studies in animals have provided evidence that the appetite-suppressive effects of L-arginine analogues may be due to central as well as peripheral NO blockade. For example, food intake is suppressed when rats are treated with intra-cerebroventricular (ICV) L-NO Arg (Squadrito *et al.*, 1993). In fact, doses that do not suppress food intake upon peripheral administration produce hypophagia after central administration (Squadrito *et al.*, 1993). Choi *et al.*, (1994) have used L-NO-arg to demonstrate a similar effect in chickens. This suggests that, at least in animals, L-NO Arg may not cross the blood brain barrier. We have no comparable information for L-NAME and L-NMMA and it is unclear as to whether these NOS antagonists actually cross the blood brain barrier in humans. In the present study we were limited by the route of administration of L-arginine analogues and did not have the capacity to assess central NOS activity. Therefore, despite the apparent peripheral suppression of NOS activity, a lack of central suppression could explain the absence of effect of these L-arginine analogues on human feeding.

In summary, despite exerting peripheral effects on the cardiovascular system consistent with inhibition of endothelial NOS, neither L-NMMA nor L-NAME had any effect on short-term appetite or feeding in humans. This suggests that peripheral NO is not involved in the short-term control of feeding. While central NO activity may play a role in appetite regulation, the present study provides no evidence for a role of endogenous NO in the regulation of human appetite.

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Table 12.1 Weight, energy content and macronutrient content (percentage of the total energy intake) of buffet meal consumed by healthy subjects during iv infusion of saline and L-NMMA (4 mg/kg/h) (n = 13).

	Saline	**L-NMMA (4 mg/kg/h)**
Weight (g)	1159.2 ± 61.5	1156.8 ± 88.8
Energy intake (kJ)	5350 ± 351	5296 ± 418
Carbohydrate (% of total)	48.2	48.0
Protein (% of total)	19.7	19.4
Fat (% of total)	32.1	32.6

Values are means ± S.E.M. Analysis by Student's paired t-test showed that there were no significant differences for any parameters.

Table 12.2 Weight, energy content and macronutrient content (percentage of the total energy intake) of buffet meal consumed by healthy subjects during iv infusion of saline and L-NAME (75 and 180 μg/kg/h) (n = 8).

	Saline	**L-NAME (75 μg/kg/h)**	**L-NAME (180 μg/kg/h)**
Weight (g)	1451.5 ± 199.9	1391.1 ± 175.5	1457.1 ± 219.9
Energy intake (kJ)	6295 ± 744	6178 ± 690	6370 ± 764
Carbohydrate (% of total)	60.6	58.2	59.3
Protein (% of total)	24.1	25.3	24.8
Fat (% of total)	15.3	16.5	15.9

Values are means ± S.E.M. Analysis by one-way ANOVA showed that there were no significant differences for any parameters.

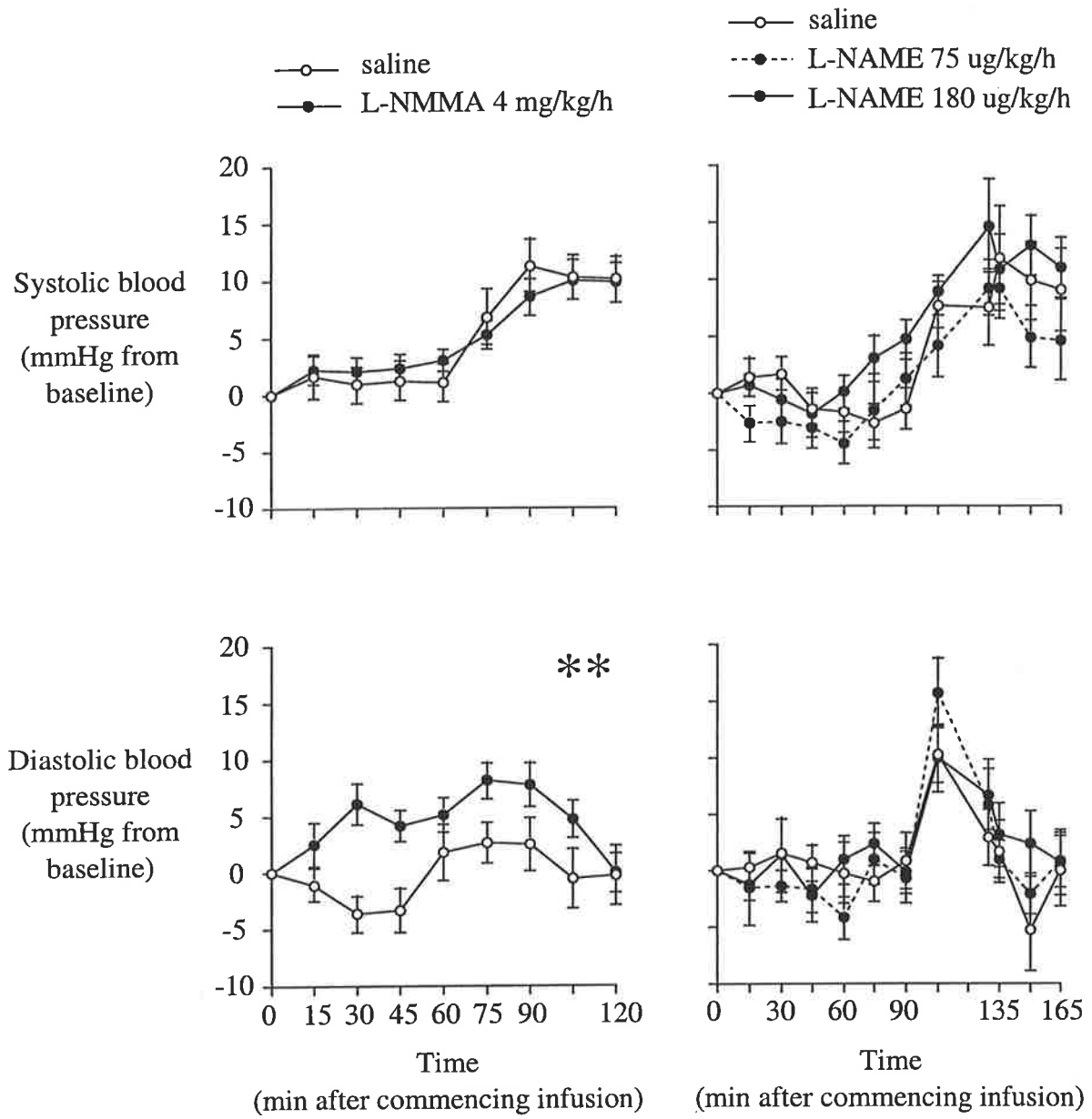

Figure 12.1 Effect of acute intravenous administration of NG-monomethyl-L-arginine (L-NMMA) (n = 13) and NG-nitro-L-arginine methyl ester (L-NAME) (n = 8) on systolic and diastolic blood pressure from baseline. Values shown are mean ± SEM. ** $P < 0.01$ for L-NAME curve v saline.

