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THE EFFECT OF MANIPULATING THE MACRONUTRIENT COMPOSITION OF MEALS ON POSTPRANDIAL LIPID METABOLISM

BY

JANET BENNOSON

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

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THE EFFECT OF MANIPULATING THE MACRONUTRIENT COMPOSITION OF MEALS ON POSTPRANDIAL LIPID METABOLISM

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Dietary lipid intake is directly related to the levels of circulating lipids and lipoproteins, and is associated with the onset of obesity, cardiovascular disease and insulin resistance. Current evidence suggests that the magnitude and duration of postprandial lipaemia (PPL), and subsequent effects on substrate oxidation following a meal may be manipulated by altering the macronutrient composition of the meal, yet few studies have examined both circulating lipids and lipid oxidation in one study. The aims of the research were to examine the effect of manipulating the macronutrient composition of a meal on postprandial lipid metabolism paying particular attention to the control of lipid oxidation.

Indirect calorimetry and plasma concentrations of lipids were used in conjunction with stable isotope tracer methodology to examine the postprandial metabolism of dietary lipid following a series of modified testmeals (lipid and carbohydrate content) containing a labelled triacylglycerol ([1,1,1-^{13}C]tripalmitin) in a group of healthy, young subjects. Stool and breath specimens were collected and analysed for their ^{13}C-enrichment, whilst plasma specimens were collected and analysed for lipid concentrations and ^{13}C-enrichment.

The magnitude of intra-individual variability in all measures was small compared to previous studies, and was used to conduct power analysis. The magnitude of PPL increased with the consumption of a high fat meal, and a low sucrose meal, with subsequent increases in lipid oxidation. In contrast, PPL decreased with the addition of 50g of sucrose, whilst the duration of PPL was extended with 100g of sucrose. The addition of sucrose suppressed endogenous lipid oxidation, whilst exogenous lipid oxidation decreased only with 100g of sucrose. The mechanism relating PPL and substrate oxidation remains unclear, however, the results suggest that the rate of fatty acid oxidation may have a 'negative feedback' effect on circulating lipid concentrations resulting in raised PPL when lipid oxidation is suppressed. By manipulating the macronutrient composition of the meal, it is possible to alter the rate of lipid oxidation and therefore the circulating lipid response, which may in turn manipulate the relationship between dietary lipid and disease.

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PUBLICATIONS

Unless otherwise indicated by acknowledgements or references to published literature, the work contained in this thesis is the work of the author. The findings reported in this thesis have been reported, in part, in the following publications:-

Bennoson J., Sassi K., Jones A.E. & Wootton S.A. (1999). Alteration in postprandial lipid metabolism with increasing sucrose content of testmeal. *Proc. Nutr. Soc.* ***(in the press)***

Humayun M.A., Jones A.E., Bennoson J. & Wootton S.A (1999). Impaired postprandial lipid metabolism in middle aged men. *Proc. Nutr. Soc.* ***(in the press)***

Bennoson J., Humayun M.A., Jones A.E., Hounslow A. & Wootton S.A. (1999). Within-individual variability in the gastrointestinal handling and metabolism of [1,1,1-^{13}C]tripalmitin. *Proc. Nutr .Soc.* **58: 25A**

Humayun M.A., Bennoson J., Jones A.E., Stolinski M., Hounslow A. & Wootton S.A. (1999). Within-individual variability in postprandial lipaemia following oral ingestion of [1,1,1-^{13}C]tripalmitin in healthy, young subjects. *Proc. Nutr. Soc.* **58: 35A**

Bennoson J., Jones A.E., Humayun M.A. & Wootton S.A. (1998) Influence of lipid content of testmeal on gastrointestinal handling and metabolic partitioning of dietary lipid in young men. *Biochem.Soc.Trans.* **26: S188**

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MAIN ABBREVIATIONS USED IN THE TEXT

ATP	adenosine triphosphate
AUCi	area under the curve measured from baseline
AUC_0	area under the curve measured from zero
BMI	body mass index
BMR	basal metabolic rate
CETP	cholesteryl ester transfer protein
CF	continuous flow
CHD	coronary heart disease
CHO	carbohydrate
CO_2	carbon dioxide
CVD	cardiovascular disease
FABP	fatty acid binding protein
FFA	free fatty acid
GC	gas chromatography
GI	gastrointestinal
HDL	high density lipoprotein
HSL	hormone sensitive lipase
IRMS	isotope ratio mass spectrometry
LBM	lean body mass
LDL	low density lipoprotein
LPL	lipoprotein lipase
MAG	monoacylglycerol
MUFA	mono-unsaturated fatty acid
NEFA	non-esterified fatty acid
NIDDM	non insulin dependent diabetes mellitus
O_2	oxygen
PDB	pee dee belemnite
PUFA	polyunsaturated fatty acid
SFA	saturated fatty acid
TAG	triacylglycerol
TRL	triglyceride rich lipoprotein

TUFA	trans unsaturated fatty acid
VLDL	very low density lipoprotein
VO_2	Whole body oxygen consumption
VCO_2	Whole body breath carbon dioxide excretion

CHAPTER 1

Introduction

1.1 Background to research

Epidemiological, prospective and clinical evidence suggests that plasma triacylglycerol (TAG) concentration is an important risk factor in the pathogenesis and progression of cardiovascular disease (CVD) [Groot *et al.*1991; Patsch *et al.*1992; Hokanson and Austin, 1996], although the precise way in which TAG metabolism may affect CVD remains unclear. The magnitude and timecourse of postprandial lipaemia appears to be affected by age, gender and body composition [Cohn *et al.*1988; Lewis *et al.*1990], and has been shown to be increased in patients with abnormalities of lipid and carbohydrate metabolism such as insulin resistance [Akanji *et al.*1992; Griffin & Zampelas, 1995; Jeppesen *et al.*1995]. One of the primary reasons why the mechanisms underlying the perturbations of lipid metabolism and disease remain unclear is due to a lack of differentiation between dietary and endogenous lipid in the circulation.

The magnitude and timecourse of postprandial lipaemia and subsequent substrate oxidation has been shown to be affected by dietary factors [Roche, 1999]. By manipulating the macronutrient content of foods, it may be possible to change the direction and magnitude of lipaemia, thereby in turn offering a possible mechanism to decrease the risk of disease. The studies in this area have produced conflicting results depending upon the amount and type of macronutrient examined. Increasing the amount of lipid within a meal has been shown to increase postprandial lipaemia, with the magnitude dependant upon the amount of lipid consumed [Murphy *et al.*1995; Dubois *et al.*1998], however, the effect of increasing the lipid in a meal on substrate oxidation has not been extensively studied. The majority of literature has concentrated on high-fat diets [Flatt, 1985], suggesting an inability of lipid oxidation to rise in response to increased lipid intake. The addition of carbohydrate to a meal may have profound effects on the lipaemic responses to a meal over the postprandial period [Roche, 1998], however the direction and magnitude of change appears to be dependant upon the type and amount of carbohydrate consumed. The addition of fructose or sucrose to a meal has been shown to amplify postprandial TAG concentration [Grant *et al.*1994], whilst glucose has been

reported to have a hypotriglyceridemic effect on lipaemia [Cohen & Berger, 1990]. None of the studies reported in the literature have examined postprandial lipaemia and substrate oxidation with the additional use of stable isotopes to differentiate between the meal lipid and endogenous lipid, to make assumptions as to the role of meal composition in lipid metabolism.

The underlying central hypothesis to the present research was that:-

Manipulation of the amount and type of macronutrient within a meal will affect the residence time of exogenous and endogenous lipids in the circulation in terms of appearance, clearance and entrapment of fatty acids to the tissues. As a consequence the rate and extent of oxidation of substrates will differ in the manipulation of the ratio of exogenous and endogenous fatty acid oxidation. This information will enable the development of a conceptual framework in which it would be possible to determine how altering the lipid and carbohydrate components of the meal or diet may alter lipid metabolism, and by inference the possible resultant effects on cardiovascular disease, insulin resistance and obesity can be considered.

The research described in the thesis examined how altering the macronutrient composition of meals would affect the magnitude and duration of postprandial lipaemia, and the subsequent partitioning of lipid towards oxidation or storage. With the use of stable isotopes, further examination of the role of exogenous and endogenous lipids in the process was conducted. In particular, attention was directed towards the effect of increasing the lipid content of a meal, and manipulating the sucrose content of a meal, and the resultant effects on the appearance and clearance of both exogenous and endogenous lipid in the circulation, and lipid oxidation. The aim of the research was to develop an understanding of the metabolic responses to meals with various macronutrient compositions in healthy adults.

1.2 Thesis Outline

The thesis is divided into chapters beginning with a concise introduction to the field of research (chapter 1); a review of the literature concentrating on dietary lipid,

lipid metabolism and previous work examining manipulation of the macronutrient composition of foods, and highlighting the limitations of existing knowledge (chapter 2); methods used during the research and validation work to justify the use of the methods (chapter 3); an account of the determination of the magnitude of intra-individual variability in measures of postprandial lipid metabolism and the use of power analysis in metabolic trials (chapter 4); an account of the effect of increasing the lipid content of a meal on measures of postprandial lipaemia and substrate oxidation (chapter 5); an account of the effect of increasing the sucrose content of a meal on measures of postprandial lipaemia and substrate oxidation (chapter 6) and a general discussion (chapter 7). Additional data are shown in the appendices (chapter 8).

CHAPTER 2

Review of the Literature

2.0 Introduction

The purpose of the following chapter is to introduce the area of research presented in the thesis, and to identify areas of our understanding which remain unclear or studies which offer conflicting interpretations. The review of literature is presented in sections discussing dietary lipid; the role of dietary lipid in disease; the digestion, absorption and postprandial metabolism of lipid; macronutrient balance and substrate oxidation, the effect of the postabsorptive state and postprandial state on lipid metabolism; the effect of altering meal composition on lipid metabolism, and finally a section on the use of stable isotopes in research. The review is completed with the aims of the present research and a central working hypothesis.

2.1 Dietary Lipid

The average UK male consumes 102.3g of dietary lipid on a daily basis, and an average female 73.5g per day. Of the total lipid consumed, the majority is in the form of triacylglycerols (TAG), which account for 90g per day in men. In contrast phospholipids provide 4-8g, glycolipids 1g, and cholesterol 350-450 mg per day [Gregory *et al.* 1990]. The major contributors to lipid in the typical diet are meat and meat products, dairy products, spreads and cooking oils. Table 2.1 shows the percentage contribution of food groups to the average daily intake of total fat and fatty acids in the UK. Dietary lipid contributes 37.6% of total energy for men, and 39.3% for women, or 40.4% and 40.3% respectively of food energy. Of the fatty acids found in TAG, saturated fatty acids (SFA) account for 41% of the total, monounsaturated (MUFA) 30%, polyunsaturated (PUFA) 15%, and trans unsaturated fatty acids (TUFA) 5.5% [Carey 1983; Gurr 1988; Gregory *et al.* 1990].

Table 2.1 Average daily contributions of fatty acid type to typical foods eaten in the UK divided into saturated and unsaturated fatty acids (% of total fatty acids) [Gregory *et al.*1990]

	Total Lipid	SFA	TUFA	MUFA	PUFA	
					n3	n6
Milk/milk products	15	23	10	12	2	6
Fat spreads	16	17	30	11	23	15
Meat/meat products	24	23	18	31	17	19
Fish	3	2	1	3	4	14
Eggs	4	3	2	5	4	2
Cereals	19	18	27	18	22	17
Vegetables	11	6	6	12	24	22
Fruit/nuts	1	0	0	1	2	1
Sugar/sweet products	3	4	3	2	1	1

There are over forty fatty acids found in nature, which give diversity and chemical specificity to the natural fats similar to the specificity given to proteins via amino acids, which are categorised depending on their chain length and saturation. In table 2.2, the more abundant fatty acids are shown with the chain length shown followed by the number of double bonds. Of the major dietary long chain fatty acids palmitic acid is the most common SFA and is present in the majority of lipids, particularly abundant in palm oil and animal products [Padley *et al.*1986].

The amount of lipid in our diets has decreased over recent years with a decrease in the consumption of milk and cream, cheese, eggs and meat [British Nutrition Foundation, 1992]. This has been in association with a decrease in total energy intake such that lipid intake as a proportion of energy has remained fairly constant. The contribution made by the various fatty acids to the diet has changed, however, with the SFA intake decreasing and the PUFA intake increasing, resulting in an increase in the polyunsaturated to saturated (P:S) fatty acids ratio of the diet to the current value of 0.4 for mean and 0.38 for women [Gregory *et al.*1990].

Table 2.2 Types of fatty acids found in the diet, shown by name and nomenclature

Systematic name	Common name	Abbreviation
Saturated:		
Butanoic acid	Butyric acid	$C_{4:0}$
Hexanoic acid	Caproic acid	$C_{6:0}$
Octanoic acid	Caprylic acid	$C_{8:0}$
Decanoic acid	Capric acid	$C_{10:0}$
Dodecanoic acid	Lauric acid	$C_{12:0}$
Tetradecanoic acid	Myristic acid	$C_{14:0}$
Hexadecanoic acid	Palmitic acid	$C_{16:0}$
Octadecanoic acid	Stearic acid	$C_{18:0}$
Icosanoic acid	Arachidic acid	$C_{20:0}$
Docosanoic acid	Behenic acid	$C_{22:0}$
Monunsaturated:		
Cis-9-hexadecenoic acid	Palmitoleic acid	$C_{16:1}$
Cis-9-octadecenoic acid	Oleic acid	$C_{18:1}$
Trans-9-octadecenoic acid	Elaidic acid	$C_{18:1}$
Cis-9-eicosanoic acid	Gadoleic acid	$C_{20:1}$
Cis-13-docosenoic acid	Erucic acid	$C_{22:1}$
Polyunsaturated:		
9,12 octadecadeienoic acid	Linoleic acid	$C_{18:2}$
9,12,15 octadecatrienoic acid	α- Linolenic acid	$C_{18:3}$
5,8,11,14 eicosatetraenoic acid	Arachidonic acid	$C_{20:4}$
Eicosapentaenoic acid		$C_{20:5}$
Docosahexaenoic acid		$C_{22:3}$

[From Padley *et al.*1986; Passmore and Eastwood, 1986]

Despite the decrease in the consumption of dietary lipid, and the increase in the polyunsaturated to saturated fatty acid ratio, the prevalence of dietary lipid associated diseases such as obesity, cardiovascular disease and insulin resistance continue to increase. The following section will introduce the association between dietary lipid and disease and in particular will show how little is known regarding the mechanisms underlying such disease.

2.2 Lipids and Disease

In recent years a substantial amount of research has focused on possible links between dietary lipid and diseases such as obesity, cardiovascular disease and insulin resistance. There are many studies available in the literature examining the role of dietary lipid in disease, in particular cardiovascular disease (CVD), and several authors have suggested mechanisms for the involvement of lipid in such disease. The following section will concentrate on the role of lipids in disease and examine the evidence for a mechanism linking the two.

2.2.1 Obesity

Currently in the UK 16% of men, and 17.5% of women are obese (BMI>30kg/m^2), whilst 45% of men and 35% of women are overweight (BMI 25-30 kg/m^2) [Colhoun & Prescott-Clarke 1996]. This represents a doubling in obesity since the early 1980's, with an increase in the recognition of obesity as an important contributor to morbidity and diminished quality of life [Jebb, 1999]. Obesity will only develop if energy intake exceeds energy expenditure over a prolonged period of time, but the mechanisms underpinning increases in energy intake or decreases in energy expenditure remain elusive [Jebb, 1997]. However two important questions remain unanswered. Firstly, will changes in dietary composition alter the gain in fat stores and therefore result in a tendency toward obesity? Secondly, are diet-induced adaptations in energy expenditure defective in some subjects, predisposing them to obesity [Danforth, 1985]? The consumption of lipid within the diet is central to the onset of obesity, with different individuals having different metabolic capabilities to cope with lipid loads [Golay and Bobbioni 1997], however what is not known are the factors underlying the partitioning of lipid. Why does one individual become a 'fat storer' whilst another is a 'fat burner'?

High carbohydrate diets have been shown to be protective against obesity, and help to increase weight loss in those already suffering from obesity [Astrup and Raben 1995], although this should be aimed at increasing consumption of complex carbohydrates rather than simple sugars [Prentice and Poppitt 1996]. The introduction of

a high carbohydrate diet would also imply a decrease in lipid consumption, which may account for the weight loss although this point is often overlooked in such studies. The gain in weight seen in obesity is not due to a decreased metabolic rate in the obese as is often thought as the obese have an increased rate of energy expenditure. The reason for the increase in energy expenditure may be due to the increased muscle mass [Ravussin, 1982], but other mechanistic reasons have not been extensively studied. Higher rates of energy expenditure would suggest that the obese also display a higher rate of substrate oxidation. Obese and overweight women were shown to have significantly higher postprandial lipid oxidation in a previous study [Jones, 1996] which was due to an impairment of the postprandial insulin mediated suppression of lipolysis of adipose tissue TAG in the obese resulting in an increased supply of endogenous NEFA for oxidation, with exogenous non esterified fatty acids (NEFA) channelled towards storage rather than oxidation. Despite the observations from the study, the mechanisms involved in the process were not tested fully as no data on circulating NEFA levels was available. In addition, there was no differentiation between endogenous and exogenous lipids to justify the argument. What is clear from other work is that NEFA concentrations are elevated in the postprandial period in the obese, and that this could provide a mechanism for the enhanced lipid oxidation observed [Coppack *et al.*1992]. The obese have a reduced capacity for adipose tissue hormone sensitive lipase (HSL) and lipoprotein lipase (LPL) to respond to insulin in the postprandial state suggesting a failure to suppress the release of NEFA into the circulation [Coppack *et al.* 1992]. It has been suggested that the increased NEFA release could contribute to glucose intolerance seen in the obese [Roust and Jensen, 1993] The obese have an increased fat mass, which with the increased lipolysis provides a constant surplus of free fatty acids, which compete with glucose for oxidation and may contribute to insulin resistance.

Individuals with a genetic predisposition to obesity have been found to respond with a more marked weight gain than control subjects when exposed to an increased dietary fat content. The discovery of the *ob* gene has created a vast amount of research into the genetics of obesity [Farooqi *et al.* 1998]. Studies have shown that a genetically determined preference for high fat foods may result in consumption of a high fat diet, and that obesity has been shown to be caused as an adaptation to a high-fat diet [Astrup *et al.* 1994]. However, it has been shown that an increase in dietary fat intake does not

result in an increase in fat oxidation suggesting a channelling of lipid to storage, increased adipose tissue mass, and a greater possibility of obesity occurring [Schutz *et al.*1989; Astrup *et al.*1994].

The magnitude and duration of postprandial lipaemia is raised in obese patients when compared to healthy controls, with an increased secretion of NEFA to the circulation due to the insulin insensitivity described before [Coppack *et al.* 1992]. Obesity impairs health and longevity through an effect on related diseases such as insulin resistance and CVD [Garrow, 1988]. Raised postprandial lipaemia may result in the development of atherosclerosis by a longer residence time of TAG in the circulation permitting a greater opportunity for neutral lipid exchange, which is discussed in section 2.2.3.

The obese have an exaggerated postprandial lipaemic response resulting in an increased residence time for lipids in the circulation, with an increase in net lipid oxidation. What is unclear is how does obesity result in an increased lipaemia? Is this due to an increase in endogenous NEFA release from adipose tissue, a decreased clearance of dietary TAG due to insulin resistance effects on LPL, an impairment in the entrapment of NEFA from the circulation, an impairment in the oxidation of lipid or a combination of all of these suggestions? Are there disturbances in the control of lipid oxidation in the obese? By understanding the mechanisms underlying obesity, it may be possible to suggest ways of decreasing the prevalence and decrease the risk of CVD and insulin resistance.

2.2.2 Insulin Resistance

Diabetes mellitus is a disorder of metabolic control arising either through a lack of insulin (Type I) or an insensitivity of tissues to insulin (Type II). Type II diabetes, or non-insulin dependant diabetes mellitus (NIDDM) usually appears in middle age or later in patients who are obese, and in whom hyperglycaemia can usually be controlled by dietary means alone [Frayn, 1996]. When obesity occurs, tissues become resistant to the actions of insulin, therefore glucose concentration in plasma rises and insulin is released in increased quantities from the pancreas [Coppack *et al.*1992]. Epidemiological evidence has shown that hyperinsulinaemia is a risk factor for morbidity and mortality in

cardiovascular disease [Smith, 1994] but that this can be controlled by dietary modulation alone. NIDDM patients have a spectrum of lipid abnormalities that may confer an increased risk of developing disease, but the pattern of dyslipidemia is different from the non-diabetic population [Coppack, 1997].

Studies of postprandial lipid metabolism have shown an increased TAG response to a meal in NIDDM patients [Lewis *et al.*1991; Chen *et al.*1993; Svyanne *et al.*1994]. The observation was independent of whether the patient was normotriglyceridemic or hypertriglyceridemic in terms of fasting blood measurements. A significant proportion of the elevation in TAG postprandially was shown to be of hepatic origin (VLDL-TAG), resulting in a competition between chylomicron-TAG (CM-TAG) and VLDL-TAG for removal by LPL [Lewis *et al.*1991]. The NEFA response to a meal in NIDDM patients resulted in a late postprandial surge of NEFA in the circulation that lasted for up to 14 hours following a meal, probably derived from the lipolysis of CM-TAG [Lewis *et al.*1991] but an impairment of entrapment. The ability of tissues to re-esterify incoming NEFA rapidly and to inhibit the lipolysis of stored TAG appears to be an important determinant of the rate of clearance of NEFA from the circulation. Insulin is an important determinant of the rate of NEFA esterification once they enter adipose cells or hepatocytes [Boden *et al.*1993], and therefore a key determinant of NEFA disposal postprandially. In NIDDM patients the insulin resistance observed may result in an impairment of this process resulting in an elevation of circulating NEFA. Coppack *et al.*[1992] support this theory by demonstrating a greater postprandial plasma concentration of NEFA and increased adipose tissue release of NEFA in obese insulin resistant patients, suggesting resistance to the antilipolytic effect of insulin in these individuals. An elevation of NEFA in the circulation could contribute to the production of VLDL-TAG in the liver, and therefore result in an elevated postprandial TAG response. Lewis *et al.*[1995] demonstrated that plasma NEFA elevation acutely stimulates hepatic VLDL production. NIDDM patients therefore enter a 'vicious cycle' where elevated VLDL-TAG competes with chylomicron TAG for removal, leading to elevated postprandial TRL, resulting in an elevated NEFA concentration which results in a further increase in hepatic VLDL-TAG secretion [Lewis and Steiner, 1996].

In summary, NIDDM patients are known to have an elevated postprandial TAG and NEFA response, which it has been suggested may be due to a lack of suppression of

NEFA release from adipose tissue resulting in enhanced VLDL-TAG secretion. However, the importance of the NEFA elevation in stimulating VLDL-TAG production has not been determined. In addition, circulating NEFA have not been quantified to examine the relative contribution from dietary and endogenous sources. Several questions remain unanswered; Is the elevation in NEFA due to a lack of suppression of NEFA release from adipose tissue, or a failure of tissues to entrap dietary derived NEFA? Is the raised glucose concentration observed in NIDDM due to a failure in glucose uptake or merely due to the excess NEFA available in the circulation? How does the excess circulating NEFA affect postprandial lipid oxidation? Does an increase in circulating NEFA result in an increased oxidation, and by what mechanism? Do NIDDM patients demonstrate an 'upregulation' of lipid oxidation?

2.2.3 Cardiovascular Disease

Cardiovascular disease is the collective term for diseases affecting the cardiovascular system and includes coronary heart disease (CHD) and stroke. Mortality from CHD in the UK is amongst the highest in the world, accounting for 30% of deaths in males, and 23% in females in 1990 [Department of Health, 1994]. The underlying basis for CVD is a combination of atherosclerosis and thrombosis [Department of Health, 1994]. Atherosclerosis occurs when the arterial wall becomes thickened with plaques as a result of excessive accumulation of cholesterol-rich lipid deposits, resulting in endothelial damage and the narrowing of the lumen, resulting in reduced blood flow. The discovery that cholesterol was integral to the formation of fatty plaques resulted in much research based on the role of dietary cholesterol in the progression of CVD. More recently, epidemiological evidence has suggested that it is not cholesterol which is important in CVD but the concentration of TAG in the circulation leading to the plasma TAG being classed as an independent risk factor for CVD [Hokanson & Austin, 1996]. However, despite the importance of measuring post-absorptive TAG levels, the complex processes involved in the absorption and metabolism of dietary TAG should not be overlooked. It has been recognised that postprandial events may be paramount in influencing fasting TAG levels and the lipid transfer reactions which modulate the risk of developing heart disease [Griffin, 1997].

Recently studies have been published comparing lipid metabolism in normal individuals, and in those with coronary heart disease [Groot *et al.*1991; Patsch *et al.*1992]. Individuals with little or no history of CVD exhibited a peak of lipaemia at 6h postprandially, followed by a sharp decline by 8h returning to fasting states. Subjects positive for CVD showed an elevation in the magnitude and duration of lipaemia, with the differences persisting on the removal of subjects with raised fasting TAG levels [Shepherd and Packard, 1996].

Similar results were found when normotriglyceridemic and hypertriglyceridemic patients were compared with an oral fat tolerance test [Karpe, 1994]. Braun *et al.*[1997] suggested that the rise in postprandial lipaemia seen in such patients is due to a delayed clearance of TRL because of an accumulation of TAG in the circulation in the latter part of the study. However, it is not clear from this study whether the accumulation of TAG is the product of delayed entry of chylomicron TAG, delayed clearance or upregulation of endogenous TAG synthesis i.e. VLDL-TAG. Without differentiating between the classes of lipoproteins it is difficult to clearly understand the mechanisms behind raised lipaemia in disease states, and raises a series of questions. How does the elevation of plasma TAG concentration result in atherosclerosis? What effect would increasing the lipid within a meal have on the postprandial TAG response in the CVD population? Is the accumulation of TAG due to a decrease in clearance of TAG from a resistance of LPL for insulin? Or is the increase in TAG due to an impairment of entrapment of fatty acids resulting in an enhancement of VLDL-TAG secretion? In addition, there are a lack of studies in the literature which examine the role of lipid oxidation in CVD. Is the raised lipaemia due to a impairment in lipid oxidation, or does the elevation in circulating NEFA result in increased oxidation?

One question, which has at least in part been answered in the literature, is how the elevation of TAG leads to atherosclerosis. Confusion has arisen in this field of research from the discovery that it is cholesterol that accumulates in atherosclerotic plaques yet TAG, which appears to be the predominant risk factor. The processes thought to be involved in the relationship between TAG rich lipoproteins and cholesterol-rich lipoproteins, and which may explain the link between elevated TAG and accelerated atherogenesis are complex [Sethi *et al.*1993; Patsch, 1994]. Over a typical day, we spend 16-20 hours in the postprandial state with plasma TAG values above

postabsorptive levels, which will be dependent on age, gender and disease progression. Therefore the concentration of TAG in the circulation is constantly changing, whilst the cholesterol concentration remains constant [Durrington, 1998], suggesting that it is the TAG levels in plasma which are associated with the varying risk of disease.

An exaggerated response to a lipid-containing meal may be due to overproduction of chylomicrons or VLDL, or may reflect a slower clearance of these particles by the rate-regulatory step in TAG hydrolysis and removal mediated by lipoprotein lipase [Williams, 1997]. Accumulation of partially hydrolysed remnant particles may also contribute to the elevated TAG response, since due to their poor recognition by hepatic receptors, there is a reduced uptake of the cholesterol-enriched particles by receptor mediated pathways [Havel, 1994; Karpe, 1997]. The net consequence of these defects is an increased retention of TRL within the circulation, providing greater opportunity for neutral lipid exchange [Williams, 1997]. In normal human plasma, neutral lipids i.e. cholesteryl ester and TAG, are redistributed between lipoproteins by cholesteryl ester transfer protein (CETP) [Mann *et al.* 1991]. CETP mediates net transfer of cholesteryl esters from HDL to TAG containing lipoproteins such as VLDL and LDL which are probably removed by receptor mediated uptake in the liver. However the accumulation of cholesteryl esters in VLDL or chylomicron remnants may lead to the formation of atherogenic cholesteryl-ester rich remnant particles [Tall *et al.* 1986]. The net consequence of these transfers is TAG accumulation on HDL and LDL, and cholesterol on CM, VLDL and their remnants. The TAG enriched HDL and LDL act as good substrates for hepatic lipase resulting in the formation of small dense HDL and LDL [Williams, 1997]. Under normal conditions of postprandial lipaemia, i.e. normal plasma TAG concentrations, the majority of chylomicron TAG is hydrolysed and remnants are taken up by the liver for degradation, with little cholesteryl-ester transfer occurring.

However, in situations where postprandial lipaemia is raised by dietary modulation or disease, more TAG is present in the circulation for a longer duration, resulting in a greater level of cholesteryl-ester transfer and a greater production of small dense HDL and LDL particles. The small dense LDL and the cholesterol-enriched remnant are reputed to have greater atherogenic potential due to their prolonged retention in the circulation [Nigon *et al.* 1991], their ability to induce foam cell formation

[Parthasarathy *et al.* 1989], and in the case of LDL, their greater oxidisability [Chait *et al.* 1993]. Chylomicron remnants have also been shown to penetrate the endothelium in much the same way as LDL and HDL [Mamo and Wheeler 1994]. In the postprandial state, with an elevated TAG pool, the TAG content of HDL and LDL is increased via neutral lipid exchange suggesting a greater hydrolysis of these TAG rich lipoproteins and an increase in the levels of small dense LDL and HDL. In addition, the exchange and net mass transfer of neutral lipids in normolipidemic plasma are significantly influenced by the presence of NEFA [Lagrost *et al.* 1995].

In summary, CVD is known to be characterised by the formation of cholesterol enriched plaques in the arterial lumen, which result in a reduction in blood flow and ultimately a blockage in the lumen and myocardial infarction. Plasma TAG concentration, and in particular the magnitude and duration of postprandial lipaemia has been shown to be a risk factor for CVD. Despite suggested mechanisms for the role of TAG in the process of neutral lipid transfer, there is little evidence to provide a mechanism for the role of TAG in CVD and several questions remain unanswered. Is the raised lipaemia observed in CVD due to a decrease in clearance of CM-TAG, an increased secretion of VLDL-TAG or a competition between the two for hydrolysis? Is the raised lipaemia due to an impairment of entrapment of NEFA leading to an overproduction of VLDL-TAG in the liver? How does the increased availability of NEFA in the circulation affect postprandial lipid oxidation?

The progression of the diseases discussed in this section are clearly associated with dietary and endogenous lipid, and appear to be dependent upon the appearance, clearance and residence time of lipids in the circulation. In addition the role of lipid oxidation has not been extensively studied in such patient groups. In order to begin to understand the role of dietary lipid in the process of disease, it is important to understand the process of the digestion, absorption and metabolism of dietary lipid in normal, healthy individuals and to suggest ways in which these processes may be disturbed in order for disease to occur. The following section introduces what is known regarding the digestion, absorption and metabolism of lipid both in the post-absorptive and postprandial states.

2.3 The Digestion, Absorption and Postprandial Metabolism of Dietary Lipid

An overall summary of the digestion and absorption of dietary lipid is shown in figure 2.1. The events of the digestion and absorption of lipid in the postprandial state, and lipid metabolism and utilisation in the post-absorptive state will be discussed.

2.3.1 Digestion of dietary lipid

Fatty acid esters in food (mainly TAG, phospholipids and cholesteryl esters) need to be hydrolysed in the gastrointestinal (GI) tract before efficient absorption can occur. GI lipid digestion consists of three sequential steps; dispersion of fat globules, enzymatic hydrolysis and dispersion [Carey, 1983]. The major site of lipid digestion is the small intestine although the stomach is also reported to play a role with up to 30% being digested due to a prolonged storage period (2 to 4 hours) of lipid compared with other nutrients [Carey *et al.*1983]. Lipid emulsification is initiated in the stomach by shearing forces produced by muscular contractions along with potential emulsifiers such as peptic digests of dietary proteins, complex polysaccharides and membrane derived phospholipids [Carey *et al.*1983]. Enzymes in the stomach, both lingual and gastric lipases, initiate the enzymatic hydrolysis of TAG [Masoro,1977; Carey *et al.* 1983]. In the mouth lingual lipase is secreted from the glands in the tongue and soft palate, which is not thought to produce much hydrolysis in adults but may play a more important role in neonates [Carey *et al.*1983], whereas gastric lipase is secreted from the gastric glands and catalyses the cleavage of short and medium length chain fatty acids [Masoro, 1977]. Lingual lipase catalyses the conversion of TAG to free fatty acid, and di or mono-acylgycerols [Carey *et al.*1983].

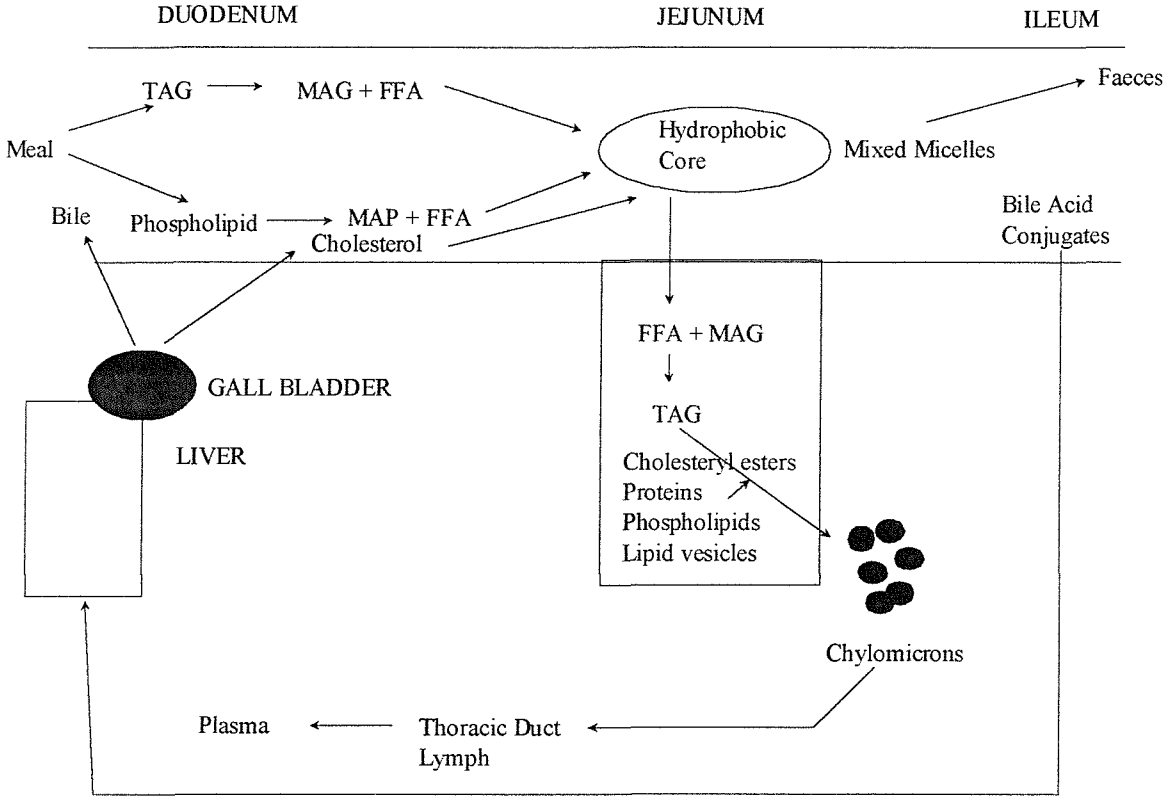
Enzymatic hydrolysis of dietary lipid in the stomach facilitates duodenal-jejunal hydrolysis in that a coarse fat emulsion is produced and binding of pancreatic lipase is encouraged. The fat emulsion enters the small intestine and is modified by mixing with bile and pancreatic juice [Carey, 1983]. The emulsion particles in the upper small intestine are generally less than 0.5μm in diameter and are extremely stable. Armand *et al.* [1994] showed for the first time that dietary lipids are present in the human stomach in the form of emulsified droplets, that gastric lipolysis may modulate the extent of

emulsification and, that at the same time, the extent of emulsification may control the rate of lipolysis. This work highlights the importance of the process of emulsification of lipids in their digestion processes. When stomach chyme is propelled through the small opening of the pyloric canal into the duodenum, the strong shear forces tear the liquid interfaces apart [Elkes *et al.* 1944].

Entry of fat into the duodenum plus the presence of acid causes the release of secretin and cholecystokinin into the bloodstream where it reaches the gallbladder and pancreas. This in turn leads to a stimulation of the flow of bile and pancreatic juice, which mix with the coarse emulsion [Patsch, 1987]. In the duodenum, peristaltic and segmental contractions continue to supply mechanical energy that increases the interfacial area of the fat droplets and as a consequence decreases the size of the particles [Senior, 1964]. The digestion and absorption of dietary lipid from the small intestine is shown in figure 2.1.

Pancreatic lipase attacks TAG molecules at the surface of large emulsion particles but before lipolysis can occur, the surface and the enzyme must be modified to allow interaction to take place. Primarily, bile salt molecules accumulate at the surface of the lipid droplet, displacing other surface-active constituents. The non-polar tails of the amphipathic molecules that stabilise small groups of non-polar molecules; predominantly TAG and cholesterol brings about emulsification, with their polar aspects facing outwards to the aqueous intestinal contents. A net repulsive action of the outward-facing polar groups also tends to split further the lipid droplets, resulting in a finer and finer emulsion [Berne & Levy, 1990]. The presence of bile salts denotes a negative charge to the oil droplets, which attracts a protein to the surface, known as colipase. The function of colipase is to attract and anchor pancreatic lipase to the surface of the droplets. Therefore, bile salts, colipase and pancreatic lipase interact in a ternary complex which also contains calcium ions necessary for the full lipolytic activity, which digests the lipid to monoglycerides and free fatty acids [Gurr *et al.* 1989].

Figure 2.1 **The digestion and absorption of dietary lipid, from small intestine to excretion of lipid in the faeces or absorption across the enterocyte into the circulation [adapted from Gurr *et al.*1989]**



In the final stages of digestion, the monoglycerides, fatty acids, bile salts and monoacylphospholipids pass into large molecular aggregates called mixed micelles. Micelles are multimolecular aggregates of bile acids (bile salts and lecithin) and the products of digestion (FFA, 2-MAG, lysophosphatides and cholesterol) [Berne & Levy, 1990]. 2-monoacylglycerols and lysophosphatides have their hydrophobic acyl chains in the interior of the micelle and their polar portions facing the surrounding water whilst the water insoluble compounds, long chain fatty acids and cholesterol are solubilised in the hydrophobic core [Harrison & Leat, 1975; Gurr, 1988]. Unabsorbed lipids are generally modified by micro-organisms in the large intestine and therefore the composition of faecal fat can be quite different to that of the diet [Carey, 1983]. Although lipid digestion has been characterised, it remains unclear how the composition of a meal affects digestion and gastric emptying. Does increasing the lipid or carbohydrate content of a meal result in an alteration in the rate or extent of lipid digestion?

2.3.2 Absorption of Dietary Lipid

Lipid absorption in man occurs predominantly in the jejunum [Gurr, 1988], but can also take place in the ileum of the small intestine. Fatty acids of chain lengths less than 12 carbon atoms are absorbed directly into the portal blood, are metabolised chiefly by beta oxidation in the liver and do not contribute to plasma lipids or adipose tissue stores [Sickenger, 1975]. However there is some evidence to suggest fatty acids of 12 carbon length are present in chylomicrons. The luminal surface of the small intestine consists of microvilli, which form a brush border, providing a large surface area for substrate absorption. The micelles diffuse among the microvilli, which allows the large surface area of the brush border membrane to participate in lipid absorption. The presence of micelles tends to keep the aqueous solution along the brush border saturated with fatty acids, 2-monoglycerides, cholesterol and other micellar contents [Berne & Levy, 1990]. The main limitation to the rate of lipid uptake by the epithelial cells of the upper small intestine is the diffusion of the micelles through an unstirred layer on the luminal surface of the brush border plasma membrane [Masoro, 1977; Berne & Levy, 1990].

Micelles dissociate at the brush border to release FFA, 2-MAG, lysophosphoglycerols and cholesterol which pass into the enterocyte. Although the uptake of MAG and FFA by the intestinal epithelial cells appears to occur by simple diffusion, specific carriers for fatty acids have been identified in a number of other tissues. It is thought that a fatty acid binding protein carries fatty acids to the smooth endoplasmic reticulum for processing [Shiau, 1981]. What is not known is whether there are specific types of fatty acid binding protein, and whether different classes of fatty acids are bound to different proteins? Also, it is unclear whether the composition of the meal consumed will affect the rate of fatty acid binding protein mediated diffusion of lipid into the enterocyte? How is the process of fatty acid binding and transport related to the extent of fatty acid oxidation and storage?.

Within the smooth ER, which is saturated with lipid following a meal, fatty acids and monoacylglycerols are re-esterified to form new TAG molecules. This occurs mainly (70%) by the *monoacylglycerol esterification pathway,* which begins with monoacylglycerol, unlike most other tissues in which the formation of TAG occurs by the phosphatidic acid pathway, which begins with glycerol-3-phosphate [Tso & Weidman, 1987]. The smooth ER generates a chylomicron from TAG, phospholipids, cholesterol and cholesterol esters along with apolipoprotein B. These large spherical vesicles (0.1um in diameter) migrate to the golgi vacuoles and are secreted from the cell by exocytosis to the intestinal fluid, secreted into the lymphatic vessels and pass via the thoracic duct into the jugular vein [Masoro, 1977; Gurr *et al.*1989; Berne & Levy, 1990]. The apolipoproteins not only help to stabilise the lipid particles in their aqueous environment but also provide a means whereby lipoproteins are recognised by tissues and their metabolism directed and controlled [Havel, 1986; Sethi *et al.* 1993]. The process of lipid absorption into the body is complex and several questions remain unanswered. How does the rate of digestion affect micellar formation and chylomicron secretion? What determines the size and quantity of chylomicrons released? How does the composition of the food consumed in the meal affect chylomicron formation? The rate and quantity of secretion of chylomicrons has the potential to influence the magnitude and duration of postprandial TAG appearance in the circulation. However, although the mechanism by which chylomicrons are formed and secreted has been examined, the factors which regulate and influence this process remain unclear.

Lipid which is not absorbed across the GI tract is excreted in stool, reported to be 5-6g/day in healthy adults [Wollaeger *et al.*1947; Southgate & Durnin, 1970; Wrong *et al.*1981; Murphy,1991]. This is equivalent to approximately 5% of lipid intake and has led to the conclusion that lipid absorption is relatively complete [Wrong *et al.*1981], and relatively constant [Walker *et al.*1973]. What is unclear is the extent to which altering the meal composition will affect the absorption of lipid, and are there upper gastrointestinal capacities for healthy individuals to absorb lipid?

2.3.3 Transport of Lipid

Water insoluble lipids are transported in plasma via protein carriers. NEFA are transported bound to albumin, whilst the other lipids are transported in lipoprotein complexes derived from several apoproteins synthesized in the liver and intestine [Berne & Levy, 1990]. The lipoprotein complexes consist of a non-polar core of TAG and cholesterol surrounded by phospholipids, cholesterol and apolipoproteins. Different classes of lipoprotein exist and are separated according to their density from chylomicrons (TAG rich); VLDL; intermediate density lipoproteins (IDL); low density lipoproteins (LDL) and high density lipoproteins (HDL).

Chylomicrons are the major transport form of dietary lipid and consist mainly of TAG [Shiau, 1981]. TAG is hydrolysed from chylomicrons by the action of lipoprotein lipase (LPL) on capillary endothelial surfaces of adipose tissue, skeletal and cardiac muscle [Frayn *et al.*1995]. The resultant fatty acids are believed to be entrapped by the adipose tissue, and re-esterified to TAG for storage, or in times of excess, are taken up by the muscle for oxidation or liver for re-esterification to TAG [Frayn *et al.*1995]. The residual lipoprotein particles, now relatively higher in cholesterol content, known as chylomicron remnants, are taken up by the liver for further degradation [Berne & Levy, 1990]. Postprandial lipaemia is a term used to describe the concentration of TAG in the circulation following a meal. What is unclear is the extent to which the transport of dietary lipid is controlled, and by what mechanism? Also what determines the uptake of fatty acids to the tissues, and is this regulated by the supply of fatty acids or demand for fatty acids?.

Very low density lipoproteins carry predominantly endogenously derived lipids

synthesised in the liver from *de novo* lipogenesis or by re-esterification of NEFA. In the circulation, VLDL are metabolised by LPL much like chylomicrons, but are believed to be suppressed in the initial postprandial period to allow efficient clearance of CM-TAG from the circulation [Potts *et al.*1995]. The extent to which LPL has a specificity for either lipoprotein is unclear. In addition, the role of VLDL-TAG in the disease process has not been examined. Low density lipoproteins act as cholesterol transporters from the liver to peripheral tissues. The uptake of LDL-cholesterol by cells has important regulatory actions on intracellular cholesterol metabolism, whereby the LDL receptor is down regulated reducing further entrance of the sterol [Berne & Levy, 1990]. Non-receptor mediated LDL degradation by macrophages also takes place and can account for two thirds of cholesterol uptake at 'normal' cholesterol levels. Macrophages which become overloaded with cholesterol form foam cells which are components of atheromatus plaques.

High density lipoproteins are synthesised in the liver and facilitate the major steps in chylomicron, VLDL, IDL and LDL movement. In the circulation they acquire apolipoproteins A and C and free cholesterol. The cholesterol is esterified by lecithin-cholesterol acyltransferase increasing the density of HDL. Some cholesterol esters remain in HDL and are transported to the liver directly whilst much is transferred to CM,VLDL and LDL and reaches the liver indirectly. Cholesterol is secreted in bile and after metabolism in bile salts [Marshall, 1988]. Despite the characterisation of the various lipoprotein classes, little is known regarding control over their movement in the circulation or their metabolism. Also how does altering the composition of a meal affect the way in which TAG is delivered to tissues by the lipoproteins, and does this affect the action of LPL?

Prior to discussing what is known regarding the transport and metabolism of lipid following a meal, it is important to understand the processes involved in lipid metabolism in the post-absorptive state. Of particular importance is the shift from the post-absorptive to the postprandial state and the complex homeostatic mechanisms controlling this event. The following section will focus on the metabolism of lipid in terms of circulating lipids, oxidation and storage of lipids in the post-absorptive state and in the postprandial state.

2.3.4 The post-absorptive state

In the post-absorptive state, the lipid from the previous meal will have been hydrolysed and taken up by the cells for storage or oxidation purposes. Non-esterified fatty acid concentration in the plasma will be around 0.5 mmol/l, and the total TAG concentration about 1 mmol/l. In contrast blood glucose concentration will remain at about 5 mmol/l, insulin varies widely between individuals but will usually be about 60 pmol/l. Glucagon will be present in the circulation at a level of approximately 20-25 pmol/l [Frayn, 1996]. Despite the small concentrations of the lipid components, this does not reflect the rapid turnover of the substrates, which are constantly being used and replaced. The concentration of NEFA in the plasma reflects their rate of release from adipose tissue, and the regulation of hormone sensitive lipase and the process of re-esterification. NEFA contribute 66% of the energy in the post-absorptive state compared to only 33% supplied by glucose.

Fasting NEFA concentrations reflect primarily the rate of liberation of fatty acids from hydrolysis of adipose tissue by the intracellular enzyme hormone sensitive lipase [Samra *et al.* 1996]. Adipose tissue hormone sensitive lipase is highly active in the post-absorptive state resulting in the release of NEFA, thought to be due to a release of the insulin-mediated suppression of the enzyme. In addition, the enzyme may be activated by the influence of glucagon and adrenaline in the plasma, and noradrenaline released from sympathetic nerve terminals within adipose tissue. The rate of release of NEFA is also regulated by the process of fatty acid re-esterification within the adipose tissue. As glycolysis will be occurring at a reduced rate in the post-absorptive state, the glycerol 3-phosphate required for re-esterification will be unavailable, suggesting that most of the fatty acids will escape from the adipocyte.

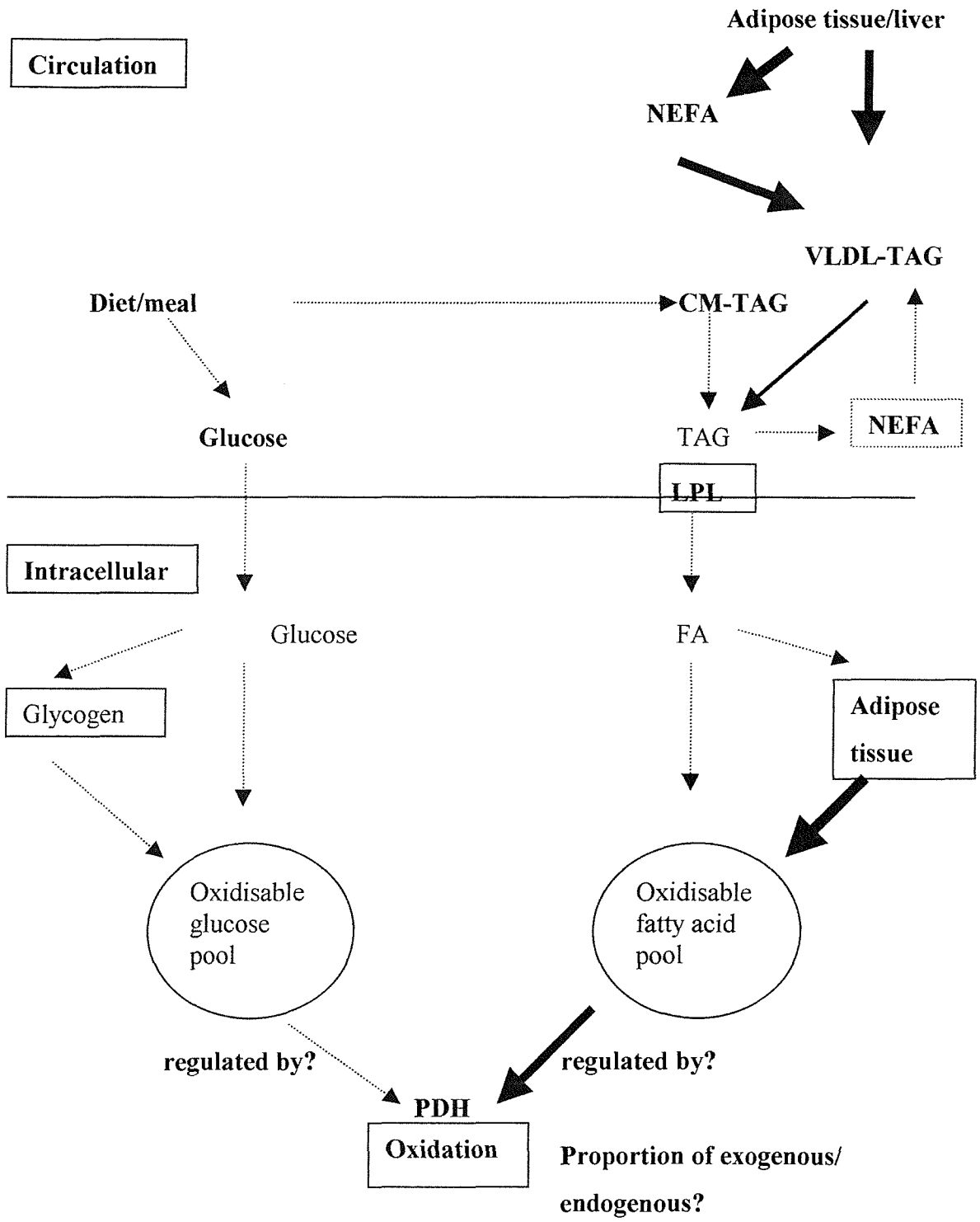
In the post-absorptive state, HSL has been shown to be more active than LPL, which is unsurprising as little substrate is available for LPL to act upon. LPL is not however inactive, suggesting the lack of re-esterification of LPL derived NEFA resulted in only 10% of NEFA being retained in adipose tissue [Frayn *et al.* 1994]. The lack of entrapment of fatty acids in adipose tissue suggests that NEFA released from adipose tissue are re-esterified in the liver, and released as VLDL-TAG to be hydrolysed by adipose tissue LPL [Elia *et al.* 1987; Wolfe *et al.* 1990]. What is unclear is the rate of

turnover of fatty acids from adipose tissue to liver for re-esterification, and how the whole process is regulated? As NEFA are present in relatively large quantities, and glucose concentrations are low, the process known as the glucose-fatty acid cycle operates. Basically, when NEFA concentrations are high, glucose uptake and oxidation are inhibited whilst fatty acid oxidation occurs in the muscle. A high rate of fatty acid oxidation (acetyl CoA formation) results in a high rate of citrate formation. At the same time the NADH/NAD+ and ATP/ADP ratios will be increased. The high acetyl CoA and NADH/NAD+ ratios inhibit pyruvate dehydrogenase resulting in an inhibition of glycolysis. The pathway of glucose breakdown and oxidation are inhibited and accumulation of free glucose is assumed to occur in the cell inhibiting further uptake of glucose [Randle *et al.*1963]. Activation of fatty acids on the outer mitochondrial membrane is necessary before the oxidation process can occur. ATP drives the formation of a thioester linkage between the carboxyl group of a fatty acid and the sulfhydryl group of CoA, where it is catalysed by acyl CoA synthase. Activated fatty acids are carried across the inner mitochondrial membrane by carnitine, and acyl carnitine is formed. Acyl carnitine is then taken across the inner mitochondrial membrane by a translocase. Carnitine then returns across the membrane leaving acyl CoA in the mitochondrial matrix [Stryer, 1988]. Activated fatty acids then undergo a series of reactions leading to the shortening of the fatty acyl chain by two carbon atoms. This process is known as beta-oxidation. The first reaction is the oxidation of acyl CoA by a dehydrogenase to give enoyl CoA. Following this hydration occurs, to produce hydroxyacyl CoA. The third reaction converts the hydroxyl group into a keto group and generates NADH, which enters the electron transport chain generating 3 molecules of ATP by oxidative phosphorylation, catalysed by L-3-hydroxyacyl CoA dehydrogenase. The acetyl CoA enter the TCA cycle yielding 12 molecules of ATP and 2 molecules of CO_2 if lipid and carbohydrate degradation are appropriately balanced [Stryer, 1988]. What is unclear is whether it is the presence of NEFA in the circulation or the decrease in glucose concentration which results in an increase in β-oxidation initially? From previous literature, the basic mechanisms underlying the association between glucose and fatty acid utilisation can be ascertained, however, the factors that influence this process such as genetics, body composition, meal composition, physical activity and exercise are poorly understood. How is TAG hydrolysis regulated? What regulates the balance of

glucose and fatty acid oxidation – is it due to supply of substrates or demand for energy? The current understanding of glucose and fatty acid utilisation in the post-absorptive state are summarised in figure 2.2.

In summary, it appears that a lack of insulin suppression and glucagon stimulation results in the release of NEFA from the adipose tissue by the action of HSL. NEFA cannot be re-esterified because of a lack of glycerol-3-phosphate and are therefore released into the plasma. The increase in NEFA concentration, and decrease in glucose concentration result in an increased uptake of NEFA to the liver and muscle, and an enhanced fatty acid oxidation. It may be that the glucose-fatty acid cycle inhibits the uptake and oxidation of glucose resulting in an maintenance of blood glucose concentrations. What remains unclear is what is it that controls the processes overall? Does the concentration of NEFA in the circulation directly affect fatty acid oxidation? Is β-oxidation occurring preferentially for fatty acids from exogenous or endogenous sources?

Figure 2.2 Interaction between glucose and fatty acids in the post-absorptive state



2.3.5 The postprandial state

2.3.5.1 The first meal of the day

In the postprandial state, chylomicrons are triglyceride rich lipoproteins (TRL) originating from the small intestine, which are hydrolysed by LPL and HSL. Therefore the main feature of the postprandial state is that endogenous TRL (VLDL) and intestinally derived TRL (chylomicrons) will compete for hydrolysis by the same enzymes and for removal by hepatic and peripheral cells [Lairon, 1996]. As the meal is absorbed as described in section 2.3.2, the carbohydrate component of the meal will result in rising glucose concentration stimulating insulin secretion, which suppresses HSL, leading to a suppression of the release of fatty acids from the adipose tissue. The declining plasma NEFA concentration removes the drive for muscle to oxidise fatty acids and glucose uptake is stimulated by the increasing glucose and insulin concentration, leading to an increase in glycolysis, glycogen synthesis and the production of glycerol 3-phosphate. This leads to re-esterification of fatty acids within the tissue. Insulin in the circulation also has a suppressive effect on the oxidation of fatty acids [Frayn, 1996].

Chylomicrons begin to appear in the plasma within 1 hour of ingestion of dietary lipid and are usually removed from the plasma 5-8 hours following the last meal [Patsch, 1987], but this may depend upon the composition of the meal, and also on the individual. The current understanding of the interaction between glucose and fatty acid metabolism in the postprandial state is shown in figure 2.4. The increase in insulin concentration following the meal leads to an activation of lipoprotein lipase on the endothelial surface of the capillaries, which results in the hydrolysis of TAG in chylomicrons and the release of NEFA. The products of lipolysis, free fatty acids and monoacylglycerols, move readily along and across cell membranes and aqueous spaces in the tissues, therefore the result of lipase activity is to make lipids available for tissue metabolic reactions. NEFA's are taken up by tissues by a process of diffusion which is now thought to be facilitated by a fatty acid binding protein and possibly a fatty acid transporter. In addition, up to 50% of LPL-derived fatty acids are released directly into the plasma, as opposed to entrapment or re-esterification at the site of action [Frayn, 1997]. This suggests that there may be

some purpose to the apparent inefficiency of LPL action in adipose tissue, creating a metabolic 'branch point' conferring greater precision of the regulation on the storage and mobilisation of fatty acids in adipose tissue [Frayn *et al.*1995]. The liver takes up the delipidated chylomicrons (chylomicron remnant) with proportionately decreased TAG and increased cholesterol where the cholesterol is used for membrane, or new lipoprotein, synthesis [Karpe, 1997]. LPL activity can change rapidly in response to physiological and patho-physiological conditions [Olivecrona *et al.* 1993]. Where carbohydrate is the primary source of dietary energy, the liver will synthesise a limited amount of lipid by a process known as *de novo* lipogenesis, which are exported to the blood as VLDL-TAG. VLDL particles are similar to chylomicrons, but smaller, denser, and containing increased phospholipids, cholesterol and protein. An exaggerated response to a fat-containing meal may be due to an overproduction of CM or VLDL, or may reflect a slower clearance of these particles by the rate regulatory step in TAG hydrolysis and removal, mediated by lipoprotein lipase. The resultant fatty acids may not be entrapped by the tissues and may be taken up by the liver for re-esterification to VLDL-TAG. Accumulation of partially hydrolysed remnant particles may also contribute to the elevated TAG response, with a net consequence of increased retention of TRL within the circulation, providing greater opportunity for neutral lipid exchange [Williams, 1997]. As discussed in section 2.2.3 neutral lipid exchange is thought to be a mechanism by which exaggerated TAG responses to meal my be associated with CVD.

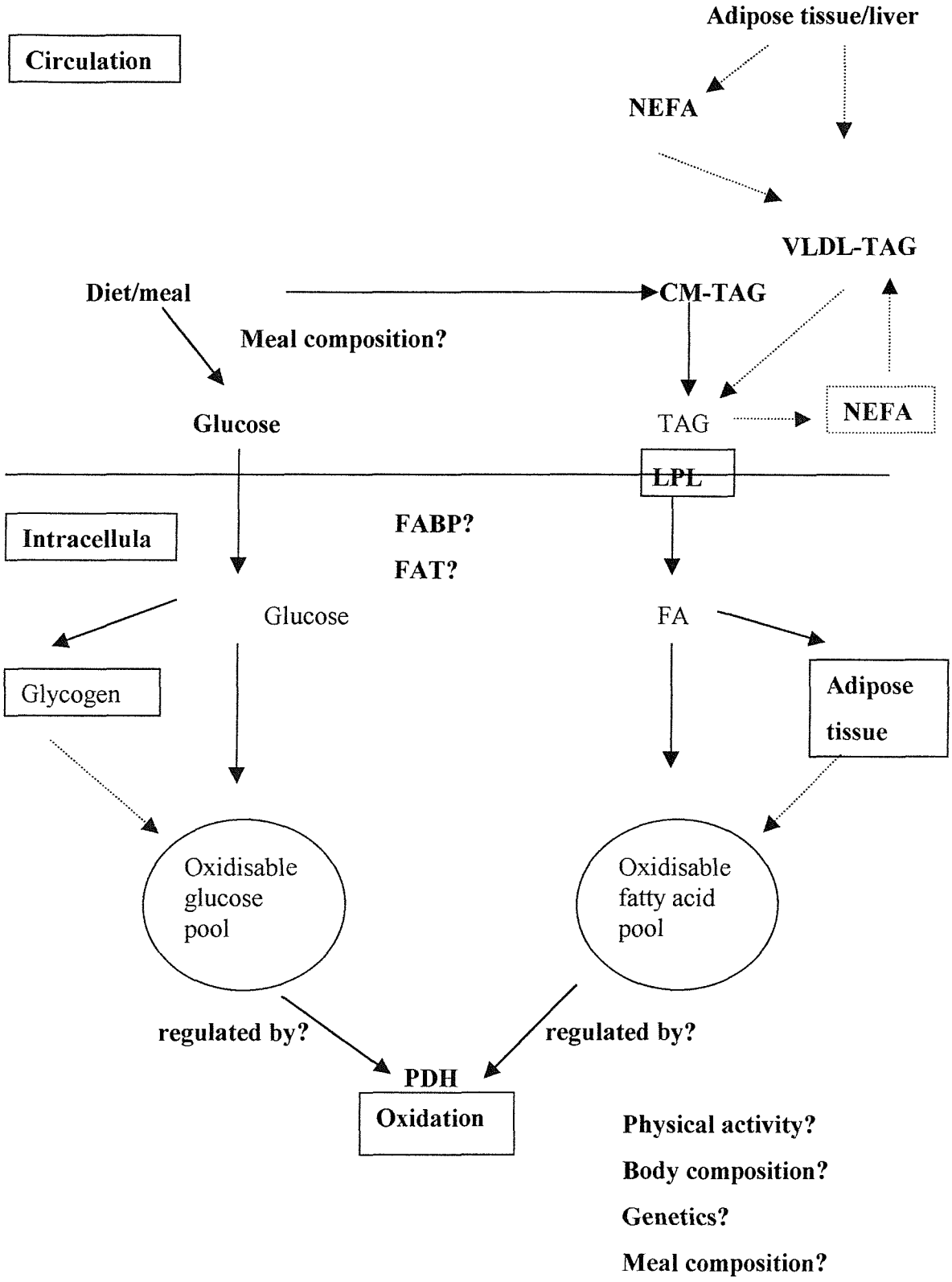
Following a meal, the suppression of NEFA release from adipose tissue, and the entrapment of resultant NEFA from CM-TAG, results in a decrease in NEFA concentration in the plasma. The decrease in NEFA concentration results in a decrease in fatty acid oxidation coupled with an increase in glucose oxidation. The fall in plasma NEFA concentration with a meal affects the metabolism of tissues which use fatty acids as oxidative fuel in the post-absorptive state. For example in skeletal muscle, the uptake of fatty acids is dependant upon fatty acid delivery, i.e. blood flow and plasma concentration. Therefore, if the concentration of NEFA in the plasma decreases, the uptake of fatty acids to tissues will decrease. It is not known, however, whether fatty acid uptake decreases merely because of a concentration effect or whether there is an inhibitory factor (possibly insulin), which 'switches off' fatty acid uptake for oxidation following a meal? In addition, is there a similar mechanism by which NEFA released by

hydrolysis of meal TAG are taken up by adipose cells for storage in the postprandial state?. There is some evidence that the hydrolysis of TAG by LPL in the postprandial state is regulated by the concentration of plasma NEFA, and that a high concentration of NEFA results in the dissociation of LPL from the endothelial surface therefore preventing its action on TAG [Karpe *et al.*1992]. The introduction of lipid to the circulation at a rate exceeding the removal capacity may result in a substantial increase in plasma NEFA concentration [Peterson *et al.*1990], which operate a feedback system to dissociate LPL, and therefore prevent further hydrolysis of TAG. A rise in the concentration of NEFA in the circulation could, therefore result in a decrease in TAG hydrolysis and eventually hypertriglyceridemia, although this has not been examined fully. To what extent the dissociation of LPL is complete, and the extent to which this will affect the clearance of TAG in the postprandial period remains unclear.

In summary, it appears that following a meal, chylomicron-TAG is hydrolysed by LPL attached to the endothelial surface of capillaries. The resultant fatty acids are, on the whole, entrapped by adipose cells, by a process of diffusion with fatty acid binding proteins and possibly fatty acid transporters. In circumstances where the entrapment process is impaired (disease/ excess lipid), NEFA are released into the circulation where they will either be taken up by muscle for oxidative purposes, by the liver to undergo re-esterification to VLDL-TAG, or will remain in the circulation and possibly result in the dissociation of LPL from its binding site. It is clear that the processes involved in the metabolism of dietary lipid are complex and multifactorial, and that the current literature does not provide a full understanding of the process. There are several questions which remain unanswered. What determines the size and rate of entry of chylomicrons to the circulation? Does LPL preferentially hydrolyse either CM-TAG or VLDL-TAG, and if so by what mechanism? How is the process of NEFA entrapment controlled, and do fatty acid binding proteins play a large role? At what point is the metabolic capacity to entrap NEFA overwhelmed, and does the introduction of a high fat meal lead to an elevation of NEFA concentration? How long following a meal does the action of insulin suppress endogenous NEFA release, and is this related to the amount of carbohydrate within a meal? At what point is there a switch between glucose oxidation and fatty acid oxidation, and what controls this process-is it due to a decrease in glucose and insulin concentration or an increase in NEFA concentration? What controls the flux of

substrates in figure 2.3? Is it influenced by the availability of substrates and substrate delivery (blood flow)?

Figure 2.3 Interaction between glucose and fatty acids in the postprandial state



2.3.5.2 Subsequent Meal

The effect of a subsequent meal during a study day has not been extensively studied, despite this being a normal meal pattern. The difference between the first meal of the day (breakfast), and a second meal (lunch) is the period of time spent in a post-absorptive state prior to the meal. Prior to the first meal of the day, the body has generally been without food for ten to twelve hours, leading to a decrease in plasma glucose and insulin concentrations, and an increase in NEFA concentration. When the meal is consumed, the rise in insulin concentration triggers a series of events described in section 2.3.5.1. The consumption of a second meal may occur only four to six hours later, and it is thought that insulin-stimulated processes become 'primed ' by previous insulin stimulation [Frayn, 1996]. Evidence suggests that the consumption of a second meal results in a second TAG peak immediately following the meal, and that a proportion of the fatty acids in the plasma peak will originate from the first meal [Williams *et al.*1992; Fielding *et al.*1996]. Fatty acids released from TRL by the action of LPL may not be entrapped by tissues effectively following a second meal perhaps due to a lack of activity of the esterification pathway in adipose tissue perhaps activated by acylation stimulating protein (ASP) [Fielding *et al.* 1996]. ASP is though to be involved in TAG synthesis in adipocytes and therefore may be associated with the entrapment of NEFA from the hydrolysed TRL [Sniderman *et al.*1997]. The role of ASP in the process of TAG clearance has not been extensively studied however, and mechanisms for the entrapment of NEFA in adipose tissue are not clear.

The result of the second meal is an enhanced insulin stimulation possibly leading to more efficient TAG clearance, but a failure to entrap NEFA to the tissues. There are no known studies in the literature to examine the effects of a second meal on fatty acid oxidation, but it would appear that oxidation may increase as the availability of NEFA increases. Several questions remain unanswered. What is the magnitude of the insulin response to a second meal, and how will this affect LPL activity and TAG clearance? Is there a failure of NEFA entrapment following a second meal, and if so why does this occur? How is fatty acid and glucose oxidation affected by the consumption of a second meal? How does a second meal affect the contribution of exogenous and endogenous fatty acids in the circulation?

2.4 Factors Affecting the Digestion, Absorption and Metabolism of Dietary Lipid

The previous section has described events which occur in the post-absorptive state and how metabolism alters with the consumption of a meal. The overall process of lipid metabolism (appearance and clearance from the circulation; storage and oxidation) appears to be tightly regulated by the presence of insulin, the availability of substrates, and an individual capacity to entrap or oxidise NEFA. Many of the studies in the literature have concentrated on the role of disease on lipoprotein metabolism, but few have examined the factors which will affect the way in which an individual handles lipid within a meal. For example, what differences are there in lipid metabolism between individuals, and what factors account for differences within an individual? How does changing the balance of macronutrients in a meal affect the rate and way in which lipid is handled in the circulation? And how does this affect the channelling of lipid towards oxidation or storage? Ultimately, there is a need to know how altering the macronutrient composition of a meal may affect disease outcome? The following section will concentrate on the manipulation of the macronutrient composition of meals, and how this affects lipids in the circulation, and oxidation.

2.4.1 Between and Within-Individual Differences

Dietary recommendations suggest a decrease in the consumption of total lipid within foods for the population as a whole, regardless of age, gender, disease state or level of physical activity. There is evidence to suggest, however, that the way in which lipid from the diet is handled both at the level of digestion and absorption and in terms of postprandial metabolism differs between individuals. Jones *et al.* [1998] showed differences between men, women and children in a study examining the metabolic disposal of [1-^{13}C]palmitic acid. Children were found to have much greater lipid oxidation rates (11.89g/6 h) compared to men (9.86 g/6 h), and women (0.03 g/6 h), with lowered levels of carbohydrate oxidation, suggesting that the postprandial partitioning of dietary lipid is regulated by the interaction of intrinsic (genetics), metabolic (body size, metabolic rate) and lifestyle (diet, physical activity) factors.

As described previously, variation in lipid metabolism can also be seen between

patients with and without coronary heart disease [Groot *et al.*1991], and non-insulin dependant diabetes mellitus [Coppack *et al.*1997]. Even within a subject group with similar characteristics such as age, gender, body composition and lifestyles, differences between individuals may still occur. This suggests that when within-individual differences are studied, that is, the same subject studied on more than one occasion, well controlled conditions are required to extract meaningful results. By controlling for background diet, levels of activity, consumption of alcohol, and the preceding meal prior to a study day, it is assumed that the variation observed is due to intrinsic and metabolic factors rather than lifestyle and diet related effects [Chambless *et al.*1992]. Very few studies have examined the effect of within-individual variability on measures of postprandial lipid metabolism, yet it is difficult to suggest differences observed in longitudinal studies are due to an intervention if the magnitude of variability is not known. In addition, it is impossible to ascertain the number of subjects required to detect differences between trials, if the magnitude of intra-individual variability is not known. Although studies have examined the magnitude of variability in post-absorptive measures [Brown *et al.*1992], measures in the postprandial state are not available. Therefore, what is the magnitude of intra-individual variability in measures of postprandial lipid metabolism? How can knowing the magnitude of variation be of use in conducting human metabolic trials? How will not knowing the magnitude of variability affect the interpretation of results?

2.4.2 Presentation of Dietary Lipid within a mixed meal

Within the human stomach, most dietary lipids are present in the form of emulsified droplets, with gastric lipolysis increasing this emulsification [Armand *et al.* 1994]. The process of lipid digestion and emulsification depends on the composition of the lipid emulsions [Rubin *et al.*1994], with the clearance of lipoproteins from plasma determined by the composition of the lipoprotein particle itself [Arimoto *et al.*1998]. Deckelbaum *et al.*[1990] showed with *in vitro* preparations that medium-chain triacylglycerol emulsions are a better substrate for hydrolysis by LPL and hepatic lipase than long-chain TAG emulsions. This is partly due to the greater solubility of MCT than LCT into phospholipid bilayers, which makes them more available for the endothelial

bound enzymes. The majority of the dietary lipid is present in the form of emulsions, and *in vitro* studies have demonstrated that the composition of the emulsion will affect the rate and extent of digestion and absorption. There are no known studies in humans examining the effect of altering the macronutrient compositions of emulsions on lipid metabolism. Within the food industry, however, the macronutrient composition of foods are often altered to improve palatability or shelf life without regard for the biological consequences. How will the manipulation of the composition of foods affect the channelling of lipid towards oxidation or storage, or the residence time in the circulation? What consequence will altering the metabolism of lipid have on the risk of developing disease? The following section of this review will concentrate on the literature examining the effect of altering the macronutrient composition of meals on lipid metabolism.

2.5 Alteration of Macronutrient Composition of a Meal on the Digestion, Absorption and Metabolism of Dietary Lipid

Manipulation of the macronutrient balance of a meal or diet has been hypothesised as a mechanism of manipulating the association between dietary lipid and disease. Altering the lipid and carbohydrate balance of a meal, may affect the magnitude and duration of postprandial lipaemia or channelling of lipid towards oxidation or storage, and in turn alter the risk of disease. The following section of the review will discuss the current literature examining the role of the composition of meals on lipid metabolism.

2.5.1 Macronutrient Balance

When energy balance occurs, balance of substrate oxidation will follow, and if balance is not met changes in body weight and composition will occur [Roy *et al.* 1998]. Carbohydrates and lipids are used with similar efficiency to regenerate the ATP used by the body's metabolic activities. However, the central nervous system and other specialised cells cannot use fatty acids to reach their metabolic demands, which must be supplied with glucose. The human body adjusts to considerable differences in the relative proportions of carbohydrate and lipid in the diet, even when major changes in

the carbohydrate-fat ratio occur [Flatt, 1995]. Carbohydrate oxidation is dependant upon carbohydrate intake, however fat intake does not seem to promote fat oxidation in the same way [Schutz *et al.* 1989]. This raises the possibility that whereas immediate corrective responses to changing carbohydrate intake exist, the inability to correct fat oxidation in response to an increase in dietary fat may be a key in the development of obesity [Roy *et al.* 1998]. The mechanisms involved in the regulation of lipid oxidation are poorly understood and it appears that these processes are highly controlled and can be affected by many factors including age, gender, disease progression and diet/meal composition. In particular dietary modulation of postprandial lipoprotein metabolism is an area in the literature with conflicting interpretation of results, perhaps due to the wide range of studies with a variety of methods to provoke the system, such as the type and size of the testmeal [Karpe, 1997]. In the following section, the literature describing dietary modulation of postprandial lipid metabolism by altering the carbohydrate and fat balance of meals, as this is the focus of this thesis, but also including a discussion on long term diets.

2.5.2 Alteration in the amount of dietary lipid

An increase in the amount of fat in the diet has been implicated in the aetiology of obesity, with an increase in BMI observed in many countries over the past 50-100 years running parallel to the increase in the proportion of dietary energy derived from fat [Prentice and Poppitt 1996]. This change in energy balance has shown that the adaptation of fat oxidation to intake is not a rapid process and, it has been suggested that the lack of promotion of oxidation with intake of fat leads to the development of fat storage and in time obesity [Schrauwen *et al.* 1997]. In order to understand the processes underlying the regulation of lipid metabolism under such conditions, several studies have examined the effects of a high fat diet or meal on postprandial lipaemia and substrate oxidation. Several studies have examined the effect of high fat meals on postprandial metabolism of lipid [Cohen *et al.* 1988; Murphy *et al.* 1995; Stubbs *et al.* 1996], suggesting a raised lipaemia with increasing lipid load.

2.5.2.1 Effect of a high-fat meal on circulating lipids

Despite the potential importance of postprandial serum TAG concentrations as a factor determining serum lipoprotein concentration and composition, few studies have investigated the factors determining the concentration of TAG following a meal [Cohen *et al.* 1988]. In periods of elevated TAG concentrations in the circulation, the process of neutral lipid exchange occurs resulting in the production of small, dense atherogenic lipoproteins as described in section 2.2.3 [Williams, 1997]. This suggests that if the consumption of high-fat meals results in an increased magnitude and duration of lipaemia, this may have a derogatory effect on the clearance of lipid from the circulation, leading to an increased risk of CVD. However, what is the mechanism which results in an increased magnitude of TAG concentration? Is this due to the increase in lipid load with no change in clearance rates, or an increase in hydrolysis of TAG leading to an 'overspill' of NEFA in the circulation resulting in the formation of VLDL-TAG?.

Cohen *et al.* [1988] examined the response of serum TAG concentrations to meals containing 40g, 80g and 120g of fat in the form of dairy cream in normotriglyceridemic men, and found that the magnitude of lipaemia that follows the ingestion of fat is directly proportional to the fat content of the meal. It was also suggested that the clearance of TAG is not saturated by meals containing the amounts of fat typically eaten by men following a Western diet. If this is the case, why is the magnitude of TAG concentration raised? If the clearance of TAG is not saturated by a high-fat meal surely TAG concentration would not be raised? Several studies investigating postprandial lipaemia use very large volumes of lipid to produce a measurable lipaemia. However, it is unclear whether this merely increases the timecourse of the lipaemic response or alters its metabolism altogether. The capacity for the maintenance of circulating lipid homeostasis in response to variations in acute and chronic dietary fat intake has not been extensively studied, and therefore normal limits remain undefined. One such study fed male volunteers 20, 40 and 80g of fat and measured the response of lipids in the circulation. Peak TAG concentrations were raised with the high fat meal, and the decrease in NEFA concentration usually observed following a meal was not as marked as with the 20g and 40g fat meals. Exaggerated NEFA and TAG responses seen with the high fat meal suggest that when fat intake

exceeds utilisation need, storage cannot fully compensate and therefore fatty acids spill into the circulation, which suggests that mechanisms controlling lipid homeostasis may be overloaded or impaired following a high fat meal [Murphy *et al.* 1995].The activation of LPL may be impaired with the introduction of a meal but this does not appear to be the case. The clearance of TAG from the circulation was found to increase with an increasing lipid content of the meal, suggesting an upregulation of lipoprotein lipase. However, insulin concentrations did not differ between the trials indicating a different stimulator of LPL was acting on the enzyme. There are no other studies in the literature which have measured LPL activity following a high-fat meal, and therefore it is not possible to support or dismiss this theory. The raised lipaemia with an increase in LPL activity does suggest that there may be an influx of VLDL-TAG caused by the increased concentration of NEFA in the circulation. What is unclear is the extent to which an increase in plasma NEFA concentration would result in an enhanced VLDL-TAG secretion? Does the increased NEFA concentration result in increased fatty acid oxidation? Is the raised TAG concentration due to a decreased clearance, increased secretion of VLDL-TAG or a competition for hydrolysis by LPL between the two?

2.5.2.2 Effect of a high-fat diet on circulating lipids

High fat diets have been found to affect the glucose tolerance of individuals, inducing insulin resistance and disturbed glucose metabolism, and may be linked to the onset of diseases such as heart disease and NIDDM. This is probably also dependent on the type of fat consumed [Uusitupa *et al.* 1994]. In contrast, low fat diets in combination with a high level of carbohydrate have been shown to be associated with fasting hypertriglyceridemia and a decrease in HDL-cholesterol concentrations in humans [Cominacini, 1988]. It remains unclear how a period of high-fat feeding affects circulating lipids as the majority of such studies have concentrated on the effect of high-fat feeding on lipid oxidation. Does the consumption of a high-fat diet over a period of time result in fasting hypertriglyceridemia, and how would the same individual handle a high-fat meal after a period of high-fat feeding? Would the same pattern emerge with a raised magnitude of TAG concentrations with an increase in plasma NEFA concentration?

It is unclear why some individuals are able to 'cope' metabolically with the ingestion of high fat diets and meals, whilst others cannot. None of the papers cited examine the differences between individuals when consuming a high-fat meal. For example, do individuals have a 'metabolic capacity' for the absorption and clearance of lipid from the circulation, and how can this capacity be altered with the addition of high fat meals? As none of the original papers show individual data it is not directly possible to examine this possibility further.

2.5.2.3 Oxidation of Lipid following a high-fat meal

In human subjects, the ability to adjust fat oxidation to fat intake appears to be less effective than the ability to adjust carbohydrate and protein oxidation to intake [Abbot *et al.*1988]. The majority of studies examining the effect of increasing lipid intake on oxidation have concentrated on the diet effect, with few studies examining the acute effect of lipid intake on lipid oxidation. Meals high in fat have been shown to increase lipid oxidation significantly [Griffiths *et al.*1994; Whiteley *et al.*1997], due to an increase in plasma NEFA concentration. This is in agreement with the glucose-fatty acid cycle [Randle *et al.*1963], which suggests a reciprocal relationship between carbohydrate oxidation and fatty acid oxidation. However, the results are not in agreement with diet based studies which suggest that the NEFA arising from ingested fat will enter the tissues for storage, resulting in a decrease in lipid oxidation. As the NEFA concentration rose in both the studies indicated, it is probable that the entrapment of NEFA from hydrolysis of TAG was impaired preventing the uptake of NEFA to the tissues. As NEFA enter the circulation, fatty acid oxidation is stimulated and the NEFA are removed by oxidative processes. What is not known is why an increase in exogenous TAG should result in an impairment of the entrapment of fatty acids, or why the increase in plasma NEFA should result in an increase in fatty acid oxidation? Very few studies have examined the role of fatty acid oxidation following a high-fat meal, despite its importance in association with the onset of obesity. If a high-fat meal is consumed, and oxidation is increased, how can the consumption of high-fat meals be linked to the onset of obesity? Is it merely that there is so much lipid present that the majority of lipid is directed towards storage whilst a smaller proportion is directed towards oxidation? The

rate and extent of lipid oxidation will also depend upon the amount of carbohydrate within the meal. Whiteley *et al.* [1997] fed isoenergetic meals so that when the lipid content was high, carbohydrate content was low, suggesting that the influence of the high fat meal may merely be an influence of the low-carbohydrate element. In contrast, Griffiths *et al.*[1994] fed non-isoenergetic meals (80g CHO) with similar results, suggesting that it is the lipid content of the meal which affects lipid oxidation rather than the carbohydrate content. What is unknown is the extent to which the lipid oxidation can be attributed to exogenous or endogenous fatty acids, and whether the carbohydrate content of the meal may influence the release of endogenous fatty acids from adipose tissue following the meal?

2.5.2.4 Oxidation of lipid following a high-fat diet

Weight maintenance requires that, in the long term, energy intake matches energy expenditure. Apart from energy balance, this also requires the oxidation rate to be equal to intake for separate nutrients, and this appears to be true for both protein and carbohydrate. However, lipid balance is poorly regulated possibly due to its increased storage capacity and even with an excessive amount of fat added to a mixed meal, 24 hour energy expenditure is unaffected [Schutz *et al.* 1989]. The lack of interaction between fat intake and fat oxidation is demonstrated by the changes in fuel utilisation induced by food ingestion. An increase in fat intake was shown to lead to detectable changes in hunger when compared to a high carbohydrate meal [Stubbs *et al.*1996]. Obese individuals have been shown to be more likely to consume a high-fat diet, and that expansion of fat stores in susceptible individuals is a prerequisite to increase lipid oxidation [Astrup *et al.*1994]. On the contrary, long term low-fat feeding has been shown to lead to a significant weight loss [Lissner *et al.*1987].

Diet based studies have demonstrated that alterations in the amount of dietary lipid consumed does not lead to abrupt changes in lipid oxidation but it is unclear how long oxidation rates take to adapt to changes in intake. A previous study found that lean subjects were capable of adjusting fat oxidation to fat intake within 7 days of an increase in dietary fat content [Schrauwen *et al.* 1997]. The promotion of fat oxidation appears to depend upon the age of the individual, with total postprandial fat oxidation increasing

proportionally to meal size in young women but not in older women. The authors suggest this as a mechanism for the increase in body fat seen with age [Melanson *et al.* 1997]. Prior athletic training is not thought to be a factor affecting substrate oxidation, with diet causing a significant effect [Roy *et al.*1998]. More studies are necessary to examine the effects of increasing dietary fat levels on fat oxidation in obese subjects.

Conflicting interpretation of data from meal based and long term high-fat feeding studies have concluded that fat intake does not promote fat oxidation. However, meal-based studies have demonstrated an increase in lipid oxidation with a high-fat meal. Therefore, how does the feeding of high-fat meals over a period of time not lead to an increase in lipid oxidation, and is the lack of increase in lipid oxidation in fact a compensatory mechanism which occurs over a period of time? In addition, what happens to lipid oxidation following a high-fat meal, at the end of a period of high-fat feeding?

2.5.3 Alterations in the type and amount of carbohydrate

From the previous section, it is clear that the manipulation of the macronutrient composition of a meal or the diet leads to profound effects both on circulating lipids and on substrate oxidation. Several studies have examined the effect of increasing the lipid content of meals by utilising isoenergetic testmeals i.e. as lipid increases, carbohydrate decreases. What is unclear is the effect that altering the carbohydrate content of the meal will have on outcome, and whether isoenergetic meals actually give the correct study conditions required? Several studies have examined the effect of carbohydrate on lipid metabolism, and will be reviewed in the following section.

2.5.3.1 Effect of Insulin and glucagon on lipid metabolism

Insulin plays an important part in both lipid deposition following a meal, and lipid mobilisation in the post-absorptive state, and as such is thought to be a major regulator of both TAG and fatty acid metabolism. Glucagon is released when blood sugar levels fall, and acts in the opposite way to insulin [Stryer, 1988]. In the post-absorptive state, fuel must be mobilised from body stores for use elsewhere in the tissues. To mobilise the fuel stores of adipose tissue, stored TAG must be hydrolysed to

FFA and glycerol. The conversion of TAG to diglyceride and FFA, the rate limiting step, is catalysed by an enzyme called hormone sensitive lipase. Hormonal activation of the enzyme is mediated by the action of cAMP which acts via a protein kinase to convert the inactive lipase to a phosphorylated active form [Masoro, 1977]. The phosphorylation of HSL is though to be activated by the presence of noradrenaline, adrenaline and glucagon in the post-absorptive state. The diglyceride is broken down further into its constituent glycerol and FFA, by the action of a second lipase, monoacylglycerol lipase, whereby the fatty acids leave the cells and enter the NEFA pool. The glycerol also leaves the cells as it cannot be utilised for the esterification of fatty acids due to the lack of glycerol kinase in adipose tissue. The rate of fat mobilisation begins to increase a few hours after a meal, and gradually increases to rates observed during the post-absorptive period. Animal studies suggest that decreased levels of insulin, and increased sympathetic nervous system activity are major factors promoting fat mobilisation. However, once the post-absorptive state is prolonged neither mechanism appears important [Frayn, 1996].

Stimulation of HSL is inhibited following a meal by the action of insulin and a decrease in the presence of glucagon, resulting in an inhibition of the release of endogenous fatty acids from adipose tissue. Following a mixed meal, plasma glucose concentration increases, promoting the secretion of insulin into the circulation. Glucose must first be phosphorylated and metabolised further before causing insulin release. The phosphorylating enzyme glucokinase may therefore participate in regulating β-cell function. Glucose entry from the GI tract leads to an increased secretion of insulin, with the additional stimulus from gastric inhibitory peptide (GIP), gastrin, secretin and cholecystokinin. When digestion and absorption processes are completed, plasma glucose levels return to normal and insulin secretion subsides [Berne and Levy 1990]. In the fed state, insulin stimulates the transport of glucose from the plasma across the cell membrane into the cytoplasm where it is readily phosphorylated. In muscle and liver, insulin stimulates glycogen formation from glucose-6-phosphate, and to a lesser extent glycolysis and oxidation. In adipose tissue insulin promotes the production of alpha-glycerol phosphate from the triose phosphate intermediates of glycolysis, which is then used to esterify FFA for storage as TAG. In liver, insulin favours shunting of incoming FFA from β-oxidation and towards esterification, again by increasing the production of alpha-glycerol phosphate [Berne and Levy 1990].

In summary in the fasted state, a lack of insulin and increase in glucagon stimulates the mobilisation of fatty acids from adipose tissue, and in the fed state, an increase in insulin stimulates the uptake of fatty acids into cells to promote storage. With increased carbohydrate content within a meal, insulin concentrations increase [Grant *et al.* 1994], however this appears to be dependent upon the type of carbohydrate involved [Mann *et al.* 1971]. Several questions remain unanswered such as is the increase in insulin concentration dose-dependant on the amount of carbohydrate in the meal? How does the presence of insulin affect LPL and HSL activation? Does the presence of insulin completely abolish the effect of HSL on adipose tissue TAG, or is its activation merely 'damped down' whilst insulin concentrations are at their highest? Is there any association between the concentration of insulin in the circulation and the concentrations of circulating lipids?

2.5.3.2 Studies in the postprandial state

Studies in the postprandial state have examined the effects of glucose, fructose, sucrose, starch, dietary fibre, different fatty acids and alcohol on postprandial lipaemia [Karpe, 1997]. The effect of carbohydrate has been studied both in postabsorptive and postprandial states, with the focus previously being on the postabsorptive data following periods of high-carbohydrate feeding. Postprandial metabolism has been shown to be increasingly important in terms of risk of disease and yet there are insufficient well-controlled studies to examine the effects of carbohydrates on a single test meal. There are many similarities between these human studies but all are poorly controlled in terms of diet and test meals which makes them increasingly difficult to compare. The studies are summarised in table 2.3. In addition, none of the studies concerned with carbohydrate effects on postprandial lipaemia give any indication of within-individual error - Is the effect seen merely a consequence of within-individual error due to repeated measurements on different occasions or is it due to the nutritional intervention as shown?

2.5.3.3 Glucose studies

Several studies have examined the effect of glucose on postprandial lipaemia

[Nikkila & Pelkonen 1966; Mann *et al.* 1971; Cohen & Schall 1988; Cohen & Berger 1990]. In all these studies the conclusion reached by the majority of the investigators is that of a hypotriglyceridemic effect of glucose when added to a test meal, with one exception possibly due the dose of glucose used [Cohen & Schall 1988]. There are several mechanisms which have been suggested for the hypotriglyceridemic effect of glucose. The earliest study in 1966 by Nikkila & Pelkonen, suggested that glucose may affect the magnitude of the plasma TAG response in two opposite ways, increased clearance of TAG by the action of insulin on lipoprotein lipase or an increased inflow of endogenous fatty acids into the circulating lipid pool. Cohen & Berger [1990] suggested that the hypotriglyceridemic effect of glucose may be due to delayed gastric emptying simply by increasing glucose leading to increased osmolarity of the test meal. However it is also concluded in this study that it is more probable that this effect is due to increased clearance of TAG-rich chylomicrons, owing to the increased activity of LPL on the adipose tissue, stimulated by the presence of insulin. This is despite evidence from Sadur *et al.*[1984], which suggests that fat ingestion abolishes the insulin-mediated increase in adipose tissue lipoprotein lipase activity. In the study [Sadur *et al.*1994], the addition of lipid to a meal resulted in a decrease in LPL activity leading to an increase in postprandial TAG concentration, yet this could be explained by the presence of lipid. The majority of other studies have examined the effect of the addition of glucose to an oral fat load, resulting in a increase in clearance of lipid from the circulation. Sadur *et al.* [1994] added lipid to a carbohydrate based meal, which would be expected to decrease the glycaemic and insulinogenic responses, therefore decreasing the effect of insulin on LPL.

The effect of glucose on plasma TAG levels also appears to be dose-dependant, with a substantially decreased lipaemia with 100g of glucose as opposed to 50g [Cohen & Berger, 1990]. As the effect is dose-dependant, even a small amount of glucose should limit postprandial lipaemia and may be of relevance to those with an increased risk of CHD. In addition, the authors suggest that for the first 2-4h of increased plasma TAG concentrations following a meal, the effects of glucose are merely due to delayed gastric emptying and not due to increased activity of LPL. However, Grant *et al.* [1994] dispute this fact as they have demonstrated that the initial postprandial rise in plasma TAG concentrations was of the same magnitude whether in the presence or absence of

carbohydrate. What this study does not indicate is that gastric emptying may be affected by the addition of glucose but the decrease in lipid concentrations is not dependant upon gastric emptying as similar initial timecourses of lipid concentrations are observed despite glucose concentrations. What is unclear is the role of glucose in the insulinogenic response, and how the insulinogenic response affects the activation of LPL? Also the insulin mediated inactivation of HSL will result in a decrease in endogenous NEFA concentration, but how is this reflected in the balance of oxidation between exogenous and endogenous substrates? As none of the studies in the literature have examined substrate oxidation following a meal with added glucose, it is not possible to comment on the role of oxidation.

Table 2.3 Studies addressing the effect of carbohydrate on circulating plasma TAG concentration

Reference	Testmeal	N	Type of sugar	Amount of sugar	Type of lipid	Amount of lipid	Change in TAG concentration	Details
Nikkila & Pelkonen 1966	Oral lipid and CHO load	8 5 9	Fructose Glucose glycerol	0g/20g	cream	40g	↑ ↓ ↑	No dietary control, small CHO doses, blood samples for 6 hours
Mann *et al.*1971	Formula breakfast	10	Glucose sucrose	60g	Sunflower oil	25g	↓ ↑ 54%	Two groups of males, blood samples for 4h. No control (fat only) meal
Cohen & Schall 1988	Oral lipid load	21	 Fructose Glucose sucrose	0g 50g 50g 50g/10g	cream	40g	 ↑ no change no change/ ↑	No alcohol and limited exercise for 3d before tests, last meal 12 h before test. Blood samples for 7h
Cohen & Berger 1990	Testmeal	55/18 55 18	glucose	0g 50g 100g	cream	40g	 ↓ 25% ↓ 39%	Each subject studied twice, no alcohol and limited exercise for 3d before, blood samples for 6h
Grant *et al.*1994	Oral lipid load	19	sucrose	0g 1.5g/kg BW	cream	1g/kg BW	↑	Blood samples for 8h, subjects pursued daily activities.
Jeppesen *et al.*1995	Oral lipid load	11	fructose	0g 50g	cream	40g	↑	No dietary control, male and female subjects, blood samples for 10h

2.5.3.4 Fructose Studies

Fructose appears to have an opposite effect on postprandial lipaemia to that of glucose. Increasing fructose consumption in the diet can significantly increase fasting plasma TAG and cholesterol concentrations and therefore increase the risk of CVD [Swanson *et al.* 1992; Hollenbeck, 1993]. In meal based studies, when fructose is added to a test meal in varying amounts, an increase in the lipaemic response is seen [Nikkila & Pelkonen 1966; Cohen & Schall 1988; Jeppessen *et al.* 1995]. The increase observed may be due to inhibition of plasma TAG removal because fructose is a less 'glycaemic' sugar and therefore will produce a decreased insulinogenic response, or stimulation of the re-esterification of VLDL-TAG in the liver. The fructose-induced accentuation of postprandial lipaemia was primarily due to accumulation of chylomicrons, possibly due to a decrease in the removal rate of intestinal TRL from plasma [Jeppesen *et al.*1994]. Fructose induced re-esterification of FFA and de novo lipogenesis may lead to an increase in liver synthesis and secretion of VLDL-TAG. As both intestinally derived and endogenous TRL are cleared by the same mechanism a competition for clearance of both TRL may result in the CM-TAG remaining in the circulation for a longer duration [Hollenbeck, 1993; Jeppesen *et al.*1994].

More recently, the replacement of glucose with fructose in the diabetic diet has been discussed as fructose is a less 'glycaemic' sugar, with concerns expressed as to the hypertriglyceridemic effects of fructose [Abraha *et al.*1998]. However an increase in postprandial lipaemia was seen in both diabetic and non-diabetic subjects, with a decrease in plasma NEFA concentration, suggesting that fructose induced an acute increase in the sensitivity of lipolysis to suppression by insulin. In turn, the changes in NEFA dynamics may underlie the changes in TAG metabolism perhaps by regulation of hepatic TAG secretion rate [Abraha *et al.* 1998]. The study suggests that as there is a decrease in NEFA concentration following the fructose meal, due to an increased sensitivity of lipolysis inhibition, that this in some way regulated the secretion of TAG from the liver. How can a decrease in NEFA concentration lead to an increase in VLDL-TAG secretion? Does fructose provide a necessary component for the synthesis of VLDL-TAG? How is secretion of VLDL-TAG controlled? The few studies carried out in this area add to the conflicting opinions associated with the effect of carbohydrates on

lipid metabolism, as there is no agreement in the mechanisms which may be involved in the hypertriglyceridemic effects of fructose. In a review of the literature, Hollenbeck [1993] states that there is an unequivocal need for additional studies before a clear understanding of the effects of dietary fructose will appear.

2.5.3.5 Sucrose Studies

Sucrose is a disaccharide, which forms from molecules of glucose and fructose, and is the common form of sugar available in most processed foodstuffs, for example, in confectionery. As described previously, studies have demonstrated a hypotriglyceridemic effect of glucose on postprandial lipaemia [Nikkila & Pelkonen 1966; Mann *et al.* 1971; Cohen & Schall 1988; Cohen & Berger 1990], yet a hypertriglyceridemic effect of fructose on lipaemia [Nikkila & Pelkonen 1966; Cohen & Schall 1988; Jeppessen *et al.* 1995]. Few studies have examined the effect of sucrose on postprandial lipaemia, and subsequently the effects that this may have on the risk factors for CHD, diabetes and obesity. What effect does a combination of glucose and fructose have on the magnitude and duration of postprandial lipaemia? The ingestion of sucrose with a lipid rich meal has been shown to increase the postprandial excursion of the serum TAG concentration when compared to a meal of lipid alone [Mann *et al.* 1971; Cohen & Schall 1990], which is possibly an effect of the fructose component of the sugar. It is possible that the presence of fructose in sucrose outweighs the hypotriglyceridemic effects of glucose but the mechanisms underlying this possibility remain unclear.

Grant *et al.* [1994] undertook a study to investigate the effect of the inclusion of sucrose in a lipid-rich meal on the serum TAG concentrations, and to aim to elucidate the mechanism by which this took place. Postprandial lipaemia was increased with the addition of sucrose by 29%, above values seen for a lipid rich meal alone, which was smaller than the amplification of 54% reported by Mann *et al.* [1971], and 56% by Cohen & Schall [1988]. The study in 1994 used a much larger amount of lipid in the test meal compared to the two earlier trials, which may contribute to the differences observed. The authors also suggest that this anomaly may be due to their small subject population, or within-individual variability, which was not measured. Cohen and Schall [1988] found an increase in PPL with 100g of sucrose added to their lipid meal and

suggest that the fructose component of sucrose causes an increase in the re-esterification and secretion of endogenous VLDL-TAG rather than an decrease in clearance in intestinally derived CM-TAG.

The increase in the magnitude and duration of lipaemia could be due to three mechanisms. Firstly increased absorption of lipid, secondly stimulation of endogenous TAG synthesis or thirdly decreased lipolysis of TAG rich lipoproteins. Grant *et al.* [1994] suggest that it is the third point that is of importance, an inhibition of LPL clearance of TAG from chylomicrons. Clearly LPL is stimulated by insulin so how would a decrease in LPL activity occur with the addition of sucrose?. However, the testmeal consumed in the study by Grant *et al.*[1994] contained a total of 1g lipid/kg body mass and 1.5g sucrose/kg body mass, resulting in an average testmeal for the subject group (76.1±9.7 kg) of 76g of lipid and 114.15g of sucrose. This testmeal contains substantially more lipid and sucrose than normally consumed within a meal and therefore it is possible that the excess carbohydrate is utilised in the process of re-esterification of NEFA via the synthesis of glycerol from alpha-glycerophosphate, which would result in an increased secretion of VLDL-TAG. CM-TAG and VLDL-TAG would compete for hydrolysis by LPL and therefore the overall clearance of total TAG would be decreased, resulting in a raised magnitude of plasma TAG concentration. Esterification of NEFA to VLDL-TAG is also stimulated by the presence of insulin suggesting an increase in esterification with the addition of sucrose to a meal. The extent to which carbohydrate overfeeding induces hepatic TAG synthesis from glucose (*de novo* lipogenesis) and VLDL-TAG secretion depends on the supply of carbohydrate which exceeds NEFA oxidation requirements for energy production [Roche, 1999]. Carbohydrate feeding stimulates *de novo* lipogenesis by increasing the flux of either glucose or fructose along the glycolytic pathway, thereby increasing the availability of acetyl CoA and stimulating fatty acid synthesis [Frayn and Kingham, 1995]. Carbohydrate feeding also upregulates the activity of the enzymes which synthesise fatty acids. The activity of hepatic synthases and the NADPH- generating enzymes were significantly increased in rats fed on glucose and fructose, which in turn led to a significant increase in the rate of TAG secretion [Kazumi *et al.*1997]. All the evidence relating carbohydrate feeding with *de novo* lipogenesis is based on high-carbohydrate diets. It is unlikely that a single high-carbohydrate meal will result in a substantial

amount of lipogenesis, as the majority of excess glucose will be utilised for fuel by oxidative purposes. What is unclear, however, is the extent to which fructose will affect the secretion of VLDL-TAG, and how this may affect the residence time of lipids in the circulation? Also are the effects of fructose dose-dependant i.e. is there an amount of sucrose needed to provide sufficient fructose to stimulate enhanced re-esterification or *de novo* lipogenesis?

There are no known studies in the literature to examine a dose-response effect of sucrose when added to a lipid based mixed meal, as Cohen and Berger [1990] have studied with glucose. The studies in this area provide conflicting interpretations of results having used various lipid and sucrose loads, yet all have included sucrose concentrations above that normally included in a single meal, and leave many questions unanswered. Is it possible that the high sucrose loads actually have an adverse effect on lipid metabolism by causing an additional lipogenesis or re-esterification, leading to delayed clearance of both dietary and endogenous TAG and therefore elevated TAG levels? Does sucrose merely have an inhibitory affect on LPL activity or would a decreased amount of sucrose (0-50g) have the same effect? Would a decrease in lipaemia be observed due to the action of insulin on LPL?

2.5.3.6 Substrate oxidation following a high-carbohydrate meal

Table 2.4 shows the studies examining the effect of carbohydrate on postprandial substrate oxidation. Whiteley *et al.* [1997] observed an increase in carbohydrate oxidation accompanied by a decrease in lipid oxidation with an increasing carbohydrate content of the testmeal, but suggests that the reciprocal relationship is not seen when the meals are not isoenergetic. In other words, the study suggests that the decrease in lipid oxidation does not occur when the amount of lipid within the testmeal remains constant. The decrease in lipid oxidation with isoenergetic meals is probably due to the decrease in lipid intake rather than any effect of carbohydrate. As expected similar results were seen when a low fat, high carbohydrate meal was compared to a high fat, low carbohydrate meal [Griffiths *et al.* 1994]. The respiratory exchange ratio over the postprandial period was significantly raised with consumption of increased carbohydrate. Once again, the decrease in lipid oxidation could be attributable to the decrease in lipid

intake rather than effects of carbohydrate. What is unclear is how lipid oxidation will be affected if the amount of lipid remains constant but carbohydrate is increased? How will the addition of carbohydrate affect the glucose/fatty acid cycle? Will carbohydrate exert its affect on exogenous or endogenous fatty acid oxidation? Although many studies have examined how the addition of carbohydrates can affect lipid metabolism at the level of the circulation, only the two studies cited have also included oxidation measures.

Table 2.4 Studies addressing the effect of carbohydrate on postprandial substrate oxidation

Reference	Testmeal	n	Type of sugar	Amount of sugar	Type of lipid	Amount of lipid	Lipid Oxidation	Details
Griffiths *et al.* 1994	High-fat meal Low-fat meal	8	mixed	80g	mixed	80g <1g	↑ ↓ *	No dietary control, overnight fast, calorimetry and blood samples hourly for 6h
Whiteley *et al.*1997	3 isoenergetic mixed meals (+ lipid only meal)	8	mixed	121g 70g 50g 0g	mixed	48g 70g 80g 108g	↓ ** ↓ ** ↑ *** ↑ ***	Fat-free meal night before test, calorimetry 30 minutes of each hour, blood samples every 30 minutes for 5h

* $p<0.05$ compared to high-fat meal

** $p<0.05$ compared to high-fat meal

*** $p<0.05$ compared to low-fat meal

2.5.3.7 Nature of the Postprandial Response

There is evidence to suggest that the inclusion of carbohydrate within a testmeal may affect the nature of the postprandial response. Shishehbor *et al.* [1998] examined the acute effect of carbohydrate on the mono-and biphasic nature of the postprandial response. While the overall magnitude of the TAG response was not different between the low and high carbohydrate meals, the high carbohydrate meal caused a biphasic increase in TAG concentrations. The biphasic response occurred following the ingestion of a high-carbohydrate meal after an overnight fast, with an increase in plasma TAG concentration between 0-2 hours following the meal, and again between 4 and 6 hours. One explanation for such a response could be that the formulation of the meal led to a rapid rate of gastric emptying and fat digestion which could only be absorbed in two waves because of a limiting factor conditional on chronic fatty acid intake. Other studies have shown that gastric emptying of lipids is not affected by the carbohydrate within the meal [Grant *et al.* 1994], but this could be dependant upon the type and amount of carbohydrate present. The biphasic response seen in Shishehbor *et al.* [1998] has not been demonstrated in previous studies with similar or greater amounts of carbohydrate [Van Amelsvoort *et al.*1989; Murphy *et al.*1995]. Clear biphasic responses have been noted, however, in studies using fructose as the carbohydrate source [Cohen & Schall, 1988; Jeppesen *et al.*1995], although other studies using sucrose have also demonstrated clear monophasic responses [Mann *et al.*1971; Grant *et al.*1994]. There are clearly conflicting opinions as to the mechanisms underlying the biphasic responses observed in some studies and several questions remain unanswered. What factor or factors is controlling the appearance/ clearance of lipid from the circulation to cause a biphasic response? How does altering the amount of carbohydrate in a dose-dependant way affect the nature of the TAG response?

2.6 Stable Isotope Tracer Methodology

The research conducted into lipid metabolism has been constrained by the lack of understanding of the role of exogenous and endogenous lipids in circulating lipids and substrate oxidation. Several studies have introduced measurements of lipoprotein classes by density separation but this does not differentiate between exogenous and endogenous fatty acids. The introduction of stable isotope tracers has permitted the separation of the metabolism and oxidation of exogenous and endogenous lipids, and has allowed a better understanding of dietary lipid and disease. The following section reviews the current literature on stable isotopes, with a brief introduction to their chemical structure and use in human research.

2.6.1 Introduction

Over the past twenty years the use of stable isotope tracers in metabolic studies has become increasingly popular. Increased availability of a variety of substrates labelled with stable isotopes, improvements in analytical procedures, and increasing awareness of the potential hazards of using radioactive tracers in humans have all contributed to the increased use of stable isotope tracers [Wolfe *et al.* 1984]. The stable isotope of carbon, ^{13}C, has been used as a tracer to determine substrate oxidation rates and to develop clinical tests for the rapid discrimination of digestive and metabolic disorders. These breath tests entail oral or intravenous administration of ^{13}C labelled substrate followed by measurement of ^{13}C enrichment in breath CO_2 [Jones *et al.* 1985].

The stable non-radioactive isotope of carbon, ^{13}C has a wide number of applications in human nutrition research. Most notably $^{13}CO_2$ breath tests have been used to examine the digestion, absorption and metabolism of substrates (carbohydrates, lipids and proteins), to detect bacterial colonization and overgrowth, and to measure organ function and oxidative capacity.

2.6.2 Stable Isotopes

Although chemical processes depend essentially on how the electrons in atoms and molecules interact with each other, the internal nature of the nuclei, and changes in nuclear composition play an important role in the study understanding of chemical processes. Atomic nuclei consist of a certain number of protons (p) called the atomic number, and a certain number of neutrons (n). The masses of these particles are each approximately equal to one mass unit and the total number of nucleons (p + n) is called the mass number. These two numbers designate a given nuclear species. It is the number of protons that express the element that is present. For a given atomic number, different values of atomic mass can be seen, resulting from different numbers of neutrons. This is responsible for the existence of different isotopes of that element [Cotton and Wilkinson, 1976].

An element normally exists in a dominant stable form with a less dominant stable form, and a radioactive isotope. This is true in the case of carbon. The dominant stable isotope is carbon 12 (^{12}C), a less dominant stable isotope as carbon 13 (^{13}C) and a radioactive isotope, carbon 14 (^{14}C) which decays emitting radioactivity.

2.6.3 Use of Isotopes in Biomedical Research

Radioactive isotopes have been used for many years in biomedical research to act as tracers to follow metabolism and cellular processes. However, the use of radioactivity in human studies of this nature has come under scrutiny due to the growing knowledge of radiation hazards associated with the use of radiotracers. Therefore, the use of stable isotope tracers has been introduced as a safer and more acceptable method. The advantages of using this method are that stable isotopes are non-toxic at levels used in clinical studies, they do not decay quickly and that multiple stable isotopes labelling different or identical nutrients enable simultaneous investigation of nutrients within various body compartments [Jones, 1990].

The stable isotopes of carbon (^{12}C and ^{13}C) are determined by isotope ratio mass spectrometry. This method utilises the different masses of the two isotopes due to the neutron content, to differentiate between ^{12}C and ^{13}C, and to show the ratio of the two.

The concentration of ^{13}C when expressed as a percentage of total carbon is known as the abundance in atom units of percent (atom %). This is therefore the measurement of atoms of ^{13}C in 100 atoms of the element. The natural abundance of ^{13}C to ^{12}C is 1.11%. Therefore approximately 1% of all carbon is ^{13}C and 99% is ^{12}C.

2.6.4 Natural Abundance of ^{13}C

Stable carbon isotope analysis of human breath CO_2 has been used to measure the *in vivo* oxidation of various drugs, metabolic fuels and products of intermediate metabolism. In each of these analyses, excess $^{13}CO_2$ produced by oxidation of the labelled substrate must be measured in the presence of a natural background of ^{13}C. This natural ^{13}C comprises about 1.1% of all carbon but ^{13}C abundance varies slightly depending on the carbon source [Schoeller *et al.* 1984]. This abundance is not constant but has a natural variation that reflects the enrichment or depletion of ^{13}C relative to ^{12}C due to isotope fractionation [Schoeller *et al.*1980].

There are two major food chains stemming from the photosynthetic processes by which carbon dioxide is converted to carbohydrate. One originates in plants that fix CO_2 into a three carbon intermediate through the Calvin-Benson reaction, the other plants that have a four carbon intermediate (C_4), in the Hatch-Slack pathway. These two pathways differ in their isotopic discrimination against ^{13}C and will result in differing ratios of ^{13}C to ^{12}C [Schoeller *et al.* 1980]. The chain of reactions in photosynthesis is cyclical, known as the Calvin Cycle. In the first step the CO_2 combines with a five carbon organic compound known as Ribulose Phosphate. This serves as a CO_2 acceptor and fixes the CO_2. The combination of CO_2 with ribulose phosphate gives an unstable six carbon compound which splits immediately into two molecules of a 3 carbon compound, phosphoglyceric acid (PGA). However, some plants - particularly cane type plants such as sugar cane and maize use another compound, phosphoenol pyruvic acid as the substrate for CO_2 fixation. Experiments in the leaves of such plants have shown that the immediate product of carbon dioxide fixation is not 3-carbon PGA but the 4 carbon compound oxaloacetic acid. The oxaloacetic acid formed in C_4 plants is subsequently converted into malic acid, from which the CO_2 is fed into the Calvin cycle to form carbohydrate. This is known as the Hatch-Slack pathway [Roberts, 1987]. These

reactions proceed with different kinetic isotope effects, consequently the C_4 plants are more enriched with ^{13}C than the C_3 plants [Scmidt and Metges 1985]. Therefore ^{13}C is found naturally enriched in C4 plants such as corn products, maize and tropical fruits.

2.6.5 Units of Notation

Tracer results are usually expressed as abundance in units of atom percent. This is an absolute measurement of the number of atoms of the isotope in 100 atoms of the element. Atom Percent Excess (APE), therefore represents the increase in isotopic abundance above background levels. APE is used to indicate the extent of the abundance of highly artificial enriched compounds with the heavy isotope ^{13}C to a level of 99% [Barrie *et al.* 1989]. In the literature, the abundance of ^{13}C is expressed as Delta units (δ) [Schoeller *et al.* 1980; Nakamura *et al.* 1982]. The delta value represents the relative difference between the isotopic ratio of the sample against a reference sample with a known isotopic composition. A common standard for ^{13}C is Pee Dee Belemnite, a limestone, which has an abundance of 1.1112328 atom % ^{13}C and a $^{13}C/\ ^{12}C$ ratio of 0.0112372. Secondary or working standards are calibrated against PDB to allow delta values to be quoted against PDB, for example sugar beet. This must be done otherwise measurements made at different times on the same instrument and on different instruments would not be comparable [Schoeller *et al.* 1980]. Delta values are used because this method allows the detection of extremely small amounts of ^{13}C, and produces figures as whole numbers to allow easier calculation. The values for ^{13}C enrichment are therefore either positive or negative from the standard according to the formula :-

$$\delta\ ^{13}C = \frac{^{13}C/\ ^{12}C_u - {}^{13}C/\ ^{12}C_s}{^{13}C/\ ^{12}C_s}$$

where u = unknown

s = standard [Schoeller *et al.* 1980]

The natural abundance of ^{13}C, when excreted in breath or stool in delta units is approximately -27 to –25, and when a sample is enriched, this number becomes less negative to -14, for example. The use of ^{13}C tracers at low enrichments or at high dilutions has occasionally resulted in a final isotope composition that is within the range of natural isotope composition of various tissues and metabolic products. These isotopic variations are generally attributed to differences in the isotopic composition of the diet [Nakamura *et al.* 1982]. Unlike ^{14}C tests in which the isotope background is low, ^{13}C tests are performed against a large background or natural abundance of approximately 1.1% ^{13}C. This abundance is not constant but has a natural variation (1.06% to 1.12%) which reflects the enrichment or depletion of ^{13}C relative to ^{12}C due to isotopic fractionation. Because there is a natural background of $^{13}CO_2$, the labelled CO_2 is actually the amount of $^{13}CO_2$ in excess of the abundance before the labelled substrate was administered. The test signal is therefore the difference between the initial abundance of ^{13}C and that after ingestion of the labelled substrate [Schoeller *et al.* 1980]. In these situations it has been suggested that ^{13}C abundance be measured for a breath test period after consumption of the test meal or diet alone. This background ^{13}C-abundance profile can then be subtracted from the enrichment profile subsequently generated when the labelled test substrate is administered under identical dietary and environmental conditions [Jones *et al.* 1985]. Schoeller and colleagues documented the isotopic abundance of ^{13}C in common foodstuffs, and illustrated the effect that the constituents can exert on the basal breath $^{13}CO_2$ abundance during a ^{13}C breath test and how the effects can be minimised. Samples of the foodstuffs were combusted and the CO_2 formed was isotopically analysed on a mass spectrophotometer. The ^{13}C isotopic abundance was expressed as the per mil relative difference from the reference standard PDB.