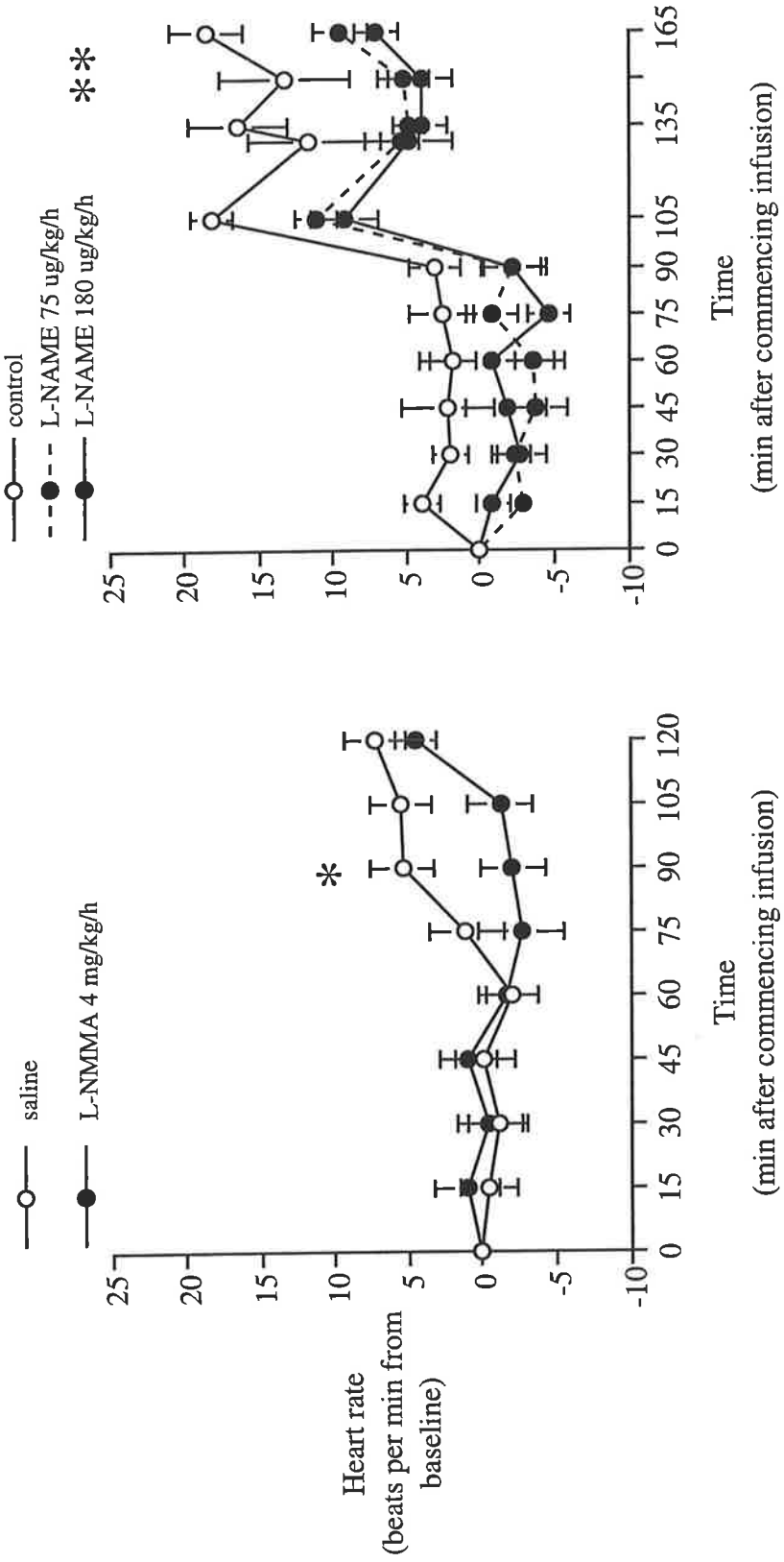

Figure 12.2 Effect of acute intravenous administration of saline, NG-monomethyl-L-arginine (L-NMMA) (n = 13) and NG-nitro-L-arginine methyl ester (L-NAME) (n = 8) on heart rate from baseline. Values shown are mean ± S.E.M. * $P < 0.05$ for specific timepoint v saline. ** $P < 0.01$ for L-NAME curves v saline.

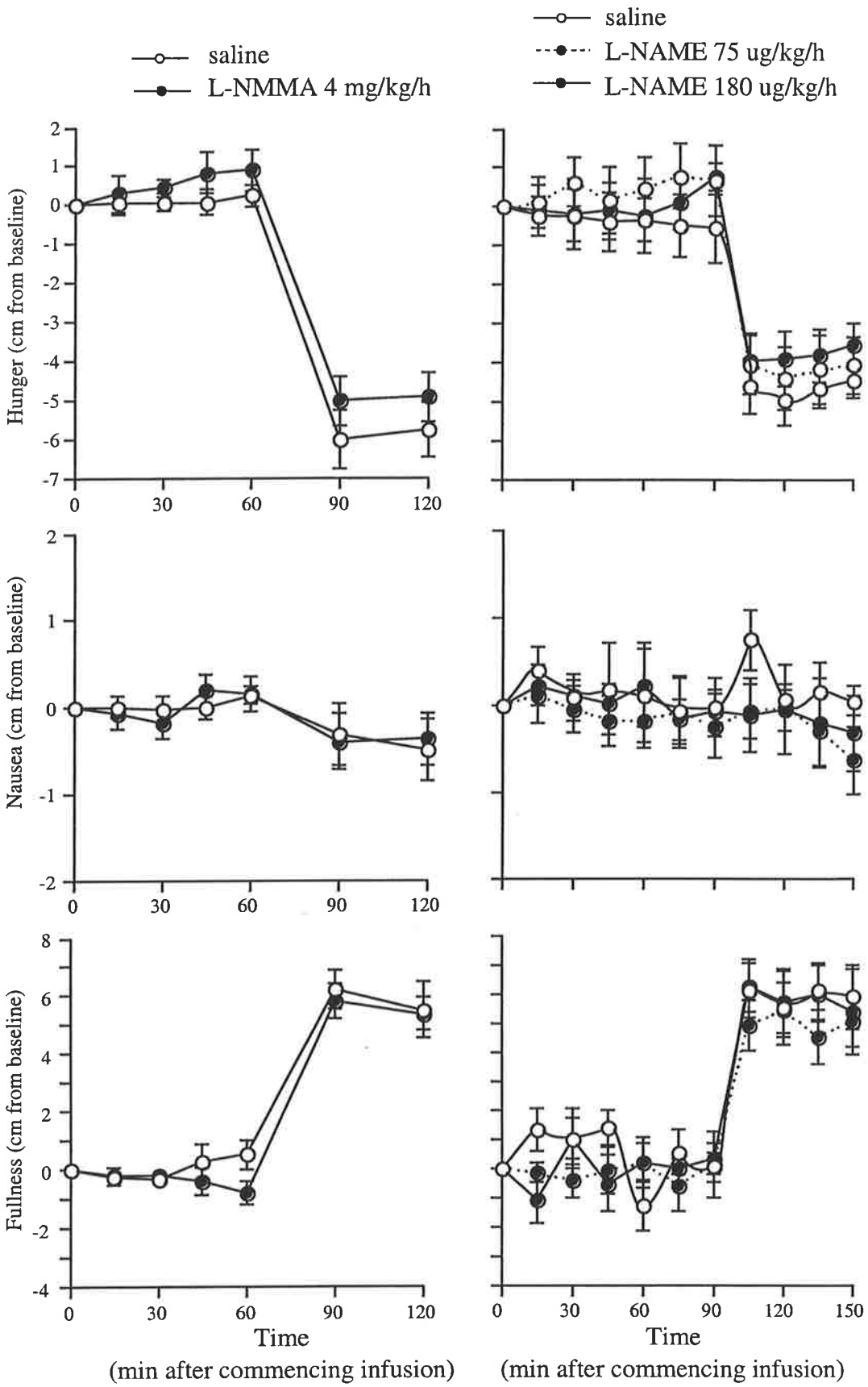

Figure 12.3 Effect of acute intravenous administration of NG-monomethyl-L-arginine (L-NMMA) (n = 13) and NG-nitro-L-arginine methyl ester (L-NAME) (n = 8) on hunger, nausea and fullness from baseline. Values shown are mean ± SEM.

Chapter 13

EFFECTS OF NITRIC OXIDE SYNTHASE INHIBITOR NG-NITRO-L-ARGININE (L-NAME) ON ANTROPYLORODUODENAL MOTILITY AND APPETITE IN RESPONSE TO INTRADUODENAL LIPID INFUSION IN HUMANS

13.1 SUMMARY

Studies in animals indicate that endogenous nitric oxide (NO) is an important inhibitory neurotransmitter in the gastrointestinal tract and also modulates food intake. To evaluate the role of NO mechanisms in mediating the effects of small intestinal nutrients on antropyloroduodenal motility and appetite in humans, 8 healthy adult men received intravenous L-NAME 180 μg/kg/h or 0.9% saline (0 - 150 min), on two separate days between 30 - 120 min an intraduodenal lipid infusion (8.36 kJ/min) was administered, and at 120 min subjects were offered a buffet meal (120 - 150 min). Antropyloroduodenal pressures were measured with a sleeve / sidehole manometric assembly. Hunger and fullness were assessed with visual analogue questionnaires and the amount and macronutrient content of food consumed at the buffet meal were quantified. Blood pressure and heart rate were monitored. Intraduodenal lipid infusion increased fullness ($P < 0.05$), the frequency of isolated pyloric pressure waves ($P < 0.05$) and basal pyloric pressure ($P < 0.05$); and decreased hunger ($P < 0.05$), and the frequency of antral ($P < 0.05$) and duodenal ($P < 0.05$) pressure waves. L-NAME increased diastolic blood pressure ($P = 0.08$) and decreased heart rate ($P < 0.05$), consistent with an inhibitory effect on endothelial NO synthase, but had no effect on antropyloroduodenal pressures or food intake. Intravenous administration of the systemic NO synthase inhibitor, L-NAME, in a dose that affects cardiovascular function in healthy humans does not modify the antropyloroduodenal motor and appetite responses to intraduodenal lipid infusion.

13.2 INTRODUCTION

The interaction between nutrients with receptors in the small intestine plays a major role in the regulation of both gastric emptying and appetite in humans (Andrews *et al.*, 1998; Cook *et al.*, 1997; Heddle *et al.*, 1989; Heddle *et al.*, 1988; Tougas *et al.*, 1992) (Chapter 2.3). Observations in animals suggest that nitric oxide (NO) may be an important modulator of gastroduodenal motility (Lingenfelser *et al.*, 1997; Orihata and Sarna, 1994; Sarna *et al.*, 1993) and food intake (Morley and Flood, 1991; Squadrito *et al.*, 1993) in animals (Chapter 2.2 and 5). There is little information about the role of NO mechanisms (if any) in the regulation of gastric motility and appetite (See Chapters 5 and 12) in humans. In healthy humans, NO donors inhibit both phasic and tonic pyloric motility stimulated by intraduodenal triglyceride infusion (Sun *et al.*, 1996), relax the proximal stomach (Thumshirn *et al.*, 1999) and slow gastric emptying (Sun *et al.*, 1998). The NOS inhibitor, L-NMMA, stimulates small intestinal phase 3 activity (Russo *et al.*, 1999), decreases proximal gastric relaxation (Hirsch *et al.*, 1998), stimulates antral pressure waves (Konturek *et al.*, 1999), but in contrast to the effects of NOS inhibition in animals (Orihata and Sarna, 1994; Orihata and Sarna, 1996), accelerates gastric emptying (Konturek *et al.*, 1999) and appears to have no effect on acute food intake (Chapter 12). Furthermore, L-NMMA does not affect the number of isolated pyloric pressure waves (IPPWs) or basal pyloric pressure in the fasted state in humans (Luiking, 1999).

The studies summarised above provide evidence that NO mechanisms modulate proximal stomach, antral, pyloric and duodenal motility, gastric emptying and appetite in various animal species. However, the known effects of NOS inhibition on gastric emptying and appetite are inconsistent with observations in animals, particularly in relation to the role of NO mechanisms in the regulation of pyloric motility and appetite. The effects of NOS inhibition on pyloric motility and food intake during small intestinal nutrient stimulation have not been examined in humans. The aims of this study were to determine whether the effects of intraduodenal lipid infusion on antropyloroduodenal motility and appetite are modulated by NO mechanisms.

13.3 METHODS

13.3.1 Subjects

Eight healthy male subjects (22 - 40 y; BMI: 21-28 kg/m^2) were recruited according to Chapter 6.2.

13.3.2 Protocol

Each volunteer participated in two studies in randomized order separated by at least 7 days. On the day of each study, subjects arrived at the Department of Gastrointestinal Medicine, Royal Adelaide Hospital at 0830, following an overnight fast. A manometric catheter (4 mm diameter) was introduced into the stomach via an anaesthetised nostril. The catheter was allowed to pass through the pylorus into the duodenum by peristalsis while the subject was in the recumbent position. Another cannula was inserted into a vein of the opposite arm for intravenous infusion. When the manometric catheter was positioned correctly, and 15 min after the end of an episode of duodenal phase 3 activity (or in the absence of phase 3 activity, 90 minutes later), an intravenous infusion of either saline (0.9%) or L-NAME (180 μg/kg/h) (Clinalfa AG, Switzerland) was commenced, in single blind fashion (t = 0 min); the infusion was continued for 150 minutes. At t = 30 minutes an intraduodenal lipid infusion (Intralipid 10%; 8.36 kJ/min) (Fresenius Kabi, Baxter Ltd, NSW, Australia) was commenced, and continued for 90 minutes (i.e. t = 30 - 120 min). At t = 120 minutes the intraduodenal infusion was ceased and the catheter removed. The volunteer was then offered a cold, buffet meal, prepared in excess of what they would normally be expected to consume, and allowed to eat until comfortably full. At t = 150 min the intravenous infusion was stopped and the intravenous cannula and reference electrode removed.

Antropyloroduodenal pressure waves were recorded continuously until the manometric catheter was removed. Systolic, diastolic, mean blood pressure and heart rate were measured every 15 minutes from the start of intravenous infusion until the end of the study (Chapter 6.7.3). In both studies, if systolic blood pressure increased by $\geq$ 30 mm Hg from baseline or diastolic blood pressure increased $\geq$ 20 mm Hg from baseline, the infusion was to be terminated. If blood pressure did not return to pre-treatment levels within 30 min, an anginine tablet would be administered. All subjects were tested for possible adverse reactions to anginine at their screening. Subjects were informed of this possible side effect. Perceptions of fullness, hunger and nausea were assessed at the same time points by visual

analogue scales (VAS) (Chapter 6.7.1). The amount and macronutrient composition of the food eaten at the buffet meal were quantified (Chapter 6.7.1).

13.3.3 Measurement of antropyloroduodenal pressures

Antropyloroduodenal pressures were measured as described in Chapter 6.7.5

13.3.4 Statistical analysis

Pre-treatment antropyloroduodenal pressures, haemodynamic parameters, and perception scores were compared using a one-way analysis of variance (ANOVA) (with treatment as a factor). Two-way repeated measures analysis of variance (ANOVA) (with time and treatment being the two factors) was used to compare these parameters during each study. Potential differences in energy consumption and macronutrient content between L-NAME and saline treatment was analyzed by one-way analysis of variance (ANOVA) (SigmaStat, Jandel Scientific, Australia). All data are expressed as mean ± S.E.M.M. A P value of < 0.05 was considered significant in all analyses.

13.4 RESULTS

All subjects tolerated the studies well. TMPD measurements indicated that the sleeve sensor was positioned correctly across the pylorus for 1843 min of a total recording time of 1920 min (96 %). On both the day of the L-NAME infusion and the saline infusion day, the intravenous infusions were initiated after phase 3 activity in four subjects and at 90 minutes in the other four.

There was no difference in pre-treatment systolic, diastolic or mean blood pressures and heart rate between the two study days. L-NAME tended to increase diastolic ($P = 0.08$), but not systolic or mean blood pressure, and decreased heart rate ($P < 0.05$), when compared to saline (Figure 13.1).

13.4.1 Antropyloroduodenal pressures

There were no significant differences between intravenous saline and L-NAME with respect to the pre-treatment frequency or amplitude of isolated pyloric pressure waves (IPPWs). Intraduodenal lipid infusion was associated with an increase in the frequency of IPPWs ($P < 0.0001$ for time) and basal pyloric pressure ($P < 0.05$ for time), but not the

amplitude of IPPWs, on both days. There were no significant differences between L-NAME and saline in the frequency, or amplitude of IPPWs, or basal pyloric pressure (Figure 13.2). Pre-treatment antral and duodenal pressure wave frequency was not significantly different between the two days. During the intraduodenal lipid infusion there was a decrease in the frequency of antral pressure waves ($P < 0.05$ for time), without any difference between saline and L-NAME treatments. Intraduodenal lipid infusion was associated with a decrease in the frequency of duodenal pressure waves ($P < 0.0001$ for time) without any difference between L-NAME and saline treatments (Figure 13.3).