2.6.6 ^{13}C-labelling studies

In order to understand the complexities of lipoprotein metabolism, and to differentiate between diet- derived and endogenous fatty acids, ^{13}C-labelling of dietary lipids has been introduced [Packard, 1995; Jones *et al.*1999]. ^{13}C-labelling offers a safe alternative to traditional radio-labelling of substrates, and the use of various ^{13}C-labelled

substrates is becoming increasingly widespread. Such approaches have been used to examine the handling of dietary lipid at the level of the GI tract and subsequently, in the circulation. Schoeller *et al.*[1981] were the first to quantify the absorption of lipid by measuring the excretion of ^{13}C-label in stool. Since then work on the GI handling of lipids has been carried out in Southampton [Murphy *et al.*1995; Stolinksi *et al.* 1997; Jones *et al.*1998/1999]. This work has shown that if ^{13}C-labelled TAG is presented in a dietary form, which is within an emulsified meal, absorption in normal subjects is between 95 and 100% complete.

Within the circulation, ^{13}C-labelled fatty acids can be differentiated from non-labelled acids and assumptions can be made regarding the metabolism of dietary lipids, although there are no published studies in the literature relating oral administration of a ^{13}C-labelled lipid and its appearance in the circulation. The appearance of $^{13}CO_2$ on the breath can be used as a marker for the oxidation of the labelled substrate. Such techniques have been used to examine the effects of different fatty acids on oxidation [MacDougall *et al.*1996], fatty acid chain length and saturation [Jones *et al.*1999], β-oxidation [Baurle *et al.*1998], age and gender of subjects [Jones *et al.*1998], and disease states such as cystic fibrosis [Amarri *et al.*1998]. As the use of ^{13}C-labelling techniques is relatively novel, there is much scope in the area for examining how differences in dietary and endogenous lipids can account for changes noted by traditional measures. For example, as the total carbohydrate oxidation increases and lipid oxidation decreases with increasing carbohydrate load [Whiteley *et al.* 1997], how does the balance of meal and endogenous fatty acid oxidation alter? Also do the changes in postprandial lipaemia caused by dietary modulation occur because of variation in CM-TAG, VLDL-TAG or both?

2.7 Summary of literature and aims of research

Lipid is known to be an important nutrient in the diet and the consumption of lipid has been associated with cardiovascular disease, obesity and insulin resistance. Previous evidence focusing on the link between lipids and disease has been based on large-scale epidemiological studies with lipid metabolism measured in the postabsorptive state emphasising the role of the liver and cholesterol metabolism. More recently the focus has moved towards events in lipid metabolism which occur in the postprandial state, and how the consumption of lipid is associated with disease through the biological processing of dietary lipid at the level of triacylglycerol, in particular chylomicron TAG, and its clearance. The way in which the body handles dietary lipid has been studied from two different perspectives. Firstly by examining differences in lipid handling between individuals with disease to understand the underlying mechanisms in disease processes, and secondly by manipulating the biological process, initially through diet or drug therapy to elicit a change in lipid metabolism, which may affect a chosen outcome variable such as TAG clearance or oxidation. Although several studies have examined the role of meals on postprandial circulating lipids, and to a lesser extent, substrate oxidation, several questions remain unanswered and conflicting interpretation of results has occurred. What is clear is that the manipulation of the composition of meals can have a profound effect on lipid metabolism even if the underlying mechanisms remain unclear. There are few studies, if any, in the literature which systematically examine the effect on lipid metabolism of manipulating the macronutrient balance of an individual meal and comparing the outcome within individuals. Research that has taken place in this area has resulted in conflicting results and interpretations depending upon the type and amount of macronutrient used. Clear statements regarding the effect of altering the macronutrient composition of meals on postprandial circulating lipids and substrate oxidation are not available, and the effect of altering meal composition on disease outcome cannot be ascertained.

The research reported in this thesis employed traditional measures of lipid metabolism; indirect calorimetry and plasma lipid concentrations, and innovative stable isotope tracer methodologies; ^{13}C-lipid oxidation and ^{13}C-TAG and NEFA concentrations in the circulation, to examine the effect of manipulating the

macronutrient balance of a meal on the postprandial metabolism of dietary lipid. The central hypothesis to this research was that:-

Manipulation of the amount and type of macronutrient within a meal will affect the residence time of exogenous and endogenous lipids in the circulation, in terms of appearance, clearance and entrapment of fatty acids to tissues, by manipulation of the activities of both lipoprotein lipase and hormone sensitive lipase. As a consequence, the rate and extent of oxidation of substrates will differ in the manipulation of the ratio of exogenous and endogenous fatty acid oxidation. This information will enable the development of a conceptual framework in which it would be possible to determine how altering the lipid and carbohydrate components of the meal or diet may alter lipid metabolism and by inference the resultant effects on cardiovascular disease, insulin resistance and obesity can be ascertained.

The specific aims and sub-hypotheses of the research were:-

1) To validate the study protocol used throughout the studies reported in this thesis by examining the repeatability of measures of postprandial lipid metabolism. This was to test the hypothesis that once extrinsic factors such as diet and lifestyle were controlled for that there would be little residual variability. In addition faecal losses would be minimal with the emulsification of dietary lipid.

2) To examine the effect of increasing the amount of lipid in a testmeal to test the hypothesis that increasing the amount of exogenous lipid would result in increased concentrations of circulating TAG and NEFA by saturating the clearance capabilities of LPL. The raised NEFA concentration would in turn result in an overall increase in total lipid oxidation.

3) To investigate how altering the carbohydrate content of a meal will influence postprandial lipid metabolism. This was to test the hypothesis that the addition of carbohydrate will lead to an increased insulinogenic response. Insulin will act to stimulate LPL and therefore lead to a decrease in circulating lipid concentration.

Excess glucose and suppressed NEFA concentrations will result in an increase in carbohydrate oxidation and decrease in lipid oxidation. In particular, insulin will inhibit the release of endogenous fatty acids from adipose tissue and result in a decrease in endogenous lipid oxidation.

4) To determine how the manipulation of the macronutrient composition of meals will affect lipid metabolism, and to provide evidence to develop a conceptual framework to consider how altering the components of the meal may affect disease.

CHAPTER 3

Methodology

3.0 Introduction

The methodology described in this chapter is divided into two sections. Section 1 describes the study and laboratory techniques which have been used throughout the studies including testmeal composition, general study protocols and laboratory techniques. Section 2 describes the evidence provided to validate the methods used, focusing on the testmeal composition, and the introduction of laboratory techniques for the assessment of ^{13}C-enrichment in plasma samples.

3.1 Background

In previous ^{13}C-labelling studies conducted in Southampton, the labelled fatty acid, palmitic acid, was consumed in an unemulsified form, with a large proportion of the ^{13}C-label recovered in stool (up to 71%), suggesting poor absorption of dietary lipid [Murphy *et al.* 1995]. Dietary lipid which is not absorbed, reported to be 5 to 6g/day in normal healthy adults [Wollaeger *et al.*1947; Southgate and Durnin, 1970; Wrong *et al.*1981; Murphy, 1991], is excreted in stool, equivalent to approximately 5% of dietary lipid. As ^{13}C-label excretion in stool was of a greater proportion of administered dose than dietary lipid excretion reported in stool, this method for the administration of the fatty acid did not give a true representation of the GI handling of dietary lipid. Lipids in the diet are generally consumed as emulsions such as within cheese or milk [Dickinson, 1992]. A glucose: sucrose:casein:lipid emulsion was developed, based on Emken *et al.*[1993] to act as a vehicle to deliver the tracer and tracee to the body, resulting in the excretion of substantially less labelled fatty acid (< 5%). The reference testmeal (emulsion and meal component) used throughout the studies reported in this thesis was designed to reflect the typical composition of an average UK male diet [Gregory *et al* 1990] (40% of total energy from dietary lipid, 45% from carbohydrate and 15% from

protein), assumed to be representative of the diet of the subject group. The components of the reference testmeal and emulsion can be seen in table 3.1.

Table 3.1 Components of the reference testmeal (emulsion and meal component)

MEAL	EMULSION
100.0g white bread	22.0g double cream
38.0g ham	3.5g olive oil
19.0g Clover margarine	3.0g sunflower oil
	12.0g casein
	9.0g glucose
	4.5g beet sugar
	10.0g milkshake powder
	10mg/kg body weight [1,1,1-^{13}C]tripalmitin

The test meal and emulsion were developed to provide the subjects with approximately one third of their daily average energy requirements. 10mg of [1,1,1-^{13}C]tripalmitin per kg body weight was administered to the subjects, as this amount allowed ^{13}C-label to be recovered in detectable quantities in breath, stool, and plasma samples following the study, and was proportional to body size. The details of the composition of the testmeal and emulsion are shown in table 3.2.

Table 3.2 Macronutrient composition of testmeal (emulsion and meal component)

	MEAL	EMULSION	TOTAL
Energy	1735 kJ	1206 kJ	**2941 kJ**
Lipid	18.1 g (39.7% en.)	17.4g (53.4% en.)	**35.5g (45% en.)**
Carbohydrate	49.5g (45.3 % en.	23.2g (30.8% en.)	**72.7g (40% en.)**
Protein	15.5g (15.2% en.)	11.2g (15.8% en.)	**26.7g (15% en.)**
SFA	13.3% energy	23.1% energy	**17.2% energy**
MUFA	13.5% energy	18.1% energy	**15.3 % energy**
n-3 PUFA	0.9% energy	0.3% energy	**0.5 % energy**
n-6 PUFA	4.7% energy	6.0% energy	**5.2 % energy**
TUFA	2.4% energy	1.9% energy	**2.2 % energy**

The studies reported in this thesis have examined the effect of manipulating the macronutrient composition of the reference testmeal by altering the composition of; the reference meal, the reference emulsion, or the meal and the emulsion. In the high-fat study, the amount of lipid in the emulsion and meal component was increased, whereas in the carbohydrate study, the emulsion and meal component remained constant and an additional carbohydrate drink was added. The unaltered reference testmeal was consumed to examine the repeatability of measures of postprandial lipid metabolism (chapter 4), and as a reference testmeal in the high fat study (chapter 5).

3.2 ^{13}C-labelled substrates

A single tracer, [1,1,1-^{13}C]tripalmitin (Masstrace Inc., Woburn, USA), was used throughout the studies reported in this thesis, to trace the metabolism of the dietary palmitic acid within the testmeal. Each fatty acid in the TAG was labelled at the carboxyl end of the chain. The labelled TAG was enriched to 99 APE, indicating that 99% of the carbon atoms in the carboxyl position would be ^{13}C. The remaining carbon atoms in the molecule were labelled with ^{13}C at a natural abundance equivalent to baseline breath $^{13}CO_2$ values.

Tripalmitin was chosen as the labelled substrate for these studies as it is the predominant saturated fatty acid present in the UK diet [Gregory *et al.* 1990]. The composition of the lipid emulsion was chosen as the predominant fatty acids were saturated, including both palmitic and stearic acids present in the cream (see section 3.8.1).

3.3 Subject Group

Young and healthy male subjects were chosen as the subject group to take part in these studies as they had no age or health related impairment of lipid metabolism, and it was easier to detect differences in lipid metabolism with meal modification in such a group. Female subjects were not included to avoid having to take account of hormonal effects on lipid metabolism. The inclusion criteria for these studies were: an age between 18-32 years as TAG metabolism has been shown to be impaired with increasing age (45+

years – Humayun *et al. In the press*), BMI between 20-30 kg/m^2 to avoid body fat related disorders of lipid metabolism, non-smokers, non-vegetarian and have no history of cardiovascular disease or insulin resistance. A total of six subjects were recruited for each study (study 1 or study 2; section 3.4), with three repeated trials for each study. Power analysis calculation of the number of subjects required for the studies is discussed in section 3.5.10, and the subject details are provided in the relevant results chapters.

Studies were fully explained to the subjects, both in writing and verbally, with written consent obtained and the subjects GP also contacted. The subject retained the right to withdraw at any point throughout the study without prejudice. Ethical committee approval for all studies was granted by the Southampton and South West Hampshire Health Commission.

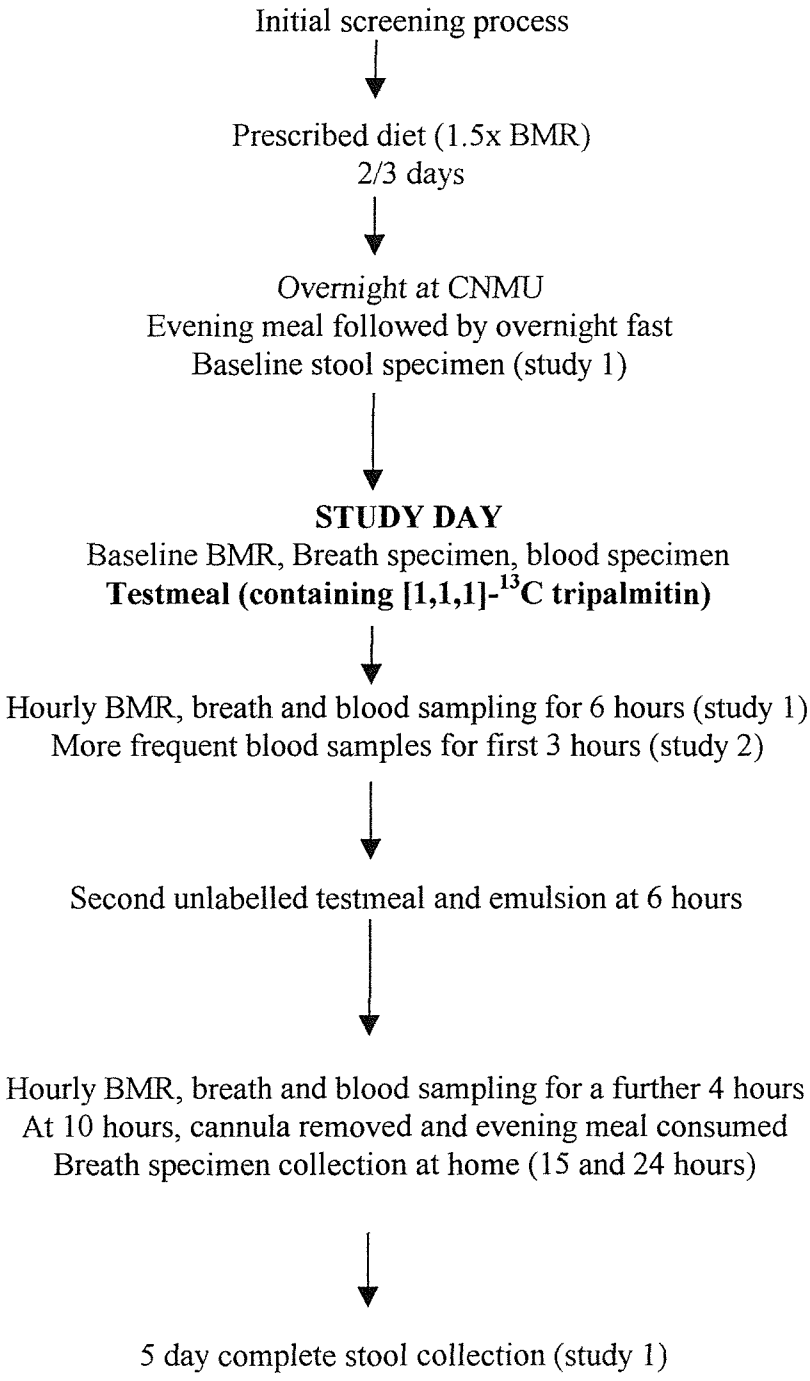
3.4 General Protocol

All studies followed a general protocol, with only the reference testmeal composition altering on each trial. In the following chapters, study 1 will refer to three trials (reference testmeal 1, reference testmeal 2 and high-fat testmeal), and study 2 will refer to three trials (0g sucrose, 50g sucrose or 100g sucrose testmeals). On completion of study 1, minor alterations were made to the general protocol to improve future trials. Subjects were required to complete a short screening process before beginning a study where basal metabolic rate was measured to estimate the average daily energy requirement and anthropometric measurements were made (weight, height, body mass index (kg/m^2), and body composition) to assess the suitability of the individual for the study. The subjects performed a weighed food intake over a 5 day period before beginning the study.

The general protocol for the studies is shown is figure 3.1. For two (study 2) or three days (study 1) prior to the study day the subjects were fed a fixed and prescribed diet. The diets were identical in energy/macronutrient composition with an incremental component of similar composition to achieve 1.5 x BMR for each individual. Subjects were permitted to consume diet drinks and water freely, and asked to consume no other foods over the prescribed diet feeding period. On the evening of the final feeding day (day 2, study 2 and day 3, study 1), subjects were admitted to the Clinical Nutrition and

Metabolism Unit, Southampton General Hospital, where they consumed a standard evening meal at 7-7.30 pm , and refrained from food overnight. From admission to the unit, subjects were supervised and remained on the unit at all times. On the morning of the study day, basal metabolic rate was measured by indirect calorimetry followed by collection of an end tidal, expired air, breath specimen. Following the insertion of an indwelling cannula into a forearm vein, a baseline blood specimen was taken. The lipid:casein:glucose:sucrose emulsion containing [1,1,1-^{13}C]tripalmitin was prepared over a 20 minute period to achieve optimum emulsification and temperature on consumption by the subject. The emulsion was consumed at a temperature of 55-65°C to keep the ^{13}C-substrate above its melting point to prevent recrystallisation of the tracer fatty acid, with the pre-prepared meal component and beverage (orange juice in study 1; carbohydrate drink in study 2). Hourly metabolic rate measures, breath and blood specimen collection were performed until 6h when a second unlabelled emulsion and testmeal was consumed. The second meal was introduced to provide a habitual meal pattern for the subject group indicated by food diary analysis. The study continued for a further four hours with hourly metabolic rate, blood and breath specimen collection. The cannula was removed 10 hours after the breakfast was consumed, and an identical evening meal served to each subject. Expired air breath specimens were collected by the subjects following their return home at 15 and 24 hours post testmeal consumption. In study 1 subjects were asked to collect a baseline stool specimen before consuming the testmeal, and all stools passed for 5 days following each study and to refrain from consuming foods naturally enriched with ^{13}C including corn and maize products, sweetcorn, exotic fruits and cane sugar during this time. Subsequent trials were carried out at approximately four-week intervals to allow breath, stool and plasma ^{13}C-enrichment values to return to baseline.

Figure 3.1 Flow diagram of general study protocol used to examine the effect of altering meal composition on the GI handling and postprandial metabolism of dietary lipid



3.5 Specific Methods

The methods used throughout the studies reported in this thesis are described in the following section. Study protocol methods are described initially followed by laboratory procedures.

3.5.1 Preceding Diet Composition

The subjects were fed a prescribed diet (study 1 for three days; study 2 for two days) prior to the study day on each occasion to ensure that each subject was consuming identical foods before each trial. The diet was matched to the energy requirements of the individual as BMR x 1.5, which allows for habitual physical activity [Department of Health, 1989]. The composition of the diet was based upon the typical UK diet [Gregory *et al.*1990], for males of a similar age group. The diets were designed to give a typical days food intake (from food diary analysis for the group) in terms of a breakfast, lunch and evening meal with snacks, and were in the form of ready meals and pre-packed items. All subjects received identical diets with an incremental component of similar composition (white bread, ham and margarine) to attain an energy intake of 1.5 x BMR. All subjects were fed an identical evening meal prior to each study day to reduce both between and within subject variability in outcome measures on the study day. The content and composition of the diets and preceding meal is shown in appendix 1.1 and 1.2.

3.5.2 Food Intake Assessment

All foods and drink (except water) consumed over a five-day period, was weighed and recorded by subjects before commencing a study. This was necessary to determine the habitual pattern of food intake of the subjects to determine an appropriate meal plan for the feeding period before each trial. Dietary analysis of the diaries was performed using a computerised diet composition program (FOODBASE, Institute of Brain Chemistry and Nutrition, London, UK).

All food intended for consumption, and waste, was weighed on digital operated scales (Soehnle, CMS Weighing Equipment Ltd., London., UK) to 1g, and recorded in a diary. Subjects were required to provide as complete information as possible regarding the type of food e.g. full fat or semi-skimmed milk, and the cooking method e.g grilling or frying.

3.5.3 Anthropometry

Anthropometry (height, weight and body composition) was performed on all subjects to determine whether subjects met the inclusion criteria for the studies. All measurements were made following urination and prior to consumption of breakfast. Weight was measured in kilogrammes on digital scales (Soehnle Digital S, CMS Weighing Equipment Ltd, London, UK) with shoes and heavy clothing removed. Height was measured in metres to the nearest 1cm using an electronic, portable stadiometer (Digi-Rod, CMS Weighing Equipment Ltd., London, UK), without shoes and with subjects standing as straight as possible. Body mass index was calculated from measures of weight and height (weight (kg)/ height (m^2)). Bioelectrical impedance was used to estimate both total body fat and lean body mass (Bodystat 1500,Bodystat Ltd., Isle of Man, UK), with subjects measured in the supine position, with legs and arms splayed so not touching other parts of the body. Percentage body fat was determined by the Bodystat 1500 from gender, age, weight, height and impedance. Lean body mass was determined from the proportion of the total body weight not accountable for by body fat.

3.5.4 Stool Collection and Analysis (Study 1 Only)

A baseline stool specimen, and all stools passed over the five day period following testmeal consumption were collected for each trial. Stools were collected individually into polythene bags, labelled and frozen immediately at -20°C. All stools passed on an individual day were pooled (total weight recorded) and homogenized with distilled water (100-200g). An aliquot of wet stool (10-20g) was dried on a rotary evaporator (Genevac Ltd., Ipswich, UK) to a constant weight, whilst a further 100-150g was frozen at -20°C and stored. Dried stool was removed from the evaporator tube, and

ground to a fine powder using a mortar and pestle. Duplicate samples, to prevent anomalous results, from each day of collection were weighed (1.8 ± 0.2mg) into tin capsules (Elemental Microanalysis, Okehampton, UK), and analyzed for the ^{13}C-enrichment of each specimen. The analysis of the ^{13}C-enrichment of the stool samples was carried out by continuous-flow isotope ratio mass spectrometry (CF-IRMS) (20/20 IRMS – GSL interface, Europa Scientific Ltd, Crewe). Analysis by CF-IRMS determines the total ^{13}C-enrichment of a solid sample, and the process is described fully in section 3.5.9. The total ^{13}C-label in the whole sample was expressed as a proportion of that administered in the testmeal as calculated using the equations of Schoeller *et al* [1981] (section 3.5.10).

3.5.5 Breath Collection and Analysis

Specimens of expired air were analysed to determine the excretion of ^{13}C-label, as $^{13}CO_2$, on breath to provide a measure of the oxidation of the [1,1,1-^{13}C]tripalmitin. End tidal expired air breath specimens were collected before testmeal consumption, hourly for 10 hours, and at 15 and 24 hours postprandially. Subjects exhaled fully into a 750ml alveolar breath bag (Quintron, Milwaukee, USA) to collect an end tidal breath specimen. The breath bag had a mouth piece with a one way valve to prevent the loss of breath from the bag. Three consecutive 10ml breath specimens were transferred from the bag to evacuated gas sample containers (Isochem Ltd., Berks., U.K.) for storage, using a system of two way taps, a 10ml syringe and a needle.

Two breath specimens were analysed for ^{13}C-enrichment by CF-IRMS (20/20 IRMS- GSL interface, Europa Scientific Ltd., Crewe, UK) and the third stored to prevent single anomalous results, and to ensure specimens were available in the event of CF-IRMS failure. The $^{13}CO_2$ excreted on breath expressed as a proportion of the administered dose of [1,1,1-^{13}C]tripalmitin, was calculated from the equations of Watkins *et al.* [1982] (section 3.5.10).

3.5.6 Indirect Calorimetry

Indirect calorimetry is a method by which substrate oxidation and metabolic rate can be estimated by measurements of the consumption of oxygen and the excretion of carbon dioxide on breath. Indirect calorimetry utilises the known proportionality between oxygen consumption ($\dot{V}O_2$) and carbon dioxide excretion ($\dot{V}CO_2$) to estimate metabolic rate. This method assumes that all oxygen consumed is used to oxidise substrates, and all the carbon dioxide evolved is recovered on breath. Measurements of the percentage of O_2 and CO_2 in inspired air (FiO_2/ $FiCO_2$), and expired air (FeO_2/ $FeCO_2$), are used to determine the rate of oxygen uptake ($\dot{V}O_2$) and carbon dioxide excretion ($\dot{V}CO_2$).

The Gas Exchange Monitor (GEM, Europa Scientific Ltd., Crewe, UK) consists of a ventilated hood which is placed over the subjects head to provide a constant flow of room air (approx. 40l/min) to prevent asphyxiation, and to collect expired breath in the calorimeter for gaseous analysis. Measurements were made with subjects rested in the supine position, but not sleeping. Discontinuous measurements were made over the study period, at the same time intervals as breath specimen collection to prevent subjects from becoming restless. Indirect calorimetry measurements were performed for 30 minutes prior to consumption of the testmeal, and for 15 minute periods at every hour during the study. Measurements were calibrated every hour against a 5% CO_2, 95% O_2 gas mix, and the equipment validated each month by the burning of pure ethanol.

Calculations of carbohydrate and lipid oxidation rates were made using measurements of $\dot{V}O_2$, $\dot{V}CO_2$ and urinary nitrogen [Frayn, 1983], both in the postabsorptive and postprandial states. The equation is derived from the total oxygen used and carbon dioxide produced if 1 g of a substrate was oxidised. The reference values used are those for glucose and a typical TAG found in adipose tissue, Palmityl Stearyl Oleyl Glycerol (PSOG). From this information rearrangement of the equations derives two further equations to calculate substrate oxidation (g/min):-

$$\text{Carbohydrate oxidation} = (4.55 * \dot{V}CO_2) - (3.21 * \dot{V}O_2) - (2.87 * \text{nitrogen})$$

$$\text{Lipid oxidation} = (1.67 * \dot{V}O_2) - (1.67 * \dot{V}CO_2) - (1.92 * \text{nitrogen})$$

Nitrogen oxidation was not measured in these studies but was estimated from the calculation of the average daily intake of nitrogen over the days preceding the study. As all the subjects were healthy and weight stable, it was assumed that they were in nitrogen balance such that nitrogen intake should equal nitrogen oxidation. Therefore the calculation of nitrogen intake (g/min) was used as a figure for nitrogen oxidation which differed for each subject due to the differences in preceding diet composition (1.5 x BMR).

3.5.7 Blood Collection and Analysis

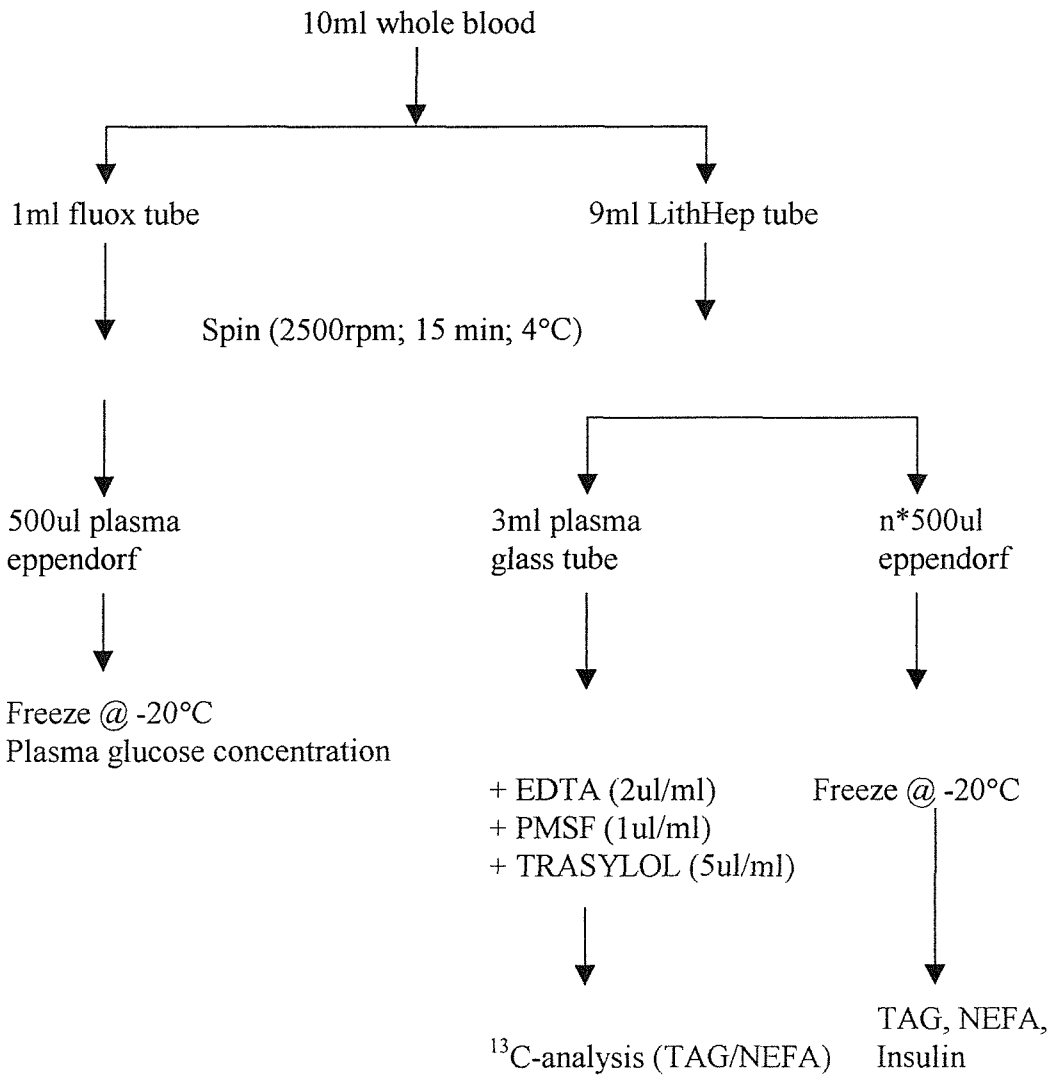
Postprandial changes in plasma lipid concentrations were determined by the collection of sequential blood specimens throughout the study day. An indwelling cannula (Insyte, Becton Dickinson Ltd., Oxford, UK) was inserted into the forearm vein, permitting repeated sampling of blood with no added discomfort to the subject. The cannula was inserted following the initial indirect calorimetry measurement, prior to consumption of the testmeal.

Figure 3.2 shows the process involved in the separation of whole blood specimens to plasma for analysis. A blood specimen (10 ml) was taken at each timepoint (baseline and hourly for 10 hours postprandially) into a syringe and then aliquoted into vacutainer tubes for centrifugation. One aliquot of blood (9ml) was transferred to a Lithium Heparin tube, while the remaining blood (1ml) was transferred to a sodium fluoride oxalate tube, and both tubes were centrifuged at 2500rpm (15 minutes; 4°C) to separate plasma and cells. Aliquots of plasma from the lithium heparin tubes were transferred to glass tubes, for ^{13}C analysis (3ml); study 1 for chylomicron separation and study 2 for plasma ^{13}C analysis, and to eppendorf vessels (0.5ml) for enzymatic determination of total TAG, NEFA, and insulin concentrations. Plasma from the sodium fluoride tube was removed for analysis of glucose concentration, and the remaining red cells discarded.

Plasma for enzymatic assays (TAG, NEFA, glucose and insulin) was frozen immediately at -20°C. Due to time constraints, the concentrations of plasma TAG, NEFA and glucose were measured by Dr. A. Jones (Institute of Human Nutrition, University of Southampton) for study 1, and with the assistance of Katja Sassi (Institute

of Human Nutrition, University of Southampton) for study 2. The methods used for the analysis of plasma TAG, NEFA and glucose are shown in appendix 1.3. Due to the radio-immuno assay involved plasma insulin concentrations were analysed by Clinical Endocrinology at Southampton General Hospital. Plasma collected for ^{13}C analysis was frozen immediately at –20°C, and subsequently thawed for the extraction of lipid and derivatisation of fatty acids described in section 3.5.8 (study 2 only).

Figure 3.2 Separation of whole blood to plasma for analysis of ^{13}C-enrichment, and concentrations of TAG, NEFA, Glucose and Insulin



3.5.8 Analysis of the ^{13}C-enrichment of plasma samples

Total plasma concentrations of TAG and NEFA were used to determine the pattern of total lipid appearance and clearance from the circulation, but by using these methods alone it was not possible to differentiate between endogenous lipid, i.e. from adipose tissue or excreted from the liver (VLDL-TAG), and exogenous lipid i.e. from the meal (TAG rich chylomicrons). By labelling the lipid within the testmeal, it was possible to trace the ^{13}C-label through circulating lipids and to calculate concentrations of ^{13}C-lipid in the circulation. To analyse ^{13}C-labelled lipids by GC-IRMS, it was necessary for fatty acid methyl esters to be generated by 3 distinct phases; lipid extraction, lipid separation and fatty acid derivatisation, which will be described in turn, shown in appendix 1.4. Validation of these techniques is described in section 3.10.

3.5.8.1 Lipid Extraction

Plasma (1ml) collected at each timepoint during the studies was pipetted into a clean glass tube with TAG (triheptadecanoin; 60ug/60ul chloroform: methanol 2:1 v/v) and NEFA (Heneicosanoic acid; 30ug/30ul chloroform: methanol 2:1 v/v) surrogate standards. Chloroform: methanol (2:1 v/v; 5ml) was added to the lipids. After mixing, 1M sodium chloride solution (1ml) was added and the samples spun at 2000rpm (10 minutes; 4°C) to separate the solvent and aqueous phases. Three distinct phases were visualised: a top layer (aqueous phase); a protein rich interphase plug, and a lower layer (solvent phase). The aqueous phase was removed and discarded, and the solvent layer was removed to a clean tube by passing a pasteur pipette through the interphase plug. The process was repeated using the interphase protein plug as extraction substrate to ensure a high recovery of lipid from the extraction. The solvent extract was sealed under nitrogen and stored in the freezer at -20°C until required.

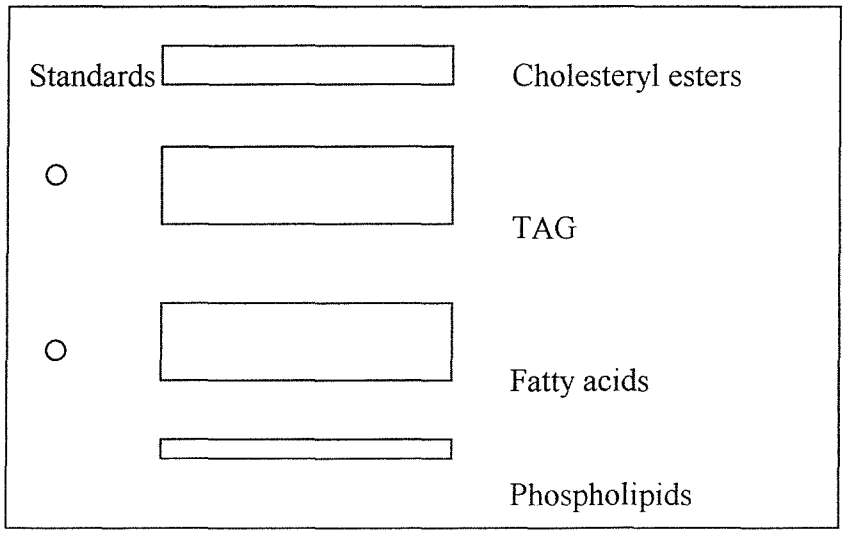
3.5.8.2 Separation of Lipid Classes

The lipid classes such as free fatty acids, TAG and phospholipids range in polarity and therefore it is possible to separate them using a thin layer chromatography

(TLC) system. The lipid extracted from plasma as described in section 3.5.8.1 was removed from the freezer and gently dried down under nitrogen at 40°C, to leave a lipid residue in the glass tube. Chloroform (100ul) was added to the residue, and agitated for the lipid to be dissolved. 15ul aliquots of the sample were pipetted onto the TLC plate (20cm x 20cm; Silica Gel 60, Merck, Germany) using a Hamilton syringe. Additional chloroform (100ul and 50ul) was added to the tube to ensure complete transfer of the lipid to the TLC plate. TAG (C17:0; 1ug/ul chloroform: methanol 2.1 v/v) surrogate standard (30ul) and NEFA surrogate standard (C21:0; 1ug/ul chloroform:methanol 2.1 v/v) standard (15ul) were pipetted onto the plate to allow visual identification of lipid classes.

The TLC plate was placed in a solvent tank (Hexane 70ml; Diethyl ether 30ml; acetic acid 1.8ml), and left to develop for 30-40 minutes. In principle, TLC acts due to the polarity of the lipid classes. TAG and cholesterol esters are both neutral compounds and are therefore uncharged or non-polar. The solvent tank contains non-polar hexane and diethyl ether, and polar acetic acid. The non-polar molecules travel the furthest distance on the plate and can be identified by standard markers (surrogate standards as described previously). Phospholipids and fatty acids, however are charged molecules, and their polarity prevents movement on the plate resulting in four distinct bands appearing on the plate (figure 3.3).

Figure 3.3 Diagrammatic view of a TLC plate following separation of the lipid classes



On completion, the plate was removed and allowed to dry before spraying with a fluorescent dye (Fluoresein 0.25% in ethanol (w/v)). Under ultraviolet light, the lipid bands fluoresced and surrogate standard bands allowed the identification of TAG and FFA classes. The silica powder from the bands was scraped from the plate and transferred to a glass tube. Toluene (1ml) and 2% sulphuric acid in methanol (2ml; v/v) were added to the silica in the tube.

3.5.8.3 Formation of fatty acid methyl esters

The samples containing silica and 2% sulphuric acid in methanol (2ml; v/v) were placed in a heating block at 50°C for 18 hours to allow complete methylation of the fatty acids. After 18 hours incubation, the samples were removed from the heating block and allowed to cool. Neutralising reagent (25g $KHCO_3$ + 34.55g K_2CO_3 in 500ml distilled water; 2ml) was added to each sample to prevent further reaction. Hexane (2ml) was added to each sample and samples were centrifuged (2500rpm; 14°C; 10 minutes) to separate the aqueous (upper phase) and solvent (lower phase) phases. The upper, solvent phase was completely removed to a round-bottomed tube, hexane (2ml) added to the remaining aqueous phase, and the process repeated. The solvent layer was gently dried down under nitrogen until completely dry. Round-bottomed tubes were washed with dry hexane (200ul), vortexed and the solvent transferred to GC-mini vials. The process was repeated three times and the solvent dried under nitrogen in the mini-vials. When dry, an internal standard (C23:0; 1ug/ul dry hexane w/v; 60ug TAG/ 30ug NEFA) was added to each vial for the determination of the recovery of the surrogate standards and to act as an internal ^{13}C-enrichment standard when analysed by GC-IRMS.

3.5.9 ^{13}C-enrichment analysis of samples by Isotope Ratio Mass Spectrometry

The ^{13}C-enrichment of stool and breath specimens was measured using continuous flow- isotope ratio mass spectrometry (20/20 IRMS with GSL interface). CF-IRMS integrates the sample preparation with the mass spectrometer, to give a 'sample-in, results-out' operation [Barrie *et al.*1989] which consists of three sections; sample preparation, capillary interface and mass spectrometer. Liquid, solid and gaseous

samples were analysed using the CF-IRMS system to determine both total carbon and ^{13}C enrichment of samples. In addition, ^{13}C-enrichment of plasma samples was analysed using the ORCHID GC-IRMS (Europa Scientific Ltd, Crewe, UK) which indicates the proportions of fatty acids present in the sample and the enrichment of each fatty acid.

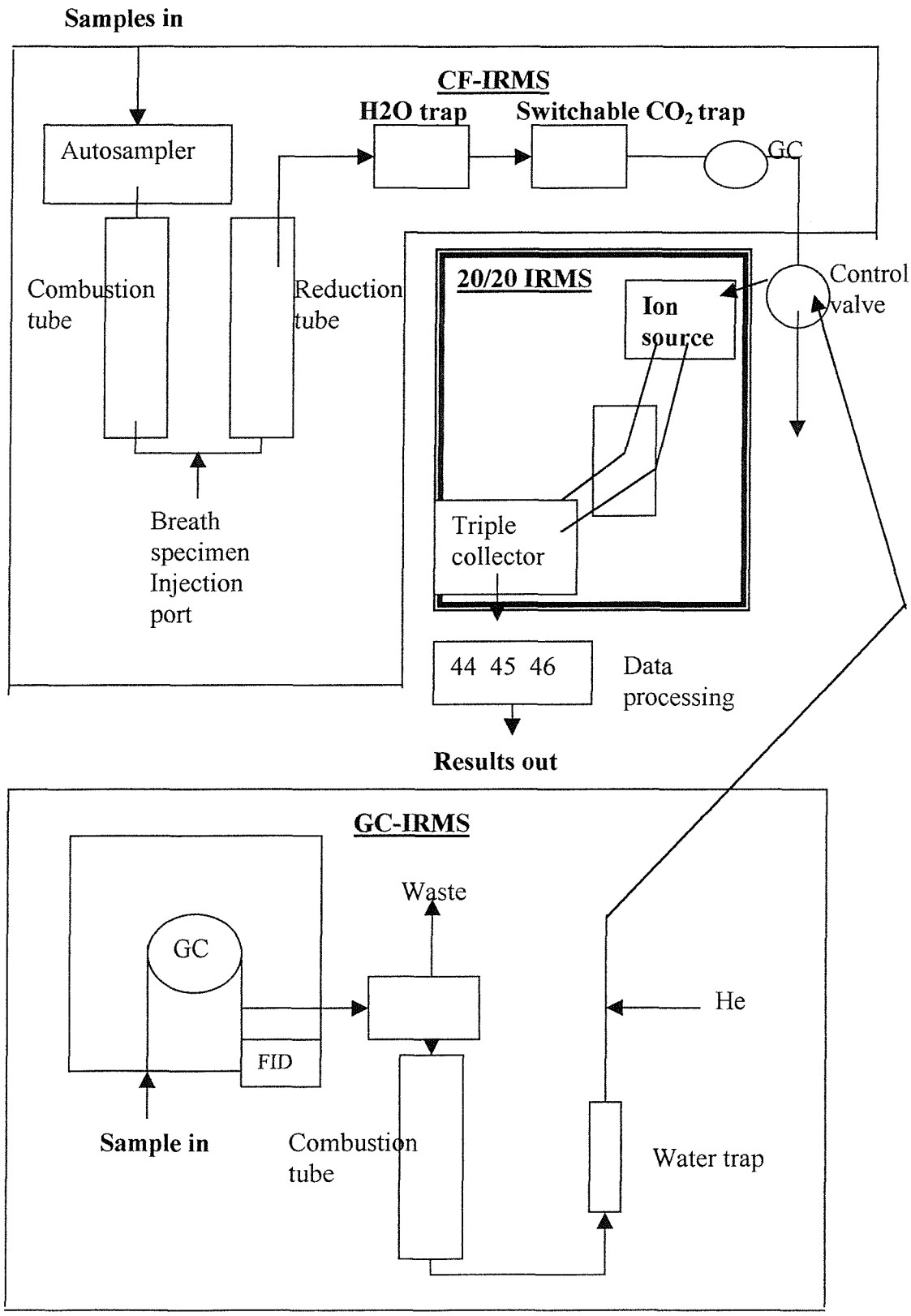
The 20/20 IRMS (GSL) system for analysis of gaseous, solid or liquid samples is described in figure 3.4. Solid (e.g stool) or liquid samples were prepared in tin capsules (Elemental Microanalysis, Okehampton, UK) and loaded into a 65 space carousel with 3 reference samples initiating the run, followed by 6 samples then 2 reference samples repeated until completion with 2 reference samples. Reference samples were 1.8 (± 0.2) mg beet sugar for stool samples. The type and amount of reference sample was chosen as it had similar ^{13}C-enrichment and carbon content as baseline stool samples. Samples loaded in the carousel were dropped into a quartz combustion chamber (1000°C) consisting of chromium oxide granules, which act as an oxidation catalyst, followed by copper wire for oxidation of hydrocarbons, and silver wool to trap sulphur and halogens. A pulse of pure oxygen combusts the sample which generates a gaseous mixture containing CO_2 and H_20, which is then carried in a flow of pure helium (60ml/second) through a reduction tube (600°C). Water was removed from the sample and the sample then passed through a GC column (125°C) to separate the gases.

Breath specimens were loaded onto a 220-tube rack. Three reference samples initiated the run, followed by 6 samples and 3 references gases which was repeated until completion with 2 reference samples. The reference gas used was 10ml of 5% CO_2 , 95% N_2 mix (BOC gases, Manchester, UK). Breath specimens were injected into the system after the combustion tube by an autosampler needle, and carried in a flow of helium through the reduction tube, water scrubber and GC column. Only a small proportion of sample (1%) passed to the mass spectrometer, and the remainder entered the atmosphere. The mass spectrometer bombarded the sample with electrons to generate positive CO_2 molecules which passed through a magnetic field and were deflected depending upon their mass to charge ratio. The mass spectrometer detects three different molecules, masses 44 ($C_{12}0_{16}0_{16,}$) 45 ($C_{13}0_{16}0_{16}$) and 46 ($C_{12}0_{18}0_{16}$). The fully automated system generates, via a computer, results indicating the carbon mass and the ^{13}C-enrichment of the sample, which can be used in derived equations to determine the actual ^{13}C-enrichment of the specimen.

Plasma lipid samples were prepared as described previously (section 3.5.8). The ORCHID system consists of a gas chromatograph with a combustion/ reduction interface coupled to the Europa Scientific 20-20 Isotope ratio mass spectrometer (figure 3.4). This system was used as only nanomoles of sample are required, and compounds can be separated from a mixture [Barrie *et al.* 1989]. Samples were resuspended in dry hexane (5ul-100ul) and vortexed to ensure they were completely dissolved. A small aliquot (1ul-5ul) of sample was removed from the vial with a Hamilton syringe and immediately injected through the injection port to the GC-oven (140°C). Calibration of the equipment was against an internal standard (C23:0) in the samples, and by reference gas injection. The solvent (dry hexane) was removed from the mix immediately prior to combustion of the fatty acids . The fatty acid methyl esters were separated by gas chromatography on a fused silica capillar column. The FAMEs were combusted to CO_2 and water by heating in the presence of platinised copper, and the CO_2 and water passed through the Nafion membrane where the water was removed. The dried carbon dioxide then passed into the 20/20 MS system as described previously. Data collection and isotope post processing was performed by ORCHID software (ORCID GC-IRMS training manual, Europa Scientific Ltd, Crewe, UK).

The ^{13}C-enrichment of samples was expressed as the isotopic ratio unit delta ($^{0}/_{00}$). In order for delta units to make physiological sense the results were then expressed as a percentage of the administered or absorbed dose of ^{13}C-label for stool and breath, and calculated as the ug of ^{13}C-label present in plasma samples (section 3.5.10)

Figure 3.4 Schematic diagram of the 20/20 IRMS system for analysis of ^{13}C-enrichment of stool and breath specimens by CF-IRMS, and plasma specimens by GC-IRMS [adapted from Barrie et al.1989]



3.5.10 Calculations and Data Analysis

Excretion of ^{13}C in stool was expressed as a proportion of the administered dose of [1,1,1-^{13}C]tripalmitin determined using the equations of Schoeller *et al.* [1981] (appendix 1.5). The total excretion of ^{13}C-label in stool was calculated for the study period as the sum of ^{13}C-label excretion on the individual days. Excretion of $^{13}CO_2$ on breath was expressed as a percentage of administered dose determined using the equations of Watkins e*t al.* [1982] (appendix 1.6). Total excretion over the 24-hour study period was calculated as the area under the time versus percentage administered dose per hour graph. Recovery of ^{13}C-labelled fatty acids in plasma samples was determined as a proportion of total palmitic acid in the specimen using a series of equations (appendix 1.7), calculated as the area under the time versus fatty acid concentration curve GC-IRMS chromatograms compared to a surrogate standard of known concentration. A series of equations was then used to determine the ^{13}C-enrichment of the specimens.

The data from the studies was prepared and analysed using a computerised data package (SPSS for Windows, SPSS Inc., Chicago, USA). Results were expressed as the mean for the group plus or minus standard deviation from the mean to show the within-group variability. Statistical analysis using two way repeated measures analysis of variance on both factors (time and trial) with post-hoc tests (Tukey's Honestly Significant Difference) was used to determine the significant difference between trials and over the study period. One way analysis of variance was also used to determine the statistical difference between the trials for the complete study period. Statistical significance was assumed at the 5% level ($P<0.05$), above which values were considered to be non-significant (NS). The graphical data shown within the results chapters were illustrated using the mean (± standard deviation) for the group to determine an overall pattern of results. The individual subject data was also included in appendices to illustrate the differences between individuals.

Power analysis was performed on all outcome variables to calculate the number of subjects required to detect a difference in means between trials. Sample size can be calculated using statistical equations and also by using computer programs to achieve the same result (Samplepower™, SPSS Inc., Chicago, USA). In order to do this several entities must be known, which include the power required, the observed between and

within individual variances, an assumed variability in response to an intervention and the statistical significance required. It is then possible to determine the smallest difference between trials that could be shown to be statistically significant for different numbers of subjects.

Power analysis was conducted on all outcome variables using a computerised analysis program (SamplePower, SPSS Inc., Chicago, USA). The standard deviation of the differences between trials was calculated from the intra-individual differences shown in chapter 4. The effect size required to achieve a power of above 80%, above the level of intra-individual variability was determined.

Section 2

The methods described in section 1 were used throughout the studies reported in this thesis. In section 2, evidence is presented to justify why such techniques were used and how methods could be improved.

3.6 Validity of CF-IRMS to measure breath $^{13}CO_2$ and current breath collection methodologies

Previous work examining the precision and repeatability of the CF-IRMS in measuring the ^{13}C-abundance of stool, urine and breath samples [Jones, 1996], indicated that the CF-IRMS system was able to measure the correct ^{13}C-abundance of specimens on repeated occasions. The repeatability of breath specimen analysis was re-tested by examining the variation in delta values of ten consecutive specimens. The frequency and duration of the collection of breath specimens was also examined to justify the use of hourly sampling during the study day.

3.6.1 Repeatability of breath specimen collection

Within-batch variability in breath specimens was determined from a single subject using 10 consecutive specimens from a single expiration at baseline and at four hours postprandially. At baseline a mean of the 10 samples of -26.5 ($\pm$0.06 $^{0}/_{00}$) was found, and at four hours a mean of the 10 samples of -11.8 ($\pm$ 0.1$^{0}/_{00}$) which suggests that within-batch variation is minimal (SD less than 1%), suggesting precision both in collection methods and analysis. These observations demonstrate the ability of the CF-IRMS system to repeatedly measure the ^{13}C-abundance of baseline samples and ^{13}C-enrichment of postprandial samples.

3.6.2 Validity of breath sampling protocol

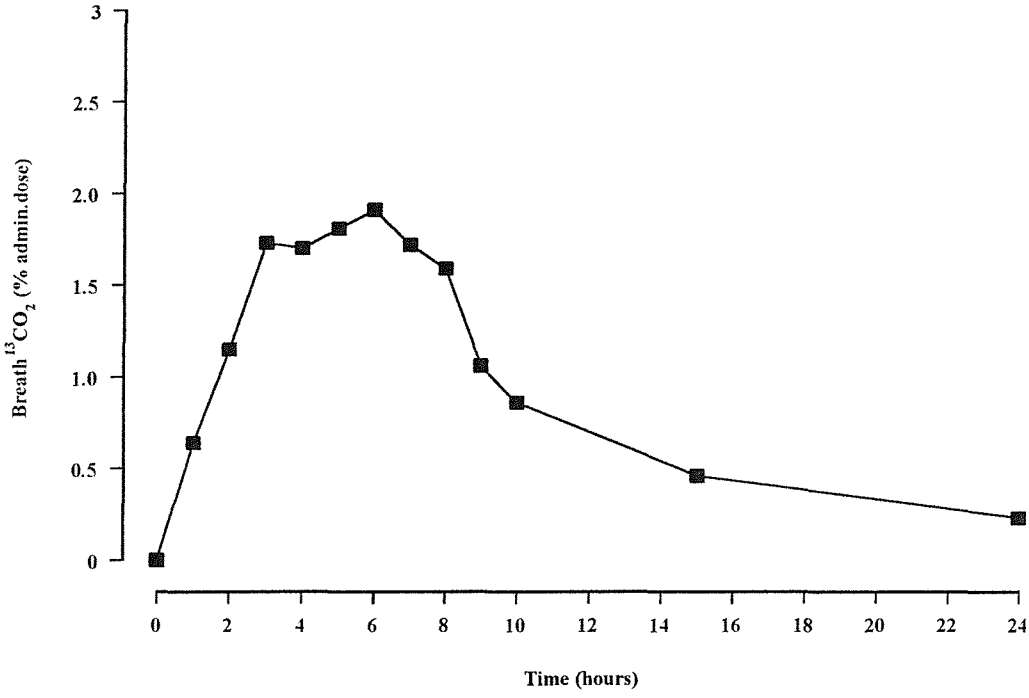
Breath $^{13}CO_2$ calculation requires two measurements: end tidal expired air specimens and $\dot{V}CO_2$ by indirect calorimetry. In previous trials, indirect calorimetry and

breath specimen collection was performed on an hourly basis throughout the study day. The use of this protocol had not been examined to determine whether measurements were made frequently enough during the study day resulting in a reduced recovery of breath $^{13}CO_2$. In order to examine the frequency of measurements required, a single subject followed the general protocol for ^{13}C-labelling studies described in section 3.5 with alterations in the timing of the breath and indirect calorimetry measurements. A 20-minute BMR and expired air breath specimen collection was performed, followed by the consumption of the reference testmeal. Subsequently, indirect calorimetry was performed for 10-minute periods every 30 minutes for 10 hours. Breath specimen collection was performed every 15 minutes for 10 hours postprandially, and specimens were also taken from 11-15 hours and at 24,36,48,60 and 72 hours postprandially. The total $^{13}CO_2$ excreted on the breath and expressed as a percentage of administered dose is shown in figure 3.5 for both hourly and 15 minute measurements.

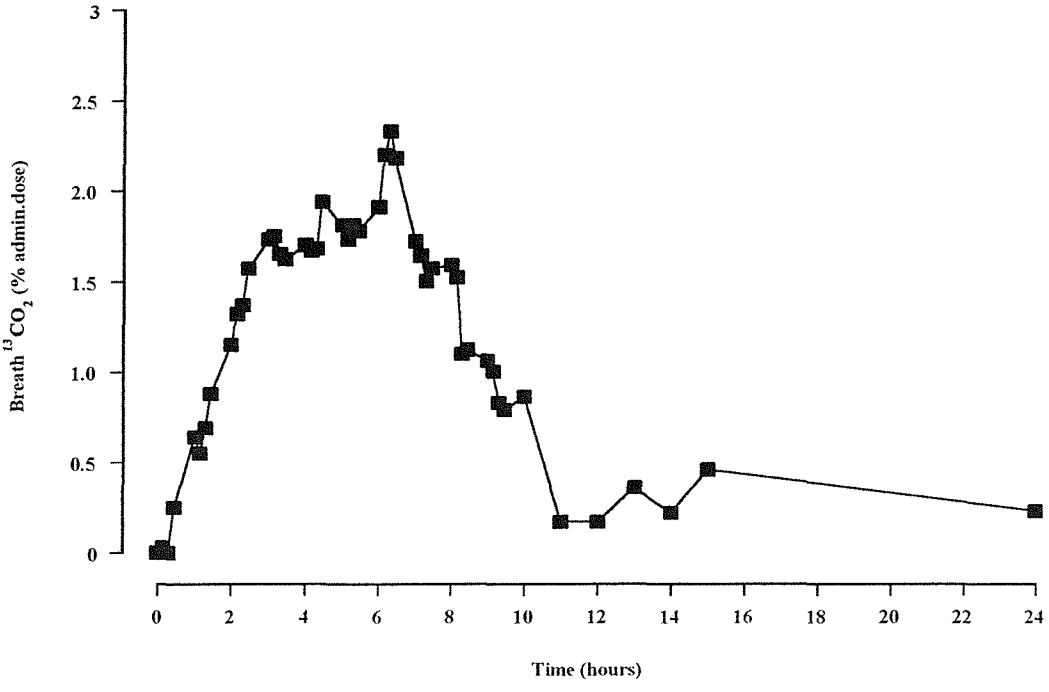
The results show that by increasing the frequency of breath sampling and $\dot{V}CO_2$ measurements, the total breath $^{13}CO_2$ over the 24 hour period decreases from 19.97% to 18.17% of administered dose of [1,1,1-^{13}C]tripalmitin. Over the 25-72 hour period there is little change in the breath $^{13}CO_2$ above baseline with values in the range of natural variation. By comparing the graphs, only two differences were seen over the timecourse: immediately after the second meal, and between 10 and 15 hours. The increased excretion at these timepoints did not, however, alter the total $^{13}CO_2$ excreted on breath over the 24 hour period. The evidence suggests that the current methods used in the collection of breath specimens and in indirect calorimetry do not lead to discrepancies in the measurement of breath $^{13}CO_2$. By increasing the frequency of breath specimen collection and $\dot{V}CO_2$ measurements, no difference in the timecourse of breath $^{13}CO_2$ excretion is seen indicating that the current breath collection protocol is suitable for use in the research presented in this thesis.