13.4.2 Appetite and food intake

There were no differences in pre-treatment ratings of hunger, fullness and nausea. Intraduodenal lipid infusion was associated with increases in sensations of fullness ($P < 0.005$ for time) and nausea ($P < 0.05$ for time), and a decrease in hunger ($P < 0.05$ for time) on both days. There were no differences between L-NAME and saline treatments with respect to sensations of hunger, fullness or nausea (Figure 13.4). There was also no difference between L-NAME and saline treatments, in either the energy content, or the macronutrient composition of the food consumed at the buffet meal (Table 13.1).

13.5 DISCUSSION

This study establishes that the NOS inhibitor, L-NAME, which has been reported in animal studies to be more potent than L-NMMA in modulating blood pressure (Rees *et al.*, 1990) has no effect on lipid-induced antropyloroduodenal motor activity. This lack of an effect does not modify the effects of intraduodenal lipid infusion on antropyloroduodenal pressures, perceptions of appetite or food intake, even at a dose that affects cardiovascular function in humans. The dose of intraduodenal lipid that was used was selected to produce a clear-cut, but submaximal, stimulation of pyloric motility and thus allow the potential for either increased or decreased motor activity in response to L-NAME (Cook *et al.*, 1997). On both study days, intraduodenal lipid infusion was associated with stimulation of phasic and tonic pyloric pressures, suppression of antral and proximal duodenal pressure waves, a reduction in hunger, and an increase in fullness. The absence of any effect of L-NAME is consistent with previous observations in humans that, in contrast to animals, NOS inhibition does not affect pyloric pressures in the fasted state (Luiking, 1999).

In interpreting these observations, other factors, including the dose of the NOS inhibitor used, the neurohumoral response to small intestinal nutrient exposure, and the potential effects of systemic NOS inhibition, must be considered. It must be recognised that the doses of L-NAME previously shown to affect gastric motility and gastric emptying in animals are substantially greater than that used in the current study (Allescher *et al.*, 1992; Hallgren *et al.*, 1995; Martinez *et al.*, 1993; Orihata and Sarna, 1994). For example, in dogs, the dose of L-NAME required to slow gastric emptying and affect antropyloroduodenal motility is some 14 times greater than the dose administered in the current study (Orihata and Sarna, 1994; Orihata and Sarna, 1996). With such large doses, any effects are potentially be attributable to anti-muscarinic properties of L-NAME (Buxton *et al.*, 1993). In the present study the changes in cardiovascular function were consistent with those induced by L-NMMA in a dose of 4 mg/kg/h, which induces premature small intestinal phase 3 activity in humans (Russo *et al.*, 1999). Furthermore, the same dose of L-NAME (i.e. rate of infusion) reduces renal blood flow and glomerular filtration rate (Montanari *et al.*, 2000) in humans. In a recent study, intravenous infusion of L-NMMA, in a dose of 4 umol/kg/h (~ 1 mg/kg/h), was reported to increase the frequency of antral pressure waves and accelerate gastric emptying in humans (Konturek *et al.*, 1999). In that study, antral motility was evaluated using a polyvinyl catheter, in which three perfused channels were separated by 5 cm, with the most distal channel placed 5 cm before the pylorus under fluoroscopic guidance. Because of the high probability of catheter displacement during the study (Heddle *et al.*, 1989; Heddle *et al.*, 1988), the reported effects on antral motility should be viewed circumspectly. Moreover, pyloric motility was not evaluated. These observations, are consistent with a recent study, using TMPD criteria to monitor catheter position, in which an intravenous bolus of L-NMMA (3 mg/kg), followed by constant infusion of 3 mg/kg/h, had no effect on the number of IPPWs or basal pyloric pressure in the fasted state in healthy humans (Luiking, 1999).

It remains possible that an effect of L-NAME on pyloric motility was not observed because the dose of L-NAME, at 180 μg/kg/h, was too low. Because of the potential for adverse cardiovascular effects however, the use of a substantially higher dose of L-NAME was considered ethically unacceptable. The absence of any stimulation of antral pressure waves by L-NAME is potentially attributable to the potent suppression of antral motility by small intestinal lipid infusion (Andrews *et al.*, 1998; Cook *et al.*, 1997) as well as an increase in systemic adrenergic activity during L-NAME infusion (Lundberg, 1996; Nakao *et al.*,

1998; Thumshirn *et al.*, 1999). A previous study in dogs, found that a substantially higher dose of intravenous L-NAME (2.5 mg/kg/h) increased both the frequency and amplitude of antral, pyloric and duodenal contractions, and slowed gastric emptying of a nutrient meal (Orihata and Sarna, 1994). Taken together, these findings are consistent with the concept that NOS-containing activity is higher in the pylorus than in other regions of the gastrointestinal tract (Saur *et al.*, 2000). Future studies in humans, using a specific neuronal NOS inhibitor, and thus allowing greater NOS inhibition in the absence of a systemic effect of endothelial NOS inhibition, are required to address this issue.

The presence of nutrients in the small intestine elicits an enteric neural reflex which stimulates pyloric motility and slows gastric emptying (Heddle *et al.*, 1988; Tougas *et al.*, 1992; Treacy *et al.*, 1992). *In vitro* observations in the isolated guinea-pig small intestine suggest that endogenous NO inhibits transmission from the sensory neuron to interneurons (Yuan *et al.*, 1995), while the NOS containing activity is limited to the descending interneurons (Costa *et al.*, 1992). These concepts are consistent with an *in vivo* study in dogs, in which L-NAME, when given intra-arterially, did not affect the stimulation of pyloric motility by duodenal electric field stimulation, but reduced the inhibitory response to antral electric field stimulation (Allescher *et al.*, 1992), and may partially account for the current findings and previous observations of the suppressive effects of a NO donor on pyloric motility in humans (Sun *et al.*, 1998; Sun *et al.*, 1996).

It is interesting that in the previous chapter, L-NAME infusions in the same dose had no effect on diastolic blood pressure in the fasting state, while here, in the post-prandial state, L-NAME appears to have increased diastolic blood pressure. There are no previous data reporting such an effect. The explanation for this is not apparent, it may be a result of some interaction between nutrients in the gut, and nitrergic mechanisms responsible for hemodynamic regulation.

In the present study, L-NAME had no effect on perceptions of appetite or food intake. This latter observation is consistent with a recent report that NO donors do not affect gastric perception (Thumshirn *et al.*, 1999) and the finding in Chapter 12 that neither L-NMMA nor L-NAME affect appetite or food intake in humans. As with interpretation of the absence of any effect of L-NAME on antropyloroduodenal pressures, these observations may potentially reflect the dose of the NOS inhibitor that was used. Peripherally administered NOS inhibitor may act centrally to modify gastroduodenal motility and

appetite. Although in the ferret exogenous L-NAME increases pyloric motility when given either peripherally or centrally (Lingenfelser *et al.*, 1997), in the cat microinjection of L-NAME into rostral dorsal motor nucleus has been shown to attenuate the excitatory effect of L-arginine on antral and pyloric motility (Panico *et al.*, 1995). In humans, it has been suggested that the effects of intravenous L-NMMA on esophageal motility reflect an action on the medulla (Hirsch *et al.*, 1998). Because of the lack of information as to whether L-NAME crosses the brain blood barrier in humans, the potential impact of central NOS inhibition on these findings is uncertain.

In summary, intravenous L-NAME, administered to healthy humans in a dose that increases blood pressure and decreases heart rate, has no effect on fasting or lipid-induced antropyloroduodenal motility or on appetite and food intake. It is unlikely that peripheral NO mechanisms have a major role in the regulation of pyloric motility or feeding behaviour in healthy humans.

Table 13.1 Energy intake, and macronutrient content (percentage of the total energy intake) for a buffet meal consumed by healthy subjects after intraduodenal infusion of lipid (8.36 kJ/min [8.36 kJ/min]) during intravenous infusion of L-NAME (180 μg/kg/h) or identical volume of 0.9% saline. Values are means ± S.E.M.. There were no significant differences between treatments in any of the parameters.

	L-NAME	**Saline**
Energy intake (g)	765 ± 146	866 ± 114
Energy intake (kJ)	3402 ± 798	3536 ± 714
CHO (% of total)	48.1 ± 3.6	53.9 ± 6.6
PROT (% of total)	17.5 ± 2.4	20.0 ±2.6
FAT (% of total)	28.5 ± 4.5	26.0 ± 4.2

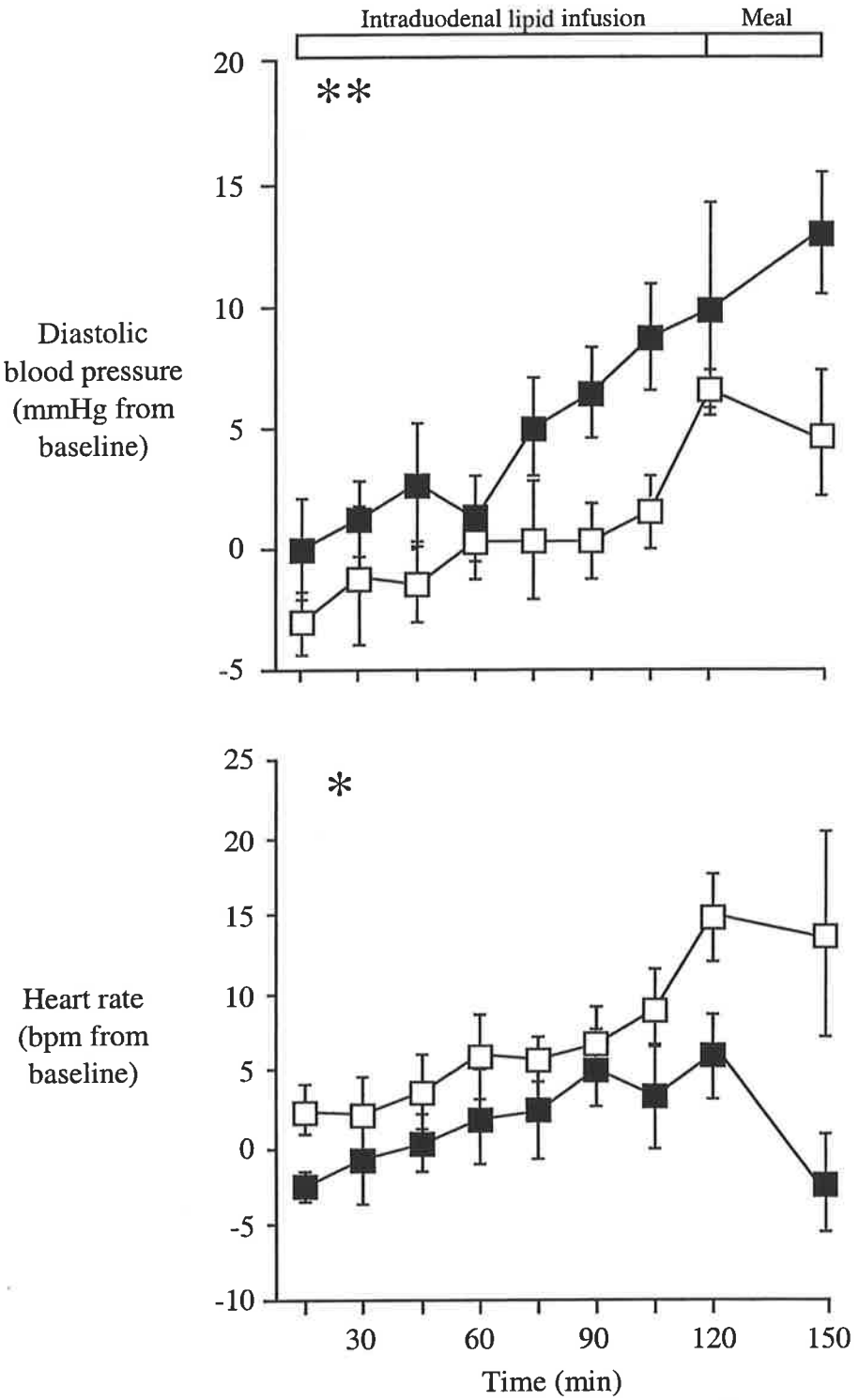

Figure 13.1 Diastolic blood pressure and heart rate during intravenous L-NAME (■) and saline (□) infusion in the fasted and fed states. Values are mean ± SEM. * $P < 0.05$ for curve, ** $P < 0.01$ for curve.

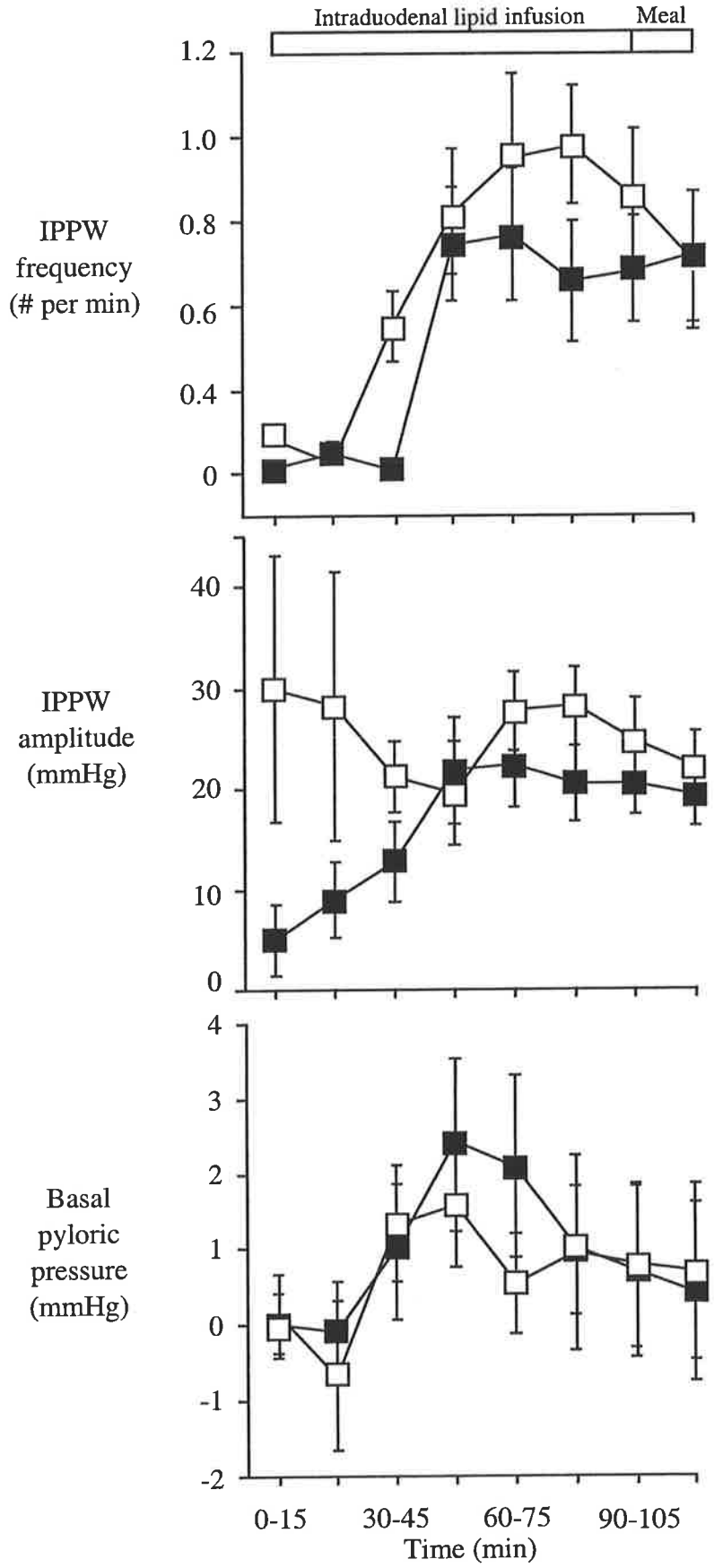

Figure 13.2 Isolated pyloric pressure wave frequency and amplitude and basal pyloric pressure during intravenous L-NAME (■) and saline (□) infusion in the fasted and fed states. Values are mean ± SEM. No significant difference between treatments.