Figure 3.5 Breath $^{13}CO_2$ expressed as a percentage of administered dose of [1,1,1-^{13}C]tripalmitin from breath specimens collected every 15 minutes for 10 hours, at 11-15 hours and at 24 hours postprandially

a) Breath specimens taken every hour:-



b) Breath specimens taken every 15 minutes:-



3.7 Justification for the testmeal and preceding diet composition

For a ^{13}C-labelled lipid to act as a tracer for dietary lipid, a known amount of the lipid must be added to the testmeal (10 mg/kg body weight). As this known amount is used in calculations of the ^{13}C-enrichment of breath and stool specimens it is vital that the testmeal itself is not naturally enriched with ^{13}C, and will not influence postprandial breath $^{13}CO_2$ [Schoeller *et al.*1977]. Previous studies have demonstrated that the consumption of ^{13}C rich foods can influence breath $^{13}CO_2$ excretion [Hiele, 1990; Murphy *et al.*1991], and if such foods are used as a component of the testmeal, this could lead to an anomaly in breath $^{13}CO_2$. To determine the ^{13}C-abundance of the reference and modified testmeals, all foods consumed on the preceding day to the study and as part of the testmeal were analysed for their ^{13}C-abundance. The three unlabelled testmeals were also consumed and breath $^{13}CO_2$ measured to provide evidence that consumption of the testmeals did not alter postprandial breath $^{13}CO_2$ excretion.

3.7.1 ^{13}C-Abundance of diet and testmeal

The ^{13}C-abundance of the components of the evening meal and testmeals was measured by homogenising a complete portion of the individual foods with 100g of distilled water using a blender. Two aliquots of the homogenate were dried to a constant weight using a rotary evaporator (Genevac Ltd., Ipswich, UK). The dried sample was ground using a pestle and mortar and transferred to an airtight container. Duplicate 1.8 mg (± 0.2) samples of the dried food was weighed into tin capsules (Elemental Microanalysis, Okehampton, UK) to determine the total ^{13}C-abundance by CF-IRMS (20/20 IRMS with GSL interface, Europa Scientific Ltd., Crewe). The results from this can be seen in table 3.3.

Table 3.3 ^{13}C-abundance of preceding diet and testmeals

Food	^{13}C-abundance ($^{0}/_{00}$) Mean of duplicate	Food	^{13}C-abundance ($^{0}/_{00}$) Mean of duplicate
Emulsion	-25.16	Greek yoghurt	-25.24
Ham sandwich	-26.21	Lemon cheesecake	-23.27
Pasta and salad	-26.07	Lemon squash (conc.)	-23.06
Pizza and chips	-27.03	Lemon squash (dilute)	-24.99

The ^{13}C-abundance of the foods used both within the testmeal and for the preceding evening meal were all of similar ^{13}C-abundance to breath and stool samples at baseline, suggesting that the use of such foods will not affect calculations of ^{13}C-enrichment of breath and stool samples.

3.7.2 Effect of unlabelled testmeal consumption on postprandial breath $^{13}CO_2$ excretion

Postprandial breath $^{13}CO_2$ excretion was examined following consumption of unlabelled reference and modified testmeals. Two subjects consumed the reference testmeal, with one subject consuming both the high-fat and 100g sucrose meal on two separate occasions. Subjects consumed the standard evening meal on the day preceding the study day, consumed no further food and were rested from 8pm. On the study day, a baseline expired air breath specimen was collected before consumption of the unlabelled testmeal and the general study protocol described in section 3.4 was followed. The results are shown in table 3.4.

Table 3.4 Effect of unlabelled testmeals on the recovery of $^{13}CO_2$ on breath

Time (hours)	^{13}C-enrichment of breath expressed in delta values ($^0/_{00}$)			
	Reference testmeal subject 1	Reference testmeal subject 2	High fat meal	100g sucrose meal
Base	**-25.46**	**-25.23**	**-26.47**	**-27.25**
1	-25.19	-24.71	-26.42	-26.66
2	-24.84	-24.76	-26.30	-26.94
3	-24.74	-24.41	-26.54	-27.64
4	-24.68	-24.37	-26.57	-27.50
5	-25.04	-24.60	-26.60	-27.50
6	-24.94	-24.76	-26.90	-27.64
7	-24.73	-24.51	-27.02	-28.27
8	-24.47	-24.44	-26.78	-27.69
9	-24.40	-24.31	-26.93	-28.06
10	-24.68	-24.93	-26.80	-26.72
15	-25.36	-25.65	-26.90	-25.15
24	-25.24	-25.55	-27.26	-25.91
Postprandial Mean	**-24.78**	**-24.75**	**-26.75**	**-27.14**
SD	**0.38**	**0.44**	**0.27**	**0.91**

Consumption of the evening meal and testmeals result in an increase in ^{13}C-enrichment of less than $1^0/_{00}$ over the study period for the reference testmeals and 100g sucrose meal, however this was not statistically significant. The baseline and postprandial ^{13}C values do not differ from natural baseline variation in agreement with that observed previously using this study protocol [Jones, 1996].

The results demonstrated that consumption of unlabelled testmeals did not result in an increased ^{13}C-enrichment on breath with $^{13}CO_2$ remaining constant throughout the study day irrespective of the meal consumed. Postprandial breath $^{13}CO_2$ did not differ to baseline breath $^{13}CO_2$ suggesting that consumption of the testmeals would not influence postprandial breath $^{13}CO_2$ with the addition of a ^{13}C-labelled substrate.

3.8 Validation of the use of lipid emulsion as a vehicle to deliver ^{13}C-lipid

The fatty acid profile of the reference lipid emulsion was determined by GC-FID (Europa ScientificLtd., Crewe, UK) to compare with the composition based on a computerised dietary analysis program [FOODBASE, Institute of Brain Chemistry and Nutrition, London, UK], which indicated that the fatty acids oleic acid, palmitic acid and stearic acid were present in the lipid emulsion in a 3:2:1 ratio. The method of administering the emulsion to the subjects was examined to determine the proportion of ^{13}C-label which remains on the glassware following consumption of the emulsion, and to suggest ways of increasing the amount of label entering the body if necessary.

3.8.1 Composition of lipid emulsion

The composition of the lipid emulsion used throughout the studies was analysed using GC-FID (Europa Scientific Ltd., Crewe, UK). A reference lipid emulsion was prepared, and 8 aliquots were transferred to glass tubes. Lipid extraction, separation and derivatisation as described in appendix 1.4 was carried out simultaneously on all samples.

Analysis by GC-FID resulted in a fatty acid profile of the lipid emulsion containing lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid and linoleic acid. Each sample was analsyed in duplicate and the results for the proportions of fatty acids present and the enrichment of the individual fatty acids is shown in table 3.5 and 3.6. A ratio of 3:2:1 in the proportions of oleic acid to palmitic acid to stearic acid were found when the lipid emulsion was analysed by GC-IRMS which is in agreement with figures determined from FOODBASE. Therefore the composition of the lipid emulsion used in these studies is comparable to the composition of fatty acids found in a typical UK diet [Gregory *et al.*1990]. Although the emulsion overall has been shown to be not naturally enriched with ^{13}C, individually lauric acid and myristic acid were enriched with ^{13}C to a greater extent than normally seen with baseline breath sampling. The analysis of the fatty acid profile of the lipid emulsion does not include measurements for the testmeal as a whole. Obviously the emulsion and meal component are consumed simultaneously during studies, but it was important to determine the lipid emulsion

profile primarily to ensure palmitic acid was present to act as a tracee for the [1,1,1-^{13}C]tripalmitin.

Table 3.5 Proportions of individual fatty acids present in a reference lipid emulsion

	Proportions of fatty acids (% of total fatty acids)					
Sample	**C12:0**	**C14:0**	**C16:0**	**C18:0**	**C18:1**	**C18:2**
A1	3.1	8.5	24.0	8.2	37.0	19.2
A2	3.4	8.6	24.0	8.2	36.6	19.2
B1	3.8	8.5	23.2	8.6	36.5	19.5
B2	3.7	8.4	23.4	8.5	36.4	19.7
C1	3.8	8.5	24.3	8.7	37.7	17.0
C2	4.3	8.5	22.5	8.7	38.2	17.7
D1	3.7	7.3	21.6	8.1	38.2	20.9
D2	3.6	6.5	19.9	8.4	41.5	20.2
E1	4.0	7.5	17.9	8.4	42.0	20.2
E2	3.9	7.6	17.5	8.4	42.6	20.0
F1	4.0	7.6	17.7	8.2	42.3	20.1
F2	4.3	8.0	17.0	8.0	42.4	20.4
G1	3.5	6.8	17.8	8.4	43.8	19.8
G2	3.6	6.8	18.3	8.3	42.9	19.7
H1	3.7	7.2	17.5	8.3	43.2	20.1
H2	4.4	9.0	17.3	7.8	41.4	20.1
Mean	**3.8**	**7.8**	**20.2**	**8.3**	**40.2**	**19.6**
SD	**0.35**	**0.77**	**2.9**	**0.24**	**2.8**	**0.99**

Table 3.6 **^{13}C-abundance of individual fatty acid classes present in a reference lipid emulsion**

	^{13}C-abundance of individual fatty acids ($^{0}/_{00}$)					
Sample	**C12:0**	**C14:0**	**C16:0**	**C18:0**	**C18:1**	**C18:2**
A1	-18.07	-19.24	-22.38	-27.89	-25.61	-26.98
A2	-17.93	-21.38	-22.84	-28.31	-25.15	-26.77
B1	-17.04	-21.33	-22.94	-28.47	-24.14	-26.28
B2	-16.74	-21.58	-23.01	-27.91	-24.46	-26.10
C1	-17.28	-22.83	-22.30	-26.71	-24.74	-26.49
C2	-15.68	-22.02	-22.68	-26.31	-24.71	-25.95
D1	-14.63	-20.56	-22.72	-25.56	-25.10	-25.96
D2	-13.00	-17.79	-23.94	-25.46	-23.12	-22.59
E1	-10.43	-15.10	-26.47	-25.76	-21.98	-21.98
E2	-11.25	-14.99	-27.31	-25.36	-21.39	-21.72
F1	-11.02	-15.55	-27.38	-25.78	-21.19	-21.71
F2	-10.77	-14.97	-27.20	-25.61	-21.93	-22.18
G1	-12.43	-16.03	-27.05	-24.64	-20.14	-21.55
G2	-12.08	-16.15	-26.95	-24.94	-20.28	-21.65
H1	-11.15	-15.64	-27.30	-25.33	-20.92	-21.91
H2	-9.35	-13.40	-24.60	-25.59	-22.29	-22.70
Mean	**-13.68**	**-18.07**	**-24.82**	**-26.23**	**-22.95**	**-23.91**
SD	**3.03**	**3.14**	**2.15**	**1.24**	**1.89**	**2.27**

3.8.2 Administration of [1,1,1-^{13}C]tripalmitin during consumption of the lipid emulsion

Once the subject has consumed the emulsion, hot water was used to rinse the beaker to ensure as much of the label as possible was consumed. There was a need to know whether a proportion of the ^{13}C-label remained in the beaker to determine a correction factor for use in calculations or to derive a method to improve the amount of label consumed. Therefore a validation study was conducted to assess the amount of ^{13}C-label remaining in the beaker following label administration. After the preparation and consumption of reference lipid emulsions containing [1,1,1-^{13}C]tripalmitin, by a series of 8 subjects, beakers were washed with 50ml of solvent (chloroform methanol 2:1v/v), and the washings refrigerated at 1-5°C. The solvent containing the beaker residue was dried to a constant weight on the rotary evaporator (Genevac Ltd., Ipswich, UK), and the dry weights recorded. The dry lipid was re-suspended in chloroform, and the amount required to give 1mg of residue was calculated for each sample. The correct volume was pipetted into a tin capsule (Elemental Microanalysis, Okehampton, UK) and analysed as described previously (section 3.5.9). The results are shown in table 3.7.

Table 3.7 The ^{13}C-enrichment of beaker washings following administration of [1,1,1-^{13}C]tripalmitin via a lipid emulsion in eight samples

Sample	^{13}C-enrichment (% administered dose)
A	0.04
B	0.04
C	0.03
D	0.04
E	0.01
F	0.12
G	0.06
H	0.10
Mean	**0.06**
SD	**0.04**

Less than 0.1% of the administered dose of [1,1,1-^{13}C]tripalmitin remained in the beaker after hot water rinsing, which equates to approximately 0.6-0.7mg of labelled substrate. When data was recalculated to take this into account, the difference in the $^{13}CO_2$ recovery on breath was of the order of 0.01% of administered dose. Therefore it can be concluded that administration of the labelled lipid is almost complete, and that if this method of hot water washing is continued, washing analysis is unnecessary and correction factors do not have to be included to compensate for losses.

3.9 Repeatability of Enzyme Assays

The repeatability of the methods used to measure the concentration of plasma TAG, NEFA and glucose were determined by repeated measures carried out on single specimens. Plasma glucose, TAG (10) and NEFA (5) concentrations were measured repeatedly on a single sample. From this the mean, standard deviation and coefficient of variation was calculated shown in table 3.8.

Table 3.8 Repeatability of plasma TAG, NEFA and glucose concentrations measured on 10 consecutive specimens by enzymatic methods

Sample	Plasma concentration (mmol/l)			
	Glucose	TAG Base	TAG Lipaemic	NEFA
1	6.00	0.62	0.99	0.618
2	6.15	0.61	0.97	0.628
3	6.22	0.66	0.98	0.607
4	6.22	0.63	1.01	0.580
5	6.18	0.7	1.00	0.534
6	6.42	0.69	1.03	
7	6.44	0.68	0.97	
8	6.55	0.67	0.97	
9	6.44	0.68	0.98	
10	6.37	0.76	0.97	
Mean	**6.3**	**0.67**	**0.98**	**0.59**
SD	**0.17**	**0.04**	**0.02**	**0.04**
CoV	**2.7**	**6.6**	**2.1**	**6.4**

For plasma glucose a mean of 6.3 mmol/l (SD, 0.17; CoV, 2.7%) was measured. For plasma TAG, a mean of 0.67 mmol/l (SD, 0.04; CoV, 6.6%) was found for a baseline sample. When a lipaemic sample was analysed, the mean rose to 0.99 mmol/l (SD, 0.02; CoV, 2.1%). For plasma NEFA a mean of 0.59 mmol/l (SD, 0.04; CoV, 6.4%) was seen. This indicates that measurement of glucose and lipaemic TAG concentrations are more repeatable than measures of baseline TAG and NEFA concentrations, however no indication is available to suggest how many replicates are necessary to determine a correct or 'true' measurement.

3.10 Validation of lipid extraction and derivatisation techniques currently used to determine ^{13}C-TAG and ^{13}C-NEFA concentration in plasma

Initial lipid extraction and chromatography work carried out to determine the levels of ^{13}C TAG and NEFA in plasma usually resulted in low (~ 20%) and very variable (5-60%) recoveries of fatty acid standards from the plasma, whilst the recoveries of plasma TAG standards were higher (~50%) and generally more consistent (45-70%). One possible explanation was that the lipid extraction method used was ineffective in isolating all fatty acids present. Therefore a series of validation methods were carried out to examine the hypothesis and to ascertain the most appropriate method for the extraction and separation of fatty acids from plasma.

Eight plasma samples were prepared for GC-C-IRMS injection using the method described in appendix 1.8. At the points shown in *italics* in appendix 1.8 surrogate TAG (C17:0; 60μg) and NEFA (C21:0; 30μg) standards were added to each of the samples, for example, sample 1 contained standards added between steps 1 and 2, sample 2 contained standards added between steps 3 and 4. The process of lipid extraction, separation and derivatisation was completed for all samples simultaneously, with an internal standard added to the samples prior to injection. Recovery of surrogate standard was compared to recovery of the internal standard and results can be seen in table 3.9.

Table 3.9 Recoveries of TAG and NEFA standards following addition at different points during the lipid extraction and derivatisation method

Step in method	TAG recovery (% of added C17:0) Mean of duplicate	Step in method	NEFA recovery (% of added C21:0) Mean of duplicate
1	52.3	1	43.9
2	36.2	2	46.0
3	48.3	3	31.5
4	53.5	4	33.5
5	33.4	5	25.0
6	65.0	6	37.0
7	74.0	7	82.5
8	80.0	8	76.7

Recovery of TAG standard ranged from 33.4% to 80.0% of added standard, and NEFA from 25% to 82.5% of added standard. The range in recoveries during this process indicated that recovery of standards improved when added in the last phase of the method (steps 7 and 8), suggesting that loss of fatty acids occurs prior to this step in the lipid extraction process.

The lipid extraction method was examined to determine the most appropriate method for the recovery of fatty acids from plasma. The four methods used are shown in appendix 1.9. Lipid extraction was performed by the four methods and subsequent separation and derivatisation was completed by an identical method for all samples (appendix 1.4). The results are shown in table 3.10.

Table 3.10 Recoveries of NEFA standards added to plasma samples prior to lipid extraction by four different methods

Method	Recovery (% of added standard) Mean of duplicate
1 – Traditional	39.5
2 – Modified traditional	63.7
3 – Modified Folch	12.1
4 – Folch	17.5

Recovery of standard increased from 39.5% using the traditional method, to 63.7% with the addition of a second protein interphase solvent wash. Methods 3 and 4 were more complicated methods, which resulted in much lower recoveries of standard. Therefore the most effective method for the extraction of NEFA from plasma was method 2, with the separation and derivatisation procedure as described previously (appendix 1.4).

Following a meal NEFA levels in the plasma decrease to virtually undetectable levels. During the separation process, Rhodamine B was traditionally used as a dye to identify lipid bands on the TLC plate but NEFA bands were undetectable visually. An alternative to Rhodamine B known as Fluorescein was used in a direct comparison of the suitability of the dyes. A TLC plate with identical lipid bands was cut in half. One side of the plate was sprayed with Rhodamine B dye and the other with Fluorescein (0.25% in ethanol). Identification of the NEFA bands was enhanced with the Fluorescein dye as background colour decreased. In addition, the new dye was found to be insoluble in hexane and therefore less likely to cause damage to the GC column.

The methodology for the determination of ^{13}C-TAG and ^{13}C-NEFA concentrations has been improved to allow the highest recoveries of fatty acids as possible from samples. These results demonstrated that:-

1) The highest recoveries of plasma NEFA could be gained by following the method described in appendix 1.4.
2) Solvent washing of the protein interphase plug appeared to increase the recovery of NEFA from sample.
3) Fluorescein dye appeared to improve the identification of lipid bands on the TLC plate and reduces the contamination of the GC-column.

CHAPTER 4

Within- and Between-Individual Variability in the Gastrointestinal Handling and Postprandial Metabolism of [1,1,1-^{13}C]Tripalmitin

4.0 Introduction

Variability in repeated measures made on one person has two broad sources, firstly within-individual biological variability, and secondly, sample processing and assay variability [Chambless *et al.*1992]. Variability in sample processing and assays can be reduced with skilled personnel and standard operating practices, but biological variability, at least in part, cannot be controlled. Within-individual variability may be due to intrinsic factors such as genetics, body composition or metabolism, or to extrinsic factors such as diet, lifestyle or physical activity levels.

Intra-individual variability in measures of the GI handling of dietary lipid was previously studied in a group of healthy young women [Murphy *et al.*1995]. The greatest variability was observed in the absorption of the ^{13}C-labelled lipid across the GI tract with a group mean of 14% of administered dose recovered in stool in the first trial, and a group mean of 35% in the second trial, which accounts for over an 80% difference between the trials. In one particular subject, stool ^{13}C-label recovery following the first trial was 8% of administered dose, which rose to 71% of administered dose following the second trial. Breath $^{13}CO_2$ excretion between trials was found to be more repeatable, but showed greater variation within the group (8.6 fold).

It has long been recognised that large inter-individual variability exists in postprandial lipaemia [Cohn *et al.* 1988], but intra-individual variability in postprandial lipaemia has not been extensively studied. Previous work has focused on measures in the post-absorptive state, and how variability may affect classification of coronary heart disease [Schectman and Sasse, 1993]. In the majority of such studies, plasma TAG was found to be a more variable marker than other lipid parameters such as HDL-cholesterol and LDL-cholesterol [Ortola *et al.*1992; Tsalamandris *et al.*1998]. Despite the knowledge that intra-individual variability exists in measures of lipid metabolism, it is

rarely measured and seldom taken into account when examining the effect of an intervention on lipid parameters.

Two solutions exist to attempt to both reduce intra-individual variability, and to account for the intrinsic variability that cannot be manipulated. In the majority of the published literature, measures of lipid metabolism (e.g. plasma TAG, LDL-cholesterol) have been made on more than one occasion with little or no control exerted over the subjects prior to each measure. By controlling for extrinsic variables such as diet, lifestyle, physical activity and preceding meal, it may be possible to reduce the level of intra-individual variation. The remaining variability that exists may be due to intrinsic factors such as body composition, age, gender or metabolism, and obviously it is more difficult to control for such factors. However, if the magnitude of the intra-individual variability is known it would then be possible to use this to predict the anticipated effect size required when an intervention is added, to gain a statistically significant result.

As measurements of the magnitude of intra-individual variability are rarely made due to time and financial constraints, variability of measures are often overlooked. If the magnitude of variability in a measure, when it is repeated on two occasions, is large (e.g. >50%), it may be impossible to suggest that any differences seen with an intervention are solely due to the intervention. By knowing the magnitude of variability it is possible to conduct power analysis to determine the sample size needed (number of subjects), and the effect size needed (increase or decrease in variable with intervention) to be able to correctly reject the null hypothesis of the study that the results from the control trial and the intervention trial are equal. Power analysis calculations are rarely used in metabolic trials because the magnitude of intra-individual variability is not known.

The study reported in this chapter is the first to combine measures of the intra-individual variability of the GI handling of dietary lipid, with measures of postprandial lipaemia to determine the magnitude of within-individual variability, enabling the calculation of the power of subsequent trials to detect differences in means which are solely due to the intervention applied.

4.1 Hypothesis

The hypothesis to be tested in this chapter is that measures of postprandial lipid

metabolism remain constant when identical testmeals are consumed on two separate occasions in the same individuals

4.2 Aim

The aim of the present study was to determine the magnitude of within-individual variation in the absorption, oxidation and metabolism of [1,1,1-^{13}C]tripalmitin and dietary lipid to determine the level of control required for subsequent studies and to estimate the sample size required for future trials in order to answer the following questions:-.

1) What effect does controlling extrinsic variability factors such as diet, physical activity and preceding meal have on the magnitude of variability of the GI handling of dietary lipid compared to previous work [Murphy *et al.* 1995]?

2) What is the magnitude of intra-individual variability in stool ^{13}C-label excretion, breath $^{13}CO_2$ excretion, substrate oxidation and measures of postprandial lipaemia, when identical study conditions are applied on two occasions?

3) How can power analysis be utilised to predict the magnitude of differences required following an intervention which can be solely attributable to the intervention and not to intra-individual variation?

4.3 Subjects

Six young, healthy male subjects were recruited from the staff and students of the University of Southampton. On initial screening, none of the six had any history of gastrointestinal disorders, coronary heart disease or insulin resistance. All provided written consent to take part in the study and were informed of their right to withdraw at any time. Subjects' basal metabolic rate, height, weight and body composition was measured and is shown in Table 4.1.

Table 4.1 Characteristics of six subjects recruited to examine within individual variability in measures of postprandial lipid metabolism

Subject	Age (years)	Weight (kg)	Height (m)	Body mass index (kg/m^2)	Basal metabolic rate (kJ/day)	Body fat (% of total mass)
1	22	67.7	1.76	21.9	8234	10.9
2	28	64.6	1.79	20.2	7206	8.2
3	26	74	1.78	23.4	7275	14.2
4	31	64	1.77	20.4	7368	15.0
5	22	82.1	1.81	25.1	8494	17.3
6	24	87.8	1.76	28.3	8340	16.7
Mean	**25.5**	**73.4**	**1.78**	**23.2**	**7820**	**13.7**
SD	**3.6**	**9.8**	**0.02**	**3.1**	**595.7**	**3.5**

4.4 Methods

Subjects followed the general study protocol described previously (section 3.4). Each subject completed two identical 9-day trials, 4 weeks apart. On each occasion, subjects were fed an identical prescribed diet (1.5 x BMR) for three days prior to the study (see appendix 1.1), and were asked not to eat or drink any other items except water. During this period they were asked to maintain their normal habitual activity regimen but not to take part in sport, and for this to remain constant between the trials. On the evening of the third day, subjects were admitted to the Clinical Nutrition and Metabolism Unit, fed a fixed evening meal, rested from 7.30 p.m., and were allowed no other food or drink. On the study day, a baseline end tidal breath specimen was collected and VCO_2 measurement made before consumption of the testmeal and hourly for 10 hours after the testmeal. In addition to the reference emulsion, containing [1,1,1-^{13}C] tripalmitin and testmeal (section 3.1), the subjects consumed 200g of orange juice to

provide 3.2MJ of energy, 35.7g of lipid, 90.3g of carbohydrate and 27.2g of protein. A second unlabelled emulsion and testmeal was consumed at 6 hours postprandially. A baseline stool sample and all stools passed over a 5 day period were collected. During this time, subjects were asked to refrain from consuming foods naturally enriched in ^{13}C (see chapter 3). The general protocol described in section 3.4 was followed throughout the study day and for the analysis of specimens.

In addition, power analysis was conducted on the major outcome variables from the study to evaluate the power of a study with six subjects to determine the magnitude of differences required between trials when an intervention is applied (chapters 5 and 6). Power analysis was conducted on the outcome variables using a computerised analysis program (SamplePower, SPSS Inc., Chicago, USA).

4.5 Results

Results are expressed as trial 1 (T1) and trial 2 (T2), of which trial 2 and the high-fat trial described in chapter 5 were randomly assigned to the subjects. Results are shown for recovery of ^{13}C in stool, expressed as a percentage of the administered dose of labelled tripalmitin over the five-day stool collection period. Oxidation of labelled tripalmitin is shown as the excretion of $^{13}CO_2$ on breath expressed as a percentage of administered dose and as a percentage of absorbed dose, taking stool losses into account. $^{13}CO_2$ on the breath was calculated over the 24-hour period as the area under the timecourse curve (AUC). Indirect calorimetry data was used to calculate total substrate oxidation, and with $^{13}CO_2$ excreted on breath to estimate the proportion of lipid oxidation from exogenous and endogenous sources. Plasma concentrations of triacylglycerol, non-esterified fatty acid and glucose over the postprandial period were calculated as the area under the timecourse curve measured from zero (AUC_0) and from baseline, the incremental area under the curve (AUCi).

4.5.1 Stool ^{13}C-excretion

Table 4.2 shows the recovery of ^{13}C-label in stool and the calculated availability

of ^{13}C-labelled lipid to the body. The mean excretion of ^{13}C-label in stool for the group in trial 1 was 1.2% of administered dose of [1,1,1-^{13}C]tripalmitin (0.5- 2.3%). In trial 2 there was no difference in the group mean which was 1.1% (0.4-2.5%). Despite no differences between the trials at the level of the group (mean difference = 0.16% T1-T2), there was greater variability between the individuals in the group, with differences between the trials ranging from 17% to 100%. Figure 4.1 shows the percentage difference in stool ^{13}C-label excretion between trial 1and trial 2. There was no pattern in the excretion of ^{13}C-label in stool between the trials with four subjects decreasing ^{13}C-label in stool from trial 1 to trial 2, and two subjects increasing excretion. Stool ^{13}C-label excretion did not differ significantly between the trials due to no consistent trend in the direction of excretion (increase or decrease), and no variability between group mean differences. The raw ^{13}C-data was used to calculate the availability of labelled substrate to the body. Table 4.2 shows that on trial 1, 98.8% of administered dose of label (97.7 to 99.2%) was available to the body, with 98.9% (97.5 to 99.6%) available on trial 2. There was little variability within and between- individuals with the percentage difference between trials ranging from 0.2% to 1.6%, resulting in a group mean difference of 0.9% (NS).

4.5.2 Breath $^{13}CO_2$ excretion

Figure 4.2 shows the total mean $^{13}CO_2$ excreted on breath over the 24-hour study period for the group as a whole as a proportion of absorbed dose of [1,1,1-^{13}C]tripalmitin. Baseline abundance of ^{13}C on breath before the testmeal was at the level of natural abundance, with a rise in $^{13}CO_2$ above baseline immediately following the meal. A peak value was reached at 4-6 hours (typically ~3% of absorbed dose) followed by a steady decrease in excretion until near baseline levels were reached at 24 hours. Univariate analysis of variance revealed no significant effects of trial on breath $^{13}CO_2$ excretion, but a significant effect of time ($p<0.05$). Multivariate analysis of variance revealed that there was no significant effect of the interaction between time and trial. From these graphs the total area under the timecourse curve was determined and the total breath $^{13}CO_2$ excretion over the timecourse calculated.

Table 4.3 shows the breath $^{13}CO_2$ excretion as a percentage of administered, and table 4.4 as a percentage of absorbed dose. Over 24-hours the group mean $^{13}CO_2$ excretion as a percentage of administered dose was 27.8% (23.1 to 31.3%) on trial 1, and 26.5% (17.9 to 33.1%) on trial 2. The difference between the trials was on average 6.9%, but there was no consistent trend in the direction of breath $^{13}CO_2$ excretion (3 subjects increase, 3 subjects decrease). For the group as a whole variability remained below 10% between the trials except for subject 4 who showed a greater variability of 37.3%.

Expressing the results as a percentage of absorbed dose resulted in a subsequent increase in the percentage of breath $^{13}CO_2$ recovered (T1= 28.2%, T2= 26.8%), and an increase in variability in four of the six subjects. For the group as a whole, introduction of correction factors for stool losses resulted in a decrease in the variability between trials. The percentage differences between trial 1 and trial 2 in terms of breath $^{13}CO_2$ excretion are shown in figure 4.3. One way analysis of variance revealed no significant effect of trial on total breath $^{13}CO_2$ expressed as a proportion of administered or absorbed dose.

4.5.3 Lipid Oxidation

Total mean lipid oxidation over the 10-hour study period for trial 1 and 2 is shown in figure 4.4 for the group as a whole. Individual lipid oxidation is shown in appendix 2.1. Postabsorptive lipid oxidation was typically 2.5g/hour for the group in both trials. Lipid oxidation decreased following the testmeal by 0.25g, and rose 2-3 hours after the meal. After the second meal, lipid oxidation was again suppressed but to a lesser extent, and rose again towards the end of the study period. Univariate analysis of variance revealed no significant effects of trial on lipid oxidation, but a significant effect of time ($p<0.05$). Two- way multivariate analysis of variance revealed that there was no significant effect of the interaction between time and trial. From these graphs the total area under the timecourse curve was determined and the total lipid oxidation over the timecourse calculated. Table 4.5 indicates the total lipid oxidation over 10 hours for trial 1 and trial 2. Total lipid oxidation for the group as a whole did not differ between trials with 28.2g oxidised per 10-hours on trial 1 (15.9g to 44.1g), and 31.3g oxidised per 10

hours in trial 2 (14.4g to 42.9g). Within-individual variability ranged from 1.1% (subject 3) between trials to 38.8% (subject 5) with the majority of subjects demonstrating less than a 20% variation between trials. There was no consistent trend in the pattern of lipid oxidation with four subjects demonstrating an increase in lipid oxidation and two subjects a decrease in lipid oxidation (T1-T2). One way analysis of variance revealed no significant differences between the trials for total lipid oxidation.

The metabolism of [1,1,1-^{13}C]tripalmitin was assumed to reflect that of the lipid in the testmeal. If the total palmitic acid in the testmeal is calculated, the proportion of this which is oxidised can be calculated from $^{13}CO_2$ excretion on breath and in turn the total palmitic acid oxidised over 10 hours from the meal can be determined. Metabolism of palmitic acid was assumed firstly to reflect that of all saturated fatty acids in the testmeal, and therefore all fatty acids. The total lipid consumed over the study day was 70.6g and from tracer information, the total exogenous lipid oxidised was calculated and is shown in table 4.6. The timecourse of both exogenous and endogenous oxidation over the ten hour study period is shown in figure 4.4, and proportion of total lipid oxidation from exogenous an endogenous oxidation in figure 4.5. Typically, as exogenous lipid oxidation increased following the meal, endogenous lipid oxidation decreased in both trials. This pattern remained until 8 hours postprandially when endogenous oxidation rose whilst exogenous lipid oxidation fell. Two way analysis of variance on repeated measures revealed that with univariate analysis there was no significant effect of trial but there was a significant effect of time ($p<0.05$). Multivariate analysis revealed no significant effect the interaction between time and trial. Total exogenous lipid oxidation was calculated from the area under the timecourse curve, and endogenous lipid oxidation by difference over the ten-hour study period and is shown in table 4.7 and table 4.8 respectively. Total exogenous lipid oxidation did not differ between trials (T1=15.0g, T2= 14.2g), with a mean difference of 0.8g. Figure 4.7 shows that there was no consistent trend in the pattern of exogenous lipid oxidation, with four subjects showing a decrease in oxidation from T1 to T2, and two subjects showing an increase. On the whole, there was a less than 15% difference in exogenous lipid oxidation between the trials, but this increased to 36.2% in subject four. Total endogenous lipid oxidation showed increasing variability between the trials, with a group mean difference of 4.0g.

The mean percentage difference was 39.0% (± 33.0%) with three subjects showing a greater than 50% difference in oxidation between trials. One way analysis of variance revealed that there were no statistically significant differences between the trials in exogenous or endogenous lipid oxidation.

4.5.4 Carbohydrate Oxidation

Total mean carbohydrate oxidation over the ten-hour study period is shown for the group in figure 4.6, with individual data shown in appendix 2.2. In the post-absorptive state in both trials, carbohydrate oxidation was constant (9g/ hour). On consumption of the testmeal, oxidation typically rose rapidly, peaking at 1-2 hours (15g/ hour) and sharply declining 3-5 hours postprandially. Following the second meal, the oxidation increased but to a lesser extent peaking at 12-13 g/hour. Univariate analysis of variance revealed no significant effects of trial on carbohydrate oxidation, but a significant effect of time ($p<0.05$). Two-way multivariate analysis of variance revealed that there was no significant effect of the interaction between time and trial. From these graphs the total area under the timecourse curve was determined and the total carbohydrate oxidation over the timecourse calculated.

Table 4.9 shows that total carbohydrate oxidation over the 10-hour study period. Carbohydrate oxidation did not differ between the trials with 107.9g per 10 hours (92.7 to 155.6g) on T1, and 103.8g per 10-hours (92.1g to 125.1g) on T2. Overall there was little difference between the trials with a group mean percentage difference of 3.4%. There was no consistent trend in carbohydrate oxidation with three subjects showing an increase and 3 subjects a decrease in oxidation from T1 to T2 shown in figure 4.7. On average there was a 10% or less variability between the trials except for subject 5 who displayed a 28.7% decrease in oxidation from T1 to T2. One way analysis of variance revealed no significant effect of trial on total carbohydrate oxidation.

4.5.5 Plasma Glucose

The glucose response over the time period for the group as a whole can be seen

in figure 4.8, with individual responses shown in appendix 2.3. In the postabsorptive state on T1, plasma glucose concentration fluctuated at baseline levels (typically ~5.0 mmol/l) for the first 6 hours of the study, from which there was little variation within the group. After the second meal at 6 hours, there was a sharp increase in glucose concentration peaking at 7 mmol/l, and variability within the group also increased at this point and thereafter. Glucose levels were diminished after 3 hours and had returned to baseline by the end of the study. In T2, plasma glucose appeared to follow a different pattern with concentrations increasing following the meal to 6 mmol/l. Following the second meal, concentrations rose to 8mmol/l, with a steady decrease towards baseline by 10 hours. Univariate analysis of variance revealed no significant effects of trial on plasma glucose concentration, but a significant effect of time ($p<0.05$). Two- way multivariate analysis of variance revealed that there was no significant effect of the interaction of time and trial. From these graphs the total area under the timecourse curve was determined from zero, and the total glucose concentration over the timecourse calculated and is shown in table 4.10.

Total glucose concentration per 10 hour study period did not differ between trials for the group as a whole, with 54.4 mmol/l per 10 hours (49.4 to 57.2) on trial 1, and 62.0 mmol/l per 10 hours (55.2 to 68.3) on trial 2. There appeared to be a trend between the trials with glucose concentration increasing from trial 1 to trial 2 in five of the six subjects shown in figure 4.11. The mean percentage difference between the trials was 12.9% (-2.8% to +19.4%), but despite plasma glucose tending to be greater in most subjects, one way analysis of variance revealed no significant differences between the trials.

4.5.6 Plasma TAG

The timecourse of postprandial lipaemia is shown in figure 4.9 for the group as a whole, and for individual subjects in appendix 2.4. All subjects had fasting TAG concentrations within the normal range (typically 0.5 to1.0 mmol/l). In trial 1 after consumption of the meal, TAG concentrations rose to a peak of 1.3mmol/l at 2 hours, with a steady decrease to 6 hours. Following the second meal, responses were delayed

with a decreased magnitude of lipaemia (peak=1.0mmol/l). In trial 2, the response was very similar with a smaller response following the second meal (peak=0.7 mmol/l). Univariate analysis of variance revealed no significant effects of trial on plasma TAG concentration, but a significant effect of time ($p<0.05$). Two- way multivariate analysis of variance revealed that there was no significant effect of the interaction of time and trial. From these graphs the total area under the timecourse curve was determined and the total plasma TAG concentration over the timecourse was calculated both from zero (AUC_0) and from baseline (AUCi).

Table 4.11 shows the plasma TAG concentrations (mmol/l per 10 hours) measured from zero, and table 4.12 measured from baseline. When measured from zero, plasma TAG concentrations did not differ between trials for the group as a whole (T1= 8.6 mmol/l per 10 hours; T2= 8.6 mmol/l per 10 hours), which resulted in no percentage differences in the group mean. Within-individual variation was increased ranging from 0%, or no change between trials, to a 34% increase in TAG concentrations from trial 1 to trial 2 in subject 3. There was no consistent trend in plasma TAG concentrations between trials with four subjects increasing TAG concentrations, 1 subject decreasing TAG concentrations and one subject showing no change, shown in figure 4.11. One way analysis of variance revealed that despite four of the six subjects showing an increase in plasma TAG concentrations between trials there was no statistically significant difference between the trials.

Greater variability between trials was observed following measurement of plasma TAG concentrations from baseline values, resulting in a group mean of 3.2 mmol/l per 10 hours (1.1 to 5.8) on trial 1, and 2.8 mmol/l per 10 hours on trial 2. A difference of 0.4 mmol/l per 10 hours was observed between the trials for the group as a whole. There was no consistent trend in the pattern of plasma TAG concentration between the trials with three subjects showing a decrease in concentration, and three subjects showing an increase in concentration from trial 1 to trial 2 shown in figure 4.11. The percentage difference between trial 1 and trial 2 differed greatly between individuals with subjects 4 and 5 showing a <2% decrease in concentration, whilst subject 6 showed a 126.3% decrease in concentration. Despite the between and within subject variation, one way analysis of variance revealed no statistical differences between the trials.

4.5.7 Plasma non-esterified fatty acids

Figure 4.10 shows the timecourse of mean NEFA concentration for the group, with individual timecourses shown in appendix 2.5. In trial 1 postabsorptive NEFA concentrations were uniform for the group (mean = 0.35 mmol/l). Following the meal, concentration decreased to near zero at 1 hour and steadily rose to baseline levels by 6 hours. After the second meal, there was a more pronounced nadir, with the concentration decreasing for 3 hours before a slight increase at 10 hours. Baseline levels were not reached before the end of the study period. The same pattern was seen for the group in trial 2 with a smaller decrease in concentration following the second meal. Univariate analysis of variance revealed no significant effects of trial on NEFA concentration, but a significant effect of time ($p<0.05$). Two- way multivariate analysis of variance revealed that there was no significant effect of the interaction between time or trial. From these graphs the total area under the timecourse curve was determined and the total plasma NEFA concentration over the ten hour study period was calculated both from zero (AUC_0) and from baseline (AUCi).

Table 4.13 shows the total plasma NEFA concentration over ten hours measured form zero (AUC_0). There were no differences in the group mean NEFA concentration between trial 1 and trial 2 when measured from zero with 2.2 mmol/l per 10 hours (1.9 to 2.5) on T1 and 2.2 mmol/l per 10 hours (1.9 to 2.5) on T2, which resulted in a mean percentage difference of 0.97% for the group as a whole. There was no consistent trend in the pattern of NEFA concentration with three subjects showing no change, two subjects showing a decrease, and one subject an increase in plasma NEFA concentration. Greater variability between trials was observed in subject two, with a 15% increase in plasma NEFA concentrations from T1 to T2. The lack of consistent trend, and overall small variability within the group resulted in a one way analysis of variance revealing that there were no significant differences between the trials.

Table 4.14 shows the total plasma NEFA concentration measured from baseline (AUCi). There were no differences in the group mean NEFA concentrations between T1 and T2 with –1.4 mmol/l per 10 hours (-2.0 to -0.2) on T1 and –1.7 mmol/l per 10 hours (-3.6 to 0.2) on T2, which resulted in a mean percentage difference between the trials of

0.05%. However, within-individual variability increased ranging from 61.8% difference between trials in subject 2 to 250% difference between trials in subject 1. There was no consistent trend in the pattern of plasma NEFA concentration between trials with four subjects showing a decrease in concentration from T1 and T2, and two subjects showing an increase. Despite large within-individual variability, one way analysis of variance revealed no statistically significant differences between the trials.

4.5.8 Power analysis

By knowing the magnitude of within-individual differences in postprandial lipid metabolism outcome variables when identical test conditions are applied on two separate occasions, and the expected magnitude of a change when an intervention is applied (chapter 5 and chapter 6), it is possible to calculate the power of the study. The power of a study describes the probability of a study rejecting or accepting the null hypothesis incorrectly. For example if a study has 10% power to detect differences in means of 10%, it is probable that the null hypothesis will be rejected when it is actually true. In other words if a high fat meal is consumed in comparison to a low fat meal, and a 20% increase in lipid oxidation is observed, if the study has 90% power the null hypothesis that the population means are equal can be rejected. If, however the study only has 10% power, it is unlikely that the 20% increase seen is due to the high-fat meal, but probably due to within-individual variability. The purpose of the study described in this chapter was to determine the within-group standard deviation for the difference in means between trial one and trial two, and then to determine the magnitude of differences when an intervention was applied in further chapters. The power of a study can be manipulated by changing the number of subjects (n), reducing or increasing the significance required, or by increasing the magnitude of the difference required. By completing this study, it was possible to calculate the power of the study for a range of outcome variables and magnitudes of effect. The study reported in this chapter allowed the calculation of the difference between the means and standard deviation from the means from T1 and T2. The effect size which could be expected with an intervention was calculated using standard percentage increases in value, for example, a 5% increase in plasma TAG

concentration or 50% increase in total lipid oxidation and the figures used in a computerized power analysis program (Samplepower, SPSS Inc., Chicago, USA). The results are shown in table 4.15 calculated for a sample size of six subjects and in table 4.16 for a sample size of ten subjects. The results will be discussed in section 4.6 (discussion).

Table 4.2 **Stool ^{13}C-label excretion (% of administered dose of [1,1,1-^{13}C]tripalmitin) and the availability of [1,1,1-^{13}C]tripalmitin calculated from the recovery of total ^{13}C in stool, collected at baseline and for 5 days following the administration of the labelled lipid, in six subjects. The difference between trials is reported as the actual difference (^{13}C-label recovery T1-T2) and the percentage difference (actual difference/ (T1+T2/2) x 100) between the trials.**

Subject	Stool ^{13}C (% administered dose of [1,1,1-^{13}C]tripalmitin)					
	Trial 1		Trial 2		Difference between trials (% difference = difference/T1+T2/2 x 100)	
	Recovery of ^{13}C	Availability of ^{13}C	Recovery of ^{13}C	Availability of ^{13}C	Recovery of ^{13}C	Availability of ^{13}C
1	1.2	98.8	0.4	99.6	↓ 0.8 (100%)	↑ 0.8 (0.8%)
2	1.3	98.7	1.1	98.9	↓ 0.2 (17%)	↑ 0.2 (0.2%)
3	0.5	99.5	1.0	99.0	↑ 0.5 (66%)	↓ 0.5 (0.5%)
4	0.8	99.2	2.5	97.5	↑ 1.7 (100%)	↓ 1.7 (1.7%)
5	1.3	98.7	0.7	99.3	↓ 0.6 (60%)	↑ 0.6 (0.6%)
6	2.3	97.7	0.7	99.3	↓ 1.6 (100%)	↑ 1.6 (1.6%)
Mean	**1.2**	**98.8**	**1.1**	**98.9**	**- 0.16 (73.8%)**	**0.16 (0.9%)**
SD	**0.6**	**0.6**	**0.7**	**0.7**	**1.1 (33.3%)**	**1.1 (0.6%)**

Table 4.3 **Excretion of $^{13}CO_2$ on the breath expressed as a proportion of administered dose of [1,1,1-^{13}C]tripalmitin, over two identical 24 hour study periods following consumption of a reference testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T2), and the percentage difference (actual difference/T1+T2/2 x 100).**

Subject	Excretion of $^{13}CO_2$ on breath (% of administered dose of [1,1,1-^{13}C]tripalmitin			
	Trial 1	Trial 2	Difference (T1-T2)	% difference (T1-T2/2)
1	23.1	22.1	↓ 1.0	↓ 4.4
2	28.3	28.8	↑ 0.5	↑ 1.8
3	29.5	31.2	↑ 0.7	↑ 2.3
4	26.1	17.9	↓ 8.2	↓ 37.3
5	31.3	33.1	↑ 1.8	↑ 5.6
6	28.6	26.1	↓ 2.5	↓ 9.1
Mean	**27.8**	**26.5**	**-1.45**	**-6.9**
SD	**2.9**	**5.7**	**3.6**	**15.8**

Table 4.4 **Excretion of $^{13}CO_2$ on the breath expressed as a proportion of absorbed dose of [1,1,1-^{13}C]tripalmitin, over two identical 24 hour study periods following consumption of a reference testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T2), and the percentage difference (actual difference/T1+T2/2 x 100).**

Subject	Excretion of $^{13}CO_2$ on breath (% of absorbed dose of [1,1,1-^{13}C]tripalmitin			
	Trial 1	Trial 2	Difference (T1-T2)	% difference (T1-T2/2)
1	23.5	22.1	↓ 1.4	↓ 6.0
2	28.7	29.3	↑ 0.6	↑ 2.1
3	29.8	31.6	↑ 1.8	↑ 5.9
4	26.3	18.4	↓ 7.9	↓ 35.3
5	31.7	33.3	↑ 1.6	↑4.9
6	29.3	26.2	↓ 3.1	↓ 11.2
Mean	**28.2**	**26.8**	**- 1.4**	**-6.6**
SD	**2.9**	**5.7**	**3.7**	**15.6**

Table 4.5 **Total lipid oxidation expressed as grams oxidised per ten hours estimated from area under the timecourse curve over two identical 10 hour study periods following consumption of a reference testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T2), and the percentage difference (actual difference/T1+T2/2 x 100).**

Subject	Total lipid oxidation (g/10 hours)			
	Trial 1	Trial 2	Difference (T1-T2)	% difference (difference/ (T1-T2/2) x 100)
1	20.6	25.6	↑ 4.9	↑ 21.2
2	26.7	29.9	↑ 3.2	↑ 11.3
3	35.2	35.6	↑ 0.4	↑ 1.1
4	15.9	14.4	↓ 1.5	↓ 9.9
5	26.6	39.4	↑ 12.8	↑ 38.8
6	44.1	42.9	↓ 1.8	↓ 4.1
Mean	**28.2**	**31.3**	**3.0**	**9.7**
SD	**10.1**	**10.4**	**5.5**	**18.1**

Table 4.6 Total exogenous lipid oxidation over the 10 hour study period calculated from the concentration of palmitic acid and total lipid in the testmeal, and the oxidation of [1,1,1-^{13}C]tripalmitin

Subject	Trial	^{13}C lipid absorbed/24 hours (mg)	% oxidised in 24 hours	% oxidised in 10 hours	^{13}C lipid oxidised / 10 hours (mg)	Total PA in testmeal (g)	PA oxidised/ 10 hours (g)	Total lipid in testmeal (g)	Total exogenous lipid oxidised/ 10 hrs (g)
1	1	662.2	23.4	18.7	123.6	14.1	2.6	70.6	13.2
	2	661.0	22.2	16.7	110.1	14.1	2.3	70.6	11.8
2	1	63637	28.7	22.9	145.8	14.1	3.2	70.6	16.2
	2	633.1	29.3	21.6	136.7	14.1	3.0	70.6	15.2
3	1	756.0	29.8	23.1	174.5	14.1	3.3	70.6	16.3
	2	744.0	31.6	23.8	176.8	14.1	3.4	70.6	16.8
4	1	625.7	26.3	19.4	121.2	14.1	2.7	70.6	13.7
	2	641.0	18.4	13.5	86.5	14.1	19	70.6	9.5
5	1	831.1	31.7	22.5	186.8	14.1	3.2	70.6	15.9
	2	805.2	33.3	25.8	207.7	14.1	3.6	70.6	18.2
6	1	858.4	29.3	21.1	181.1	14.1	3.0	70.6	14.9
	2	879.2	26.2	19.0	167.0	14.1	2.7	70.6	13.4

Table 4.7 **Exogenous lipid oxidation expressed as grams oxidised per ten hours estimated from area under the timecourse curve over two identical 10 hour study periods following consumption of a reference testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T2), and the percentage difference (actual difference/T1+T2/2 x 100).**

Subject	**Exogenous lipid oxidation (g/10 hours)**			
	Trial 1	**Trial 2**	**Difference (T1-T2)**	**% difference**
1	13.2	11.8	↓ 1.4	↓ 11.2
2	16.2	15.2	↓ 1.0	↓ 6.4
3	16.3	16.8	↑ 0.5	↑ 3.0
4	13.7	9.5	↓ 4.2	↓ 36.2
5	15.9	18.2	↑ 2.3	↑ 13.5
6	14.9	13.4	↓ 1.5	↓ 10.6
Mean	**15.0**	**14.2**	**-0.8**	**-7.8%**
SD	**1.3**	**3.2**	**2.2**	**16.4**

Table 4.8 **Endogenous lipid oxidation expressed as grams oxidised per ten hours estimated from area under the timecourse curve over two identical 10 hour study periods following consumption of a reference testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T2), and the percentage difference (actual difference/T1+T2/2 x 100).**

Subject	Endogenous lipid oxidation (g/10 hours)			
	Trial 1	Trial 2	Difference (T1-T2)	% difference
1	7.4	13.8	↑ 6.4	↑ 60.0
2	10.5	14.7	↑ 4.2	↑ 33.3
3	18.9	18.8	↓ 0.1	↓ 0.5
4	2.2	4.9	↑ 2.7	↑ 76.0
5	10.7	21.2	↑ 10.5	↑ 65.6
6	29.2	29.5	↑ 0.3	↑ 1.0
Mean	**13.2**	**17.2**	**4.0**	**39.0**
SD	**9.6**	**8.2**	**4.0**	**33.0**

Table 4.9 **Total carbohydrate oxidation expressed as grams oxidised per ten hours estimated from area under the timecourse curve over two identical 10 hour study periods following consumption of a reference testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T2), and the percentage difference (actual difference/T1+T2/2 x 100).**

Subject	Total carbohydrate oxidation (g/10 hours)			
	Trial 1	Trial 2	Difference (T1-T2)	% difference
1	105.6	106.4	↑ 0.8	↑ 0.8
2	97.6	92.1	↓ 5.5	↓ 5.6
3	100.3	93.9	↓ 6.4	↓ 6.6
4	113.3	125.1	↑ 11.8	↑10.0
5	138.3	103.6	↓ 34.7	↓ 28.7
6	92.2	101.9	↑ 9.7	↑ 10.0
Mean	**107.9**	**103.8**	**-4.1**	**-3.4**
SD	**16.6**	**11.8**	**16.8**	**14.4**

Table 4.10 **Plasma glucose concentration (mmol/l per 10 hours) estimated from area under the timecourse curve over two identical 10 hour study periods following consumption of a reference testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T2), and the percentage difference (actual difference/T1+T2/2 x 100).**

Subject	Plasma glucose concentration (mmol/l per 10 hours)			
	Trial 1	Trial 2	Difference (T1-T2)	% difference
1	53.1	55.2	↑ 2.1	↑ 3.9
2	49.4	62.9	↑ 13.5	↑ 24.0
3	57.2	55.6	↓ 1.6	↓ 2.8
4	57.0	66.1	↑ 9.1	↑ 14.8
5	56.2	68.3	↑ 12.1	↑ 19.4
6	53.4	64.1	↑ 10.7	↑ 18.2
Mean	**54.4**	**62.0**	**7.7**	**12.9**
SD	**3.0**	**5.5**	**6.0**	**10.2**

Table 4.11 **Plasma TAG concentration (mmol/l per 10 hours) estimated from area under the timecourse curve measured from zero, over two identical 10 hour study periods following consumption of a reference testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T2), and the percentage difference (actual difference/T1+T2/2 x 100).**

Subject	Plasma TAG concentration (mmol/l per 10 hours) AUC measured from zero (AUC_0)			
	Trial 1	Trial 2	Difference (T1-T2)	% difference
1	9.6	10.3	↑ 0.7	↑ 7.0
2	5.2	6.0	↑ 0.8	↑ 14.3
3	3.4	4.8	↑ 1.4	↑ 34.0
4	8.0	8.0	No change	No change
5	16.7	13.1	↓ 3.6	↓ 24.2
6	8.8	9.4	↑ 0.6	↑ 6.6
Mean	**8.6**	**8.6**	**-0.02**	**6.3**
SD	**4.6**	**3.0**	**1.8**	**19.0**

Table 4.12 **Plasma TAG concentration (mmol/l per 10 hours) estimated from area under the timecourse curve, measured from baseline, over two identical 10 hour study periods following consumption of a reference testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T2), and the percentage difference (actual difference/T1+T2/2 x 100).**

Subject	Plasma TAG concentration (mmol/l per 10 hours) AUC measured from baseline (AUC_i)			
	Trial 1	Trial 2	Difference (T1-T2)	% difference
1	4.5	3.9	↓ 0.6	14.3
2	1.9	1.8	↓ 0.1	5.3
3	1.0	1.7	↑ 0.7	51.9
4	2.8	2.8	No change	0
5	5.8	5.7	↓ 0.1	1.7
6	3.1	0.7	↓ 2.4	126.3
Mean	**3.2**	**2.8**	**↓ 0.3**	**16.0**
SD	**1.7**	**1.8**	**1.3**	**58.9**

Table 4.13 **Plasma NEFA concentration (mmol/l per 10 hours) estimated from area under the timecourse curve, measured from zero, over two identical 10 hour study periods following consumption of a reference testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T2), and the percentage difference (actual difference/T1+T2/2 x 100).**

Subject	**Plasma NEFA concentration (mmol/l per 10 hours) AUC_0**			
	Trial 1	**Trial 2**	**Difference (T1-T2)**	**% difference**
1	2.2	2.2	No change	0
2	1.8	2.1	↑ 0.3	↑ 15
3	2.1	2.0	↓ 0.1	↓ 4.9
4	1.9	1.9	No change	0
5	2.5	2.5	No change	0
6	2.4	2.3	↓ 0.1	↓ 4.3
Mean	**2.2**	**2.2**	**0.01**	**0.97**
SD	**0.3**	**0.2**	**0.14**	**7.2**

Table 4.14 **Plasma NEFA concentration (mmol/l per 10 hours) estimated from area under the timecourse curve, measured from baseline, over two identical 10 hour study periods following consumption of a reference testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T2), and the percentage difference (actual difference/T1+T2/2 x 100).**

Subject	Plasma NEFA concentration (mmol/l per 10 hours) AUCi			
	Trial 1	Trial 2	Difference (T1-T2)	% difference
1	-1.8	0.2	↑ 2.0	↑ 250
2	-1.9	-3.6	↓ 1.7	↓ 61.8
3	-1.0	-2.1	↓ 1.1	↓ 70.9
4	-2.0	-0.6	↑ 1.4	↑ 107.7
5	-0.2	-1.2	↓ 1.0	↓ 142.9
6	-1.3	-3.1	↓ 1.8	↓ 81.8
Mean	**-1.4**	**-1.7**	**-0.3**	**0.05**
SD	**0.7**	**1.5**	**1.5**	**148.4**

Table 4.15 Power analysis calculations for a range of anticipated effect sizes (5-50%) for all outcome variables measured in the present study for a sample size of six subjects. The power of the study determines the ability for the study to reject the null hypothesis that the mean result from the control trial and intervention trial are equal.