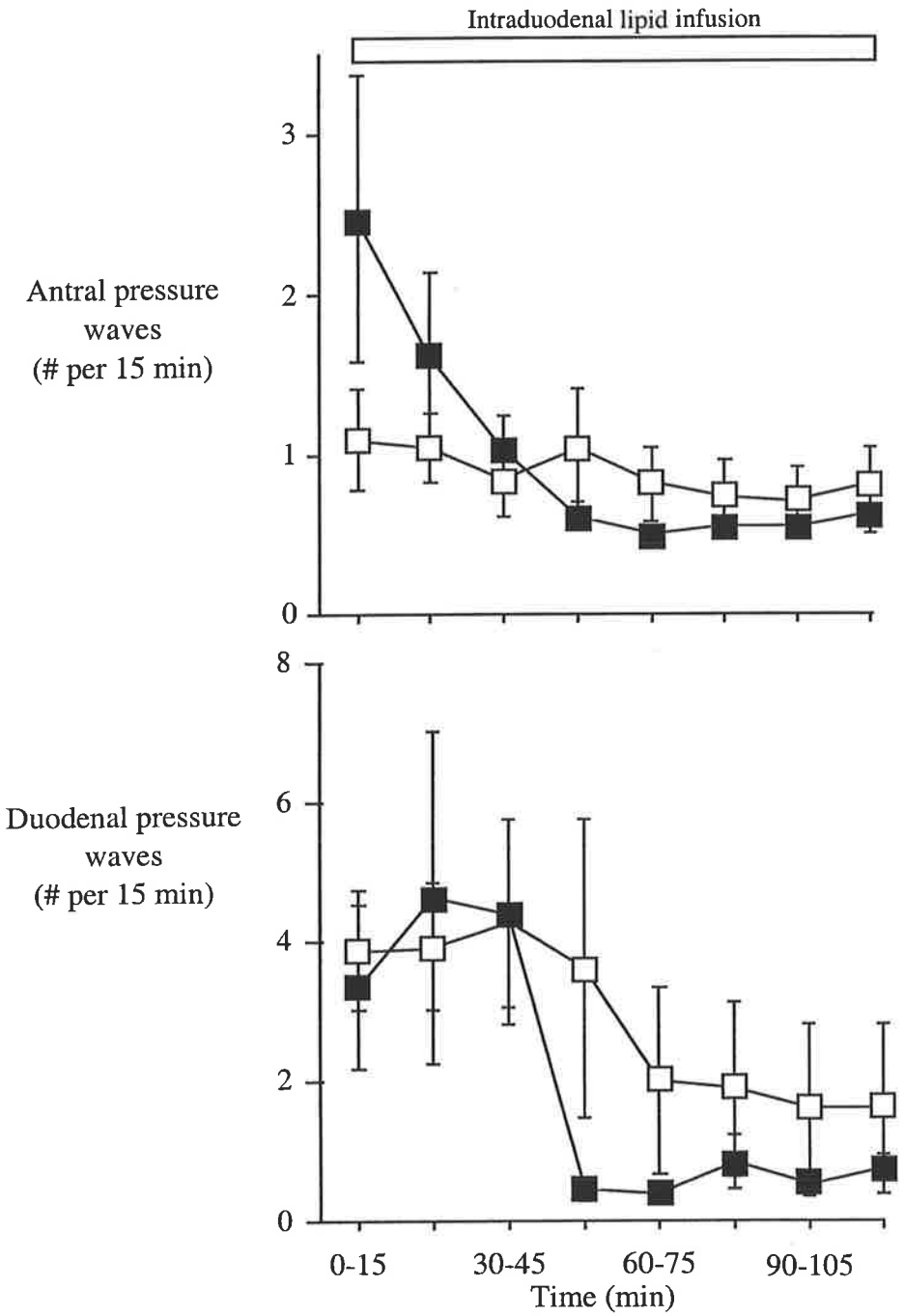

Figure 13.3 Antral and duodenal presure wave frequency during intravenous L-NAME (■) and saline (□) infusion in the fasted and fed states. Values are mean ± SEM.

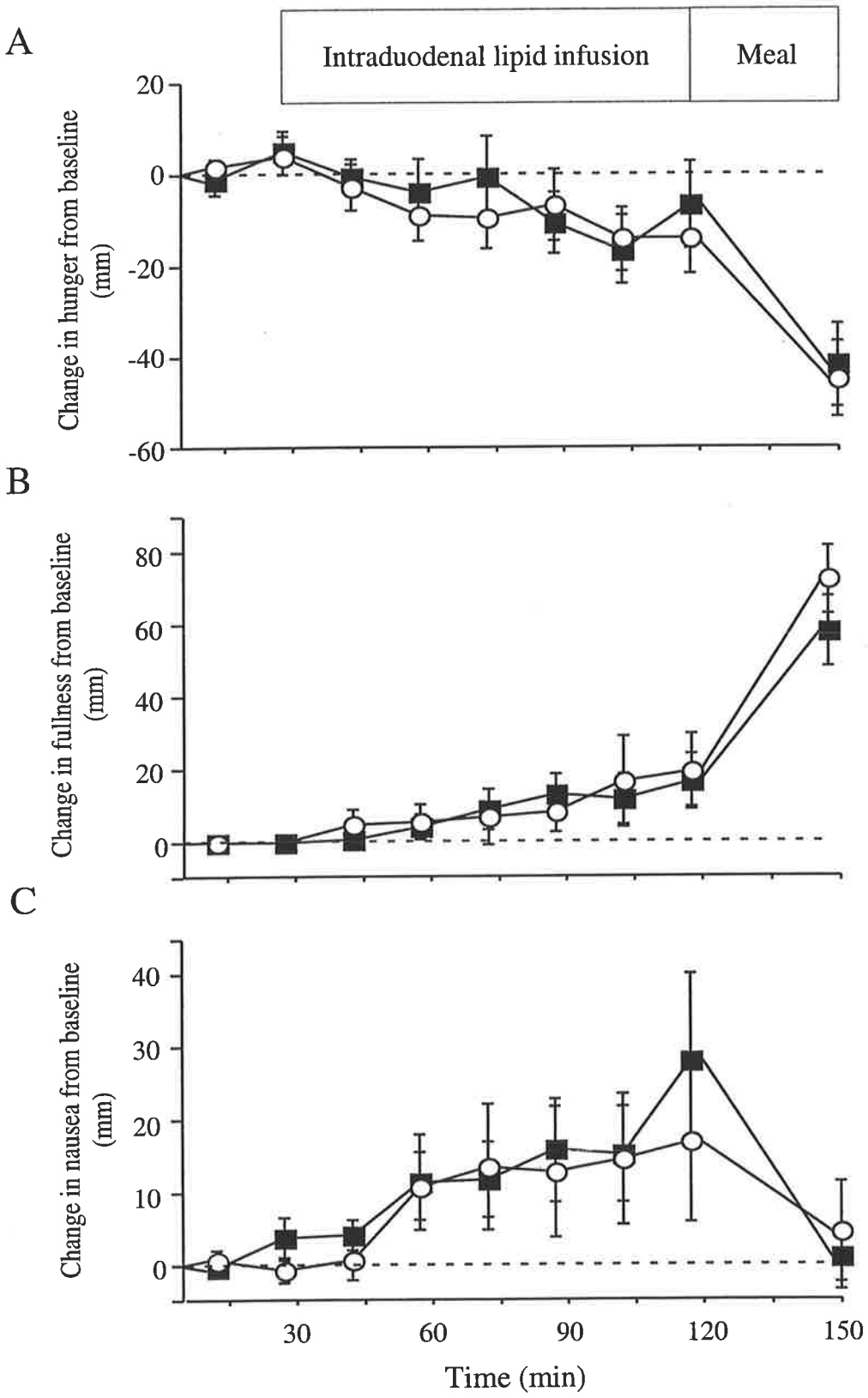

Figure 13.4 Effect of intravenous L-NAME (■) and saline (□) infusion on hunger, fullness and nausea in the fasted and fed states. Values are mean ± SEM.

Chapter 14

CONCLUSIONS

This thesis describes original studies that have evaluated the gastrointestinal regulation of appetite and food intake in humans. The potential involvement of macronutrients and nitric oxide in the regulation of feeding behaviour were investigated. The results support an effect of dietary macronutrients on specific gastrointestinal responses. The findings also suggest that in humans, in contrast to rodents, nitric oxide has only a minor role, if any, in the peripheral regulation of food intake.

The main aim of the studies reported in this thesis was to assess the role of the gastrointestinal system in the regulation of appetite and food intake in healthy humans. Two broad areas of potential interaction were evaluated (i) the effect of dietary macronutrients on gastrointestinal hormone release, upper gastrointestinal motility, appetite and food intake (Chapters 7 – 11), and (ii) the effect of nitric oxide mechanisms on gastrointestinal hormone release, upper gastrointestinal tract motility, appetite and food intake (Chapters 12 and 13).

The studies reported in Chapter 7 and 8 evaluated the effect of high fat, high carbohydrate, high protein or high protein / high fat pre-loads on appetite and food intake responses. High protein pre-loads suppressed food intake more than high fat and high carbohydrate (Chapter 7), but did not change the frequency or the time interval between subsequent meals (Chapter 8). These findings support the concept of a satiating hierarchy under strictly controlled, fixed mealtime conditions. Under less rigid conditions more akin to real life, rather than research studies, such a hierarchy appears to be less important. Future studies should be directed towards establishing the potential benefit of using such dietary manipulation in the treatment of obesity. Ideally, this would be approached by initially assessing acute food intake responses to different macronutrients in obese individuals. Additional studies to examine the chronic *ad libitum* feeding responses to interventions such as high protein diets in obese individuals would also be required. These studies would also need to assess the factors that influence compliance and adaptation to these

diets, together with potential side effects, such as exacerbation of the hyperinsulinaemia that occurs frequently in obese individuals.

The studies described in Chapter 9 assessed the feeding responses to mixed nutrient pre-loads administered at different frequencies. In this experiment, there was no effect of the pre-load on subsequent food intake. It is possible that the timing of the pre-load administration was sub-optimal, or the total amount of nutrient administered was insufficient to produce any feedback response that would lead to suppression in food intake. From these data, it is not possible to conclude whether eating frequency affects total energy intake.

A common technique used in gastrointestinal research is continuous infusion of nutrients into different parts of the gastrointestinal tract, even though nutrient delivery from the stomach into the small intestine is normally pulsatile. While this method enables different regions of the tract to be studied in isolation, it is unclear whether the responses are physiological. The study reported in Chapter 10 evaluated the effect of continuous or pulsatile intraduodenal lipid infusion on feeding, antropyloroduodenal motility and cholecystokinin release in healthy subjects. The finding that the appetite responses to intraduodenal lipid infusions are independent of the method of the delivery into the duodenum has implications for the techniques used in future research trials. These data support the continued use of continuous, intraduodenal infusions in studies that evaluate feeding responses to small intestinal lipid, a simpler technique than pulsatile delivery. The lack of a pulsatile saline infusion prevented this study from evaluating small intestinal lipid responses to pulsatile mechanical distension and pulsatile chemical exposure independently. To directly assess the antropyloroduodenal motility and feeding response to pulsatile mechanical distension independent of any chemical stimulation, future studies would need to evaluate appetite responses to pulsatile intraduodenal saline. Future studies should also assess feeding and antropyloroduodenal motility responses to other macronutrients that do not require processing before absorption.

There is considerable interest in dietary modification in patients with type II diabetes, both as a treatment for fasting hyperglycemia and obesity. Fructose has been promoted in the community as a potential alternative sweetener for diabetics. The study reported in Chapter 11 therefore evaluated the glycaemic, insulin, incretin and feeding responses to oral glucose and fructose in type II diabetics. The finding that fructose was similar to

glucose in suppressing subsequent food intake in diabetics argues against its promotion as an alternate sweetener for diabetics on the basis of suppression of food intake. It is possible that different time intervals between the pre-load and the test meal, and different pre-load concentrations could affect the extent of suppression of food intake by fructose, and these could be explored in future studies. Variations on the way in which fructose is incorporated into the diabetic diet, eg. into rather than before a meal, may be important to explore. Quantification of the plasma fructose concentrations following different concentrations of fructose in pre-loads, would establish a threshold concentration for suppression of food intake by fructose.

A growing number of studies in animals have shown that endogenously produced nitric oxide (NO) plays a role in regulating feeding behaviour and gastric motor function. The study reported in Chapter 12 assessed whether this also applies in humans using intravenous administration of two inhibitors of NO. The role of NO in lipid-induced appetite suppression and antropyloroduodenal motor responses was assessed in Chapter 13. In both studies, inhibition of endogenously produced NO had no effect on appetite, food intake or antropyloroduodenal motor function, despite systemic effects such as increases in blood pressure. Future studies in animals are needed to clarify whether the effects of NO inhibition on food intake are non-specific, and due to an aversive response, rather than a true food intake suppressive effect on feeding. It is possible that the differences in NO has a limited role in healthy, as compared to obese subjects. This requires independent evaluation.

The search for factors that regulate human appetite and feeding behaviour is hampered by the fact that appetite regulation is multifactorial, and as a consequence, appetite responses within healthy subject samples are heterogenous. Responses in obese subjects are likely to be even more varied depending on the duration and severity of obesity and any co-morbidities. In addition, the studies reported in this thesis all involved acute interventions and their extrapolation to long term appetite regulation is unknown. The application of data from the studies reported to an obese population is unclear and requires further focused and longer-term evaluation. Finally, the chances of type II statistical errors should be borne in mind when interpreting the negative studies reported.

The results reported in this thesis support and extend the conclusion that macronutrients have a role in the regulation of human appetite. They do not however, suggest that

nitrergic mechanisms are important regulators of human appetite. These data support dietary modification as a potential treatment option for diseases such as obesity, and suggest possible roles for macronutrient intake modifications. Future studies in specific areas may enable current treatments to be refined further and new therapeutic strategies to be identified.

APPENDIX 1

3 DAY FOOD DIARY

Name: _____

Please return to
Rosalie Vozzo
Department of Medicine, RAH
Telephone: 8222-5039

GUIDELINES

1. This is a diary for you to record **everything** you eat and drink for **3** days.

2. To record in the food diary we would like you to **weigh** as many foods as practical. Alternatively, use cup or spoon measures (metric) or common serves eg. **slice** of bread. **Do not** guess weights unless you are eating out and there is no alternative.

3. Record **everything** that you eat and drink from the time that you get up in the morning until you go to bed at night. **Use a separate page for each day.**

4. Fill in the diary **immediately** after eating. Try to make your eating pattern as **typical** as possible.

5. Don't forget to **record all drinks and snacks** such as tea/ coffee (with or without sugar or milk), or alcoholic of soft drinks.

6. Be as **specific** as possible eg. Specify the type of bread (white/ wholemeal), the degree of fat trimming meat, type of margarine or oil and the type of milk (whole fat or skim).

7. If you follow a **recipe**, please record it in the food diary, see **example**.

8. Indicate the **method** of cooking eg. Boiling, frying. Aslo indicate the type of oil used in frying, such as olive or canola.

9. List **separate foods on a different line** so that a ham sandwich should be recorded as bread (type), margarine (type) and ham (type) -all on separate lines.