Anticipated effect size (%)	Outcome Variable Power (%) N=6					
	Stool ^{13}C-label excretion (% of administered dose	Breath $^{13}CO_2$ (% of absorbed dose)	Total lipid oxidation (g/10 hours)	Exogenous lipid oxidation (g/10 hours)	Endogenous lipid oxidation (g/10 hours)	Total carbohydrate oxidation (g/10 hours)
5	5	9	7	8	6	8
10	5	22	13	19	8	17
20	6	66	36	57	20	52
30	9	95	68	89	45	82
40	11	100	89	99	73	98
50	14	100	98	100	90	100
	Plasma glucose concentration (mmol/l per 10 hours)	Plasma TAG concentration (AUC0- mmol/l per 10 hours)	Plasma TAG concentration (AUCi – mmol/l per 10 hours)	Plasma NEFA concentration (AUC0 – mmol/l per 10 hours)	Plasma NEFA concentration (AUCi – mmol/l per 10 hours	
5	11	6	6	35	5	
10	29	12	7	88	5	
20	81	32	14	100	6	
30	99	62	30	100	7	
40	100	84	46	100	10	
50	100	96	62	100	11	

Table 4.16 **Power analysis calculations for a range of anticipated effect sizes (5-50%) for all outcome variables measured in the present study for a sample size of ten subjects. The power of the study determines the ability for the study to reject the null hypothesis that the mean result from the control trial and intervention trial are equal.**

Anticipated effect size (%)	Outcome Variable Power (%) N=10					
	Stool ^{13}C-label excretion (% of administered dose	Breath $^{13}CO_2$ (% of absorbed dose)	Total lipid oxidation (g/10 hours)	Exogenous lipid oxidation (g/10 hours)	Endogenous lipid oxidation (g/10 hours)	Total carbohydrate oxidation (g/10 hours)
5	5	13	8	12	7	10
10	5	36	19	30	13	28
20	7	89	58	82	43	78
30	12	100	90	99	80	98
40	16	100	99	100	96	100
50	21	100	100	100	99	100
	Plasma glucose concentration (mmol/l per 10 hours)	Plasma TAG concentration (AUC0- mmol/l per 10 hours)	Plasma TAG concentration (AUCi – mmol/l per 10 hours)	Plasma NEFA concentration (AUC0 – mmol/l per 10 hours)	Plasma NEFA concentration (AUCi – mmol/l per 10 hours	
5	16	8	7	56	5	
10	48	19	9	99	5	
20	97	52	21	100	7	
30	100	86	49	100	9	
40	100	98	71	100	14	
50	100	100	87	100	17	

Figure 4.1 **Percentage difference between stool ^{13}C-label excretion, and the availability of ^{13}C between trial 1 and trial 2, calculated from the measured difference between the trials as a percentage of T1+T2/2 x 100**

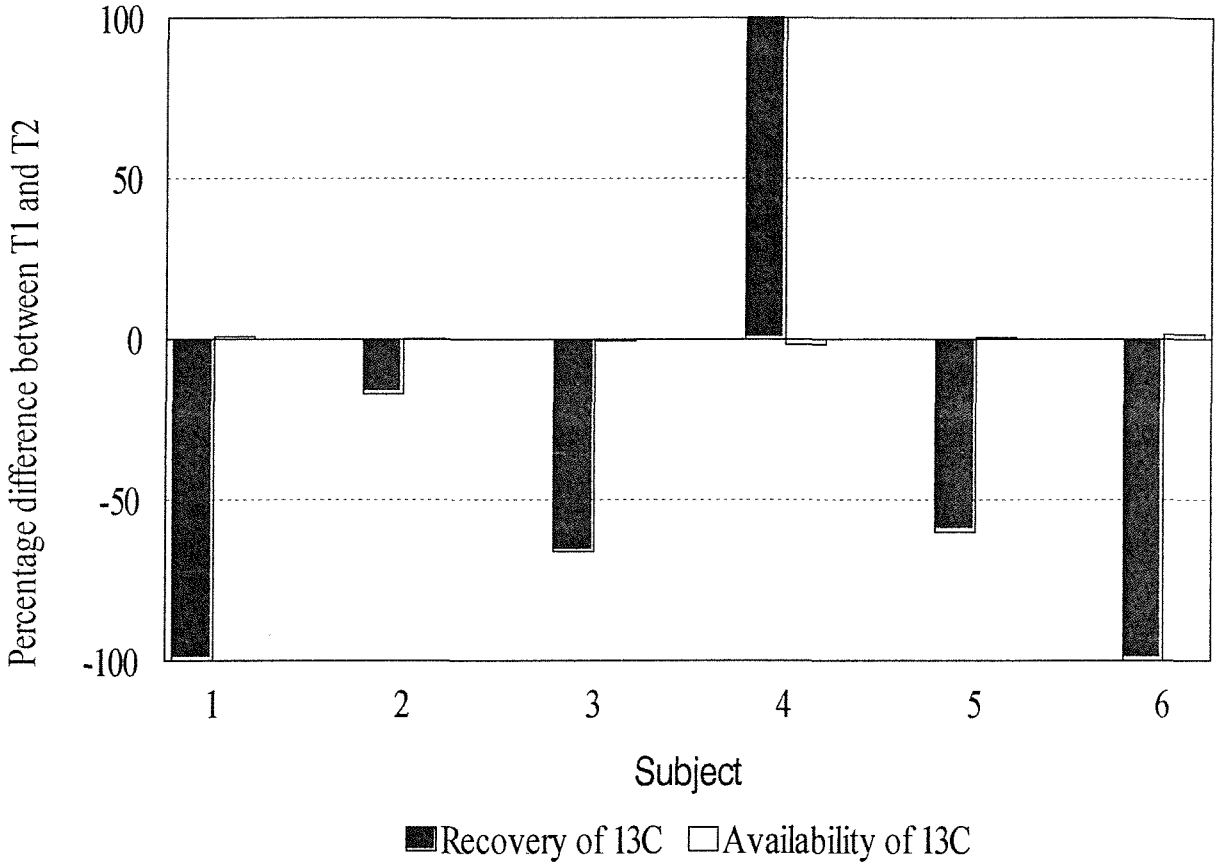


Figure 4.2 **Excretion of $^{13}CO_2$ on the breath expressed as a proportion of absorbed dose of [1,1,1-^{13}C]tripalmitin, in a group of six subjects, after consumption of two identical testmeals (group mean ± SD). Arrows indicate the timing of consumption of the testmeals (baseline and 6 hours postprandially).**

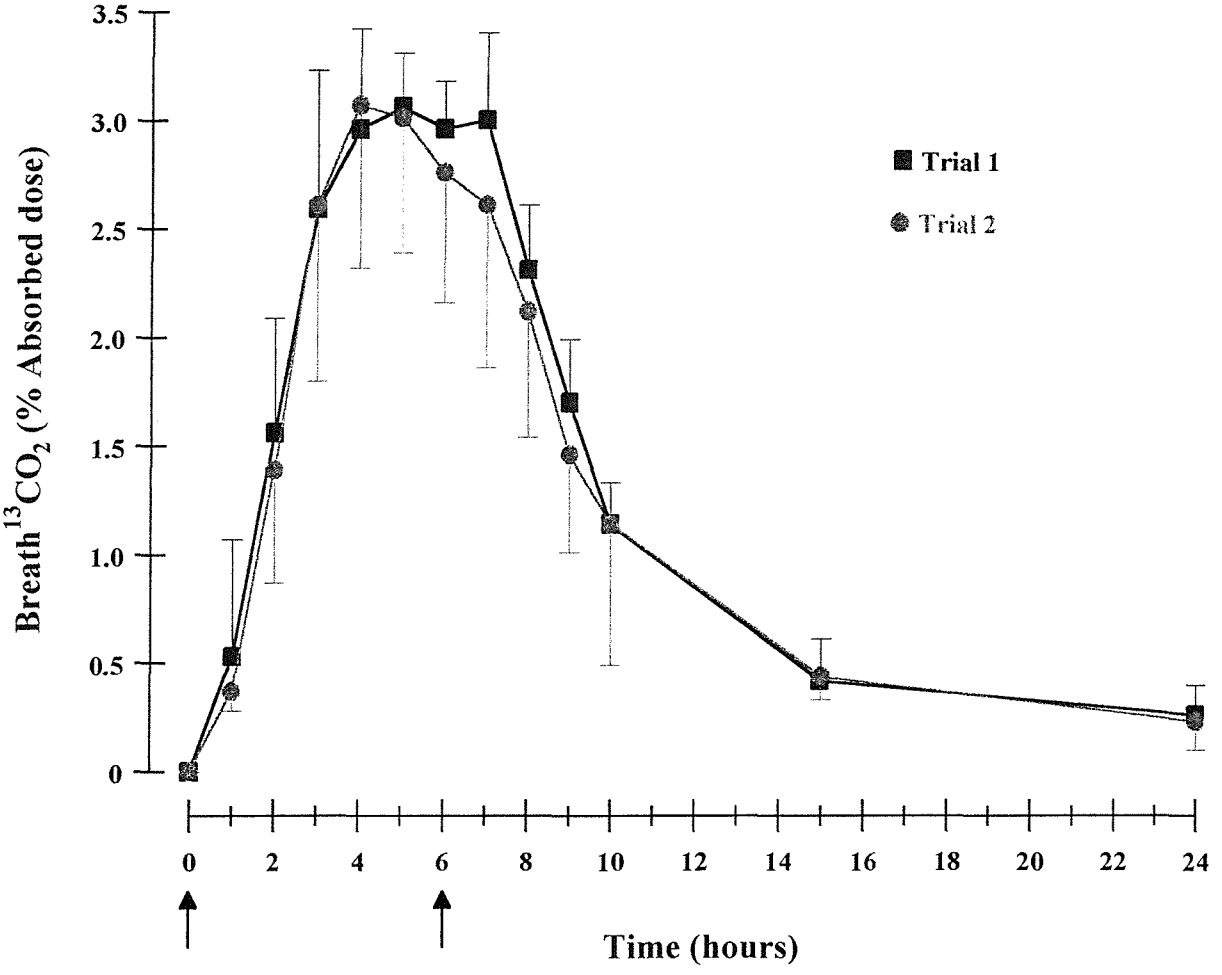


Figure 4.3 **Percentage difference between trial 1 and trial 2 for the excretion of $^{13}CO_2$ on breath expressed as a proportion of administered dose and of absorbed dose of [1,1,1-^{13}C]tripalmitin. Percentage difference was calculated as the actual difference between trial/ (T1 + T2/2) x 100, where T1 is the result from trial 1 and T2 the result from trial 2**

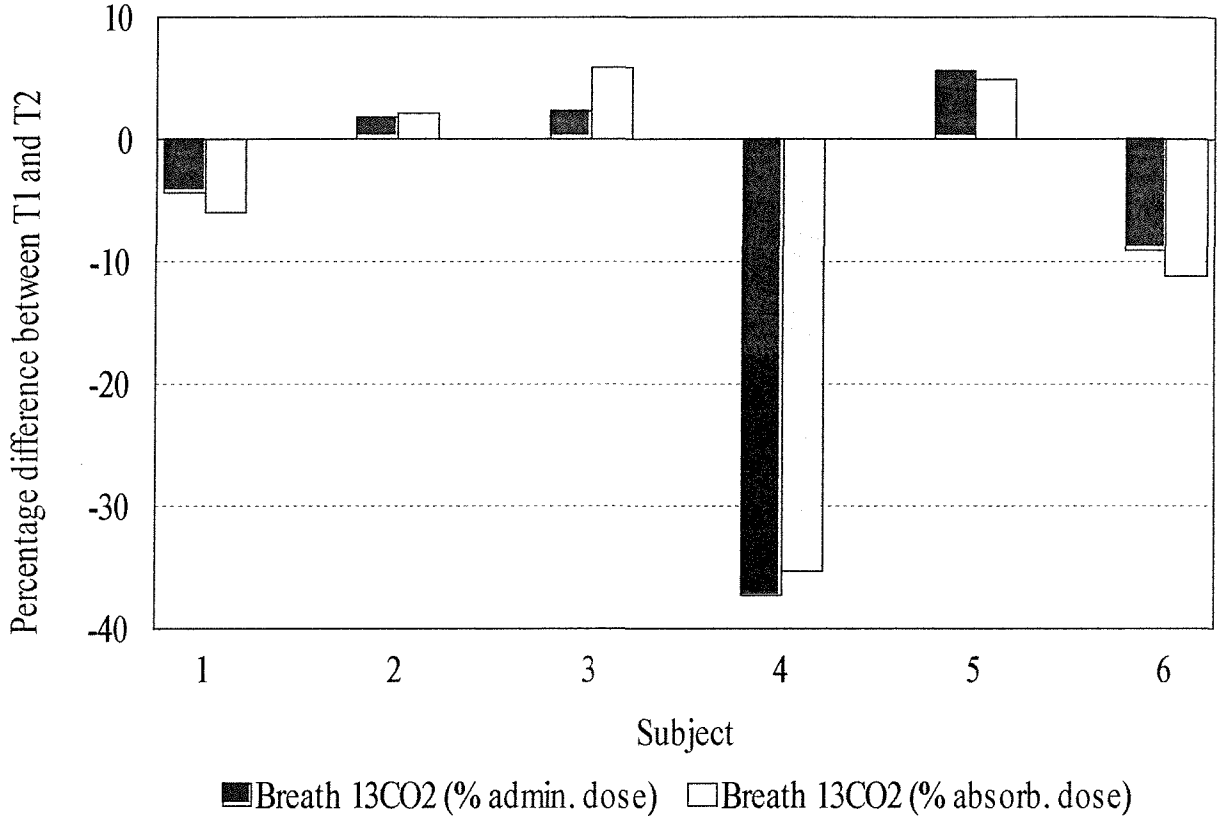
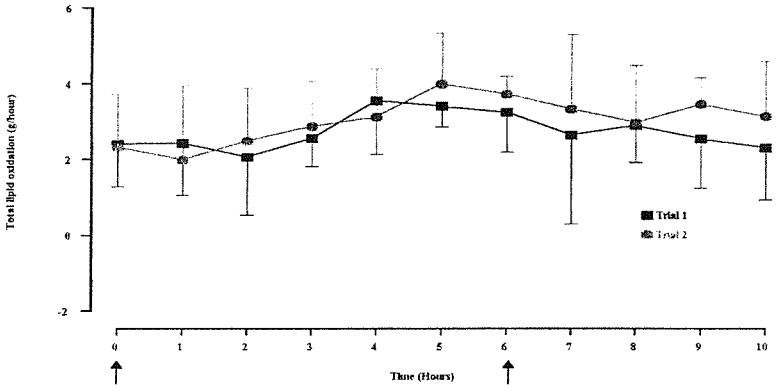
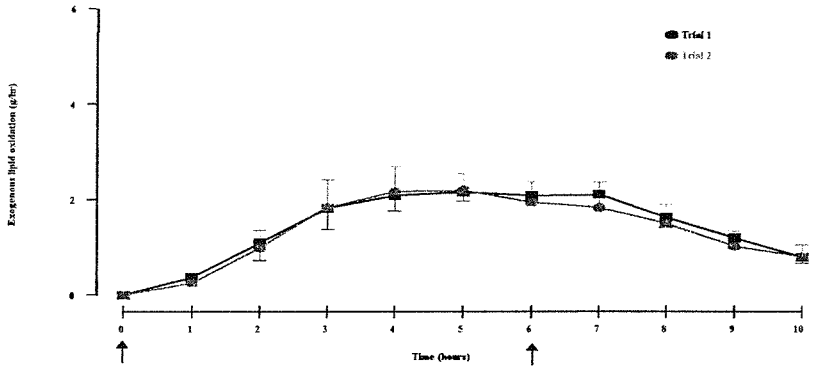


Figure 4.4 **Total, exogenous and endogenous lipid oxidation expressed in grams of lipid oxidised per hour over a ten hour study period in a group of six subjects following consumption of two identical testmeals (group mean ± SD)**

Total lipid oxidation:-



Exogenous lipid oxidation:-



Endogenous lipid oxidation:-

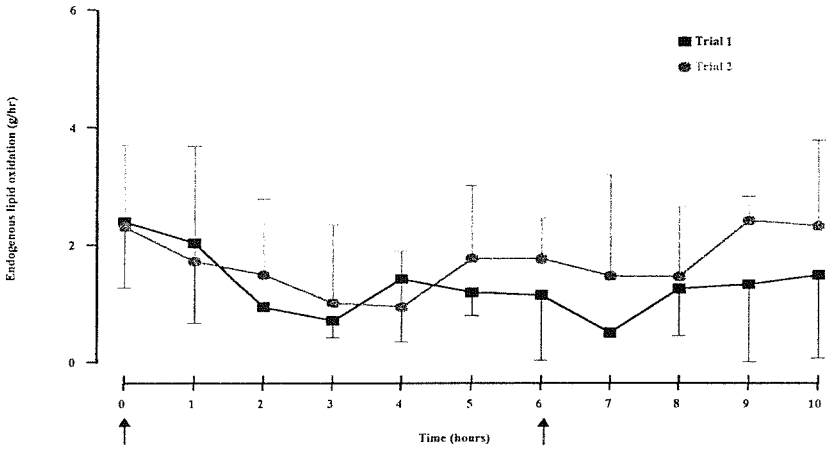


Figure 4.5 **Proportion of total lipid oxidation from exogenous and endogenous sources in g/10 hours. Individual bars show total lipid oxidation on each trial, with proportions of exogenous oxidation and endogenous oxidation indicated**

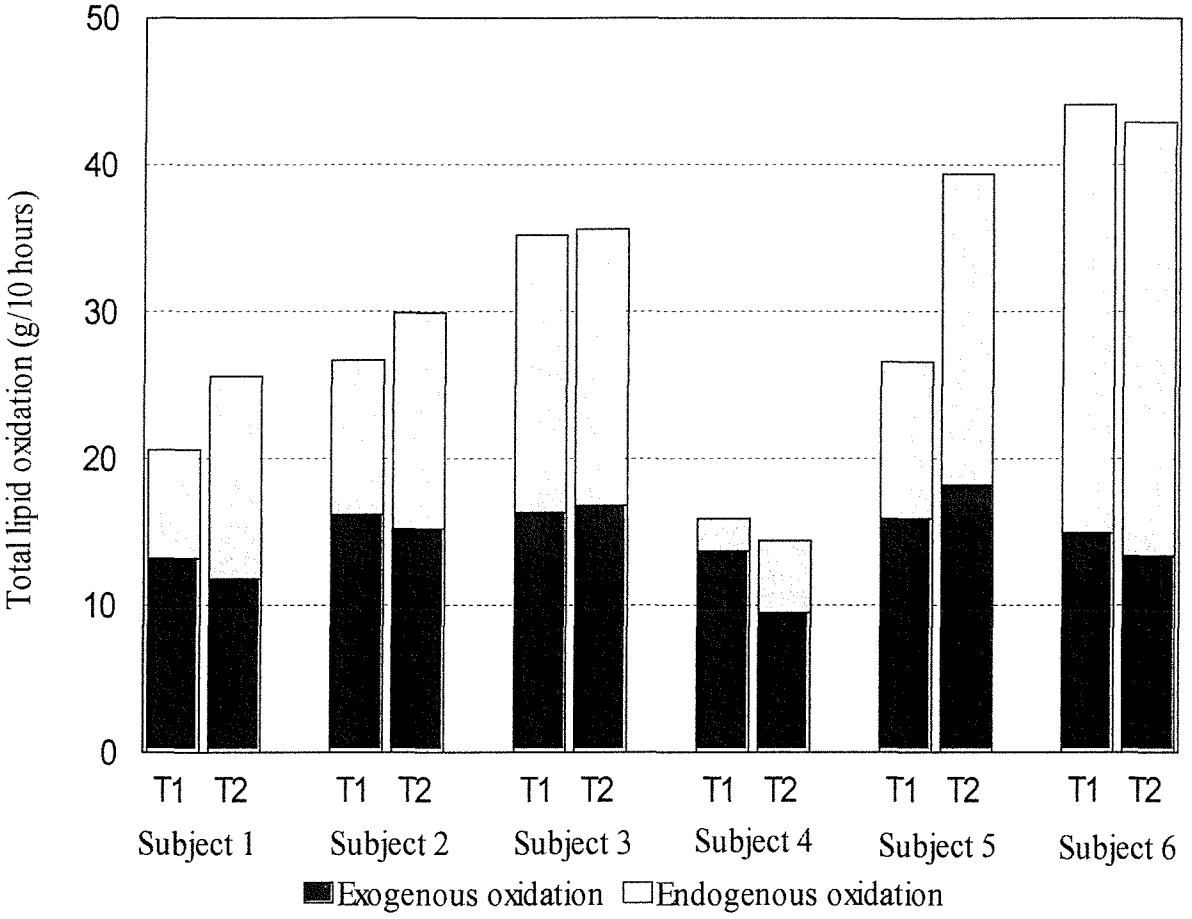


Figure 4.6 **Total carbohydrate oxidation expressed as grams oxidised per hour over a ten hour study period in a group of six subjects following consumption of two identical testmeals (group mean ± SD)**

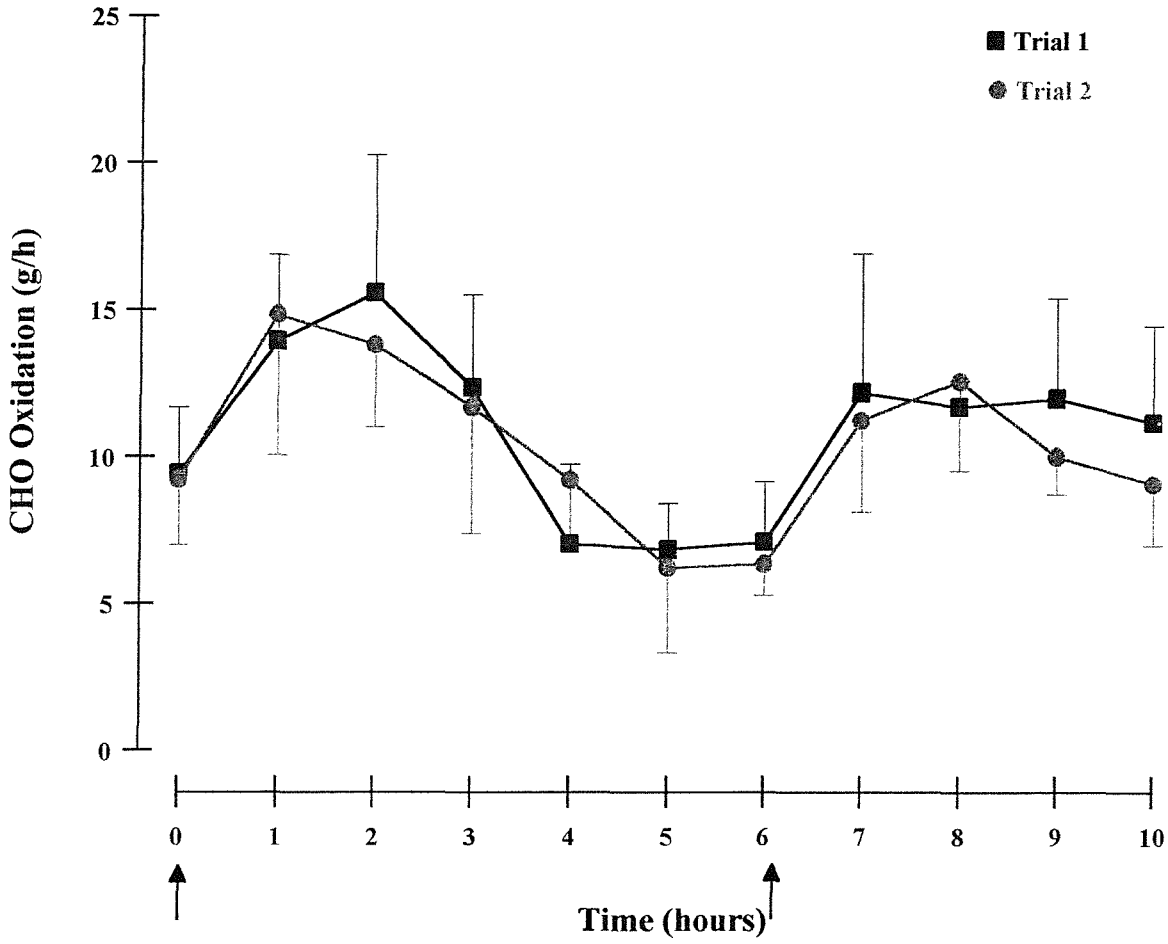


Figure 4.7 Percentage difference between trial 1 and trial 2 for total lipid oxidation, total carbohydrate oxidation, exogenous lipid oxidation and endogenous lipid oxidation. Percentage difference was calculated as the actual difference between trials/ (T1+T2/2) x 100.

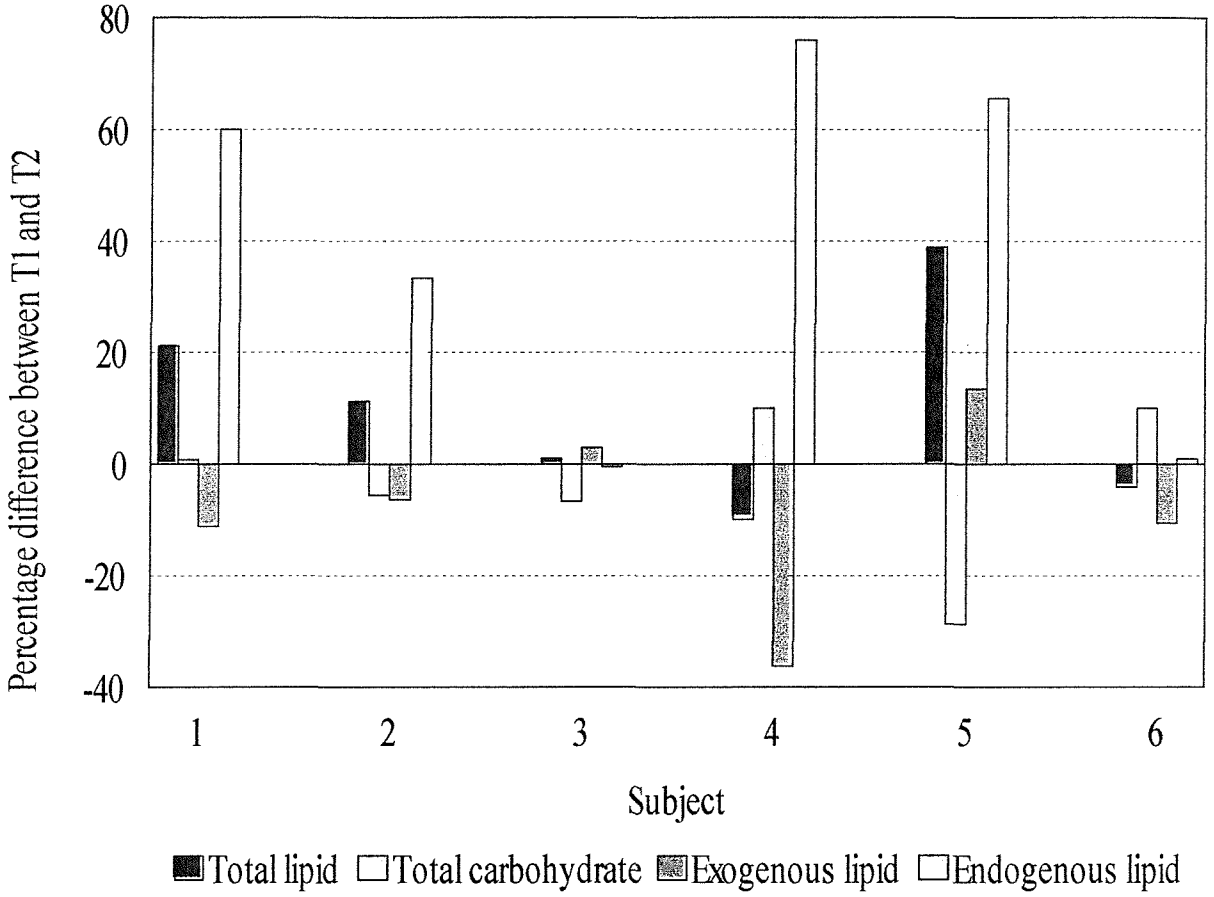


Figure 4.8 **Plasma glucose concentration (mmol/litre) over a ten hour study period in a group of six subjects following consumption of two identical testmeals (group mean ± SD)**

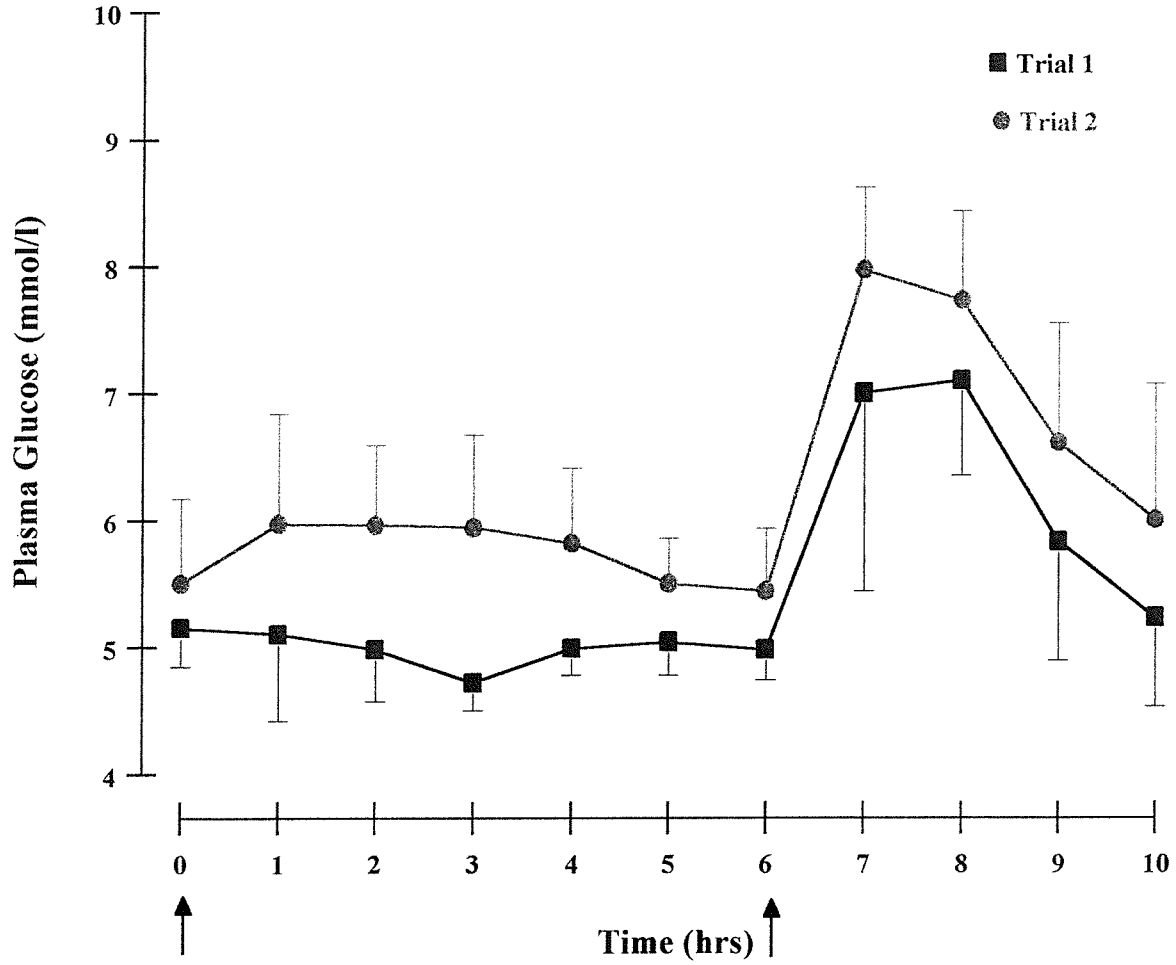


Figure 4.9 **Plasma triacylglycerol concentration (mmol/l) over a ten hour study period in a group of six subjects following consumption of two identical testmeals (group mean ± SD)**

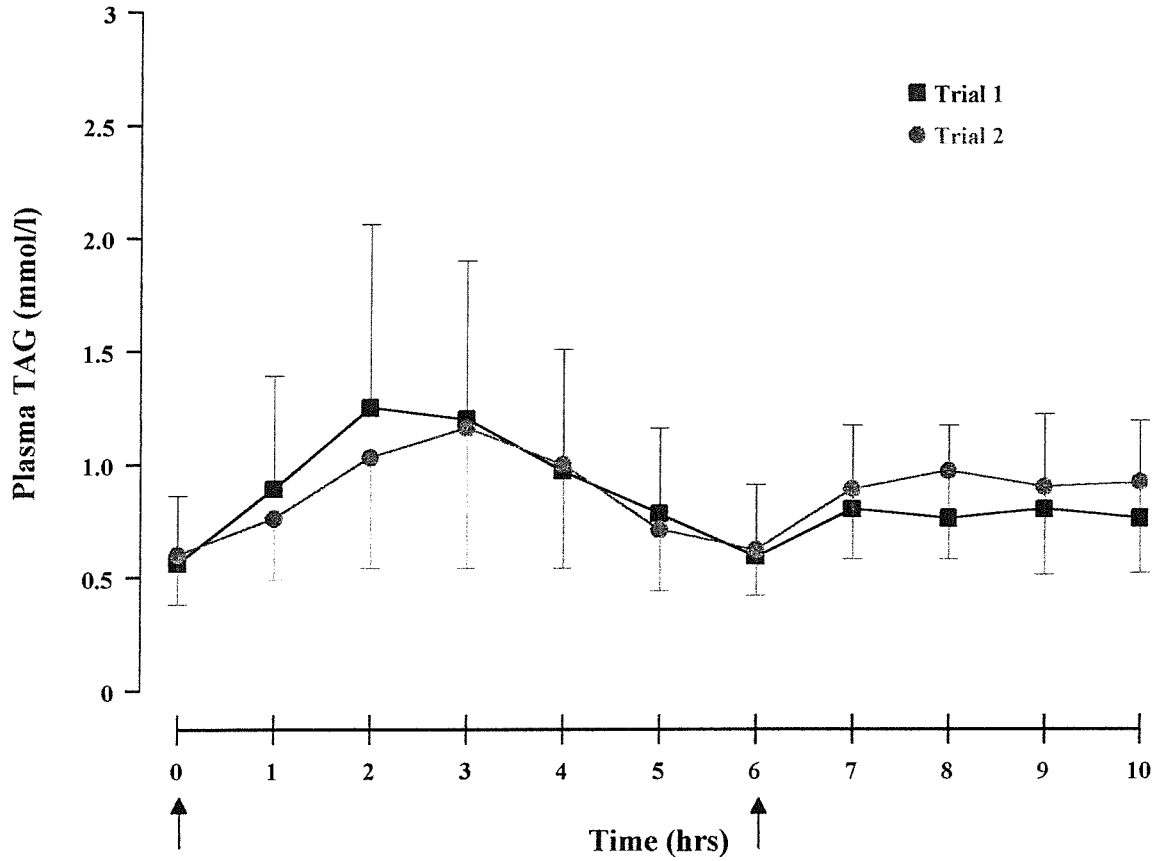


Figure 4.10 **Plasma non esterified fatty acid concentration (mmol/l) over a ten hours study period in a group of six subjects following consumption of two identical testmeals (group mean ± SD)**

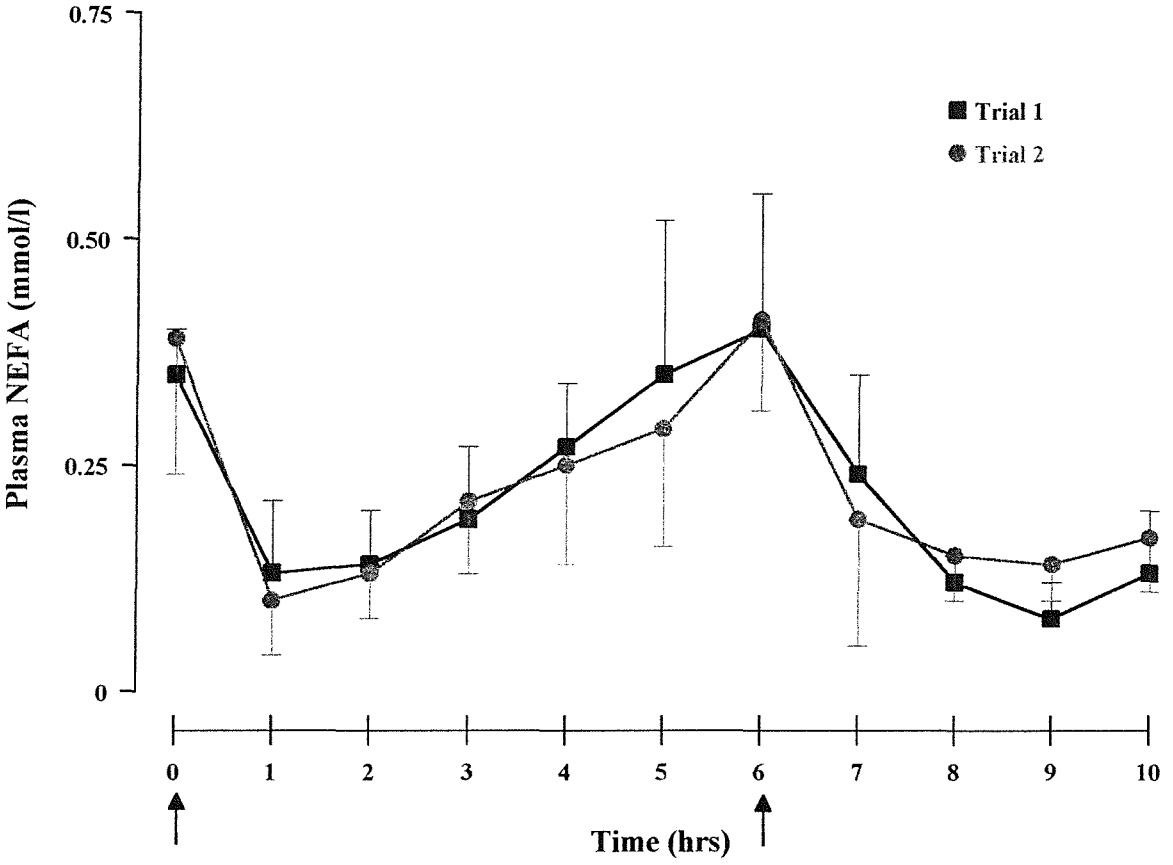
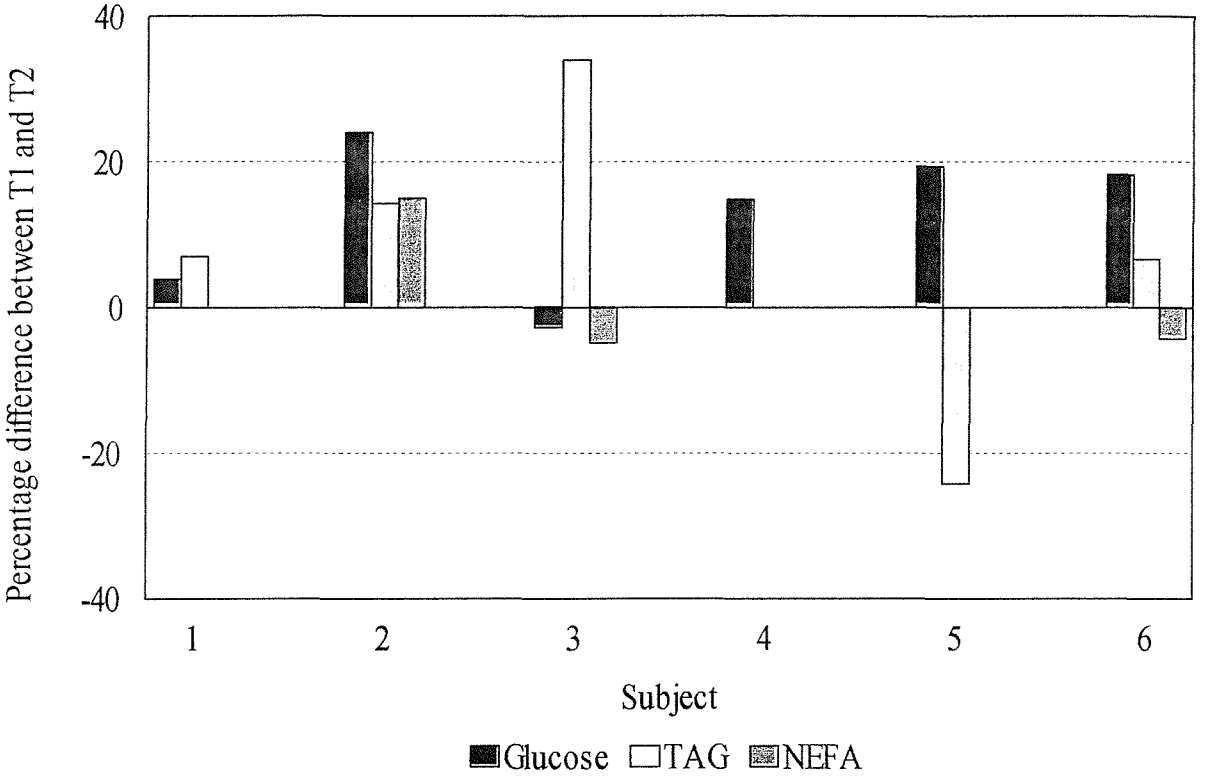


Figure 4.11 **Percentage difference between trial 1 and trial 2 for plasma glucose, plasma TAG and plasma NEFA concentrations, calculated from the actual difference between trials/ (T1+T2/2) x 100.**



4.6 Discussion

The study reported in this chapter is the first to examine the variability of measures of postprandial lipid metabolism, following a period of dietary and lifestyle control over subjects. The aim of the study was to determine the magnitude of variability in outcome measures when identical study conditions were applied on two separate occasions. The determination of the magnitude of within-individual variability (standard deviation of group within-individual differences) allowed power analysis calculations to be performed to demonstrate the power of the study to detect differences in means when an intervention is applied (chapter 5 and 6).

4.6.1 Gastrointestinal handling of [1,1,1-^{13}C]tripalmitin

The availability of meal lipid to the body was estimated by measurement of the excretion of ^{13}C-label within the stool. The results from the present study suggest that the label was almost completely absorbed across the GI tract with an average of 99% of administered label absorbed. The variability in the availability of ^{13}C-label to the body was determined as the difference between the means from trial 1(T1) and trial 2 (T2). A difference of 0.16 % of administered dose of ^{13}C-label was found between the trials, which was equivalent to a difference of 0.9% from T1 to T2, suggesting little variability between trials. This is in contrast to the only known previous study to examine the repeatability of measurements of postprandial lipid metabolism using stable isotopes, which found both a large proportion of label in stool (mean = 31.6% of administered dose) and large variation both within (trial 1=8.0%, trial 2=71.0%) and between individuals (subject 2= 8.3%, subject 6= 71.0%) [Murphy *et al.* 1995]. However this study is not directly comparable as the subjects were female, and there are known differences between the GI handling of men and women [Jones, 1996]. The decrease in label excretion in the stool was in contrast to a previous study in a similar subject group. Jones *et al* [1998] found an average of 36.4% of administered dose of label in the stool in comparison to 2-3% in this study. The result of large between and within-individual differences in the GI handling of lipid when the same study conditions are applied on two separate occasions suggest that when an intervention is applied the study will have

less power to determine differences in means. For example, power analysis of data supplied by Murphy *et al.*[1995] suggests that as variability is large, even to detect a difference in means of 50%, the study will only have a power of 7%. In other words, as variability is so great, it is unlikely that any intervention which results in a difference in means will have the ability to predict that the difference seen is due to the intervention alone. In the study described in this chapter, however, the same level of caution need not be applied. As variability between the trials decreases the power of the study increases such that for a difference in means of 50%, the power of the study increases to 14%. This suggests that using the present study protocol there is a greater likelihood of differences in stool ^{13}C-label excretion with an intervention being due to the intervention rather than variability. The reasons for the decrease in variability from Murphy *et al.*[1995], to the study described in this chapter were the emulsification of the [1,1,1-^{13}C]tripalmitin resulting in a greater absorption, and an increased control over subjects including diet, preceding meal and overnight admission to the unit. This suggests that controlling extrinsic variability factors leads to a reduction in the overall intra-individual variability.

4.6.2 Metabolic Disposal of [1,1,1-^{13}C]tripalmitin

Oxidation of the absorbed labelled lipid was determined by measuring the excretion of ^{13}C-label on breath as $^{13}CO_2$. The pattern and timecourse of ^{13}C-oxidation over the ten hour study period was similar in both trials for the group as a whole with a group mean difference between trial 1 and trial 2 of 1.45% of administered dose or 1.4% of absorbed dose of [1,1,1-^{13}C]tripalmitin. This equated to an overall decrease in oxidation of 6.6% and 6.9% respectively for the group suggesting little variability between trials for the group as a whole. On an individual basis variability increased with subject 4 demonstrating a 35% decrease in $^{13}CO_2$ excretion on breath from trial 1 to trial 2, and decreased with subject 2 demonstrating a 2.1% increase in oxidation from trial 1 to trial 2.

Differences between individuals in the oxidation of [1,1,1-^{13}C]tripalmitin may be due to differences in body composition, or in the metabolic capacity to oxidise lipids. In a similar subject group of young and healthy males, Jones *et al* [1998] found an average

of 35.1% of absorbed dose excreted on breath, which is higher than the present subject group. There was no difference in age or body composition between the two studies, and therefore the differences in $^{13}CO_2$ excretion seen may be due to the different protocols used. A proportion of the $^{13}CO_2$ formed from oxidation of the labelled substrate is not directly excreted on breath but may be retained in the body pools yet none of the values were corrected for retention of $^{13}CO_2$ in body pools. A study examining the metabolic fate of intravenous labelled sodium bicarbonate [Jones *et al* 1999- *in the press*] shows that it is unlikely ^{13}C-label is excreted in urine as previously reported by Elia *et al.* [1992]. It is believed that the body pools consist of a central rapidly turning over pool including organs with a high perfusion rate, and a more slowly turning over pool including muscle, adipose tissue and bone [Irving, 1983]. It is unlikely that over 24 hours a substantial proportion of ^{13}C-label will be retained in rapidly turning over bicarbonate pools, but is more likely to be retained in the bone, adipose and muscle. By applying a correction factor for the retention of $^{13}CO_2$ in bicarbonate pools, for example 70% of administered dose [Jones *et al.*1999- *in the press*], the $^{13}CO_2$ excretion on breath in this study would rise from 27% to 39%. However, by applying a correction factor to the present study, although the numbers would change, the overall interpretation of the data would remain unchanged.

Power analysis using the standard deviation of the mean difference between trial one and trial two as a measure of variability, and an anticipated effect size of 30% with an intervention (control trial = 28.2% of administered dose, intervention = 36.7% of administered dose) suggests that with a n of six, the study will have a 95% power to demonstrate differences in means and to reject the null hypothesis that the two population means are equal. An 80% power would be demonstrated with a difference in means of ± 6.8% of administered dose (control trial = 28.2%; intervention trial = 35.0%).

4.6.3 Substrate Oxidation

Total substrate oxidation, the sum of exogenous and endogenous oxidation and synthesis, was estimated using indirect calorimetry. The pattern of total lipid oxidation over the ten-hour study period did not differ between the trials for the group as a whole,

however greater individual variability was seen between the trials, in particular in subjects 4 and 5. The within-subject variation for total lipid oxidation was small for most subjects (< 10%), but increased in subject 5 (38.8%). Total lipid oxidation over the study day varied greatly between individuals, for example from 14.0g (subject 4) to 46.0g (subject 6). This suggests that individuals have different metabolic capacities to handle lipid loads, partitioning towards oxidation or storage, however the contributing factors to the within and between subject variability will be discussed in more detail in chapter 7 (general discussion). Power analysis calculations demonstrated that due to the variability in total lipid oxidation, that there would need to be a change in total lipid oxidation of over 30%, to allow the study to have an 80% power to detect differences in means in order to test the null hypothesis that the two populations are equal. A change of 30% equates to an increase in lipid oxidation from 28.2g (control trial) to 36.7g (intervention trial) which suggests that differences observed in the high fat study (chapter 5) would not reach a power of 80%. Variability decreased when exogenous lipid oxidation was examined, with a mean difference of 7.8% between trial one and trial two. Power analysis demonstrated that exogenous lipid oxidation would have to increase or decrease by 25% (control trial = 15.0g/10 hours; intervention = 18.8g/10 hours) to detect a difference in means to challenge the null hypothesis that the two populations are equal. Endogenous lipid oxidation was a more variable measure with a mean difference between trial one and trial two of 39.0%, resulting in a decrease in the power of the study when examining differences of 25%. Endogenous lipid oxidation was calculated from the difference between total lipid oxidation and measured exogenous lipid oxidation, which may account for the increase in variability. The repeatability of lipid oxidation has not been directly studied previously, however the use of indirect calorimetry as a method for the determination of carbohydrate and fat utilisation reported a coefficient of variation of 17.4% between trials which, it was suggested, did not provide sufficient accuracy for the measurement of substrate oxidation [Gasic *et al.*1997].

Carbohydrate oxidation was repeatable between trials with 107.9g oxidised over the ten hour study period on trial one, and 103.8g oxidised on trial two, which resulted in a difference in means of 4.1g (3.4%). The results are comparable to a similar study in healthy men over a six hour study period with a group mean of 85.0g oxidised per six

hours [Jones *et al.*1999]. Within-individual variability differed between subjects with the majority of subjects demonstrating a difference between trials of less than 10%. Subject 5, however, demonstrated a difference between trials of 28.7%, although the reasons are unclear. Power analysis calculations showed that an increase or decrease in carbohydrate oxidation of 30% with an intervention, would be sufficient for the study to have 80% power to detect differences in means to reject the null hypothesis that the two populations are equal. A difference of 30% equates to carbohydrate oxidation of 107.9g per 10 hours on the control trial, and 140.3g per 10 hours with an intervention.

4.6.4 Plasma Glucose

In normal, healthy individuals the concentration of glucose in the circulation is tightly regulated resulting in a relatively constant concentration throughout the day. In the study described in this chapter, baseline glucose concentrations were within the normal range for all subjects, and values fluctuated around baseline for the initial 6 hours of the study in both trials. Following the second meal, however, there was a substantial increase in glucose concentration, but the reasons for this remain unclear. The first meal was consumed following a 12 hour period without any food or drink, but the second meal was consumed only 6 hours following the first meal. Therefore it is possible that in the post-absorptive state glucose is rapidly taken up by the cells and glucose concentration remains constant. After the second meal, glucose is not removed as rapidly and therefore glucose concentrations are elevated, and will also depend upon the insulin sensitivity of the individual. As insulin was not measured in this study it is not possible to comment on the insulin sensitivity of the individual, although insulin sensitivity was shown to differ between individuals in chapter 6.

Plasma glucose concentrations were found to be consistently higher in trial 2 than in trial 1 but there is no experimental reason why this should be the case. The mean difference in plasma glucose concentration between trial 1 and trial 2 was 12.9% suggesting poor repeatability between trials. Within subject variability ranged from 2.8% (subject 3) to 24.0% (subject 2). Power analysis calculations demonstrated that with six subjects, a 20% change in plasma glucose concentration following an intervention trial was sufficient to provide an 80% power for the study to detect differences in means to

reject the null hypothesis that the two populations were equal. A 20% rise in plasma glucose concentrations relates to 54.4 mmol/l per 10 hours (control trial) rising to 65.3 mmol/l per 10 hours (intervention trial). There are no known studies in the literature to examine the repeatability of measurement of plasma glucose, and therefore it is not possible to compare the power analysis calculations.

4.6.5 Plasma Lipids

Measurements of the variability in postprandial lipid metabolism have not been extensively studied. Several previous studies have examined the variation in post-absorptive measures of cholesterol, TAG and fatty acid concentration [Ortola *et al.* 1992; Schectman and Sasse, 1993; Tsalamandris *et al.* 1998]. One such study found no significant differences in means for TAG and cholesterol when blood specimens were taken on two separate occasions, when subjects were measured following an overnight fast [Brown *et al* 1992]. However, the intra-individual variability of fasting triglycerides is several fold higher than that of other fasting plasma lipid and lipoproteins measurements [Brown *et al.* 1992]. The variability in plasma TAG concentrations decreased, however, when measured on two occasions in the postprandial state. The study described in the present chapter measured plasma TAG concentration for a ten hour postprandial period on two identical study days. The timecourse and magnitude of plasma TAG concentrations did not differ between the trials for the group as a whole. Area under the timecourse curve measurements which did not take account of baseline TAG concentrations demonstrated little within-individual variation between the trials (difference in group means =6.3%). However, when corrected for baseline TAG concentration was (iTAG), variation increased (16.2%) with repeatability varying between subjects (3.5% - 142.9%), suggesting that changes in baseline concentrations of TAG may account for some of the variation seen.

Power analysis calculations demonstrated that with six subjects, a 40% change in plasma TAG concentration (measured from zero; AUC_0) following an intervention trial was sufficient to provide an 80% power for the study to detect differences in means to reject the null hypothesis that the two populations were equal. The power of the study decreased when measuring TAG concentrations from baseline, where the study would

have a power of 46% with similar conditions. It was unclear how measurement of exogenous TAG alone would affect the power of the study, and the extent to which measures of ^{13}C-label in the TAG and NEFA fraction would be repeatable. Although this was not directly measured as part of the present research, concentrations of ^{13}C-chylomicron TAG were measured [Humayun *et al.*1999], in collaborative work in the study. A group mean of 18.9 ug/ml plasma per 10 hours was found on trial 1 which decreased to 18.5 ug/ml per 10 hours on trial 2, which suggests a difference in means of 0.4 ug/ml (SD = 4.99), suggesting that with six subjects, a 40% change in ^{13}C-chylomicron TAG concentrations following an intervention would result in a 66% power to detect differences in means to reject the null hypothesis. Power analysis calculations were particularly important for this outcome variable as measurements of ^{13}C-TAG and ^{13}C-NEFA concentrations were made in chapter 6.

Following identical study conditions the pattern and magnitude of plasma NEFA concentration did not differ between trials. Measurement of total plasma NEFA concentrations over the timecourse without taking baseline into account resulted in good repeatability between trials (mean difference = 0.01 mmol/l per 10 hours). Variability increased with the measurement of NEFA concentration from baseline with a mean difference between trials of 0.3 mmol/l per 10 hours. Power analysis calculations demonstrated that with six subjects, a less than 10% change in plasma NEFA concentration following an intervention trial was sufficient to provide an 80% power for the study to detect differences in means to reject the null hypothesis that the two populations were equal. The large variability demonstrated with incremental measures of plasma NEFA (from baseline) demonstrated that even a 50% change in plasma NEFA concentration with an intervention would only result in an 11% power for the study to detect differences in means appropriately. The large variability seen when taking baseline NEFA concentrations into account suggests that the method chosen to report results from graphs is extremely important when calculating anticipated effect sizes and power.