10. Please **take the diary with you** if you eat or drink anything outside of your home.

11. Use the **example** and **flow charts** given as a guide to record your foods and drinks.

Date: *20-5-00* Day of the week: *Saturday*

Time	Description of food and drink consumed	Quantity	Measure
	EXAMPLE		
0700	*Weetbix*	*3*	*biscuit*
	milk (full cream)	*1/2*	*cup*
	bread (white, toasted)	*1*	*slice*
	margarine (low-salt, polyunsat)	*2*	*tsp*
	orange juice (unsweetened)	*1*	*glass*
1000	*coffee (instant)*	*1*	*cup*
	sugar (white)	*2*	*tsp*
	biscuits (milk arrowroot)	*2*	*biscuit*
1230	*bread (white)*	*2*	*slices*
	margarine (low-salt, polyunsat)	*2*	*tsp*
	ham (shaved, shoulder)	*1*	*slice*
	cheese (low-fat chedder)	*1*	*slice*
1730	*steak (beef, grilled)*	*200*	*g*
	potato (with skin, baked)	*200*	*g*
	beans (french, boiled)	*60*	*g*
	bread (white)	*1*	*roll*
2200	*milk (full cream)*	*250*	*ml*
	'milo'	*2*	*tsp*
	biscuits (milk coffee)	*2*	*biscuits*
	EXAMPLE RECIPE		
Recipe	*TUNA MORNAY*	*2*	*serves*
	Tuna (in brine)	*250*	*g*
	flour (plain, white)	*1*	*tbsp*
	cheese (matured)	*20*	*g*
	breadcrumbs (white)	*1*	*tbsp*
	margarine (low-salt, polyunsat)	*3*	*tsp*
	milk (skimmer)	*1/2*	*cup*

Date: ______________ Day of the week: ______________

Time	Description of food and drink consumed	Quantity	Measure

APPENDIX 2 Visual analogue questionnaire

Name: **Time:**

Please indicate how you are feeling at this moment by placing a **vertical mark** at the appropriate point on each scale below. Please **do not** make a cross or a sloping mark. Mark **all** scales.

Not nauseated _____ Nauseated
Drowsy _____ Alert
How bloated do you feel ?
Very _____ Not very
Calm _____ Anxious
Tired _____ Energetic
Muddled _____ Clear-headed
Withdrawn _____ Sociable
Empty _____ Full
Happy _____ Sad
Strong _____ Feeble
Efficient _____ Inefficient
How much food do you think you could eat ?
None _____ A large amount
Antagonistic _____ Friendly
Hungry _____ Not hungry
Angry _____ Not angry
Bored _____ Not bored
Satiated _____ Not satiated
How strong is your desire to eat
Weak _____ Strong
Restless _____ Settled
Cheerful _____ Miserable
Bored _____ Interested
Confident _____ Helpless
Grouchy _____ Tolerant
How happy are you to be participating in this study
Very _____ Not happy

Yoghurt rating questionnaire

Name: **Time:**

Please indicate how you are feeling at this moment by placing a **vertical mark** at the appropriate point on each scale below. Please **do not** make a cross or a sloping mark. Mark **all** scales.

How tasty do you think the yoghurt was ?

Extremely tasty _____ Not tasty

How sweet do you think the yoghurt was ?

Extremely sweet _____ Not sweet

How much more yoghurt do you think you could eat ?

None _____ A large amount

How creamy do you think the yoghurt was ?

Extremely creamy _____ Not creamy

How strong is your desire to eat food other than yoghurt ?

Weak _____ Strong

How much food do you think you could eat ?

None _____ A large amount

How pleasant tasting did you find the yoghurt ?

Very pleasant _____ Not pleasant

The yoghurt was acceptable in texture ?

Agree _____ Disagree

Rate the colour of the yoghurt

Intense _____ Not intense

APPENDIX 3 Energy content and macronutrient composition of test foods served as a buffet lunch[1]

Food item	Weight (g)	Energy (kcal)	Energy (kJ)	Fat (g)	CHO[2] (g)	Protein (g)
White Bread	276	754	3155	6	149	25
Brown Bread	312	786	3288	7	153	26
Sliced Ham	112	103	432	4	0	17
Sliced Chicken	100	162	677	7	0	25
Sliced Cheese	80	212	886	12	0	25
Tomato	100	13	56	0	2	1
Cucumber	100	11	44	0	2	1
Lettuce	100	6	27	0	0	1
Fruit Salad	91	38	161	0	9	1
Ice Cream	84	99	416	3	16	4
Apple, Red Delicious	140	71	295	0	18	0
Banana	101	86	362	0	20	2
Orange Juice	263	85	357	0	19	2
Iced Coffee	250	152	635	4	22	8
Mayonnaise	24	89	372	8	5	0
Margarine	20	145	606	16	0	0
Milky Way Bar	35	145	606	6	22	2
Salted Peanuts	40	250	1048	21	6	10
Boiled Egg	100	151	632	11	0	13
3 Bean Mix Salad	250	213	890	1	36	16
Total - Omnivore	2228	3207	13423	95	442	148
- Vegetarian	2282	3196	13380	94	459	131

[1]Amounts given as served with the normal buffet excluding eggs and 3 bean mix salad and the vegetarian buffet excluding sliced ham and chicken. Total percentages of energy from fat, carbohydrate, and protein, respectively are 26.1%, 52.6%, & 18.8% and 25.9%, 54.9%, & 16.7% for normal and vegetarian buffets, respectively.

[2]CHO = carbohydrate

APPENDIX 4

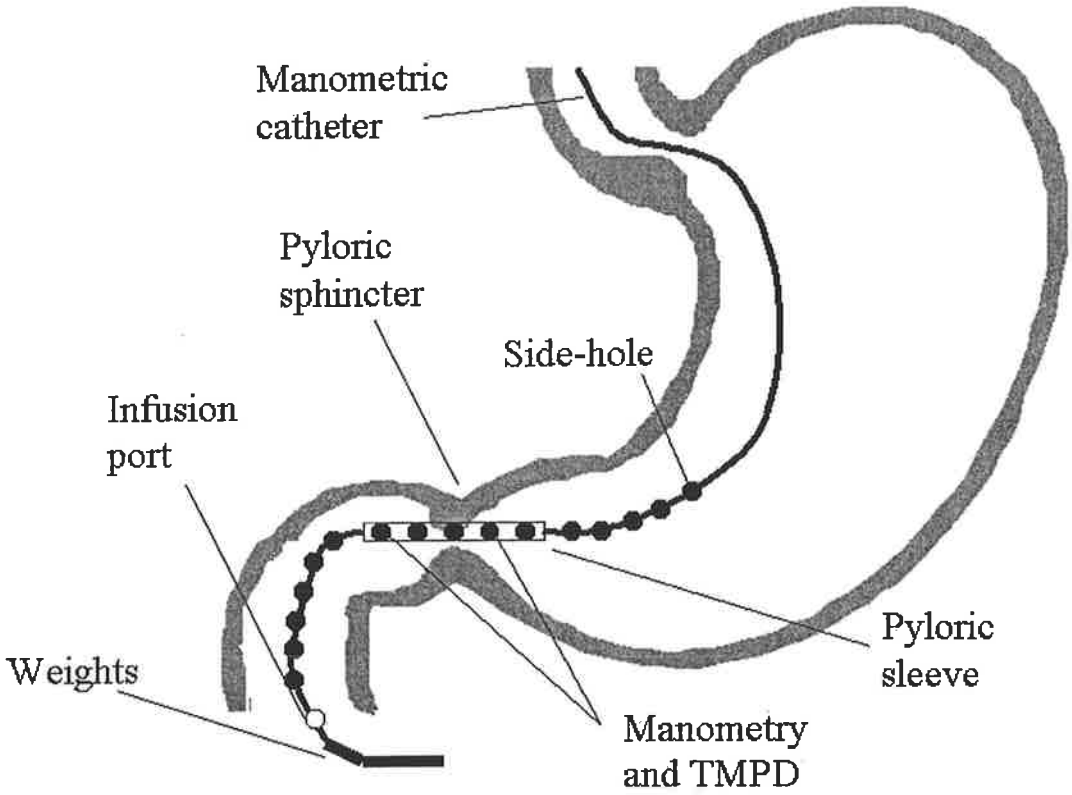

Diagram of multi-lumen catheter used in the intraduodenal nutrient infusion studies (Chapter 10 and 13). Side-holes are 1.5 cm apart. An infusion port was located 14 cm distal to the middle of the pyloric sleeve. TMPD; transmucosal potential difference.

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