4.6.6 Calculation of subject numbers using power analysis

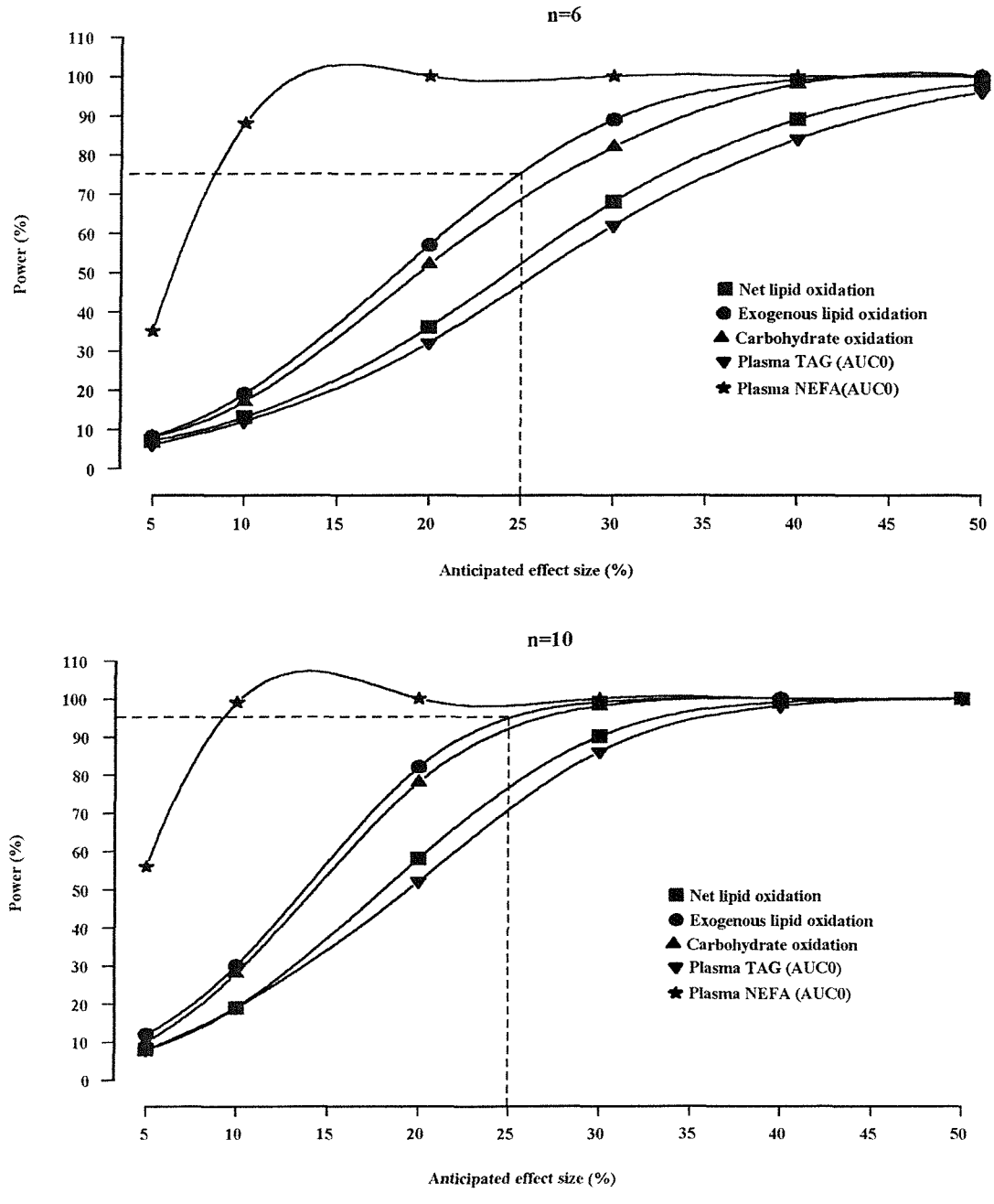
The purpose of power analysis is to determine the number of subjects required to show the smallest difference in means following an intervention, which may be of clinical or biological importance [Kirkwood, 1993]. In other words, by knowing within-individual variability of a measure (standard deviation of the mean difference between two repeated trials), and the effect size needed to be of clinical importance it is possible to manipulate both the power and the n required. For example, patients with known coronary heart disease were found to have plasma triglyceride concentrations 15% greater than healthy patients in a study of postprandial lipoprotein metabolism [Groot *et al.*1991]. If this is thought of as 'clinically significant', the values can be used in the power analysis model. In the present study a group mean of 8.6 mmol/l per 10 hours was observed on trial one (control trial). A 15% increase in plasma TAG concentration results in 9.9 mmol/l per 10 hours (intervention trial). When the figures were used in power analysis, the study demonstrated only an 18% power to detect differences in means of 15%, with a group of six subjects. Increasing the number of subjects to 20, which is the number used in the study, results in an increase in power to 54%. This suggest that it is unlikely that a difference in means would be detected at the level of significance desired ($p<0.05$), and this was found to be the case in the study.

The purpose of the study described in the present chapter was to determine the magnitude of intra-individual variability for each of the outcome measures in order for the power of the study to be calculated when an intervention was applied in further chapters. The variability of each outcome measure is shown in tables 4.1 to 4.14, as the mean difference between trial 1 and trial 2. The standard deviation describes the between subject variability in the measure. By calculating the anticipated effect size (5-50%) expected when an intervention is applied, it was possible to calculate the power of the study for an n of six or of ten. In other words, if an increase in plasma TAG of 40% is expected with a doubling of the lipid content of a meal, and the variability of the measure is known to be an SD of 1.8, the power can be calculated to be 84%. The study demonstrates that with a sample size of six, an effect size of 40% will have an 84% power to reject the null hypothesis that the two population means are equal, assuming a 2-tailed significance of 0.05. The power for anticipated effect sizes for each outcome

variable is shown in table 4.15. for an n of six, an in table 4.16 for an n of ten. The data is presented shown graphically (figure 4.12) to demonstrate that power increases with increasing anticipated effect size. From the graphs it is possible to calculate for any anticipated effect size, what the power of the study would be for 6 or 10 subjects. For example, an anticipated effect size of 25% for exogenous lipid oxidation (control trial =15.0g/10 hours; intervention trial = 18.8g/ 10 hours) would result in a 75% power with an n of six subjects, which would increase to 95% with an n of ten subjects.

The determination of the magnitude of variability of measures is rarely performed as it involves the completion of two identical studies which may be considered a waste of time and resources. By knowing the magnitude of variability in measures it is possible not only to calculate the number of subjects required for a study, but also to provide evidence that small subject numbers are effective in providing sufficient power to a study to be able to reject the null hypothesis that population means are equal. Therefore the measurement of the magnitude of variability in measurements was the single most important study to complete in order to ascertain whether the manipulation of meal composition results in significant changes in outcome above, and beyond the magnitude of intra-individual variability.

Figure 4.12 **Power analysis calculations to determine the power of a study (%) following an intervention with an anticipated effect size (%) with a known magnitude of within-individual variation for total lipid oxidation, exogenous lipid oxidation, carbohydrate oxidation, plasma TAG concentration and plasma NEFA concentration. The dashed line represents an effect size of a 25% increase in exogenous lipid oxidation with an intervention resulting in a known power.**



4.7 Summary and Conclusions

The aims of the study described in this chapter were to determine the magnitude of within-individual variability in the GI handling, metabolic disposal and lipaemic responses to a mixed meal containing [1,1,1-^{13}C]tripalmitin. The results demonstrate that: -

1) Controlling extrinsic variability factors such as preceding diet, physical activity levels and preparing the [1,1,1-^{13}C]tripalmitin within an emulsion appear to decrease the variability in the GI handling and metabolic disposal of lipid compared to that seen in previous trials.

2) The absorption of ^{13}C-label across the GI tract was almost complete (>97%) in both trials suggesting that the collection of stools in subsequent trials using this testmeal and within this subject group is not essential.

3) Substrate oxidation for the group as a whole displayed little variation between trials but large between subject differences were seen. However, the factors that underlie the marked between subject variability in lipid oxidation, and the remaining within-subject variability, require further investigation.

4) The magnitude of postprandial lipaemia variability was found to be small (<10%) when measured from zero (AUC_0). However, variability increased when baseline concentrations were taken into account for plasma NEFA concentrations.

5) By knowing the magnitude of intra-individual variability, the power of subsequent intervention studies can be calculated for any outcome variable and any anticipated effect size.

CHAPTER 5

Effect of Increasing Meal Lipid Content on the Gastrointestinal Handling and Postprandial Metabolism of [1,1,1-^{13}C]Tripalmitin

5.0 Introduction

It is well recognised that an elevation in plasma triacylglycerol and non-esterified fatty acid concentrations, particularly following a meal, can be potentially atherogenic (see section 2.2.3). Exaggerated TAG and NEFA responses to an 80g fat load [Murphy *et al.*1995] demonstrated that when acute fat intake exceeds the capacity of tissues to utilise fatty acid as an immediate energy source, storage of NEFA and TAG in adipose tissue cannot fully compensate and as a consequence NEFA 'spill' out into the circulation. This may in turn influence TAG clearance since Karpe *et al.*[1992] propose that postprandial accumulation of NEFA impedes LPL-catalyzed TAG hydrolysis and dissociates the enzyme from its endothelial surface. Jeppesen *et al* [1995] demonstrated that the concentration of postprandial plasma TAG increased in an approximately linear fashion as the oral fat load increased, however the degree of postprandial lipaemia was accentuated with increasing fat loads in those with the highest fasting TAG concentrations. Jeppesen *et al* [1995] also raise questions regarding the effect that increasing the lipid content of a meal has on the secretion and clearance of endogenous lipids.

Dietary lipid intake is also an important factor in substrate utilisation. Whilst carbohydrate oxidation appears to equal intake, intake of fat in a meal does not seem to promote fat oxidation during the postprandial hours [Schutz, 1989]. Excessive gains in glycogen stores appear to be prevented by a subsequent increase in carbohydrate oxidation yet irrespective of the consequences on fat balance, a metabolic response serving to increase fat oxidation in the presence of increased intake, in terms of a meal or the diet, appears to be lacking. This inability to adjust metabolism to attenuate the effect of changes in fat intake on fat balance provides a possible metabolic explanation for epidemiological findings that obesity is associated with increased dietary fat intake [Schutz 1989].

From previous work it is clear that consumption of increasing lipid loads leads to an increased postprandial lipaemia (see section 2.5.2.1). In addition, lipid oxidation has been shown to be disproportional to lipid intake unlike other macronutrients. There are no studies in the literature which have combined measures of the gastrointestinal handling of lipid with measures of the metabolic disposal of lipid, and measures of postprandial lipaemia. In addition none of the previous studies have been able to differentiate between the oxidation of meal or endogenous lipid, and how increasing the lipid content of a meal may alter the balance of oxidation.

The study reported in this chapter was the first time that tracer methodologies have been used to determine the effect of increasing the lipid content of a meal on the GI handling, oxidation and appearance in the circulation of dietary lipid under controlled study conditions.

5.1 Hypothesis

The hypothesis tested within the study presented in this chapter was that doubling the lipid content of a testmeal would result in an increase in lipid oxidation (greater than described by within-individual variability) with a subsequent decrease in carbohydrate oxidation, and an increase in the postprandial lipaemic response compared to a reference testmeal.

5.2 Aim

The aim of the present study was to examine the changes in postprandial lipid metabolism that occur following consumption of a high-fat meal, and to investigate individual capacities to metabolise lipid in order to answer the following questions:-

1) How complete is absorption of [1,1,1-^{13}C]tripalmitin when presented to the GI tract as part of a high-fat meal?

2) Does consumption of a high-fat meal result in changes in the oxidation of [1,1,1-^{13}C]tripalmitin, despite the consumption of identical amounts of tracer?

3) Lipid oxidation does not appear to be related to lipid intake. Does increasing the lipid content of the meal result in alterations of total lipid and carbohydrate oxidation, or the proportions of meal (exogenous) or endogenous lipid oxidised?

4) Does increasing the lipid content of the meal result in raised postprandial lipaemia in terms of plasma TAG and plasma NEFA concentrations, compared to lipaemic responses following a reference testmeal?

5) Are there differences between individuals of similar body composition in terms of the 'metabolic capacity' for the clearance and oxidation of lipid?

5.3 Subjects

The subject group recruited for the study presented in chapter 4 also completed the study presented in this chapter. Subjects completed three studies of which two studies (T1 and T2) were identical (reference testmeal) to study within-individual variability, and the third study consisted of a high-fat testmeal (trial 3). T1 was assigned as the control trial for all subjects, whilst T2 and trial 3 (T3) were assigned in a random order to the subjects. Subject characteristics are shown in table 4.1.

5.4 Methodology

Subjects followed the general study protocol described previously (section 3.4). Each subject completed two 9-day trials, 4 weeks apart (trial 1-reference testmeal, trial 3, high-fat testmeal). On each occasion, subjects were fed an identical prescribed diet (1.5 x BMR) for three days prior to the study day (see appendix 1.1), and were asked not to eat or drink any other items except water. During this period they were asked to maintain their habitual activity, and for this to remain constant between the trials. On the evening of the third day, subjects were admitted to the Clinical Nutrition and Metabolism Unit, and were fasted and rested from 7.30pm. On the study day, a baseline end tidal breath specimen was collected and VCO_2 measurement were made before consumption of the testmeal and hourly for ten hours after the testmeal. Subjects

performed 15 hour and 24 hour breath tests at home. An indwelling cannula was inserted into a forearm vein, and a 10ml blood sample withdrawn. Following the meal hourly blood samples (10 ml) were taken for 10 hours. In addition to the reference emulsion and testmeal (section 3.1) subjects consumed 200g of orange juice. The reference testmeal provided 3.2MJ of energy, 35.7g of lipid, 90.3g of carbohydrate and 27.2g of protein. The high fat testmeal described in tables 5.1 and 5.2, provided 4.4 MJ of energy, 67.0g of lipid, 91.2 g of carbohydrate and 28.3 g protein. [1,1,1-^{13}C]tripalmitin was added to the emulsion at a concentration of 10mg/kg body weight and this did not alter with the high-fat testmeal A second unlabelled emulsion and testmeal was consumed at 6 hours postprandially. A baseline stool specimen and all stools passed over a 5 day period were collected. Specimens were analysed according to methods described in chapter 3. The aim of this study was to examine the effect of feeding a high-fat meal on outcome measures, therefore the composition of the reference emulsion and testmeal (section 3.1) was manipulated to achieve this aim.

Table 5.1 Composition of a high-fat lipid emulsion and testmeal

Meal component	**Emulsion**
100g White bread	44g Double cream *
38g Ham	7 g Olive oil *
38g Clover margarine *	6g Sunflower oil *
	12g Casein
	9g glucose
	4.5g beet sugar
	10g milkshake powder
	150g mineral water
	10mg/kg body weight [1,1,1-^{13}C]tripalmitin

* denotes a doubling of the component quantity from the original reference and testmeal described in chapter 3. The composition of the high fat testmeal is described in table 5.2.

Table 5.2 Macronutrient composition of high-fat lipid emulsion and testmeal

	Meal component	Emulsion	Total
Energy:	2277 kJ	2162 kJ	4439 kJ
Lipid:	32.3g	34.7g	67.0 g
Carbohydrate:	49.8g	41.4g	91.2 g
Protein:	15.7g	12.6g	28.3g
SFA:	18.5% energy	25.7% energy	22.1% energy
MUFA:	19.2% energy	19.9% energy	19.6% energy
n-3 PUFA:	1.2% energy	0.3% energy	0.8% energy
n-6 PUFA	5.6% energy	6.7% energy	6.2% energy
TUFA:	3.6% energy	2.1% energy	2.9% energy

5.5 Results

Results are expressed as reference testmeal trial (trial 1) and high fat testmeal trial (trial 3). Results are shown for the recovery of ^{13}C-label in stool, expressed as a percentage of the administered dose of labelled tripalmitin. Oxidation of [1,1,1-^{13}C]tripalmitin is shown as the excretion of $^{13}CO_2$ on breath expressed as a percentage of administered dose and as a percentage of absorbed dose, taking stool losses into account. $^{13}CO_2$ excretion on the breath was calculated over the initial 10-hour period, and full 24-hour study period as the area under the timecourse curve (AUC). Indirect calorimetry was used to calculate total substrate oxidation, and with $^{13}CO_2$ data was used to estimate the proportion of lipid oxidation from exogenous and endogenous sources. Plasma concentrations of TAG, NEFA and glucose over the postprandial period were calculated as the area under the timecourse curve measured from zero (AUC_0) and from baseline, the incremental area under the curve (AUCi).

5.5.1 Stool ^{13}C-excretion

Table 5.3 shows the recovery of ^{13}C-label in stool and the calculated availability of ^{13}C-label to the body. The mean excretion of ^{13}C-label in stool for the group following consumption of the reference testmeal was 1.2% (0.5 to 2.3%) of administered dose which increased to 2.0% (1.0 to 3.1) following the high fat meal. Each subject

demonstrated an increase in stool ^{13}C-label excretion from the reference to the high-fat testmeal (mean difference = 0.8 % of administered dose), and the greatest difference was seen in subject 2 (1.8%). Despite the trend in stool ^{13}C-label excretion between the trials, one way analysis of variance revealed no statistical differences between the trials. The raw ^{13}C-data was used to calculate the availability of labelled substrate to the body. Table 5.3 shows that following the reference testmeal 98.8% of administered dose of label (97.7-99.2%) was available to the body which decreased to 98.0% (96.9% to 99.0%) following the high-fat testmeal.. There was little variability between or within-individuals with an increase of 0.8% between trials for the group as a whole which resulted in no significant differences between trials.

5.5.2 Breath $^{13}CO_2$ excretion

Figure 5.1 shows the total $^{13}CO_2$ excreted on breath over the 24-hour study period for the group as a whole as a proportion of the absorbed dose of [1,1,1-^{13}C]tripalmitin. Baseline abundance of ^{13}C on breath before administration of the label was at the level of natural abundance with a rise in $^{13}CO_2$ above baseline immediately following the meal on both trials. The general pattern of oxidation was the same in both trials as $^{13}CO_2$ excretion increased from baseline and peaked at 3-4 hours postprandially before returning to baseline. However there was a greater magnitude of excretion following the reference testmeal trial (peak=3% of absorbed dose), than the high-fat trial (peak=2.4% of absorbed dose). Univariate analysis of variance revealed no significant effect of trial on breath $^{13}CO_2$, but a significant effect of time ($p<0.05$). Multivariate analysis revealed that there was no significant effect of the interaction between time and trial.. Total oxidation of the ^{13}C-label was calculated by measuring the total area under the timecourse curve.

Table 5.4. shows the total breath $^{13}CO_2$ excretion as a percentage of administered and table 5.5 as a percentage of absorbed dose. Over 24 hours the group mean $^{13}CO_2$ excretion as a percentage of administered dose following the reference testmeal was 27.8% (23.1 to 31.3%) which decreased to 21.7% (14.0 to 26.9%) following the high fat trial. All subjects demonstrated a decrease in breath $^{13}CO_2$ excretion from the reference testmeal to the high fat testmeal, with an overall group decrease of 6.2% of administered

dose, which was equivalent to a 26.7% decrease in effect size. The effect of the high fat meal differed between individuals with subject 6 showing a 6.1% decrease, whilst subject 1 showed a 48.9% decrease in breath $^{13}CO_2$ (calculated as the difference between T1 and T3 divided by T1+T3/2).

Expressing the results as a percentage of absorbed dose resulted in a subsequent increase in the percentage of breath $^{13}CO_2$ recovered (reference = 28.2%; high fat = 22.0%), which resulted in a small decrease in the percentage effect for the group as a whole (26.5%). One way analysis of variance revealed a significant effect of the high-fat testmeal on total $^{13}CO_2$ excretion over 24 hours ($p<0.05$).

5.5.3 Lipid Oxidation

Figure 5.2 shows the group mean for the timecourse of lipid oxidation over the ten-hour study period. Individual lipid oxidation is shown in appendix 3.1. Postabsorptive lipid oxidation was approximately 2-2.5g/hour for the group in both trials. Following the reference testmeal, lipid oxidation decreased following the testmeal by 0.25g, and began to rise 2 to 3 hours after the meal. After the second testmeal, lipid oxidation was suppressed again but to a lesser extent, and rose again towards the end of the study period. On the high fat trial, there was no suppression of lipid oxidation following the meal but a steady increase in oxidation peaking at 5 hours postprandially, with no response following the second meal. Univariate and multivariate analysis of variance showed no statistical differences in the timecourse of lipid oxidation over the ten hour study period. Total lipid oxidation over the whole study period was calculated from the area under the timecourse curve for each individual. Table 5.6 indicates the total lipid oxidation over the ten hour study period for the reference testmeal and high fat testmeal trials. Total lipid oxidation increased from 28.2g/10 hours (15.9 to 44.1g/10 hours) following the reference testmeal to 32.9g/10 hours (24.2 to 46.4g/10 hours) following the high-fat meal. For the group as a whole total lipid oxidation increased by 4.7g per 10 hours, equivalent to a 16.7% increase. There was a trend in lipid oxidation from T1 to T3, with five subjects showing an increase in lipid oxidation and only one subject showing a decrease. The effect of the high-fat meal also differed greatly between individuals with subject 2 demonstrating a 9.8% decrease in lipid oxidation, whilst

subject four demonstrated a 38.0% increase in lipid oxidation. Despite the trend of an increase in lipid oxidation, the effect was not great enough, and variability too large to show any significant effects of trial when conducting one way analysis of variance.

The metabolism of [1,1,1-^{13}C]tripalmitin was assumed to reflect that of the lipid in the testmeal. If the total palmitic acid in the testmeal is calculated, the proportion of this, which is oxidised, can be calculated from $^{13}CO_2$ excretion on breath and the total palmitic acid oxidised over the ten hours can be calculated. Metabolism of palmitic acid was assumed firstly to reflect that of all saturated fatty acids in the testmeal, and therefore all fatty acids. The total lipid consumed over the study day on the reference testmeal trial was 70.6g (two testmeals) which increased to 133.2g on the high fat testmeal trial. From the amount of lipid in the testmeal and data from $^{13}CO_2$ excretion on the breath, the oxidation of meal lipid (exogenous) was calculated (table 5.7) and the oxidation of endogenous lipid estimated. Figure 5.3 shows the timecourse of exogenous and endogenous oxidation for the group as a whole. As exogenous lipid oxidation increased following both meals, endogenous lipid oxidation decreased. Exogenous lipid oxidation increased following the high fat meal peaking at >3g/hour between 4 and 7 hours postprandially, compared to approximately 2g/hour following the reference meal. Two way analysis of variance on both factors revealed a significant effect of time, trial and time/trial interaction ($p<0.05$) on exogenous lipid oxidation. Post-hoc analysis (Tukey's HSD) revealed that significance was reached at 4-8 hours postprandially. Endogenous lipid oxidation demonstrated a greater variance between subjects and statistical significance was not reached at the multivariate level, despite revealing a significant effect of time ($p<0.05$).

Total exogenous and endogenous lipid oxidation were calculated from the area under the timecourse curve and are shown in tables 5.8 and 5.9 respectively. The proportion of net lipid oxidation, which can be accounted for by exogenous and endogenous sources is shown in figure 5.4. For the group as a whole, exogenous lipid oxidation increased from 15.0g/10 hours (13.2 to 16.3g/10 hours) following the reference meal to 21.9g/10 hours (16.4 to 24.6g/10 hours) following the high fat meal. All subjects demonstrated an increase in exogenous lipid oxidation with the high-fat testmeal with a mean increase of 36.1%. Between subject variability was small for exogenous lipid oxidation with a range of a 21.6% to 61.4% increase in oxidation with the high fat meal.

One way analysis of variance revealed that due to the trend in the increase in exogenous oxidation, and small between subject variance that statistical significance was reached between the trials ($p<0.05$). Endogenous lipid oxidation decreased from 13.2g/10 hours (2.2 to 29.2g) with the reference testmeal to 11.0g/10 hours with the high fat testmeal. There was no trend in endogenous lipid oxidation with 3 subjects demonstrating an increase in oxidation and 3 subjects a decrease. For the group overall, however, there was a 16.4% decrease in endogenous lipid oxidation with consumption of the high-fat testmeal. Between subject variance was large with subject 3 demonstrating a 1.6% increase in lipid oxidation whilst subject 2 showed a 113.4% decrease in oxidation. No significant differences were revealed between the trials with one way analysis of variance due to the lack of trend of endogenous lipid oxidation and the large between individual variation.

5.5.4 Carbohydrate Oxidation

Total mean carbohydrate oxidation over the 10 hour study period is shown for the group as a whole in figure 5.5, with individual carbohydrate oxidation shown in appendix 3.2. In the postabsorptive state in both trials carbohydrate oxidation was constant at 9g/hour. When the reference testmeal was consumed, oxidation increased to a peak of 16g/hour at two hours postprandially before decreasing to below baseline levels. Following the second meal, a smaller peak was seen (peak =12g/hour). When the high fat testmeal was consumed carbohydrate oxidation decreased, peaking at approximately 12g/hour at two hours postprandially, with a similar pattern after the second meal. Univariate analysis of variance revealed a significant effect of time ($p<0.05$), but no significant effect of trial on carbohydrate oxidation. Multivariate analysis of the interaction between time and trial revealed no statistical differences between the trials. Total carbohydrate oxidation over the ten hour study period was calculated from the area under the timecourse curve for each subjects, and results are shown in table 5.10. For the group as a whole carbohydrate oxidation following the reference testmeal trial was 107.9g/10 hours (92.2 to 138.3g/10 hours) which decreased to 101.1g/10 hours (87.0 to 119.2g/10 hours) following the high fat meal. There was no obvious trend in carbohydrate oxidation from the reference testmeal to the high fat

testmeal with four subjects showing a decrease in oxidation, whilst two subjects showed an increase in oxidation. Between subject variability was large with subject 1 demonstrating a 12.1% increase in oxidation with the high-fat meal, whilst subject 5 demonstrated a 22.0% decrease in carbohydrate oxidation. Overall there was a decrease in 6.1% oxidation between trials, but this did not reach statistical significance following one way analysis of variance indicating no effect of trial on carbohydrate oxidation due to the lack of trend in carbohydrate oxidation and between subject variability.

5.5.5 Plasma glucose

The plasma glucose response over the time period for the group as a whole can be seen in figure 5.6, with individual responses shown in appendix 3.3. Following a twelve hour period without food, baseline concentrations of glucose were constant between the trials (~5.0mmol/l). Following the reference testmeal, there was little glycaemic response with values remaining at baseline concentrations until 6 hours postprandially. After the second meal plasma glucose concentration rose and peaked at 9 hours postprandially (~7.5 mmol/l). Following the high fat testmeal, plasma glucose concentrations increased to 6 mmol/l by 3 hours postprandially. After the second meal there was a marked glycaemic response, reaching 8mmol/l by 7 hours postprandially. Univariate analysis of variance revealed no significant effects of trial on plasma glucose concentrations but a significant effect of time ($p<0.05$). Multivariate analysis did not reveal significant effects of the interaction between time and trial. Total plasma glucose concentrations over the ten hour study period was calculated from the area under the timecourse curve, measured from zero (AUC_0), shown in table 5.11.

Total plasma glucose concentration increased from 54.4 mmol/l per 10 hours (49.4 to 57.2) following the reference testmeal to 62.1 mmol/l per 10 hours (53.3 to 77.6) with the high-fat testmeal, which accounted for an overall 12.6% increase in glucose concentration. There was an overall trend in plasma glucose concentrations as five subjects demonstrated an increase in concentration whilst one subject demonstrated a decrease. Between subject variability was large with subject six showing a 0.2% decrease in glucose concentration from the reference meal to the high fat meal, whilst subject five showed a 32.0% increase in plasma glucose concentration. One way analysis

of variance revealed no significant effects of the high-fat meal on plasma glucose concentration due to the large between subject variability seen.

5.5.6 Plasma TAG

The timecourse of postprandial lipaemia (PPL) is shown in figure 5.7 for the group as a whole, and in appendix 3.4 for individual subjects. All subjects had postabsorptive plasma TAG concentrations within the normal range (0.5 to 1.0 mmol/l) prior to commencing both trials. Following the reference testmeal trial, TAG concentrations rose to a peak of 1.3 mmol/l at two hours, with a steady decrease to six hours postprandially. After the second meal, responses were delayed with a decreased magnitude of PPL (peak, 1.0 mmol/l). On the high fat trial, the overall magnitude and duration of PPL was increased. PPL peaked at three hours postprandially (1.6 mmol/l) before decreasing to near baseline levels at 6 hours. After the second meal TAG concentrations increased, peaking at 1.2-1.3 mmol/l. Univariate analysis of variance revealed no significant effects of trial on plasma TAG concentrations but a significant effect of time ($p<0.05$). Multivariate analysis revealed no significant effects of the interaction of time and trial on plasma TAG concentrations. Total plasma TAG concentrations over the ten hour study period were calculated from the total area under the timecourse curve measured from zero (AUC_0) and from baseline (AUCi), shown in tables 5.12 and 5.13 respectively.

Total (AUC_0) plasma TAG concentrations increased from 8.6 mmol/l per 10 hours following the reference testmeal (3.4 to 16.7), to 11.4 mmol/l per 10 hours following the high fat testmeal (6.2 to 16.6), revealing an overall 34.3% increase in plasma TAG concentrations. There was a trend in plasma TAG concentrations with five subjects demonstrating an increase in TAG concentration whilst one subject demonstrated a decrease. Between subject variability was large with subject 5 showing a 0.6% decrease in concentration whilst subject 3 showed a 58.3% increase in concentration. One way analysis of variance did not, however, reveal a significant effect of trial on total plasma TAG concentrations due to the large between and within-individual variation.

Total (AUCi) plasma TAG concentrations increased from 3.2 mmol/l per 10 hours following the reference testmeal (1.0 to 5.8), to 5.7 mmol/l per 10 hours following the high-fat testmeal (3.6 to 7.4), revealing an overall increase in plasma TAG concentrations of 62.7%. All subjects displayed an increase in TAG concentration from the reference testmeal to the high-fat testmeal, despite differing magnitudes. Subject 6 demonstrated a 14.9% increase in TAG concentrations whilst subject 3 demonstrated a 134.4% increase in TAG concentrations. One way analysis of variance revealed a significant effect of the high-fat testmeal on total plasma TAG concentration (AUCi) ($p<0.05$).

5.5.7 Plasma NEFA

Figure 5.8 shows the timecourse of NEFA concentration for the group, with individual timecourses shown in appendix 3.5. Following the reference testmeal trial, post-absorptive NEFA concentrations were uniform for the group (0.35 mmol/l). On consumption of the meal, concentrations decreased to near zero by one hour postprandially, and steadily rose to baseline levels by 6 hours. After the second meal there was a more pronounced nadir, with the concentration decreasing for 3 hours before a slight increase at 10 hours (~0.1 mmol/l), however, baseline levels were not reached by the end of the study at ten hours postprandially. After consumption of the high fat testmeal, a similar pattern was seen at one hour postprandially. NEFA concentration rose with greater magnitude and reached above baseline levels by 6 hours (0.45 mmol/l). After the second meal, no nadir was seen with concentrations falling minimally below baseline levels. Repeated measures analysis of variance on both factors revealed a significant effect of time, trial and time/trial/interaction on plasma NEFA concentration ($p<0.05$). Post-hoc analysis (Tukey's HSD) revealed that differences between the trials were significant at 8, 9 and 10 hours postprandially ($p<0.05$). Total plasma NEFA concentration was calculated as the area under the timecourse curve over the ten hour study period, measured from zero (AUC_0), and from baseline (AUCi), which are shown in tables 5.14 and 5.15 respectively.

Total (AUC_0) NEFA concentration increased from 2.2 mmol/l per 10 hours on the reference testmeal trial (1.8 to 2.5), to 3.2 mmol/l per 10 hours on the high fat

testmeal trial (1.9 to 3.9), revealing an overall increase in plasma NEFA concentration of 43.5%. There was a trend in the increase in NEFA concentration with five subjects showing an increase and only one subject a decrease in NEFA concentration. There was, however, large between subject variability with subject 3 demonstrating a 10.0% decrease in NEFA concentration and subject 4, an 88.0% increase in concentration. One way analysis of variance revealed a significant effect of the high-fat testmeal on total plasma NEFA concentration ($p<0.05$).

Total (AUCi) NEFA concentration increased from –1.4 mmol/l per 10 hours on the reference testmeal trial (-2.0 to –0.2), to 0.08 mmol/l per 10 hours on the high fat testmeal trial (-1.0 to 2.0), revealing an overall increase in plasma NEFA concentration of 142.6%. All six subjects demonstrated an increase in plasma NEFA concentration with the high-fat meal. One way analysis of variance revealed a significant effect of the high-fat meal on total plasma NEFA concentration ($p<0.05$).

Table 5.3 **Stool ^{13}C-label excretion (% of administered dose of [1,1,1-^{13}C]tripalmitin) and the availability of [1,1,1-^{13}C]tripalmitin calculated from the recovery of total ^{13}C in stool, collected at baseline and for 5 days following the administration of the labelled lipid, in six subjects, within a reference testmeal and a high-fat testmeal. The difference between trials is reported as the actual difference (^{13}C-label recovery T1-T3) and the percentage difference (actual difference/ (T1+T3/2) x 100) between the trials.**

Subject	Stool ^{13}C (% administered dose of [1,1,1-^{13}C]tripalmitin)					
	Reference testmeal (T1)		High-fat testmeal (T3)		Difference between trials (% difference = difference/T1+T3/2 x 100)	
	Recovery of ^{13}C	Availability of ^{13}C	Recovery of ^{13}C	Availability of ^{13}C	Recovery of ^{13}C	Availability of ^{13}C
1	1.2	98.8	2.8	97.2	↑ 1.6 (80%)	↓ 1.6 (1.6%)
2	1.3	98.7	3.1	96.9	↑ 1.8 (82%)	↓ 1.8 (1.8%)
3	0.5	99.5	1.2	98.8	↑ 0.7 (82%)	↓ 0.7 (0.7%)
4	0.8	99.2	1.0	99.0	↑ 0.2 (22%)	↓ 0.2 (0.2%)
5	1.3	98.7	1.8	98.2	↑ 0.5 (32%)	↓ 0.5 (0.5%)
6	2.3	97.7	2.1	97.9	↓ 0.2 (9%)	↑ 0.2 (0.2%)
Mean	**1.2**	**98.8**	**3.0**	**98.0**	**0.8 (48.2%)**	**0.8 (0.8%)**
SD	**0.6**	**0.6**	**0.8**	**0.8**	**0.8 (38.8%)**	**0.8 (0.8%)**

Table 5.4 **Excretion of $^{13}CO_2$ on the breath expressed as a proportion of administered dose of [1,1,1-^{13}C]tripalmitin, over a 24-hour study period following consumption of a reference testmeal and a high fat testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T2), and the percentage difference (actual difference/T1+T2/2 x 100).**

Subject	Excretion of $^{13}CO_2$ on breath (% of administered dose of [1,1,1-^{13}C]tripalmitin			
	Reference testmeal	**High-fat testmeal**	**Difference (T1-T3)**	**% difference (T1-T3/2)**
1	23.1	14.0	↓9.1	48.9
2	28.3	22.7	↓5.6	22.0
3	29.5	22.2	↓7.3	28.0
4	26.1	17.5	↓8.6	39.4
5	31.3	26.7	↓4.6	15.9
6	28.6	26.9	↓1.7	6.1
Mean	**27.8**	**21.7***	**↓6.2**	**26.7**
SD	**2.9**	**5.1**	**2.8**	**15.6**

* Significantly different from reference testmeal trial (*ANOVA* $p<0.05$)

Table 5.5 Excretion of $^{13}CO_2$ on the breath expressed as a proportion of absorbed dose of [1,1,1-^{13}C]tripalmitin, over a 24 hour study period following consumption of a reference testmeal and a high-fat testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T3), and the percentage difference (actual difference/T1+T3/2 x 100).

Subject	Excretion of $^{13}CO_2$ on breath (% of absorbed dose of [1,1,1-^{13}C]tripalmitin			
	Reference testmeal	High-fat testmeal	Difference (T1-T3)	% difference (T1-T3/2)
1	23.5	14.4	↓ 9.1	48.0
2	28.7	23.4	↓ 5.3	20.4
3	29.8	22.5	↓ 7.3	27.9
4	26.3	17.7	↓ 8.6	39.1
5	31.7	27.2	↓ 4.5	15.3
6	29.3	27.0	↓ 2.3	8.2
Mean	**28.2**	**22.0***	**↓ 6.2**	**26.5**
SD	**2.9**	**5.1**	**2.6**	**15.0**

* Significantly different from reference testmeal trial (*ANOVA* $p<0.05$)

Table 5.6 **Total lipid oxidation expressed as grams oxidised per ten hours estimated from area under the timecourse curve over a 10 hour study period following consumption of a reference testmeal and high fat testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T3), and the percentage difference (actual difference/T1+T3/2 x 100).**

Subject	Total lipid oxidation (g/10 hours)			
	Reference testmeal	High-fat testmeal	Difference (T1-T3)	% difference (difference/ (T1-T3/2) x 100)
1	20.6	24.3	↑ 3.7	16.4
2	26.7	24.2	↓ 2.5	9.8
3	35.2	41.5	↑ 6.3	16.4
4	15.9	23.4	↑ 7.5	38.0
5	26.6	37.4	↑ 10.8	34.0
6	44.1	46.4	↑ 2.3	5.0
Mean	**28.2**	**32.9**	↑ **4.7**	**16.7**
SD	**10.1**	**10.2**	**4.6**	**17.8**

Table 5.7 **Total exogenous lipid oxidation over the 10 hour study period calculated from the concentration of palmitic acid and total lipid in the testmeal, and the oxidation of [1,1,1-^{13}C]tripalmitin following consumption of a reference testmeal and a high fat testmeal**

Subject	Trial	^{13}C lipid absorbed/24 hours (mg)	% oxidised in 24 hours	% oxidised in 10 hours	^{13}C lipid oxidised / 10 hours	Total PA in testmeal (g)	PA oxidised (g/ 10 hours)	Total lipid in testmeal (g)	Total exogenous lipid oxidised (g/ 10 hrs)
1	Std	662.2	23.4	18.7	123.6	14.1	2.6	70.6	13.2
	High	705.3	14.4	12.3	86.8	26.8	3.3	133.2	16.4
2	Std	63637	28.7	22.9	145.8	14.1	3.2	70.6	16.2
	High	615.2	23.4	16.0	98.2	26.8	4.3	133.2	21.3
3	Std	756.0	29.8	23.1	174.5	14.1	3.3	70.6	16.3
	High	740.2	22.5	16.8	124.0	26.8	4.5	133.2	22.3
4	Std	625.7	26.3	19.4	121.2	14.1	2.7	70.6	13.7
	High	632.8	17.7	14.1	89.4	26.8	3.8	133.2	18.8
5	Std	831.1	31.7	22.5	186.8	14.1	3.2	70.6	15.9
	High	812.3	27.2	18.4	149.8	26.8	4.9	133.2	24.6
6	Std	858.4	29.3	21.1	181.1	14.1	3.0	70.6	14.9
	High	940.0	27.5	21.6	203.04	26.8	5.8	133.2	28.8

Table 5.8 **Exogenous lipid oxidation expressed as grams oxidised per ten hours estimated from area under the timecourse curve over a ten hour study period following consumption of a reference testmeal and a high-fat testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T3), and the percentage difference (actual difference/T1+T3/2 x 100).**

Subject	Exogenous lipid oxidation (g/10 hours)			
	Reference testmeal	High-fat testmeal	Difference (T1-T2)	% difference
1	13.2	16.4	↑ 3.2	21.6
2	16.2	21.3	↑ 5.1	27.1
3	16.3	22.3	↑ 6.0	31.1
4	13.7	18.8	↑ 5.1	32.3
5	15.9	24.6	↑ 8.7	42.9
6	14.9	28.1	↑ 13.2	61.4
Mean	**15.0**	**21.9 ***	**↑6.9**	**36.1**
SD	**1.3**	**4.1**	**3.6**	**14.3**

* Significantly different to reference testmeal trial (*ANOVA $p<0.05$*)

Table 5.9 **Endogenous lipid oxidation expressed as grams oxidised per ten hours estimated from area under the timecourse curve a ten hour study period following consumption of a reference testmeal and a high fat testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T3), and the percentage difference (actual difference/T1+T3/2 x 100).**

Subject	Endogenous lipid oxidation (g/10 hours)			
	Reference testmeal	High-fat testmeal	Difference (T1-T3)	% difference
1	7.4	7.9	↑ 0.5	6.5
2	10.5	2.9	↓ 7.6	113.4
3	18.9	19.2	↑ 0.3	1.6
4	2.2	4.6	↑ 2.4	70.6
5	10.7	12.8	↑ 2.1	17.8
6	29.2	18.3	↓ 10.9	45.8
Mean	**13.2**	**11.0**	**↓ 2.9**	**↓ 16.4**
SD	**9.6**	**6.9**	**5.2**	**61.1**

Table 5.10 **Total carbohydrate oxidation expressed as grams oxidised per ten hours estimated from area under the timecourse curve over a ten hour study period following consumption of a reference testmeal and a high-fat testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T3), and the percentage difference (actual difference/T1+T3/2 x 100).**

Subject	Total carbohydrate oxidation (g/10 hours)			
	Reference testmeal	High-fat testmeal	Difference (T1-T3)	% Difference
1	105.6	119.2	↑ **13.6**	12.1
2	97.6	103.5	↑ **5.9**	5.9
3	100.3	93.2	↓ **7.1**	7.3
4	113.3	93.0	↓ **20.3**	19.7
5	138.3	110.9	↓ **27.4**	22.0
6	92.2	87.0	↓ **5.2**	5.8
Mean	**107.9**	**101.1**	↓ **6.8**	**6.1**
SD	**16.6**	**12.4**	**15.4**	**13.5**

Table 5.11 **Plasma glucose concentration (mmol/l per 10 hours) estimated from area under the timecourse curve over a ten hour study period following consumption of a reference testmeal and a high-fat testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T3), and the percentage difference (actual difference/T1+T3/2 x 100).**

Subject	Plasma glucose concentration (mmol/l per 10 hours)			
	Reference testmeal	High-fat testmeal	Difference (T1-T3)	% difference
1	53.1	56.3	↑ 3.2	5.9
2	49.4	65.8	↑ 16.4	28.5
3	57.2	62.5	↑ 5.3	8.8
4	57.0	57.2	↑ 0.2	0.4
5	56.2	77.6	↑ 21.4	32.0
6	53.4	53.3	↓ 0.1	0.2
Mean	**54.4**	**62.1**	↑ **7.7**	↑ **12.6**
SD	**3.0**	**8.8**	**9.0**	**14.2**

Table 5.12 **Plasma TAG concentration (mmol/l per 10 hours) estimated from area under the timecourse curve measured from zero, over a ten hour study period following consumption of a reference testmeal and a high-fat testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T3), and the percentage difference (actual difference/T1+T3/2 x 100).**

Subject	Plasma TAG concentration (mmol/l per 10 hours) AUC measured from zero (AUC_0)			
	Reference testmeal	High-fat testmeal	Difference (T1-T3)	% Difference
1	9.6	13.6	↑ 4.0	34.4
2	5.2	8.9	↑ 3.7	52.5
3	3.4	6.2	↑ 2.8	58.3
4	8.0	12.6	↑ 4.6	44.7
5	16.7	16.6	↓ 0.1	0.6
6	8.8	10.4	↑ 1.6	16.7
Mean	**8.6**	**11.4**	**↑ 2.8**	**34.3**
SD	**4.6**	**3.7**	**1.8**	**22.6**

Table 5.13 **Plasma TAG concentration (mmol/l per 10 hours) estimated from area under the timecourse curve, measured from baseline, over a ten hour study period following consumption of a reference testmeal and a high-fat testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T3), and the percentage difference (actual difference/T1+T3/2 x 100).**

Subject	Plasma TAG concentration (mmol/l per 10 hours) AUC measured from baseline (AUC_t)			
	Reference testmeal	High-fat testmeal	Difference (T1-T3)	% Difference
1	4.5	7.2	↑ 2.7	46.0
2	1.9	5.1	↑ 3.2	91.4
3	1.0	5.1	↑ 4.1	134.4
4	2.8	5.5	↑ 2.7	65.1
5	5.8	7.4	↑ 1.6	24.2
6	3.1	3.6	↑ 0.5	14.9
Mean	**3.2**	**5.7***	↑ 2.5	**62.7**
SD	**1.7**	**1.4**	**1.3**	**44.8**

* Significantly different from reference testmeal trial (*ANOVA* $p<0.05$)

Table 5.14 **Plasma NEFA concentration (mmol/l per 10 hours) estimated from area under the timecourse curve, measured from zero, over a ten hour study period following consumption of a reference testmeal and a high-fat testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T3), and the percentage difference (actual difference/T1+T3/2 x 100).**

Subject	Plasma NEFA concentration (mmol/l per 10 hours) AUC_0			
	Reference testmeal	High fat testmeal	Difference (T1-T3)	% difference
1	2.2	3.7	↑ **1.5**	50.8
2	1.8	2.8	↑ **1.0**	43.5
3	2.1	1.9	↓ **0.2**	10.0
4	1.9	3.1	↑ **2.2**	88.0
5	2.5	3.8	↑ **1.3**	41.3
6	2.4	3.9	↑ **1.5**	47.6
Mean	**2.2**	**3.2***	↑ **1.2**	**43.5**
SD	**0.3**	**0.8**	**0.8**	**31.4**

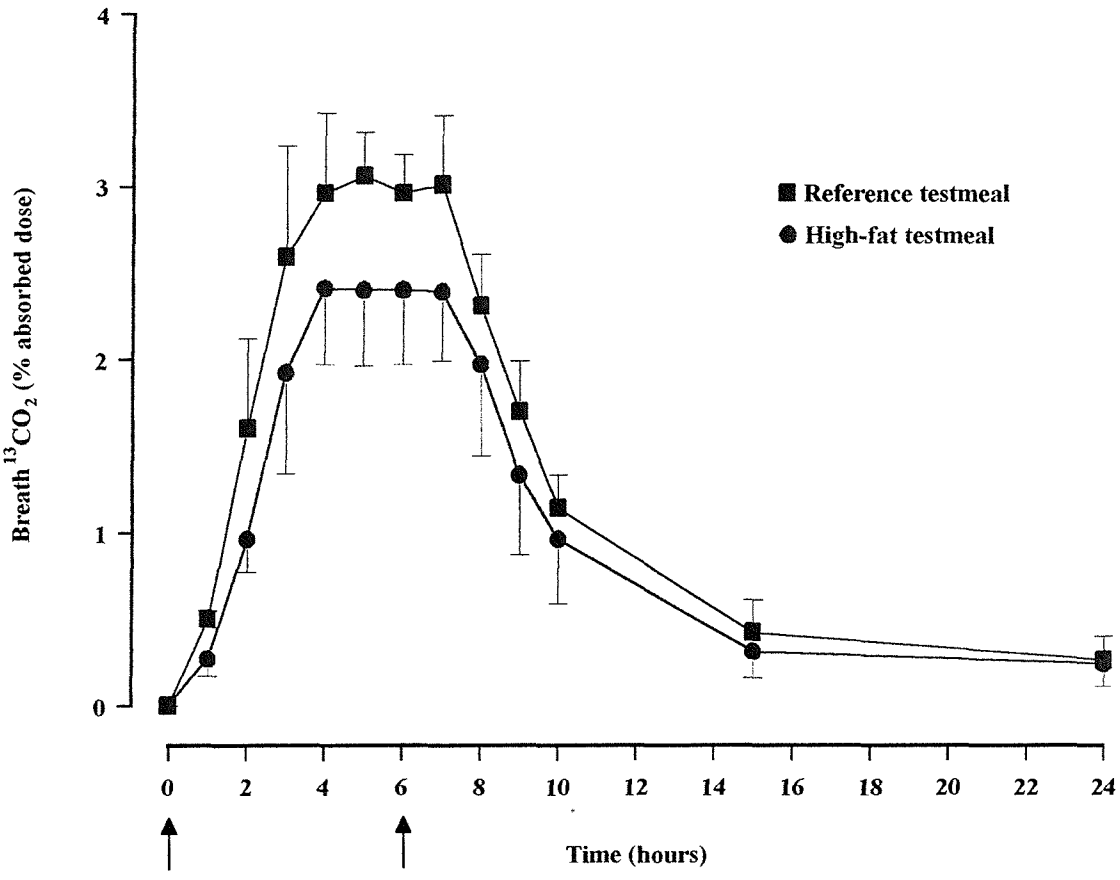
* Significantly different from reference testmeal trial (*ANOVA* $p<0.05$)

Table 5.15 **Plasma NEFA concentration (mmol/l per 10 hours) estimated from area under the timecourse curve, measured from baseline, over a ten hour study period following consumption of a reference testmeal and a high-fat testmeal, in six subjects. The difference between the trials is reported as the actual difference (T1-T3), and the percentage difference (actual difference/T1+T3/2 x 100).**

Subject	Plasma NEFA concentration (mmol/l per 10 hours) AUCi			
	Reference testmeal	High-fat testmeal	Difference (T1-T3)	% difference
1	-1.8	2.0	↑ 3.8	200.0
2	-1.9	-1.0	↑ 0.9	62.1
3	-1.0	0.1	↑ 1.1	200.0
4	-2.0	-0.5	↑ 1.5	120.0
5	-0.2	0.5	↑ 0.7	200.0
6	-1.3	-0.6	↑ 0.7	73.7
Mean	**-1.4**	**0.08***	**↑ 1.5**	**142.6**
SD	**0.7**	**1.07**	**1.2**	**65.8**

* Significantly different from reference testmeal trial (*ANOVA* $p<0.05$)

Figure 5.1 **Breath $^{13}CO_2$ excretion over a 24 hour study period following consumption of [1,1,1-^{13}C]tripalmitin within a reference testmeal or a high-fat testmeal. Excretion of $^{13}CO_2$ on breath is expressed as a percentage of absorbed dose which takes into account losses in the stool (group mean ± SD) . Arrows indicate the timing of consumption of testmeals during the study day.**



N.B. **Identical amounts of [1,1,1-^{13}C]tripalmitin were consumed within the reference testmeal and the high-fat testmeal**

Figure 5.2 **Total lipid oxidation expressed in grams of lipid oxidised per hour over a ten hour study period following consumption of a reference testmeal or a high-fat testmeal (group mean ± SD).**

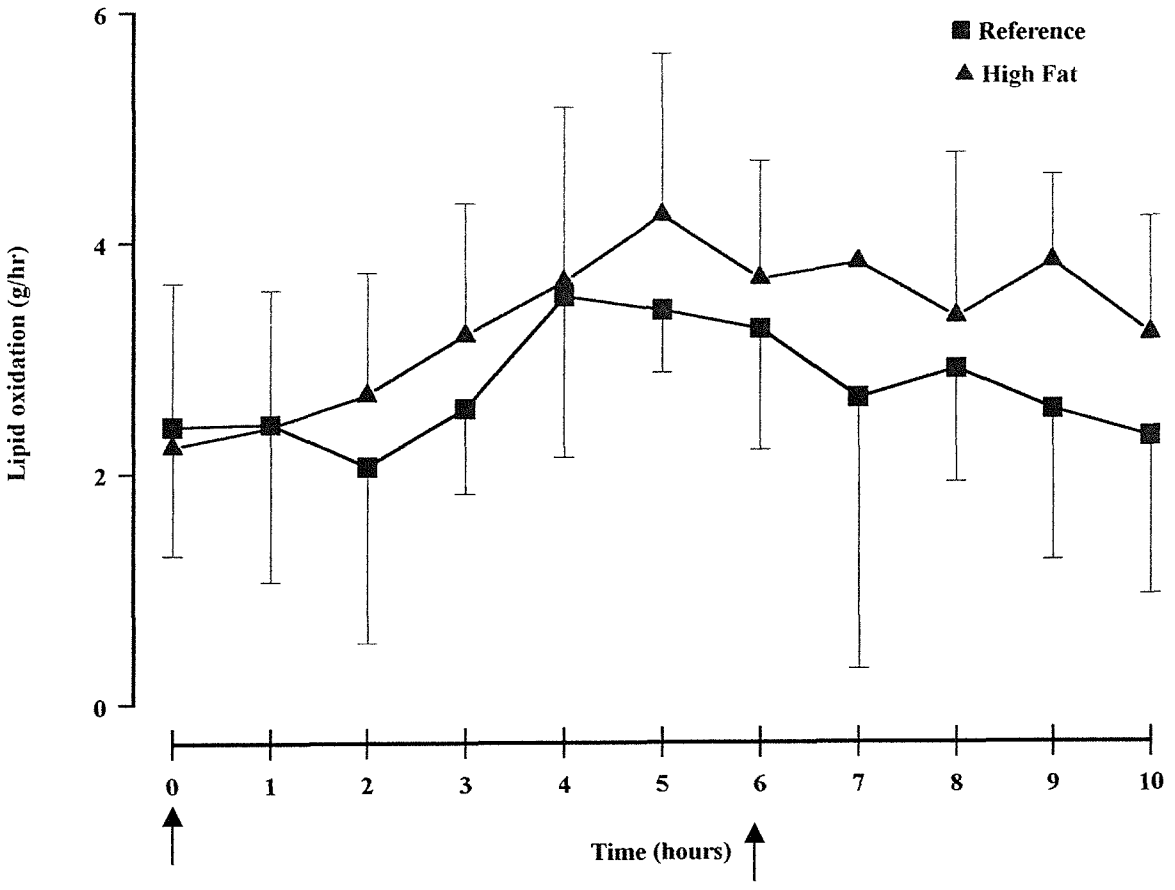
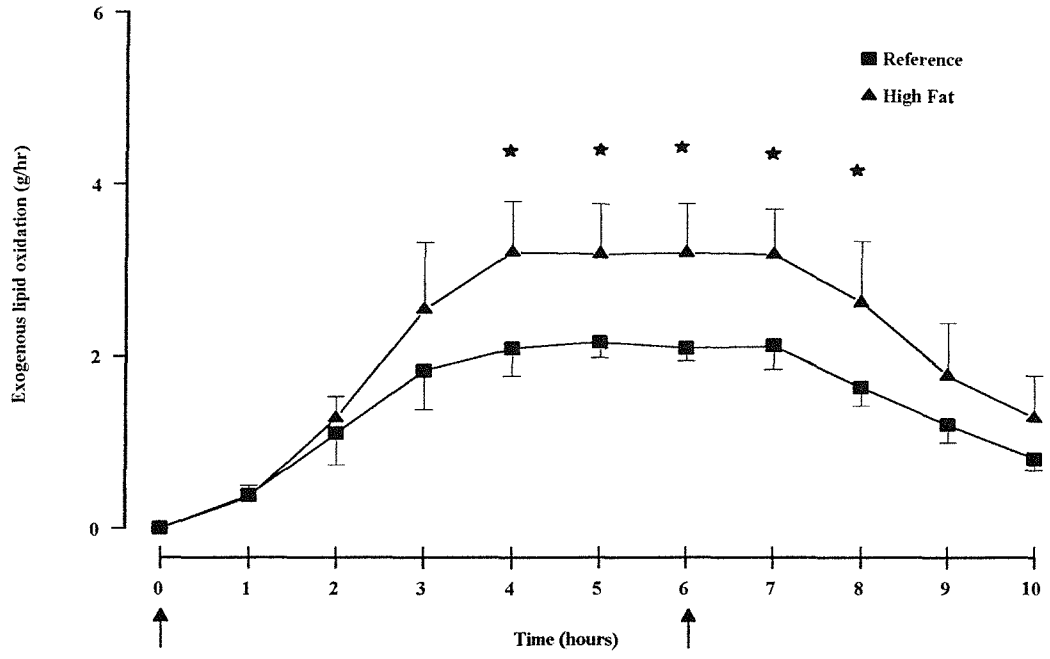


Figure 5.3 Exogenous and endogenous lipid oxidation over a ten hour study period following consumption of a reference testmeal and a high-fat testmeal. Data is shown for the group mean with error bars showing the standard deviation from the mean

Exogenous lipid oxidation:-



*Significantly different to high-fat study (*ANOVA $p<0.05$)*

Endogenous lipid oxidation:-

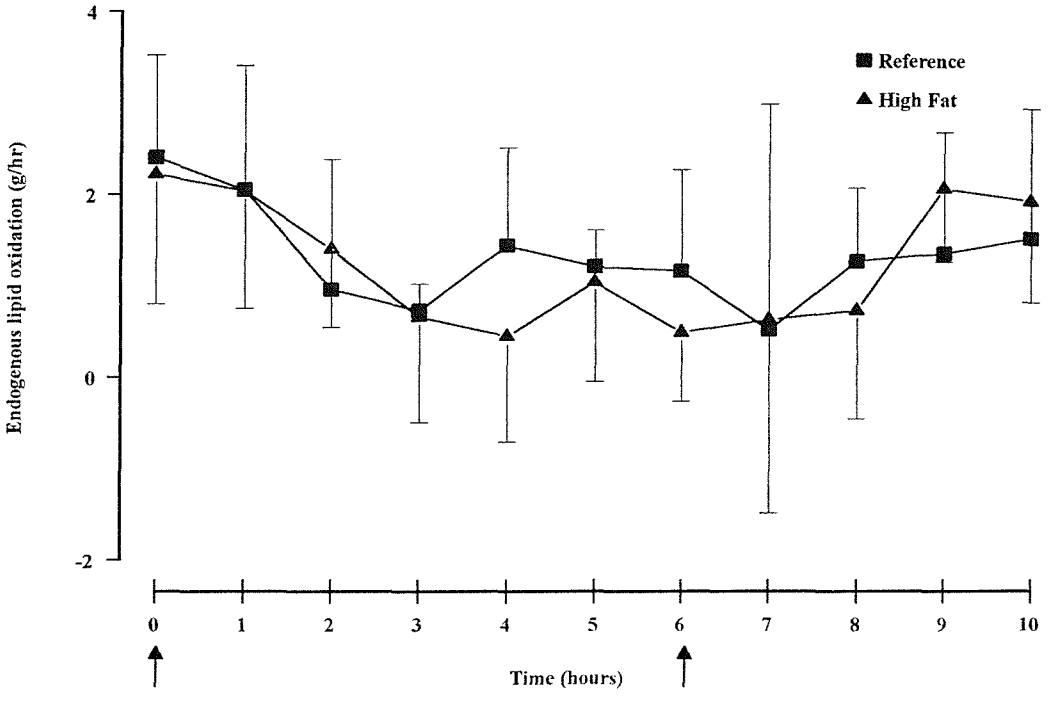


Figure 5.4 **Total lipid oxidation expressed in grams oxidised per ten hour study period accountable for by exogenous and endogenous lipid oxidation. Oxidation was calculated following ingestion of a reference (T1) and a high-fat (T3) testmeal containing [1,1,1-^{13}C]tripalmitin, shown in six subjects**

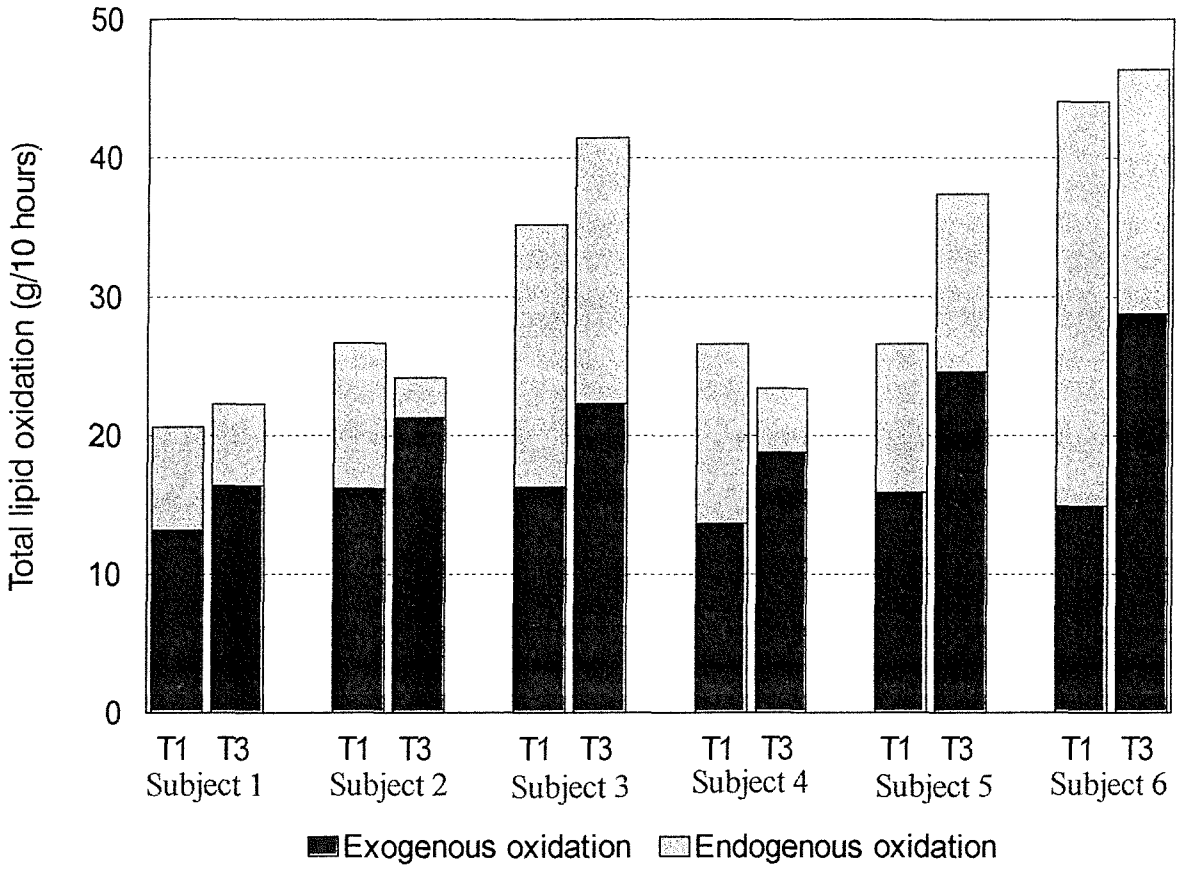


Figure 5.5 **Total carbohydrate oxidation expressed in grams of carbohydrate oxidised per hour over a ten hour study period, shown for a group of six subjects, following consumption of a reference testmeal and a high-fat testmeal (group mean ± SD)**

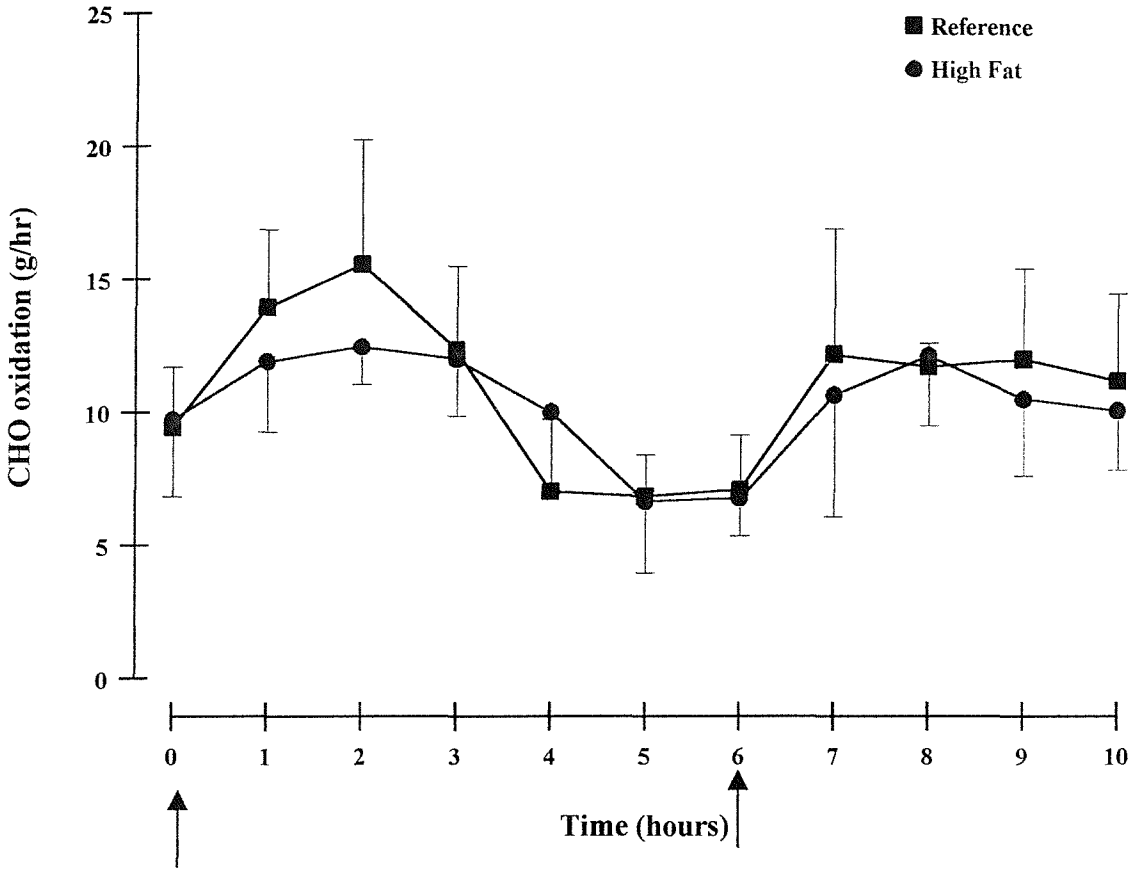


Figure 5.6 **Plasma glucose concentrations expressed in mmol/l of plasma, over a ten hour study period, shown for a group of six subjects following consumption of a reference testmeal and a high-fat testmeal (group mean ± SD)**

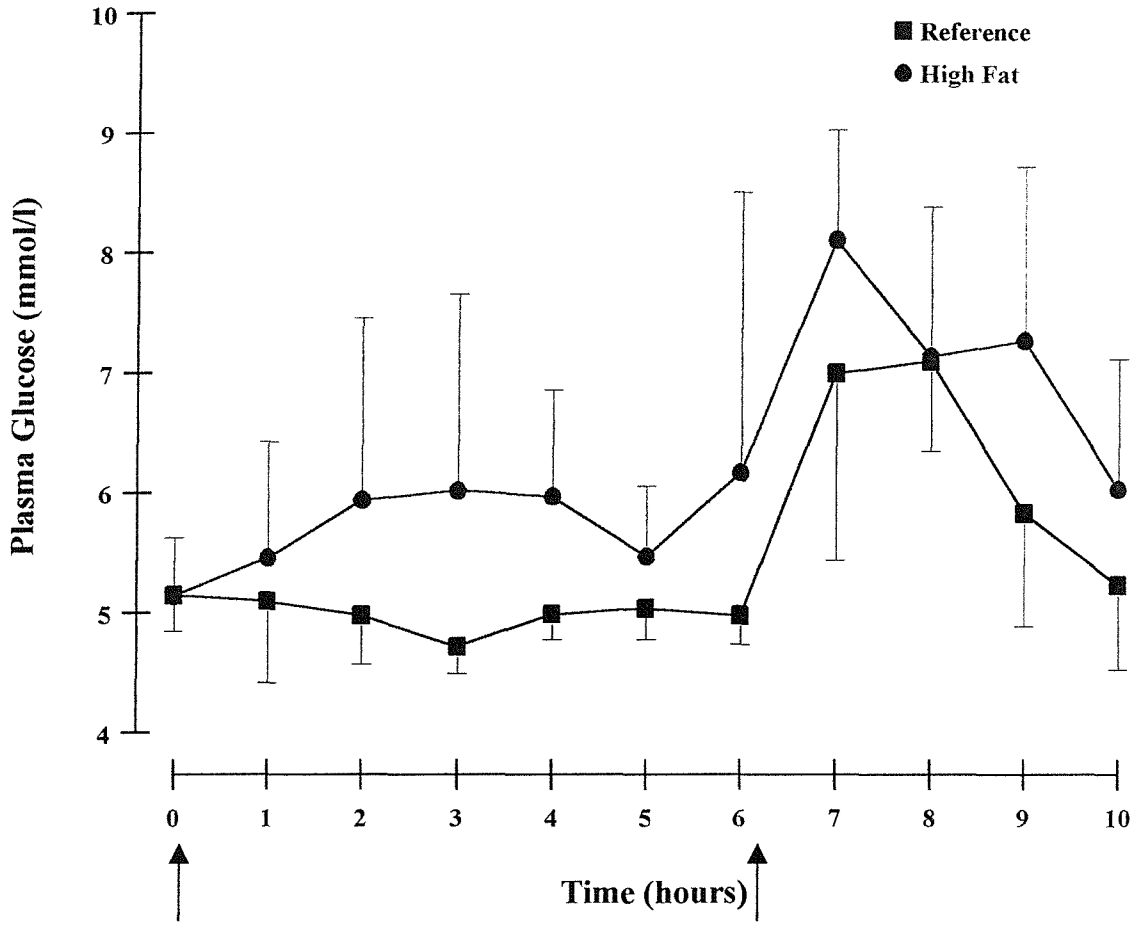


Figure 5.7 **Plasma triglyceride concentrations expressed in mmol/l of plasma, shown for a group of six subjects following consumption of a reference testmeal and a high-fat testmeal (group mean ± SD)**

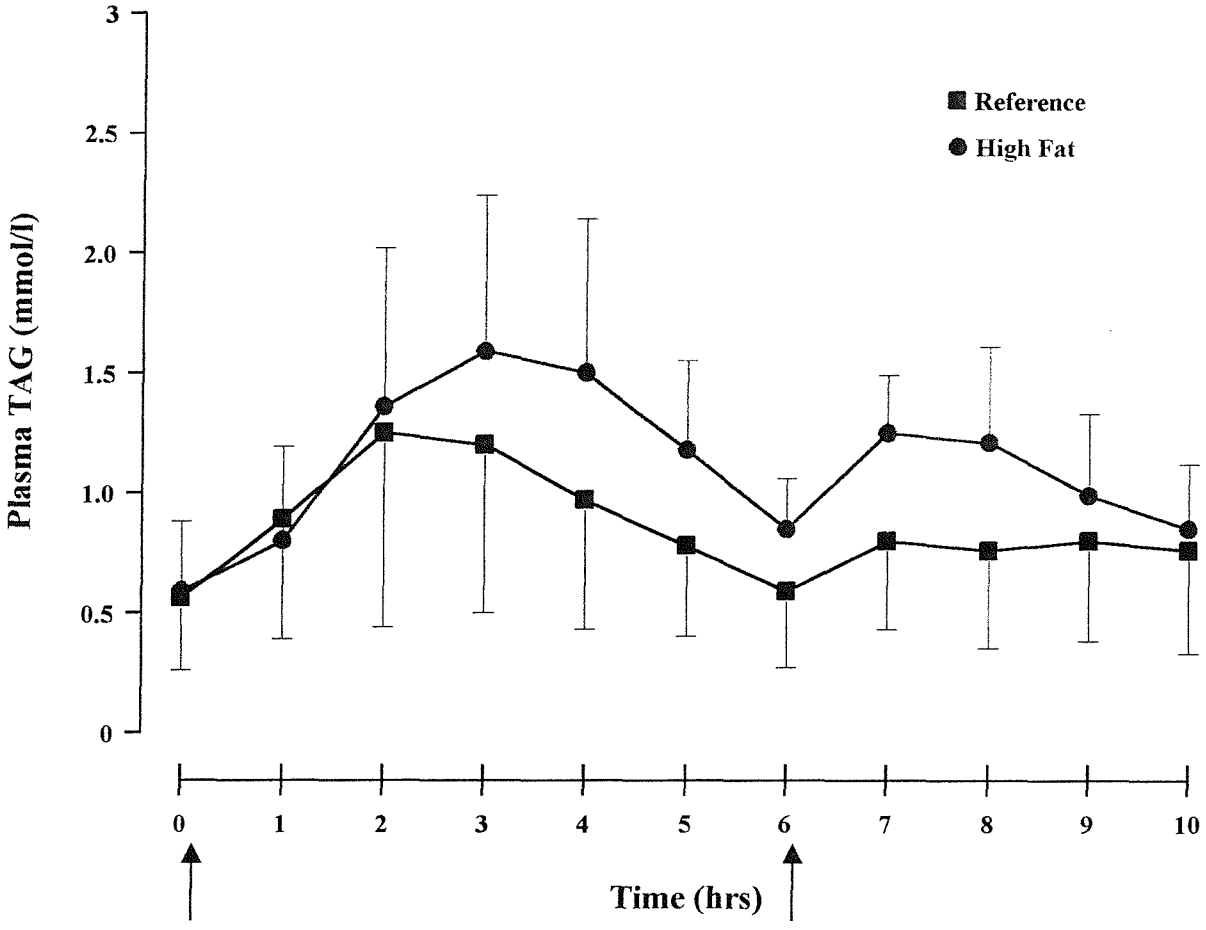
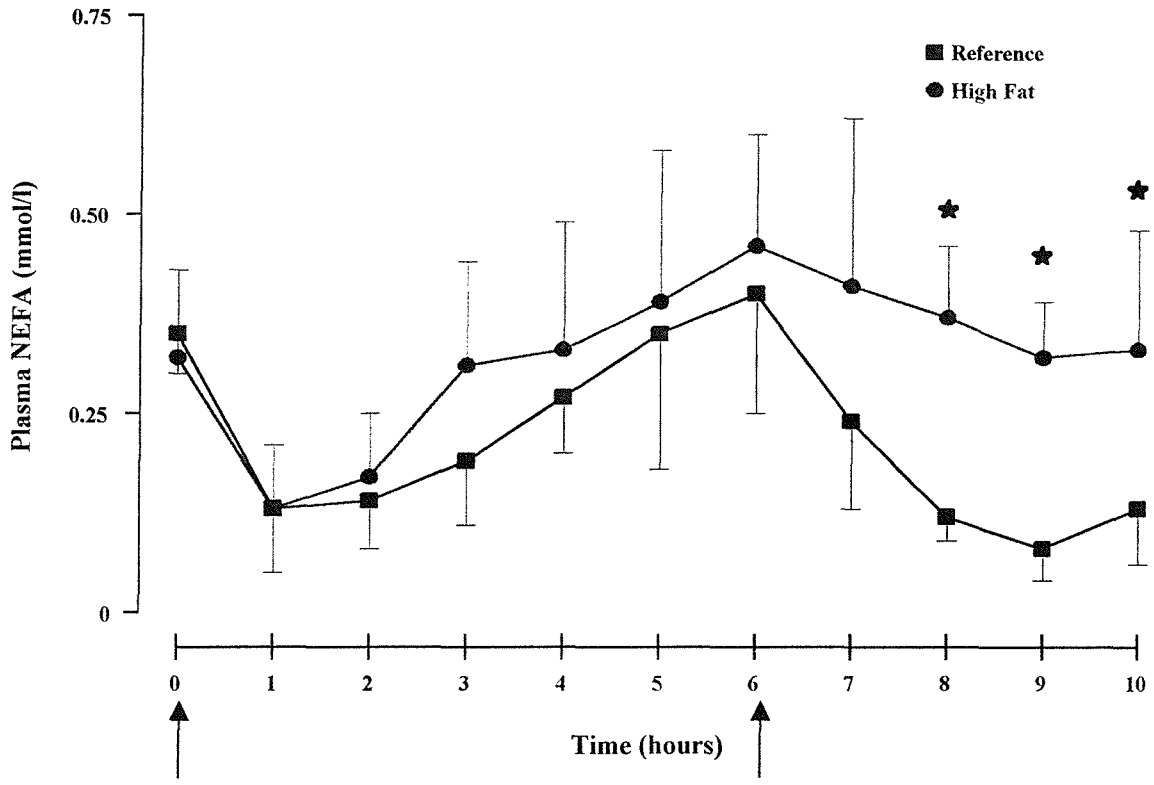


Figure 5.8 **Plasma non-esterified fatty acid concentration expressed in mmol/l of plasma, shown for a group of six subjects, following consumption of a reference testmeal and a high-fat testmeal, over a ten hour study period (group mean ± SD)**



* significantly different to high-fat trial (*ANOVA $p<0.05$*)

5.6 Discussion

The study reported in this chapter is the first study to examine the effect of a high fat testmeal on the GI handling, postprandial metabolism and metabolic disposal of dietary lipid with the use of tracers. The aim of the present study was to examine the changes in postprandial lipid metabolism that occur following consumption of a high-fat meal, and to investigate individual capacities to metabolise lipid. The following discussion will discuss the results from the present study suggesting possible mechanisms for the results observed and will compare the results with those from previous published literature

5.6.1 Gastrointestinal Handling of [1,1,1-^{13}C]tripalmitin

The availability of ^{13}C-labelled lipid to the body was estimated from the recovery of ^{13}C-label in stool, and the results from the present study would suggest that following the reference testmeal, absorption of the label was almost complete across the GI tract with an average of 99% of administered dose absorbed. By increasing the amount of lipid within the testmeal (35g to 70g) the availability of [1,1,1-^{13}C]tripalmitin to the body decreased to 98% of administered dose. Although this was not a statistically significant decrease in lipid absorption, it indicates a doubling in the excretion of the label in the stool (1-2%). The total lipid in the trial increased from 70g to 134g (2 meals), which is a substantial amount of lipid to consume within six hours. However, almost complete absorption irrespective of the amount of lipid in the meal suggests that even this large amount of lipid has been absorbed and metabolised within the body.

A previous study [Murphy *et al.* 1995] suggest that stool results should be interpreted with caution due to large variability between trials. It would appear that the same level of caution need not be applied in the present study as emulsification of the [1,1,1-^{13}C]tripalmitin provides an effective method for administration, and would suggest that the collection of stool samples in future trials, using the same study protocol and subject group, is not essential. The results from this study indicate how emulsification of [1,1,1-^{13}C]tripalmitin, and control over subjects prior to the study

reduces variability and increases the likelihood of differences observed being due to the intervention.

5.6.2 Metabolic Disposal of [1,1,1-^{13}C]tripalmitin

Oxidation of the labelled lipid to $^{13}CO_2$ indicates the oxidation of the dietary lipid within the body. By measuring $^{13}CO_2$ on the breath, the proportion of the ingested dietary lipid, which has been partitioned towards oxidation or storage, was estimated. In the present study measurement of $^{13}CO_2$ on the breath suggests that there was a decrease in the oxidation of [1,1,1-^{13}C]tripalmitin following the high fat testmeal trial compared to the reference testmeal trial (decrease by 26.5%). A decrease in the oxidation of labelled fatty acid would suggest that there was a decrease in the oxidation of all dietary saturated fatty acids, and therefore all dietary lipid. However, at this point, it should be remembered that the labelled lipid was ingested in the same quantities for both trials (10mg/kg body weight), and no correction was made for the doubling of the lipid within the meal. If the tracer was doubled in the same quantities as the tracee (lipid in the testmeal) we would expect to see an increase in tracer oxidation. Therefore, the tracer/tracee effect must be taken into account, that is, a decreased oxidation of the tracer but of a larger amount of the tracee, which had to be taken into account when calculating the oxidation of exogenous lipid from total substrate oxidation rates. There are no previous studies in the literature examining the response of a high-fat meal on the oxidation of ^{13}C-labelled substrates, and therefore no direct comparisons can be made. It was assumed that no differences in the interpretation of results would be seen if values were corrected for retention within the bicarbonate pools as discussed in section 4.4.2. However there is a possibility that the consumption of the high-fat testmeal may result in alterations in the retention of $^{13}CO_2$ in the bicarbonate pool, but any examination of the theory is outside the experiments conducted as part of the present thesis.

5.6.3 Substrate Oxidation

The purpose of the present study was to examine the effect of increasing the lipid content of a meal on subsequent lipid oxidation, and to use tracers to examine any

changes which may occur in the proportions of exogenous and endogenous lipid oxidation. Total lipid oxidation increased in all subjects except for subject two. An explanation for this was that the subject was already at his metabolic capacity for oxidising lipid following the ingestion of the reference testmeal, and therefore was unable to increase oxidation following a doubling of lipid in the meal. For the other subjects, lipid oxidation increased to varying degrees. Subjects 4 (increased by 38.0%) and 5 (increased by 34.0%) appeared to increase lipid oxidation in response to the high-fat meal more effectively than other subjects. In addition, each individual demonstrated a different pattern of lipid oxidation over the ten-hour period, which suggests that each individual has his own metabolic capacity or limit, above which oxidation may not be increased within this experimental design. Bobbioni-Harsch *et al* [1997] showed similar results with an increase in lipid oxidation when lipid was fed alone in a testmeal compared to a mixed meal. However, this is not a comparable study as the addition of carbohydrate is known to influence lipid oxidation [see section 2.5.3]. The only known study in the literature directly examining the effect of increasing the fat content of a meal on postprandial lipid oxidation [Surina *et al* 1993], found a decrease in respiratory quotient on the high-fat trial suggesting an increase in fat oxidation, with an increase in plasma FFA concentration. Several diet based studies have found a failure of increased dietary fat intake to promote fat oxidation [Schutz *et al* 1989; Astrup *et al* 1994], however others have found that the adaptation to a high-fat diet does occur but can take up to a week to be demonstrated [Schrauwen *et al* 1997]. The literature in this area is contradictory and no clear explanation is found. The present study demonstrated an increase in oxidation with an increased fat meal, yet the increase is not proportional to the amount of fat in the meal, however, by using tracers we can make further statements regarding the source of the fat which is oxidised.

Previous studies would suggest that fat oxidation is determined primarily by the difference between total energy expenditure and the energy ingested in the form of carbohydrate or protein, rather than by the amount of fat consumed. However, by using a tracer, it is possible to demonstrate the lipid oxidation accountable to the lipid within the meal. Exogenous lipid oxidation data calculated from the tracer/tracee relationship, suggests that there was an increase in the amount of dietary lipid oxidised on consumption of the high fat meal for each subject. This is the first time that the oxidation

of dietary lipid has been shown to increase following ingestion of an increased lipid meal. However, although lipid oxidation has increased, this does not reflect the doubling of lipid in the meal, suggesting that although some of the lipid is oxidised, the majority is channelled to non-oxidative disposal. It has been suggested that whilst carbohydrate ingestion seems to equal oxidation, the maintenance of fat balance does not appear to be facilitated by the same metabolic regulatory effects [Flatt *et al* 1985], although this is based on diet and not meal effects but the same principles appear to apply to the present study. For example a total of 134g of lipid was consumed over the study day on the high-fat testmeal trial, with on average 32.9g oxidised over the study day (ten hours). Of the 32.9g oxidised 21.9g was calculated as exogenous lipid and 11.0g as endogenous. Therefore of the 134g of lipid consumed, only 16.3% was oxidised, suggesting 83.7% remained in the body and was partitioned towards non-oxidative disposal. The proportion of the consumed lipid that was oxidised varied between individuals, from 12% to 18%, even within the subject group chosen for their similarities in age, gender and body composition. What is unclear is the fate of the un-oxidised lipid, as it is assumed that almost the entire dietary lipid has been absorbed. It is clear from plasma data that there is an excess of both triglyceride and free fatty acids in the circulation suggesting an incomplete lipolysis or uptake of lipid into the adipose tissue. However, as the uptake of lipid to the adipose tissue cannot be measured directly, this has to be assumed. Murphy *et al* [1995] showed an increase in lipoprotein lipase activity following a high-fat meal, suggesting that hydrolysis of chylomicron TAG could be enhanced. However, with increased hydrolysis, more NEFA are released which may not all be entrapped by the tissues, therefore being transported to the liver for VLDL-TAG secretion. The proportion of dietary lipid which can be oxidised may be a mechanism for the development of obesity, that is, the greater the proportion of dietary lipid which can be oxidised, the lesser is stored, and therefore the lesser the risk of increasing adipose stores of lipid. In addition, a greater magnitude of postprandial lipaemia was seen with the high-fat meal, which suggests that more lipid is remaining in the circulation resulting in an increased risk of cardiovascular disease.

In conclusion, increasing the lipid content of a testmeal results in an increase in total lipid oxidation, due to an increase in exogenous lipid oxidation, but a decrease in endogenous oxidation. The decrease in endogenous lipid suggests that the release of

endogenous fatty acids is suppressed possibly due to an increased insulin response although insulin concentrations were not measured in the present study. Previous work [Murphy *et al.*1995] would suggest that increasing the lipid load results in a raised insulinogenic response. However, there was an increase in circulating lipids suggesting an upper capacity to store and oxidise lipid in such great amounts. There are also clear differences between individuals, suggesting individual metabolic capacities to handle fat. Power analysis revealed that although total lipid oxidation and endogenous lipid oxidation do not reach a power of 80%, exogenous lipid oxidation demonstrates a power of 100% with an n of six. This suggests that the differences in means between the reference testmeal and the high-fat meal are sufficient to be additional to differences caused by intra-individual variability and suggest that exogenous lipid oxidation is increased with the consumption of a high-fat meal.

It has been demonstrated that the oxidation of carbohydrate is directly related to its intake [Flatt 1991]. Therefore in this study where carbohydrate intake is kept constant, it would be expected that carbohydrate oxidation would remain constant. It appears that individuals who are able to alter lipid metabolism with regard to intake, have greater alterations in carbohydrate oxidation than those subjects whose oxidation balance remains more stable. In other words it appears that individuals such as subject 5, who has a large increase in lipid oxidation between trials, also has a large decrease in carbohydrate oxidation. A possible explanation for this is the body attempting to maintain glycogen reserves by reducing glucose oxidation [Flatt, 1995], which could be supported by the increase in plasma glucose concentration following the high-fat meal. Surina *et al* [1993] found a decrease in respiratory quotient on consumption of a high-fat meal, supporting our findings. They predicted that two hours after the high-fat meal, 15-20% of the fuel burned was lipid. Therefore it can be concluded that the decrease in carbohydrate oxidation following a high-fat meal is merely an indication of the high availability of lipid in the circulation which must be metabolised supporting the glucose-fatty acid cycle hypothesis [Randle *et al.*1963]. The decrease in carbohydrate oxidation could be due to an alteration in the insulin response following a high fat meal, but may also be due to the 'overspill' of lipid into the circulation, which could in turn provide the stimulus for the oxidation of lipid to increase whilst carbohydrate oxidation is suppressed. Without having a measure of the insulin response, it is not possible to

comment on the mechanisms involved with the inhibition of carbohydrate oxidation.

5.6.4 Plasma Glucose

Plasma glucose concentrations were increased in magnitude and duration following consumption of a high fat meal despite both testmeals being identical in carbohydrate content. The rise in plasma glucose suggests that glucose is not cleared to the tissues as rapidly following the high-fat meal, due to the excess fatty acids present in the circulation. Similar results were seen with the consumption of an 80g fat meal compared to a 40g fat meal [Murphy *et al.* 1995], with an increase in total plasma glucose concentrations, but with the glycaemic response occurring at later timepoints. It has been suggested that raised postprandial NEFA concentrations reduce insulin stimulated glucose uptake by muscle [Walker *et al.* 1993; Frayn, 1994], which could be provided as an explanation of the raised glycaemia with increased fat load. In addition, a decrease in carbohydrate oxidation would suggest that glucose is not being utilised as a fuel due to the excessive supply of fatty acids.

A similar pattern of glycaemia was seen on both trials, with a greater magnitude of response seen following the second meal. The first meal was consumed following a 12 hour period without food whereas the second meal was consumed at 6 hours postprandially, suggesting that the enhanced glycaemic response following the second meal may be due to a lack of glucose uptake to the tissues for storage or for use as fuel.

5.6.5 Plasma Lipids

The purpose of the present study was to examine the effect of increasing the lipid content of the meal on the magnitude and duration of postprandial TAG concentrations. The study showed that doubling the amount of lipid within the testmeal resulted in a significant increase in the concentration of plasma TAG over the study period, however, there were differences in the extent and duration of raised lipaemia between individuals.

Our findings are supported by a number of studies in the literature. Cohen *et al* [1988] determined the dose-response relationship between serum TAG concentrations and lipid feeding by measuring the TAG response to 3 meals containing 40g, 80g and

120g of lipid. Significant differences in the magnitude and duration of lipaemia were seen in a stepwise fashion, suggesting that the appearance and/or clearance of TAG is affected in some way by consumption of a high fat meal. Our findings were surprising in that the TAG response, although significantly greater with the high-fat meal compared to the reference testmeal, did not reach concentrations equivalent to the amount of lipid in the testmeal. Murphy *et al* [1995] also found a significant increase in the serum TAG concentration following an 80g lipid meal, but also expressed surprise over the overall lack of effect of the high-fat meal compared to a standard-fat (40g) testmeal. The increase in plasma TAG concentration following a high-fat meal may be dependant on endogenous fatty acids as well as the inflow of exogenous TAG from the meal. In a similar study, Jeppessen *et al* [1995], showed that postprandial TAG concentrations increased in an approximately linear fashion as the oral fat load was increased, but that the smaller the fat load, the greater the number of particles in the VLDL fraction, and less in the chylomicron fraction, suggesting that in our study the majority of particles in the plasma would be from exogenous or chylomicron TAG rather than VLDL-TAG. However, the proportions of exogenous and endogenous lipid in the circulation were not measured as part of this study. In all the studies where plasma TAG is examined following a single high-fat meal, although a response is seen it is smaller than expected. However, all subjects in the studies were young and healthy (18-30 years), suggesting that TAG clearance was at its most effective compared to older subjects or those with known pathologies

The results from the present study and previous work suggest that consumption of high fat foods results in raised postprandial TAG concentrations and ultimately an increased risk of disease. In particular there appear to be differences in the individual capacity to handle the lipid suggesting differing risk of disease in later life. The exaggerated TAG response in the circulation may be due to increased chylomicron TAG from the meal, and also from increased VLDL TAG from the liver. It is possible that lipolysis of TAG by LPL has occurred and the subsequent overspill of fatty acids leads to an increased production of VLDL from the liver, although this is unlikely due to the observations of Jepessen *et al.*[1995]. The role of raised lipaemia in the disease process will be discussed in chapter 7 (general discussion). Power analysis confirmed the effect of the high-fat meal on postprandial lipaemia, with plasma TAG concentration (AUCi)

showing a power of 85%, suggesting that the differences between the reference and high-fat testmeals are due to the intervention and not intra-individual variability. The power of the study decreased to 68% when concentration was measured from zero, suggesting that differences in baseline TAG concentration may account for some of the differences observed between subjects.

The plasma concentration of NEFA indicates the product of the release of fatty acids from the adipose tissue by hormone sensitive lipase, and the hydrolysis of TAG by LPL with the resultant fatty acids not entrapped by the tissue. HSL is controlled by the concentration of plasma insulin, which rises following a meal, therefore the concentration of plasma NEFA decreases immediately following a meal, in some cases to near zero. In the postprandial period, the concentration of NEFA slowly rose, normally to baseline levels by 6 hours postprandially. Following the second meal, suppression occurred once again. Consumption of double the amount of lipid within a testmeal led to raised plasma NEFA levels in the postprandial period with an overshoot past baseline at 6 hours and no suppression of NEFA following the second meal. Increasing the lipid content of the meal may lead to an inability of the tissues to remove fatty acids for storage from the circulation. Murphy *et al* [1995] noted that the NEFA response to a high-fat meal was flatter than the U-shaped response seen in the lower fat meals and also demonstrated that when acute fat intake exceeded the capacity of tissues to utilise fatty acid as an immediate energy source, storage of NEFA and TAG in adipose tissue cannot fully compensate and as a consequence NEFA 'spill' out into the circulation. The increase in NEFA concentration following the second meal was directly related to the increase in lipid oxidation observed, indicating that lipid oxidation and circulating lipid concentrations may be closely interrelated. The role of NEFA concentration in lipid oxidation will be discussed further in chapter 7.

5.7 Summary and Conclusions

The aims of the study described in this chapter were to examine the effect of increasing the lipid content of a testmeal on the GI handling, metabolic disposal and lipaemic responses to a mixed meal containing [1,1,1-^{13}C]tripalmitin. The results demonstrate that:-

1) Doubling the amount of lipid within a testmeal leads to a doubling in the excretion of ^{13}C-label in stool, suggesting a decrease in the availability of dietary lipid to the body. However, the availability of lipid remained above 95% of administered dose suggesting that, in this subject group, there is no upper metabolic capacity for the absorption of dietary lipid.

2) In accordance with some previous work, total lipid oxidation increased with increased lipid intake. However, the increase was not proportional to intake, and was due to an upregulation of exogenous lipid oxidation and a decrease in endogenous oxidation. This is probably due to the increase in circulating exogenous lipids, which are removed by the tissues for oxidation or storage. Exogenous lipid oxidation accounted for only 16% of total lipid intake suggesting that high-fat meals result in an increase in lipid storage.

3) The increase in plasma TAG levels with a high-fat testmeal suggests an increase in chylomicron TAG concentration [Jepessen *et al* 1995] due to an increased secretion from the intestine or a decreased clearance by LPL. However, LPL is known to be stimulated by the consumption of a high-fat meal [Murphy *et al* 1995]. The subsequent rise of NEFA in the circulation suggests that LPL is hydrolysing the TAG to the same extent but the resultant NEFA fail to be entrapped.

4) The prolongation of postprandial lipaemia and the failure of lipid oxidation to increase proportionally to intake support the evidence that consumption of high-fat foods may result in an increased risk of cardiovascular disease and obesity.

CHAPTER 6

Effect of Altering the Carbohydrate Content of a Testmeal on the Postprandial Metabolism and Partitioning of [1,1,1-^{13}C]Tripalmitin

6.0 Introduction

Early clinical studies investigating the effect of long term low-fat, high-carbohydrate feeding on lipid metabolism [Ahrens *et al.*1967; Quarfordt *et al.*1970; Reaven *et al.*1974], found an association with fasting hypertriglyceridemia and a decrease in HDL cholesterol concentration in humans. The effect of increasing the amount of carbohydrate in a testmeal is less clear and often conflicting results have been observed. Ingestion of carbohydrate with lipid in a meal may have profound effects on the changes in the lipaemic responses to the meal over the postprandial period [Roche, 1999]. Studies examining the effect of carbohydrates on postprandial lipid metabolism have yielded conflicting results with the magnitude and direction of the effect altering with the type and amount of carbohydrate ingested. However, the magnitude and direction of the lipaemic response appears to be dependent upon the glycaemic and insulinogenic response to the meal. The major enzyme involved in the clearance of TAG from the circulation, lipoprotein lipase, is stimulated by the administration of insulin [Korn, 1955]. Mann *et al.* [1971] postulated that the extent of postprandial changes in TAG concentrations is reduced by insulin mediated activation of LPL. This observation would suggest that increasing the insulin concentration in the plasma would lead to an increase in LPL activity, and therefore an increased clearance of TAG. As the magnitude and duration of postprandial lipaemia is known to be directly related to the risk of CVD [Groot *et al.*1991; Patsch *et al.*1992], it is important to understand mechanisms by which lipaemia can be diminished. Carbohydrate within a meal is known to stimulate the release of insulin from the pancreas [Frayn 1996], and therefore understanding the role of carbohydrates in lipid metabolism is clearly important.

The effects of carbohydrate within a testmeal on lipid metabolism are discussed in section 2.5.3. Glucose has been shown to be hypotriglyceridemic , with a decrease in the magnitude of postprandial lipaemia thought to reflect increased TAG clearance

secondary to insulin-mediated increases in adipose tissue LPL activity [Cohen & Berger, 1990]. In contrast, Cohen and Schall [1988] investigated the relative effects of glucose, sucrose and fructose on postprandial TAG metabolism. Compared to a fat only meal, the addition of 50g of glucose increased postprandial TAG concentrations, while 50g of fructose caused a much larger increase, equivalent to that seen with 100g of sucrose, concluding that fructose is the hypertriglyceridemic element of sucrose. The mechanisms that underlie the changes in lipid metabolism remain unclear, and the conflicting data from previous studies hinders attempts to understand the complex processes involved. It is possible that a better understanding of the role of exogenous and endogenous fatty acids in postprandial lipid metabolism may allow the mechanisms involved with the addition of carbohydrates to a meal to be ascertained [Jepessen *et al.* 1995].

In summary, current knowledge on the effect of carbohydrate on lipid metabolism remains conflicting and unclear. If the literature to date is correct, it would appear that carbohydrate has an atherogenic effect on lipid metabolism both in the post-absorptive and postprandial states. However, the only known study to employ 'normal' meal conditions, has shown the opposite effect of carbohydrate with a decrease in plasma TAG concentrations.

6.1 Hypothesis

The addition of sucrose to a testmeal will result in a decrease in postprandial lipaemia and total lipid oxidation, with the magnitude of the response dependant upon the amount of sucrose ingested.

6.2 Aim

The aim of the present study was to examine changes in postprandial lipid metabolism that occur with the addition of varying loads of sucrose to a reference testmeal, and to investigate the role of exogenous and endogenous fatty acids in order to answer the following questions:-

1) How does increasing the carbohydrate content of a meal affect the postprandial concentrations of glucose and insulin, and does this vary from the expected outcome reported in previous literature?

2) What effect does increasing carbohydrate within a meal have on the concentration of circulating TAG and NEFA, and does increasing carbohydrate increase meal lipid clearance by stimulation of LPL?

3) How do alterations in the circulating lipid concentration determined by the carbohydrate content of the meal affect total energy balance and substrate oxidation?

4) Does increasing the carbohydrate content of a meal result in alterations in the concentrations of ^{13}C-TAG and ^{13}C-NEFA in the circulation, and what consequences could this have on substrate oxidation?

5) What effect does increasing the carbohydrate load have on the balance of lipid oxidation in terms of lipid from the meal and from the body?

6) How do the results compare to the previous studies, and does the use of ^{13}C-tracers assist in understanding the mechanisms behind changes in metabolism?

6.3 Subjects

Six young, healthy male subjects were recruited from the staff and students of the University of Southampton. All subjects gave their written, informed consent following a full explanation of the study protocol on an initial visit to the Clinical Nutrition and Metabolism Unit, and were informed of their right to withdraw at any time. All subjects were non-smokers and had no known disorders of the GI tract or lipid metabolism. On initial assessment, basal metabolic rate, height, weight and body composition was measured. The characteristics of the subjects are shown in table 6.1.

Table 6.1 Characteristics of six subjects recruited to examine the effect of carbohydrate consumption on lipid metabolism

Subject	Age (years)	Weight (kg)	Height (m)	Body mass index (kg/m^2)	BMR (kJ/day)	Body fat (% of total mass)
1	30	83.2	1.83	24.9	8026	13.6
2	28	80.6	1.83	24.1	7467	18.0
3	22	72.5	1.81	22.1	8025	11.6
4	25	78.4	1.86	22.7	8226	11.6
5	19	58.5	1.73	19.5	6426	8.2
6	20	62.6	1.80	19.2	6472	8.8
Mean	**24**	**72.6**	**1.81**	**22.1**	**7440**	**12.0**
SD	**4.4**	**10.1**	**0.04**	**2.3**	**808.6**	**3.6**

6.4 Methodology

Subjects followed the general study protocol described previously (section 3.4). Each subject completed three 4-day trials, at 4-week intervals (0g sucrose, 50g sucrose and 100g sucrose) presented randomly and single-blind. On each occasion, subjects were fed an identical prescribed diet (1.5 x BMR) for two days prior to each study day (see appendix 1.2), and were asked not to eat or drink any other items except water. During this period they were asked to maintain their habitual activity, and for this to remain constant between trials. On the evening of the second day subjects were admitted to the Clinical Nutrition and Metabolism Unit, served an evening meal and were rested without additional food from 7.30pm. On the study day a baseline end tidal breath specimen and VCO_2 measurement were made before consumption of the testmeal and hourly for ten hours after the testmeal. Subjects performed 15 hour and 24 hour breath specimen collection at home. An indwelling cannula was inserted into a forearm vein, and a 10ml blood sample withdrawn. Following the meal blood samples (10ml) were taken at 1 hour, 1.5, 2, 2.5, 3 and hourly to ten hours. Blood samples were analysed for

concentrations of TAG, NEFA, insulin, glucose, ^{13}C-TAG and ^{13}C-NEFA (see section 3.5.7). Breath specimens were analysed as described in section 3.5.5.

In addition to the reference emulsion and testmeal (section 3.2) subjects consumed 450ml of a sugar-free lemon squash drink containing the carbohydrate load. The carbohydrate load consisted of either 0g, 50g or 100g of sucrose, chosen to represent three sucrose loads within a typical dietary intake range [Gregory *et al.*1990] and to reflect previous studies presented in the literature, which was dissolved within the lemon drink and mineral water, providing 0kJ, 840kJ or 1680kJ of energy respectively. Power analysis was conducted on the outcome variables using a computerised analysis program (SamplePower, SPSS Inc., Chicago, USA).

6.5 Results

Results are expressed for the three trials as 0g sucrose, 50g sucrose and 100g sucrose. Results are shown for the oxidation of [1,1,1-^{13}C]tripalmitin expressed as a percentage of administered dose of labelled tripalmitin. $^{13}CO_2$ excretion on the breath was calculated over the initial 10 hours period, and full 24-hour study period as the area under the timecourse curve (AUC). Indirect calorimetry was used to calculate total substrate oxidation, and with $^{13}CO_2$ data was used to estimate the proportion of lipid oxidation from exogenous and endogenous sources. Plasma concentrations of TAG and NEFA were calculated as the area under the timecourse curve measured from zero (AUC_0), and from baseline, the incremental area under the curve (AUCi). Plasma concentrations of glucose and insulin were calculated as the area under the timecourse curve measured from zero only (AUC_0) ^{13}C-TAG and ^{13}C-NEFA concentrations were analysed for the initial 6-hours of the study period only due to equipment constraints, and were calculated as the total area under the curve.

6.5.1 Breath $^{13}CO_2$ excretion

Figure 6.1 shows the mean excretion of $^{13}CO_2$ over the 24-hour study period for the group as a whole. Following the trial where no added sucrose was consumed, oxidation of labelled tripalmitin increased from baseline to peak at 3.0% of administered

dose between 4 and 5 hours postprandially before decreasing steadily to baseline levels by 24-hours. As 50g of sucrose was added, oxidation increased as before but to a lower magnitude peaking at 2.5% of administered dose prior to returning to baseline by the end of the study period. With 100g of added sucrose, oxidation increased at a much slower rate, peaking at a similar level but at approximately 8 hours postprandially. Following this there was a sharp decline in oxidation following the same pattern as the other meals and returning to baseline by 24 hours. Univariate analysis of variance revealed significant effects of both time and trial on the magnitude and duration of breath $^{13}CO_2$ excretion ($P<0.05$). In addition, multivariate analysis revealed significant effects of the interaction of time and trial on breath $^{13}CO_2$ excretion ($p<0.05$). Post-hoc analysis revealed statistical significance was reached at 2, 3 , 4 and 5 hours postprandially between the 0g and 100g sucrose trials, and the 50g and 100g trials. No statistical differences were shown in the magnitude and timecourse of $^{13}CO_2$ excretion on the breath between the 0g and 50g sucrose trials.

Table 6.2 shows total $^{13}CO_2$ excretion on the breath over the initial 10-hours and complete 24-hour study period measured as the total area under the timecourse curve. Breath $^{13}CO_2$ excretion, expressed as a percentage of administered dose, decreased with increasing sucrose within the testmeal. With 0g of added sucrose, 19.3% (16.6 to 22.2%) of administered dose was excreted over the initial 10-hour study period, which decreased to 18.5% (14.8 to 23.7%) with 50g of sucrose and 13.8% (8.8 to 18.6%) with 100g of sucrose. Over the 24-hour period, a decrease was also seen from 23.4% (17.6 to 31.2%) with 0g sucrose to 22.9% (17.0 to 28.3%) with 50g of sucrose and to 17.4% (9.3 to 23.7%) with 100g of added sucrose. Table 6.3 shows that there was an overall decrease of 0.5% of administered dose from 0g to 50g sucrose trial, with two subjects showing an increase in oxidation, and four a decrease. Oxidation decreased by 5.5% from the 50g to 100g trials, and all subjects displayed a decrease in oxidation. Between the 0g and 100g sucrose trial there was a decrease of 6.1% of administered dose. Despite the overall trend in oxidation of [1,1,1-^{13}C]tripalmitin with increasing sucrose load, one way analysis of variance revealed no statistical differences in total breath $^{13}CO_2$ when measured over 24-hours between the trials due to the large between individual variability. Over the ten hour period, however, when subjects were resting on the unit, one way analysis of

variance revealed significant differences between the 0g and 100g sucrose trials ($p<0.05$).

6.5.2 Lipid Oxidation

Figure 6.2 shows the group mean for the timecourse of lipid oxidation over the ten-hour study period, with individual lipid oxidation shown in appendix 4.1. Postabsorptive oxidation was approximately 2g/hour for the group in all trials. When no sucrose was added to the meal there was little suppression of lipid oxidation (~2g/hour) immediately following the meal, which rises and peaks at 7 hours postprandially (3.5 g/hr). When 50g of sucrose was added, a small suppression of oxidation was seen at 1 hour (0.5g/hr) and 7 hours (1.5g/hr), immediately following the meals, but a similar pattern was observed overall. On addition of 100g of sucrose a large suppression of oxidation was seen with negative values directly after the meals (1 hour = -1g/hr, 7 hours = -1.5g/hr). Repeated measures analysis of variance revealed a significant effect of time, trial, and time/trial interaction on total lipid oxidation ($p<0.05$). Post-hoc analysis revealed that statistical significance was reached for the differences between the 0g and 100g sucrose trials at 1,7 and 9 hours, and between all three trials at 7 hours postprandially. Total lipid oxidation over the 10 hour study period was calculated by measuring the total area under the timecourse curve.

Table 6.4 shows the total lipid oxidation over the ten hour study period for the three sucrose trials, and the group mean difference between the trials expressed in absolute amounts (g) and as a percentage. Total lipid oxidation decreased with increasing sucrose load from 27.0g/10 hours (16.9 to 37.7g) with 0g of sucrose, to 22.0g/10 hours (6.2 to 38.5g) with 50g sucrose and to 11.1g/10 hours (-0.2 to 25.6g) with 100g sucrose. The decrease in lipid oxidation with added sucrose was found to be equivalent to a 20.5% decrease from 0g to 50g sucrose, 65.9% decrease from 50g to 100g sucrose and 83.6% decrease from 0g to 100g of sucrose. Despite all subjects displaying a decrease in oxidation from 0g to 100g sucrose trials, only four of the six subjects displayed a decrease from 0g to 50g of sucrose, and five of the six from 50g to 100g. One way analysis of variance revealed a significant difference in total lipid oxidation between the 0g and 100g sucrose trials ($p<0.05$). Statistical significance was

not reached between the remaining trials, despite the trends observed, due to the large between and within-individual variability.

The oxidation of [1,1,1-^{13}C]tripalmitin was assumed to reflect that of the lipid in the testmeal. The proportion of palmitic acid in the testmeal which was oxidised was calculated from $^{13}CO_2$ excretion in breath to determine exogenous lipid oxidation as described in chapter 5. Figure 6.3 shows the timecourse of exogenous and endogenous lipid oxidation over the ten hour study period. Similar patterns of lipid oxidation were seen with the addition of 0g and 50g of sucrose to the testmeal, with oxidation increasing following the meal peaking at 4 hours postprandially (<2g/hr). When 100g was added the rise in oxidation was delayed peaking at 7 hours postprandially (2g/hr). Repeated measures analysis of variance revealed significant time, trial and time/trial interaction effects on exogenous lipid oxidation ($p<0.05$). Post-hoc analysis revealed that statistical significance was reached at 2,3,4 and 5 hours postprandially between the 0g and 100g trials, and the 50g and 100g trials. No significant differences were found in exogenous lipid oxidation between the 0g and 50g sucrose trials.

For the group as a whole endogenous lipid oxidation remained at baseline levels for the ten hour study period following the 0g sucrose meal. With 50g sucrose, endogenous lipid oxidation was suppressed immediately following the testmeal (approximately 0g/hour), and then rose towards baseline levels by 6 hours postprandially. Following the second meal, oxidation was again suppressed with a similar pattern to the first meal. With 100g of sucrose, endogenous lipid oxidation was greatly suppressed following the first meal (approximately -2g/hour), increasing towards baseline levels by 5 hours postprandially. After the second meal, oxidation decreased to a greater extent (approximately -4g/hour), and began to rise by 10 hours postprandially. Repeated measures analysis of variance revealed a significant effect of time, trial and time/trial interaction on endogenous lipid oxidation ($p<0.05$). Post-hoc analysis revealed statistical significance was reached at 1 hour and 7 hours postprandially between the 0g and 100g trials and between the 50g and 100g sucrose trials. Total exogenous and endogenous lipid oxidation over the ten hour study period was calculated by measuring the total area under the timecourse curve and results are shown in tables 6.5 and 6.6. The proportion of total lipid oxidation accountable for by exogenous and endogenous lipid is shown in figure 6.4.

For the group as a whole, total exogenous lipid oxidation decreased from 13.6g/10 hours (11.7 to 15.7g) on the 0g sucrose trial, to 13.0g/10 hours (10.4 to 16.7g) and 9.7g/10 hours (8.8 to 13.1g) with 50g and 100g of sucrose respectively. The decrease in exogenous lipid oxidation was equivalent to a 4.4% decrease from 0g to 50g sucrose, 29.3% from 50g to 100g sucrose and 33.6% from 0g to 100g sucrose. One way analysis of variance revealed significant differences in exogenous lipid oxidation between the 0g and 100g of sucrose trial ($p<0.05$). Statistical differences were not observed between the remaining trials despite trends, due to variability in the magnitude and direction of oxidation changes between the trials. Endogenous lipid oxidation decreased from 13.4 g /10 hours (5.0 to 23.9g) with 0g sucrose to 9.0g (-4.2 to 21.8g) with 50g sucrose, and to 1.4g/10 hours (-9.0 to 14.4g) with 100g sucrose, equivalent to a 39.7% decrease (0g to 50g), 146.5% decrease (50g to 100g) and a 162.5% decrease (0g to 100g). One way analysis of variance revealed significant differences between the 0g and 100g sucrose trials only ($p<0.05$), with difference between the remaining trials not reaching significance due to large between and within-individual variability.

6.5.3 Total Carbohydrate oxidation

The mean oxidation of carbohydrate over the ten-hour study period for each trial is shown in figure 6.5 for the group as a whole, with individual graphs shown in appendix 4.2. With no added sucrose, carbohydrate oxidation increased after the first meal, peaking at 15g per hour at 2 hours postprandially, and subsequently decreased to less than baseline with a smaller increase after the second meal. Oxidation peaked at 1 hour following the 50g meal (~20g/hour), with a similar pattern for the remaining 9 hours to the 0g sucrose meal. With 100g of added sucrose, oxidation increased even further with a peak of 25g carbohydrate oxidised per hour after the first meal, which was the same after the second meal. Repeated measures analysis of variance revealed a significant effect of time, trial and time/trial interaction on carbohydrate oxidation ($p<0.001$). Post-hoc analysis revealed that statistical significance was reached at 1,3,7,8,9,and 10 hours postprandially between the 0g and 100g sucrose trials, and at 7 hours postprandially between all three trials ($p<0.001$). Total carbohydrate oxidation over the study period was calculated from the area under the timecourse curve graphs and shown in table 6.7.

Total carbohydrate oxidation increased with increasing sucrose in the testmeal from 102.1g/10 hours (88.5 to 110.1g) with the 0g sucrose meal to 124.4g/10 hours (97.3 to 155.6) and 159.7g/10 hours (143.8 to 194.2) following the 50g and 100g sucrose meals respectively. The addition of 50g of sucrose to the testmeal resulted in a 19.7% increase in oxidation, whilst 100g of sucrose resulted in a 44.0% increase in oxidation. One way analysis of variance revealed significant differences in total carbohydrate oxidation all three trials ($p<0.05$), due to five of the six subjects showing an increase in oxidation from 0g to 50g of sucrose and all subjects showing an increase from 0g to 100g of sucrose.

6.5.4 Plasma Glucose

The mean glucose response over the time period for the group as a whole for each trial can be seen in figure 6.6, with individual responses shown in appendix 4.3. In the post-absorptive state, baseline concentrations of plasma glucose were similar between the trials (approximately 6mmol/l). After consumption of the first meal on each trial there was no change in plasma glucose concentration, which remained at baseline levels up to 6 hours postprandially. After consumption of the second meal, glucose concentration increased to 9 mmol/l (0g sucrose trial) and to 10.5 mmol/l (50g and 100g sucrose trials) with all values returning to baseline concentrations by the end of the study period. Univariate analysis of variance revealed a significant effect of time on plasma glucose concentration ($p<0.05$), but no effect of trial. Multivariate analysis of variance revealed no effect of time/trial interaction on plasma glucose concentrations. Marked differences between the trials were seen in subjects 1 and 4, whereas in other subjects' glucose concentration remained relatively constant between trials. Total glucose concentration over the ten hour study period was calculated from the area under the timecourse curve measured from zero, and is shown in table 6.8.

Following the 0g sucrose meal, glucose concentration was on average 66.2mmol/l per 10 hours (56.1 to 74.7 mmol/l per 10 hours). After consumption of the 50g sucrose meal glucose concentration remained constant at 65.7 mmol/l per 10 hours (57.8 to 74.9 mmol/l per 10 hours), and was 68.3 mmol/l per 10 hours (63.2 to 82.4 mmol/l per 10 hours) with the 100g sucrose meal. There was an overall decrease in plasma glucose

concentration from the 0g to 50g sucrose meal of 0.7%, despite four of the six subjects demonstrating an increase in glucose concentration. From the 50g to 100g meal, three subjects demonstrated an increase in glucose concentration whilst three showed a decrease. Overall there was a 3.9% increase in concentration. From 0g to 100g sucrose, four of the six subjects demonstrated an increase in glucose concentration resulting in an overall group increase of 3.2%. One way analysis of variance, however, revealed no statistical differences between the trials due to a lack of effect, and to the variability in the direction and magnitude of change between individuals.

6.5.5 Plasma Insulin

The mean insulinogenic response to the meals for the group as a whole for each trial can be seen in figure 6.7, with individual data shown in appendix 4.4. In the post-absorptive state, baseline concentrations of plasma insulin were similar between the trials (approximately 7uU/l). When no sucrose was added, the peak in plasma insulin (50uU/l) was seen at 1.5 hours postprandially, with a subsequent decrease to baseline levels by 6 hours. After the second meal, there was a greater increase in insulin, peaking at 70uU/l before falling back to baseline by 10 hours. With the addition of 50g of sucrose, there was a slight increase in insulin (peak 55uU/l), but a similar pattern to the 0g sucrose meal was seen overall. The magnitude of the insulin response increased greatly after 100g of sucrose, peaking at 75 to 80uU/l after the first meal, and at 90 to 95uU/l following the second meal . Repeated measures analysis of variance revealed a significant effect of time, trial and time/trial interaction for plasma insulin responses over the timecourse ($p<0.001$). Post-hoc analysis revealed significant differences between the 0g and 100g of sucrose trials observed at 1,1.5,2,2.5,3,9, and 10 hours. In addition differences between the 50g and 100g sucrose trials were seen at 2,2.5 and 3 hours postprandially. Total plasma insulin concentration over the ten hour study period was calculated as the area under the timecourse curve measured from zero and is shown in table 6.9.

A group mean of 262.5 uU/l per 10 hours (208.3 to 301.60) was measured for the 0g sucrose trial. With 50g of sucrose this figure rose to 288.3 uU/l per 10 hours (238.5 to 333.2), and with 100g to 415.73 uU/l per 10 hours (346.5 to 482.2). The increase in

insulin concentration from the 0g to 50g sucrose trial was equivalent to a 9.5% increase. From the 50g to 100g trial the percentage difference increased to 36.0%, and increased further to 45.2% from the 0g to 100g trials. One way analysis of variance revealed significant differences in total plasma insulin concentrations between the 0g and 100g trials and 50g and 100g trial ($p<0.05$). There were no significant differences between the 0g and 50g trials due to a lack of effect and variability in the direction and magnitude of changes in insulin concentration.

6.5.6 Plasma TAG

The mean plasma TAG response to the meals for the group as a whole, for each trial can be seen in figure 6.8, with individual data shown in appendix 4.5. All subjects had post-absorptive plasma TAG concentrations within the normal range (0.5 to 1.0 mmol/l) prior to commencing all three trials. Following the 0g sucrose meal, there was a marked rise in plasma TAG peaking at 2.5 hours postprandially (1.6 mmol/l) and returning to baseline levels by 5 hours. Plasma TAG concentration increased following the second meal at 7 hours postprandially (1.3 mmol/l) but returned to baseline by 10 hours. After the 50g sucrose meal, the same pattern of postprandial TAG response was seen but with a decreased magnitude (peak= 1.15 mmol/l at 2.5 hrs). Following the 100g sucrose meal, initially plasma TAG increased as with the 50g sucrose meal to 2.5 hours postprandially (1.2 mmol/l) but the raised lipaemia persisted and remained elevated at 6 hours. After the second meal, concentrations rose again and remained elevated at the end of the study. Repeated measures analysis of variance revealed significant effects of time, trial and time/trial interactions on plasma TAG concentrations ($p<0.005$). Post-hoc analysis revealed statistical significance was reached at 1, 2 and 2.5 hours postprandially between the 0g and 100g trials. In addition, differences between the 0g and 50g trials were seen at 2 and 2.5 hours. Total plasma TAG over the time period was calculated both from zero (AUC_0) and from baseline values (AUCi) as the area under the timecourse curve shown in tables 6.10 and 6.11 respectively.

Total plasma TAG concentration (AUC_0) decreased from 11.0 mmol/l per 10 hours (8.5 to 15.5) with the 0g sucrose meal to 9.0 mmol/l per 10 hours (7.5 to 10.9)with the 50g sucrose meal. With the 100g sucrose meal, TAG concentrations increased to 9.7

mmol/l per 10 hours (6.3 to 14.7). Overall this resulted in a 20.1% decrease in concentration from 0g to 50g of sucrose, a 7.3% increase from 50g to 100g of sucrose and a 12.9% decrease from 0g to 100g sucrose. Three subjects demonstrated a decrease in plasma TAG with 50g of sucrose and an increase with 100g of sucrose, whereas the remaining subjects demonstrated a continuous decrease in plasma TAG concentration with increasing sucrose loads. One way analysis of variance revealed no significant effects of trial on plasma TAG concentrations due to he lack of effect and variability in the magnitude and direction of changes between subjects. Total plasma TAG concentration (AUCi) decreased from 3.0 mmol/l per 10 hours (0.5 to 4.2) with the 0g sucrose meal to 2.8 mmol/l per 10 hours (1.8 to 5.9) with the 50g sucrose meal. With the ingestion of the 100g sucrose meal TAG concentrations decreased further to 2.3 mmol/l per 10 hours (0.7 to 4.0). Subjects 3 and 4 demonstrated an increase in plasma TAG concentration from the 0g to 50g trials, whilst the remaining subjects demonstrated a decrease, which resulted in an overall decrease of 7.5% between the trials. Three subjects displayed an increase in concentration from the 50g to 100g sucrose trial, whilst the remaining three subjects displayed a decrease in plasma TAG concentration. One way analysis of variance revealed no significant effects of increasing the sucrose content of the meal on total plasma TAG concentration despite the trends observed, due to the variability in the magnitude and direction of changes in TAG concentrations.

6.5.7 Plasma NEFA

The mean plasma NEFA response to the meals for the group as a whole, for each trial can be seen in figure 6.9, with individual data shown in appendix 4.6. Baseline concentrations of plasma NEFA were consistent for the group as a whole prior to commencing all three trials (0.45 mmol/l). Following consumption of the 0g sucrose meal, plasma NEFA concentration decreased by 1 hour (0.1mmol/l), with a rise in concentration which overshot baseline substantially by 6 hours postprandially (0.7 mmol/l). Following the second meal NEFA concentration was suppressed once more and remained suppressed at the end of the study. With the 50g sucrose meal, a similar response occurred with a smaller overshoot above baseline levels at 6 hours (0.5 mmol/l). With the 100g sucrose meal, a similar pattern was observed but the overshoot

seen with the 0g sucrose meal was completely diminished (0.4 mmol/l at 6 hours). Repeated measures analysis of variance revealed a significant effect of time ($p<0.05$), trial and time/trial interaction was revealed ($p<0.05$). Post-hoc analysis revealed significance was reached between the 0g and 100g sucrose trial at 2,2.5,3,6,7,8 and 9 hours postprandially, and between the 0g and 50g sucrose meal at 2 hours postprandially. Total NEFA concentration in the plasma was calculated from the area under the timecourse curve for each individual subject measured from zero (AUC_0) and from baseline (AUCi) shown in tables 6.12 and 6.13 respectively.

Total plasma NEFA concentration (AUC_0) decreased from 2.8 mmol/ per 10 hours (2.1 to 3.3) with 0g sucrose to 2.4 mmol/l per 10 hours (2.0 to 3.1) with 50g of sucrose, and to 1.6 mmol/l per 10 hours (1.3 to 2.1) with 100g of sucrose. The decrease in plasma NEFA concentration observed was equivalent to a 15.3% decrease (0g to 50g), 40.7% decrease (50g to 100g) and 55.1% decrease (0g to 100g). One way analysis of variance revealed significant differences in total plasma NEFA concentrations between the 0g and 100g sucrose trial and 50g and 100g sucrose trial ($p<0.05$). No significant differences were observed between the 0g and 50g trials due to an insufficient decrease in NEFA concentration. Total NEFA concentrations (AUCi) decreased from –1.4 mmol/l/10 hours (-2.7 to 0.3) to –1.8 mmol/l/10 hours (-3.8 to 0.4) with 50g sucrose and to -2.6 mmol/l/10 hours (-4.3 to -1.6) with 100g of sucrose. The decrease in plasma NEFA observed was equivalent to a 21.9% decrease (0g to 50g), 37.6% decrease (50g to 100g) and a 58.3% decrease (0g to 100g). Despite the trends in plasma NEFA concentration observed with the addition of sucrose to the testmeal, no significant differences between trials were revealed, possibly due to the large between individual variability in the magnitude and direction of effects.

6.5.8. ^{13}C-Palmitic Acid in TAG Fraction

N.B Results of the ^{13}C-label in the TAG fraction are only shown for the initial six hours of the study due to a technical fault with the equipment resulting in a reduction in the amount of time available to process samples. Therefore the first six hours of samples were run in preference to the remainder to determine ^{13}C-TAG and ^{13}C-NEFA responses to the first meal only

The appearance of mean ^{13}C-TAG in the circulation over the 6-hour study period for the three trials is shown in figure 6.10 for the group as a whole, with individual data shown in appendix 4.7. At baseline there was no ^{13}C-TAG detectable in the circulation as subjects were fed a diet not naturally enriched with ^{13}C for two days prior to each study. With no added sucrose in the meal, a rise in the ^{13}C-TAG was seen which peaked at 2 hours postprandially (11ug/ml). A decrease was then seen with the concentration of ^{13}C-TAG returning to baseline levels by 6 hours. When 50g of sucrose was added, a similar pattern was seen but with a smaller magnitude of responses, peaking at 7ug/ml at 2 hours postprandially. With the addition of 100g of sucrose, there was a delay in the appearance of ^{13}C-TAG in the circulation, which peaked at 3 hours (8ug/ml). Following this there was no decrease in ^{13}C-TAG concentration, but a prolongation of elevated ^{13}C-TAG concentration in the circulation, which remained above 7ug/ml until 6 hours postprandially. Repeated measures analysis of variance revealed that there was a significant effect of time, trial and a time/trial interaction on the pattern of plasma ^{13}C-TAG concentration ($p<0.05$). Total ^{13}C-TAG concentration over the 6-hour period was calculated from measuring total area under the timecourse curve shown in table 6.14.

A total of 33.60 ug/ml per 6 hours (19.3 to 44.3) was recovered following the 0g sucrose trial, which tended to decrease for the group as a whole to 27.9 ug/ml per 6 hours (18.2 to 42.3) when 50g of sucrose was added. With 100g of added sucrose, plasma ^{13}C-TAG concentration increased to 34.1 ug/ml per 6 hours (17.1 to 60.5) due to the prolongation of lipaemia. The differences between the trials were equivalent to an 18.7% decrease in concentration (0g to 50g), a 20.0% increase (50g to 100g) and an overall 1.3% increase from the 0g to 100g trials. Despite the trends in plasma ^{13}C-TAG concentration, no statistical differences were observed in the ^{13}C-TAG concentration between the three sucrose trials, due to the variability in the magnitude and direction of effects with the addition of sucrose.

6.5.9. ^{13}C-Palmitic Acid in NEFA Fraction

The appearance of mean ^{13}C-NEFA in the circulation over the 6-hour study period for the three trials is shown in figure 6.11 for the group as a whole, with individual data shown in appendix 4.8. When no sucrose was added to the meal, the

concentration of labelled NEFA increased from zero and peaked at 3 hours postprandially (1.0ug/ml), before reaching a plateau for the remainder of the study. The addition of 50 g of sucrose resulted in a decrease in the magnitude of ^{13}C-NEFA concentration, with a peak of 0.75 to 1.0 ug/ml at 6 hours. After consumption of 100g of sucrose, the magnitude of response decreased further initially, but continued to rise thereafter with the concentration increasing at 5 hours to above 1.0ug/ml. All trials resulted in a similar concentration of ^{13}C-NEFA at 6 hours postprandially. Despite the trends in the appearance and clearance of ^{13}C-NEFA from the circulation, repeated measures analysis of variance revealed only a significant effect of time ($p<0.05$), but no significant effects of trial or time/trial interactions on plasma ^{13}C-NEFA concentration. Total ^{13}C-NEFA concentration over the study day was calculated by measuring the area under the timecourse curve shown in table 6.15.

A mean concentration of 4.6 ug/ml per 6 hours (2.5 to 6.0) of ^{13}C-NEFA was observed following the 0g sucrose meal. With 50g of sucrose, ^{13}C-NEFA concentration decreased to 3.82 ug/ml per 6 hours (3.0 to 5.5), and to 3.4 ug/ml/6 hours (1.5 to 5.5) with 100g of added sucrose. One way analysis of variance revealed no significant differences in plasma ^{13}C-NEFA concentration between the trials despite the overall decrease in plasma ^{13}C-NEFA concentration between the 0g and 100g sucrose meals being a decrease of 27.9%, due to the lack of effect of sucrose on ^{13}C-NEFA concentration and the variability in the direction of changes observed.

Table 6.2 **Breath $^{13}CO_2$ excretion over the initial 10 hours or the complete 24 hours of the study day expressed as a percentage of administered dose of [1,1,1-^{13}C]tripalmitin, following consumption of a reference testmeal with varying loads of sucrose**

Subject	Breath $^{13}CO_2$ excretion (%)					
	0g sucrose		50g sucrose		100g sucrose	
	10 h	**24 h**	**10 h**	**24 h**	**10 h**	**24 h**
1	19.9	26.8	15.0	19.9	14.2	19.7
2	16.6	17.6	14.0	17.0	12.6	14.1
3	19.6	22.7	21.2	28.3	15.9	20.1
4	22.2	31.2	23.7	28.0	8.8	9.3
5	20.9	24.1	14.8	19.1	12.4	17.4
6	16.6	18.2	22.1	25.2	18.6	23.7
Mean	**19.3**	**23.4**	**18.5**	**22.9**	**13.8 ***	**17.4**
SD	**2.3**	**5.2**	**4.3**	**4.9**	**3.4**	**5.1**

* Significantly different from low carbohydrate trial at 10h (*ANOVA $P<0.05$)*

Table 6.3 **Difference between breath $^{13}CO_2$ excretion over 24 hours over the three carbohydrate trials expressed as the actual difference between trials and the percentage difference (actual difference/ 0g +50g/2 OR actual difference/ 50g +100g/2OR actual difference/ 0g +100g/2)**

Subject	Breath $^{13}CO_2$ excretion difference between trials					
	0g-50g sucrose		50g-100g sucrose		0g-100g sucrose	
	Actual	% diff.	Actual	% diff.	Actual	% diff.
1	↓ 6.9	29.6	↓ 0.2	1.0	↓ 7.1	30.5
2	↓ 0.6	3.5	↓ 2.9	18.6	↓ 3.5	22.0
3	↓ 3.2	10.8	↓ 18.7	100.0	↓ 21.9	107.9
4	↑ 5.6	22.0	↓ 8.2	33.9	↓ 2.6	12.1
5	↓ 5.0	23.1	↓ 1.7	9.2	↓ 6.7	32.2
6	↑7.0	32.3	↓ 1.5	6.1	↑ 5.5	26.2
Mean	**↓ 0.5**	**2.1**	**↓ 5.5**	**28.1**	**↓ 6.1**	**29.8**
SD	**5.7**	**24.7**	**7.0**	**37.1**	**9.0**	**43.9**

Table 6.4 Total lipid oxidation expressed in grams of substrate oxidised per ten hour study period shown for six subjects, following consumption of a reference testmeal with varying sucrose loads.

Subject	Total lipid oxidation (g/10 hours)		
	0g sucrose	**50g sucrose**	**100g sucrose**
1	27.2	16.7	17.0
2	23.6	25.2	13.8
3	37.7	30.2	25.6
4	36.8	38.5	4.7
5	19.8	6.2	-0.2
6	16.9	15.1	5.59
Mean (mean difference/ mean percentage difference)	**27.0 (0g-50g ↓ 5.0g / 20.5%)**	**22.0 (50g-100g ↓ 10.9/ 65.9%)**	**11.1 * (0g-100g ↓ 15.9g / 83.6%)**
SD (SD of difference)	**8.7 (6.5)**	**11.6 (11.9)**	**9.5 (8.8)**

* Significantly different to 0g sucrose trial (*ANOVA $P<0.05$*)

Table 6.5 Exogenous lipid oxidation expressed in grams of substrate oxidised per ten hour study period shown for six subjects, following consumption of a reference testmeal with varying sucrose loads.

Subject	Exogenous lipid oxidation (g/10 hours)		
	0g sucrose	**50g sucrose**	**100g sucrose**
1	14.0	10.6	10.0
2	11.7	9.9	8.9
3	13.8	15.0	11.2
4	15.7	16.7	6.2
5	14.8	10.4	8.8
6	11.7	15.6	13.1
Mean (mean difference/ mean percentage difference)	**13.6 (0g-50g ↓ 0.6 / 4.4%)**	**13.0 (50g-100g ↓ 3.3 /29.3%)**	**9.7 * (0g-100g ↓ 3.9 / 33.6%)**
SD (SD of difference)	**1.6 (3.2)**	**3.1 (3.7)**	**2.3 (3.7)**

* Significantly different to 0g sucrose trial (*ANOVA $P<0.05$*)

Table 6.6 Endogenous lipid oxidation expressed in grams of substrate oxidised per ten hour study period shown for six subjects, following consumption of a reference testmeal with varying sucrose loads.

Subject	Endogenous lipid oxidation (g/10 hours)		
	0g sucrose	**50g sucrose**	**100g sucrose**
1	13.2	6.1	7.0
2	11.9	15.3	4.9
3	23.9	15.2	14.4
4	21.1	21.8	-1.5
5	5.0	-4.2	-9.0
6	5.2	-0.5	-7.5
Mean (mean difference/ mean percentage difference)	**13.4 (0g-50g ↓ 4.4 / 39.7%**	**9.0 (50g-100g ↓ 7.6/ 146.5%)**	**1.4* (0g-100g ↓ 12.0/ 162.5%)**
SD (SD of difference)	**7.9 (5.2)**	**10.1 (8.7)**	**9.0 (6.0)**

* Significantly different to 0g sucrose trial (*ANOVA $P<0.05$*)

Table 6.7 **Total carbohydrate oxidation expressed in grams of substrate oxidised per ten hour study period shown for six subjects, following consumption of a reference testmeal with varying sucrose loads**

Subject	Carbohydrate oxidation (g/10 hours)		
	0g sucrose	**50g sucrose**	**100g sucrose**
1	105.9	155.6	152.2
2	104.2	119.6	150.4
3	88.5	106.2	143.8
4	107.0	97.3	194.2
5	96.6	134.9	153.7
6	110.1	132.6	163.7
Mean (mean difference/ mean percentage difference)	**102.1 ‡ (0g-50g ↑ 22.3g / 19.7%)**	**124.4 * ‡ (50g-100g ↑ 35.3g/ 24.9%)**	**159.7 * † (0g-100g ↑ 57.6g / 44.0%)**
SD (SD of difference)	**8.0 (20.5)**	**21.2 (33.5)**	**18.1 (15.2)**

* Significantly different to 0g sucrose trial (*ANOVA $P<0.05$*)

† Significantly different to 50g sucrose trial (*ANOVA $P<0.05$*)

‡ Significantly different to 100g sucrose trial (*ANOVA $P<0.05$*)

Table 6.8 Plasma glucose concentration (mmol/l per 10 hours) measured over a ten hour study day, shown in six subjects, following consumption of a reference testmeal with varying sucrose loads

Subject	Plasma glucose (mmol/l/10 hours)		
	0g sucrose	**50g sucrose**	**100g sucrose**
1	74.7	74.9	82.4
2	60.2	64.6	63.2
3	75.8	72.6	69.5
4	56.1	63.5	62.5
5	71.4	57.8	65.2
6	58.7	60.7	67.0
Mean (mean difference/ mean percentage difference)	**66.2 (0g-50g ↓ 0.5mmol/l / 0.7%)**	**65.7 (50g-100g ↑ 2.6 mmol/l / 3.9%)**	**68.3 (0g-100g ↑ 2.2 mmol/l / 3.2%)**
SD (SD of difference)	**8.8 (7.4)**	**6.7 (4.9)**	**7.4 (6.8)**

Table 6.9 **Plasma insulin concentration (uU/l per 10 hours) measured over a ten hour study day, shown in six subjects, following consumption of a reference testmeal with varying sucrose loads**

Subject	Plasma Insulin (uU/l)		
	0g sucrose	**50g sucrose**	**100g sucrose**
1	301.6	287.4	482.2
2	208.3	271.7	373.6
3	236.1	333.2	401.7
4	247.8	238.5	346.5
5	290.8	298.6	427.3
6	290.3	303.4	463.1
Mean (mean difference/ mean percentage difference)	**262.5 (0g-50g ↑ 26.3 uU/l / 9.5%)**	**288.8 (50g-100g ↑ 126.9 uU/l / 36.0%)**	**415.7 * † (0g-100g ↑ 153.3 uU/l / 45.2%)**
SD (SD of difference)	**37.3 (44.3)**	**32.0 (44.9)**	**52.1 (30.6)**

* Significantly different to 0g sucrose trial (*ANOVA $P<0.05$*)

† Significantly different to 50g sucrose trial (*ANOVA $P<0.05$*)

Table 6.10 **Plasma TAG concentration (mmol/l per 10 hours) measured over a ten hour study day, shown in six subjects, following consumption of a reference testmeal with varying sucrose loads. Plasma TAG concentration was calculated from the area under the timecourse curve measured from zero (AUC_0)**

Subject	Plasma TAG (mmol/l per 10 hours AUC_0)		
	0g sucrose	50g sucrose	100g sucrose
1	12.0	9.9	8.9
2	11.2	9.6	9.0
3	9.5	8.5	11.8
4	15.5	10.9	14.7
5	8.5	7.5	7.5
6	9.5	7.7	6.3
Mean (mean difference/ mean percentage difference)	**11.1 (0g-50g ↓ 2.0 mmol/l / 20.1%)**	**9.0 (50g-100g ↑ 0.7 mmol/l / 7.3%)**	**9.7 (0g-100g ↓ 1.3 mmol/l / 12.9%)**
SD (SD of difference)	**2.5 (1.3)**	**1.3 (2.3)**	**3.1 (2.0)**

Table 6.11 **Plasma TAG concentration (mmol/l per 10 hours) measured over a ten hour study day, shown in six subjects, following consumption of a reference testmeal with varying sucrose loads. Plasma TAG concentration was calculated from the area under the timecourse curve measured from baseline (AUCi)**

Subject	Plasma TAG (mmol/l per 10 hours AUCi)		
	0g sucrose	50g sucrose	100g sucrose
1	3.6	2.3	3.1
2	4	3.2	1.1
3	0.5	1.8	3.2
4	4.2	5.9	4.0
5	2.3	1.7	1.9
6	3.3	1.7	0.7
Mean (mean difference/ mean percentage difference)	**3.0 (0g-50g ↓ 0.2 mmol/l / 7.5%)**	**2.8 (50g-100g ↓ 0.4 mmol/l / 17.0%)**	**2.3 (0g-100g ↓ 0.7 mmol/l / 24.5%)**
SD (SD of difference)	**1.4 (1.4)**	**1.6 (1.5)**	**1.3 (2.0)**

Table 6.12 **Plasma NEFA concentration (mmol/l per 10 hours) measured over a ten hour study day, shown in six subjects, following consumption of a reference testmeal with varying sucrose loads. Plasma NEFA concentration was calculated from the area under the timecourse curve measured from zero (AUC_0)**

Subject	Plasma NEFA (mmol/l per 10 hours AUC_0)		
	0g sucrose	50g sucrose	100g sucrose
1	3.0	2.7	1.9
2	3.3	3.1	1.6
3	2.7	2.0	2.1
4	2.9	2.6	1.3
5	2.9	2.0	1.3
6	2.1	2.1	1.4
Mean (mean difference/ mean percentage difference)	**2.8 (0g-50g ↓ 0.4 mmol/l / 15.3%)**	**2.4 (50g-100g ↓ 0.8 mmol/l / 40.7%)**	**1.6 * † (0g-100g ↓ 0.5 mmol/l / 55.1%**
SD (SD of difference)	**0.4 (0.3)**	**0.5 (0.6)**	**0.3 (0.5)**

* Significantly different to 0g sucrose trial (*ANOVA $P<0.05$)*

† Significantly different to 50g sucrose trial (*ANOVA* ***$P<0.05$)***

Table 6.13 **Plasma NEFA concentration (mmol/l per 10 hours) measured over a ten hour study day, shown in six subjects, following consumption of a reference testmeal with varying sucrose loads. Plasma NEFA concentration was calculated from the area under the timecourse curve measured from baseline (AUCi)**

Subject	Plasma NEFA (mmol/l per 10 hours AUCi)		
	0g sucrose	50g sucrose	100g sucrose
1	0.0	-1.0	-2.0
2	0.3	0.4	-2.2
3	-1.2	-3.8	-4.3
4	-2.7	-1.8	-2.5
5	-2.4	-2.0	-1.6
6	-2.7	-2.6	-3.2
Mean (mean difference/ mean percentage difference)	**-1.4 (0g-50g ↓ 0.4 mmol/l / 21.9%)**	**-1.8 (50g-100g ↓0.8 mmol/l / 37.6%)**	**-2.6 (0g-100g ↓ 1.2 mmol/l / 58.3%)**
SD (SD of difference)	**1.4 (1.3)**	**1.4 (1.0)**	**1.0 (1.6)**

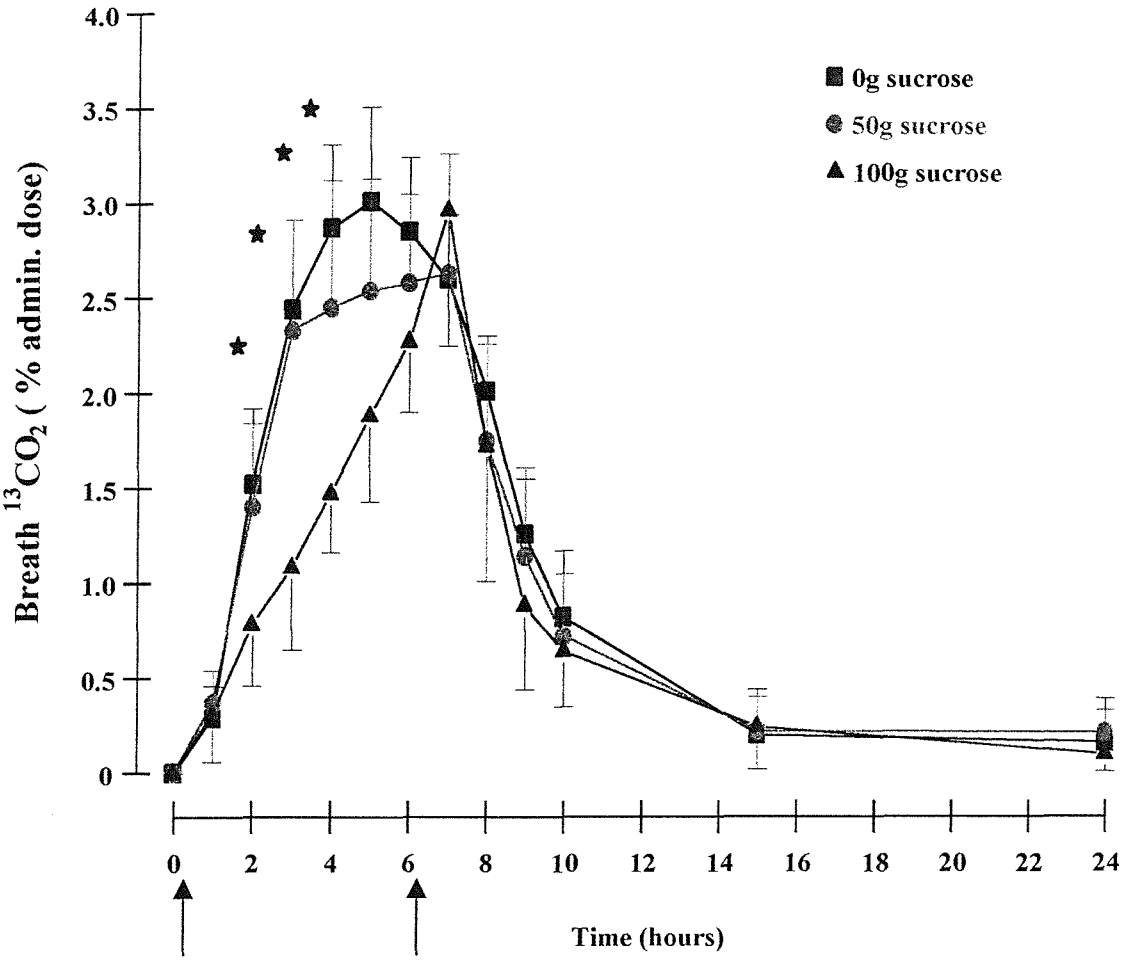
Table 6.14 **Plasma ^{13}C-TAG concentration (ug/ml plasma per 6 hours) measured over the initial six hours of a ten hour study day, shown in six subjects, following consumption of a reference testmeal with varying sucrose loads.**

Subject	Plasma ^{13}C-TAG (ug/ml per 6 hours)		
	0g sucrose	50g sucrose	100g sucrose
1	38.8	42.3	33.2
2	30.0	29.3	43.0
3	28.6	18.2	25.6
4	44.3	32.3	60.5
5	19.3	21.0	24.9
6	40.6	24.0	17.1
Mean (mean difference/ mean percentage difference)	**33.6 (0g-50g ↓ 5.8 ug/ml / 18.7%)**	**27.9 (50g-100g ↑ 6.2 ug/ml / 20.0%)**	**34.1 (0g-100g ↑ 0.5 ug/ml / 1.3%)**
SD (SD of difference)	**9.3 (8.3)**	**8.8 (13.8)**	**15.7 (14.5)**

Table 6.15 **Plasma ^{13}C-NEFA concentration (ug/ml per 6 hours) measured over the initial six hours of a ten hour study day, shown in six subjects, following consumption of a reference testmeal with varying sucrose loads.**

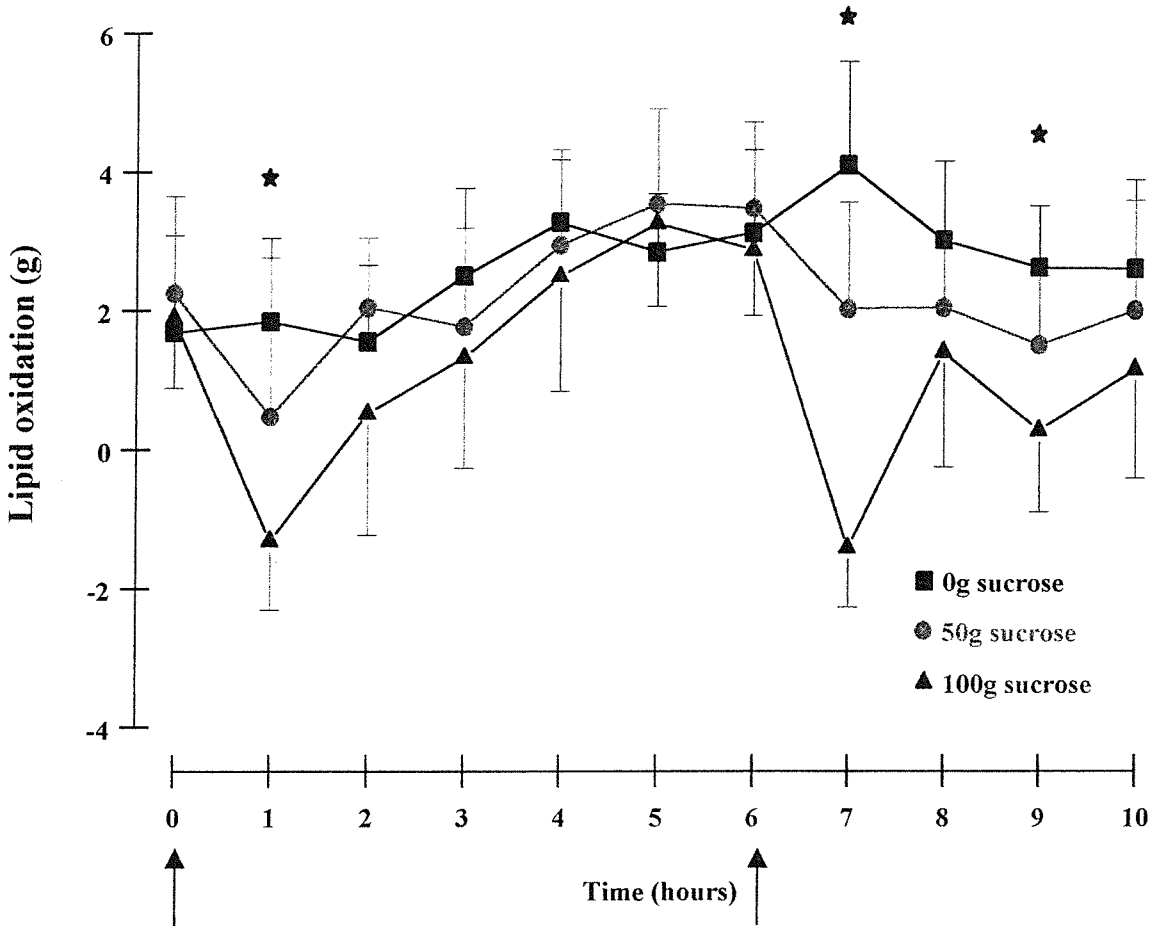
Subject	Plasma ^{13}C-NEFA (ug/ml per 6 hours)		
	0g sucrose	**50g sucrose**	**100g sucrose**
1	5.4	3.0	3.3
2	6.0	3.2	5.0
3	4.2	4.4	2.1
4	5.7	5.5	1.5
5	2.5	3.7	5.5
6	3.6	3.2	3.3
Mean (mean difference/ mean percentage difference)	**4.6 (0g-50g ↓ 0.7 ug/ml / 17.5%)**	**3.8 (50g-100g ↓ 0.4 ug/ml / 10.5%)**	**3.4 (0g-100g ↓ 1.1 ug/ml / 27.9%)**
SD (SD of difference)	**1.4 (1.6)**	**1.0 (2.3)**	**1.6 (2.4)**

Figure 6.1 **Breath $^{13}CO_2$ excretion expressed as a proportion of administered dose of [1,1,1-^{13}C]tripalmitin over a 24 hour study period. Group data for six subjects is shown following consumption of a reference testmeal with varying loads of sucrose (group mean ± SD).**



* Significant difference between 0g/100g and 50g/100g sucrose trials ($p<0.05$)

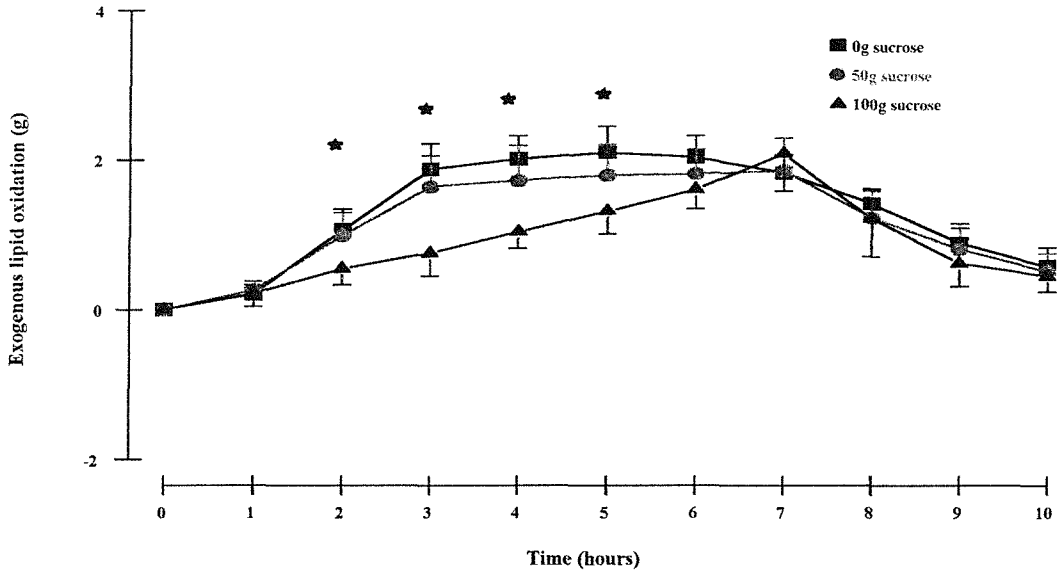
Figure 6.2 Total lipid oxidation expressed as the grams of lipid oxidised per hour over a ten hour study period, for a group of six subjects, following consumption of a reference testmeal with varying sucrose loads (group mean ± SD).



* Significant difference between 0g/100g trials, and all trials at 7h ($p<0.05$)

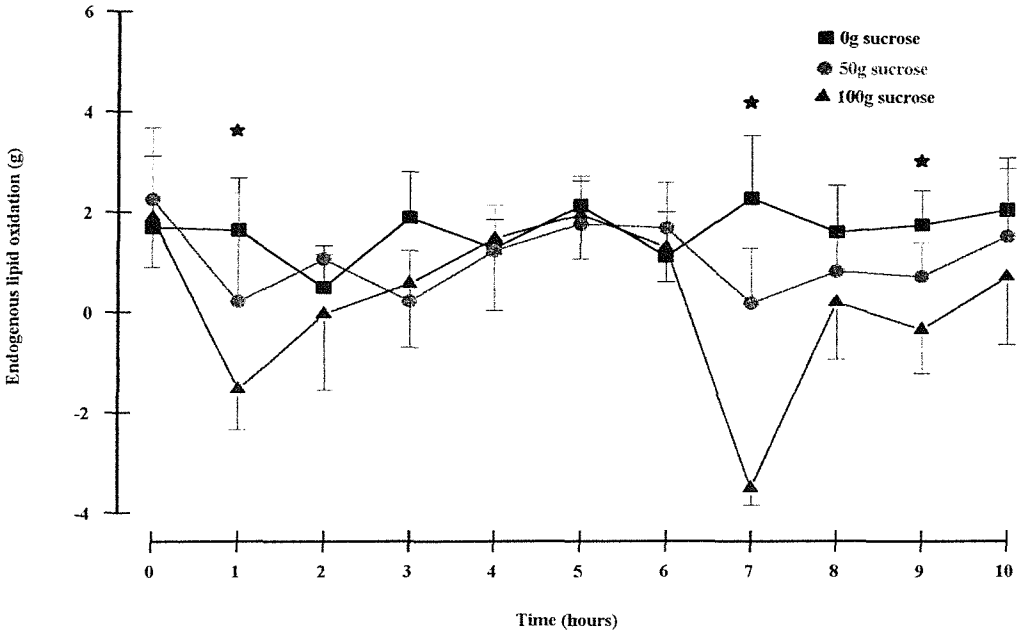
Figure 6.3 **Total exogenous and endogenous lipid oxidation expressed as the grams of lipid oxidised per hour over a ten hour study period in six subjects, following consumption of a reference testmeal with varying loads of sucrose (group mean ± SD)**

Exogenous lipid oxidation:-



* Significant difference between 0g/100g and 50g/100g sucrose trials ($p<0.05$)

Endogenous Lipid Oxidation:-



* Significant difference between 0g/100g and 50g/100g sucrose trials ($p<0.05$)

Figure 6.4 **Exogenous and endogenous lipid oxidation expressed as a proportion of total lipid oxidation (g/10 hours) in six subjects following consumption of a reference testmeal with varying loads of sucrose**

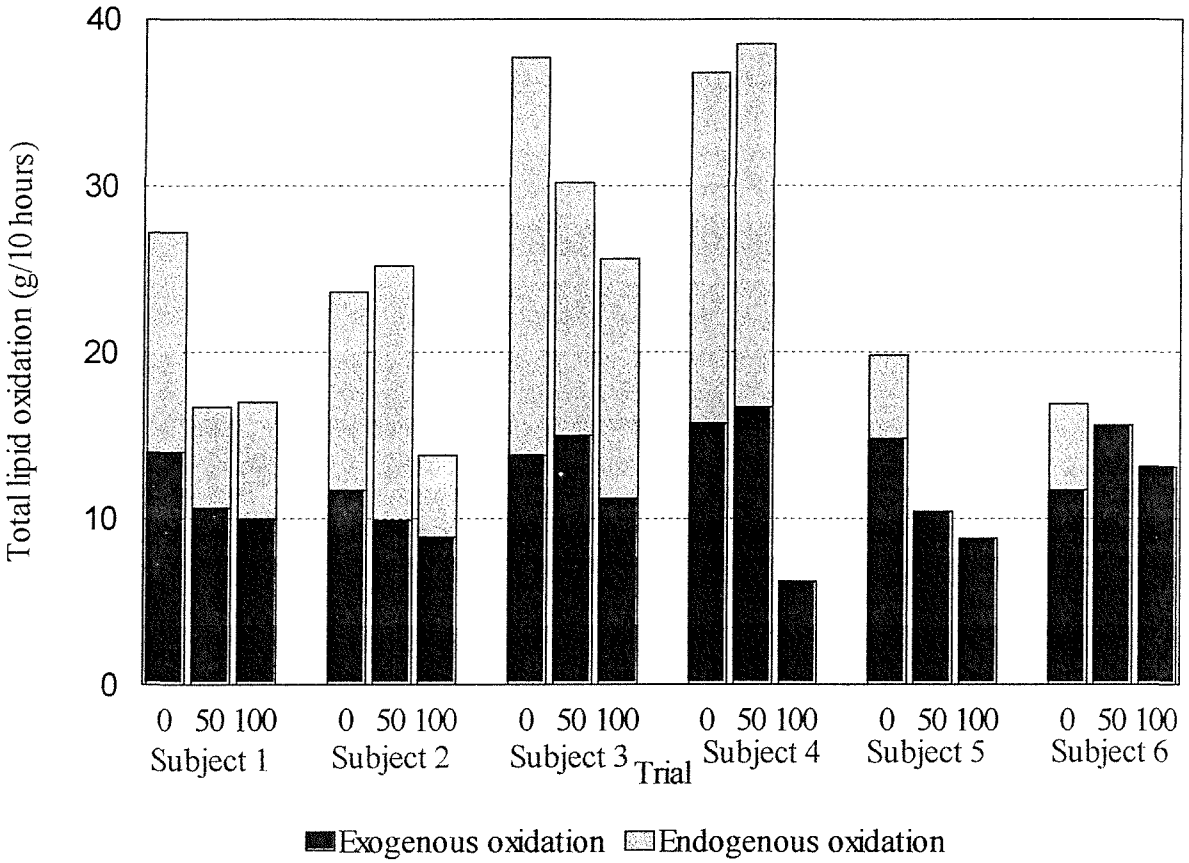
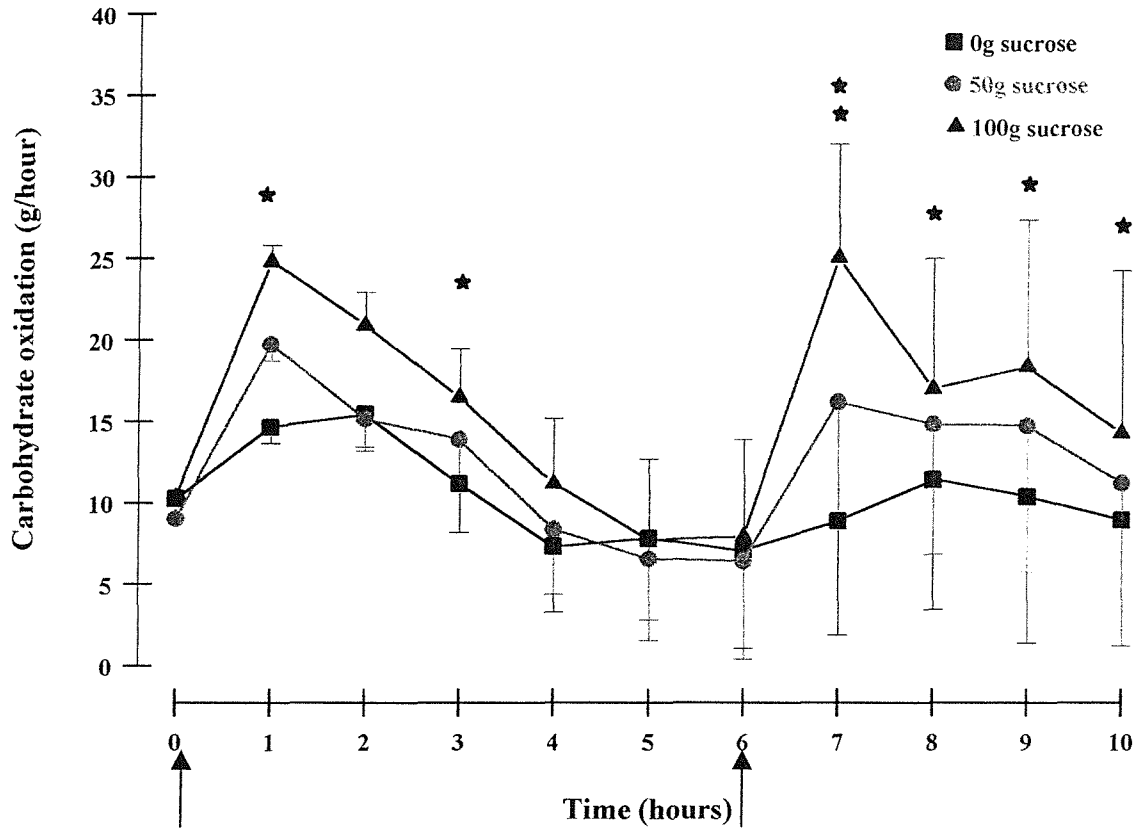


Figure 6.5 **Total carbohydrate oxidation expressed as the grams of carbohydrate oxidised per hour over a ten-hour study period, in six subjects following consumption of a reference testmeal with varying sucrose loads (group mean ± SD)**



* Significant difference between 0g/100g sucrose trial ($p<0.05$)
** Significant difference between all trials ($p<0.05$)

Figure 6.6 **Plasma glucose concentration expressed in mmol/l of plasma over a ten hour study period, shown in six subjects following consumption of a reference testmeal with varying sucrose loads (group mean ± SD).**

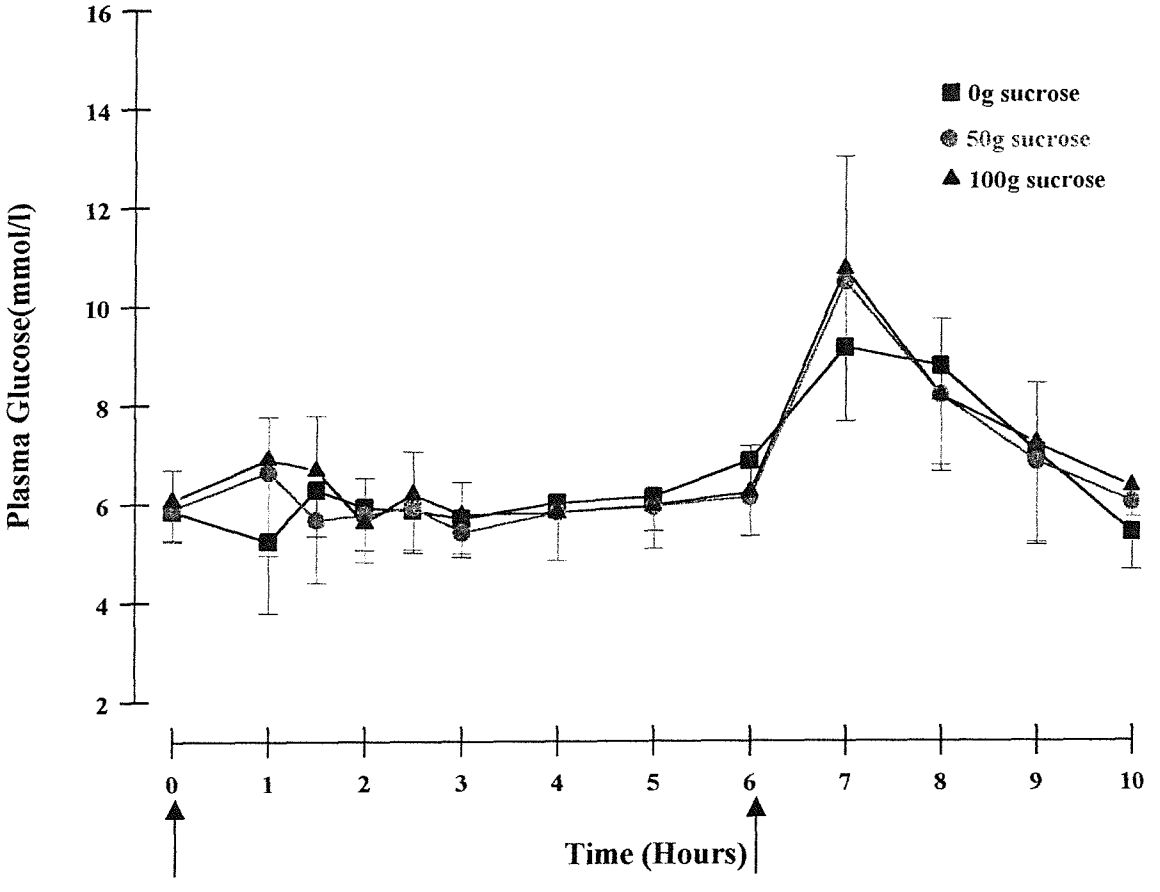
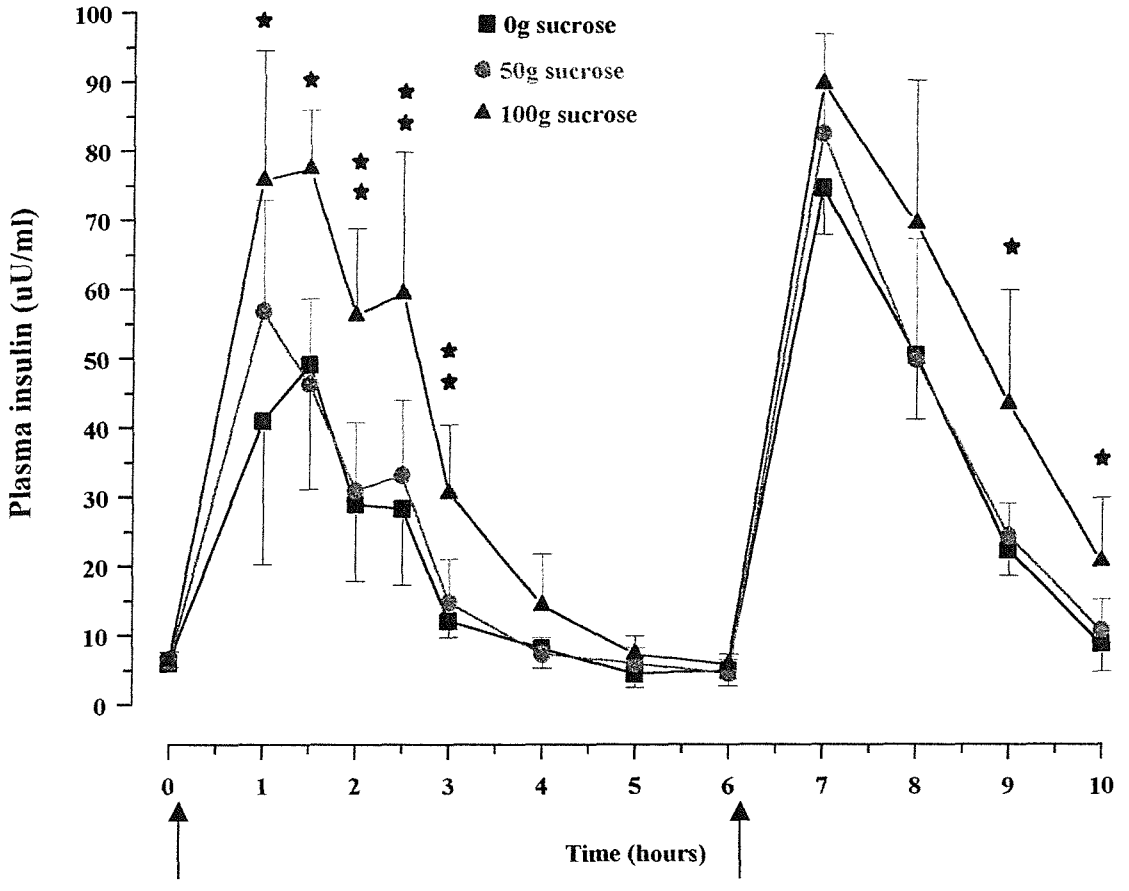
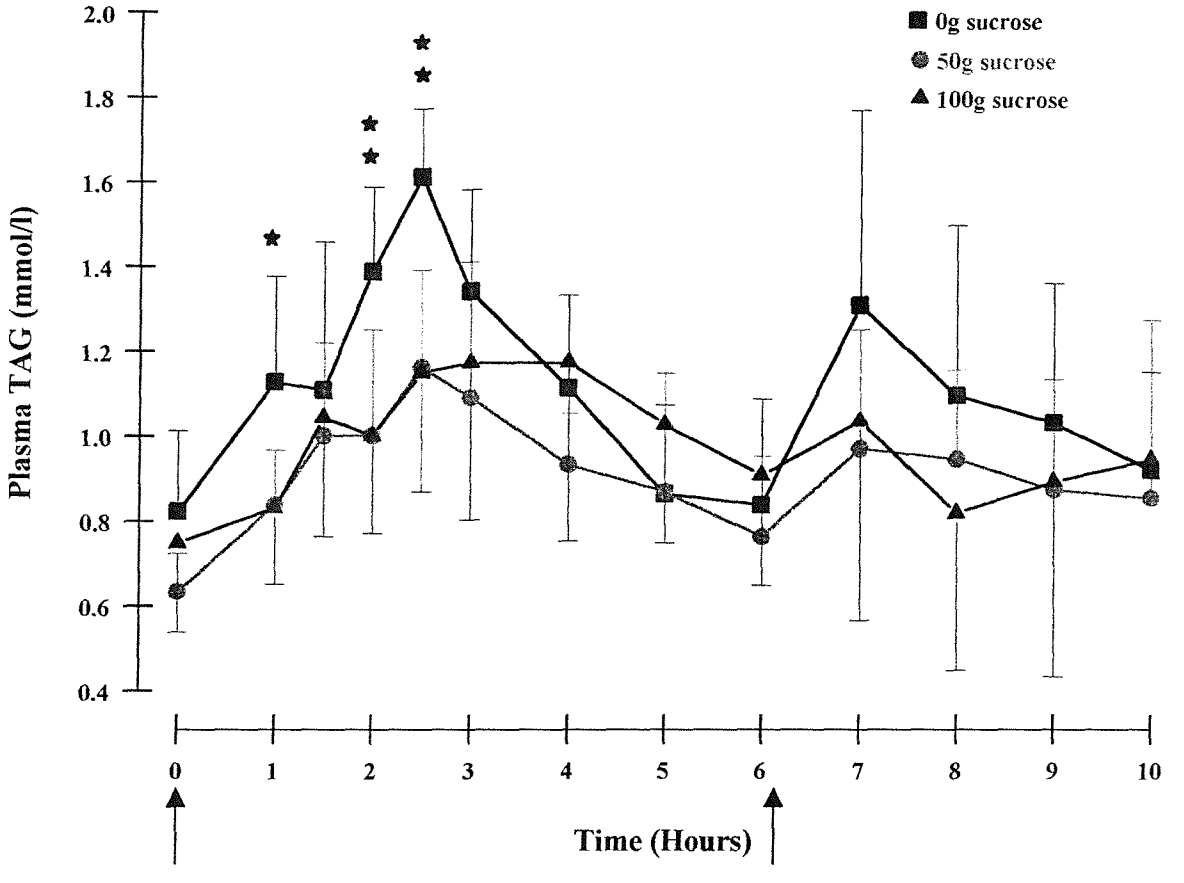


Figure 6.7 Plasma insulin concentration (uU/l) measured over a ten-hour study period in a group of six subjects following consumption of a reference testmeal with varying sucrose loads (group mean ± SD).



* Significant difference between 0g/100g sucrose trials ($p<0.05$)
** Significant difference between 0g/100g and 50g/100g sucrose trials ($p<0.05$)

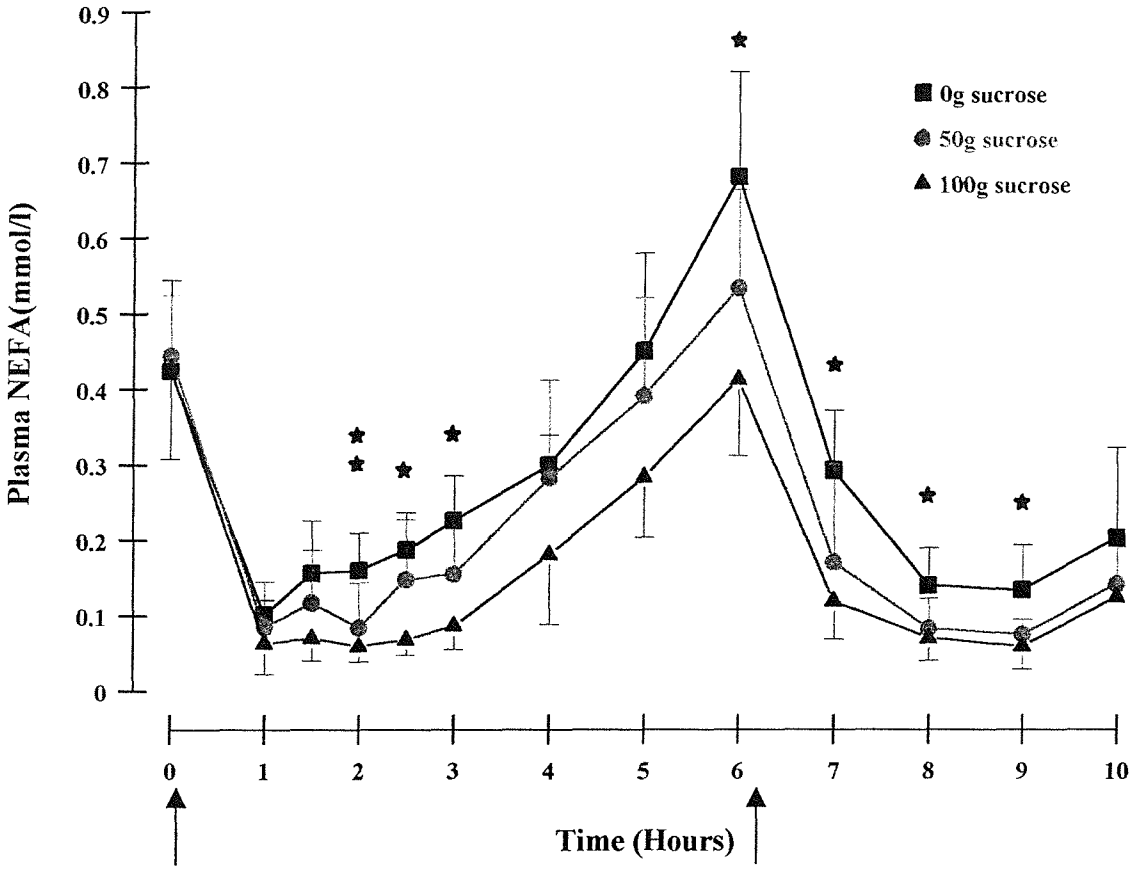
Figure 6.8 **Plasma triglyceride concentration expressed in mmol/l plasma over a ten hour study period, shown in a group of six subjects following consumption of a reference testmeal with varying loads of sucrose (group mean ± SD)**



* Significant difference between 0g/100g sucrose trial ($p<0.05$) only

** Significant difference between 0g/100g and 0g/50g sucrose trial ($p<0.05$)

Figure 6.9 **Plasma non-esterified fatty acid concentration expressed in mmol/l over a ten hour study period, shown for a group of six subjects, following consumption of a reference testmeal with varying sucrose loads (group mean ± SD)**



* Significant difference between 0g/100g sucrose trial ($p<0.05$)

** Significant difference between 0g/100g and 0g/50g sucrose trials ($p<0.05$)

Figure 6.10 **^{13}C-Palmitic Acid concentration (ug/ml plasma) in the TAG Fraction of plasma over the initial six hours of a ten hour study period, in a group of six subjects, following consumption of a reference testmeal with varying sucrose loads (group mean ± SD)**

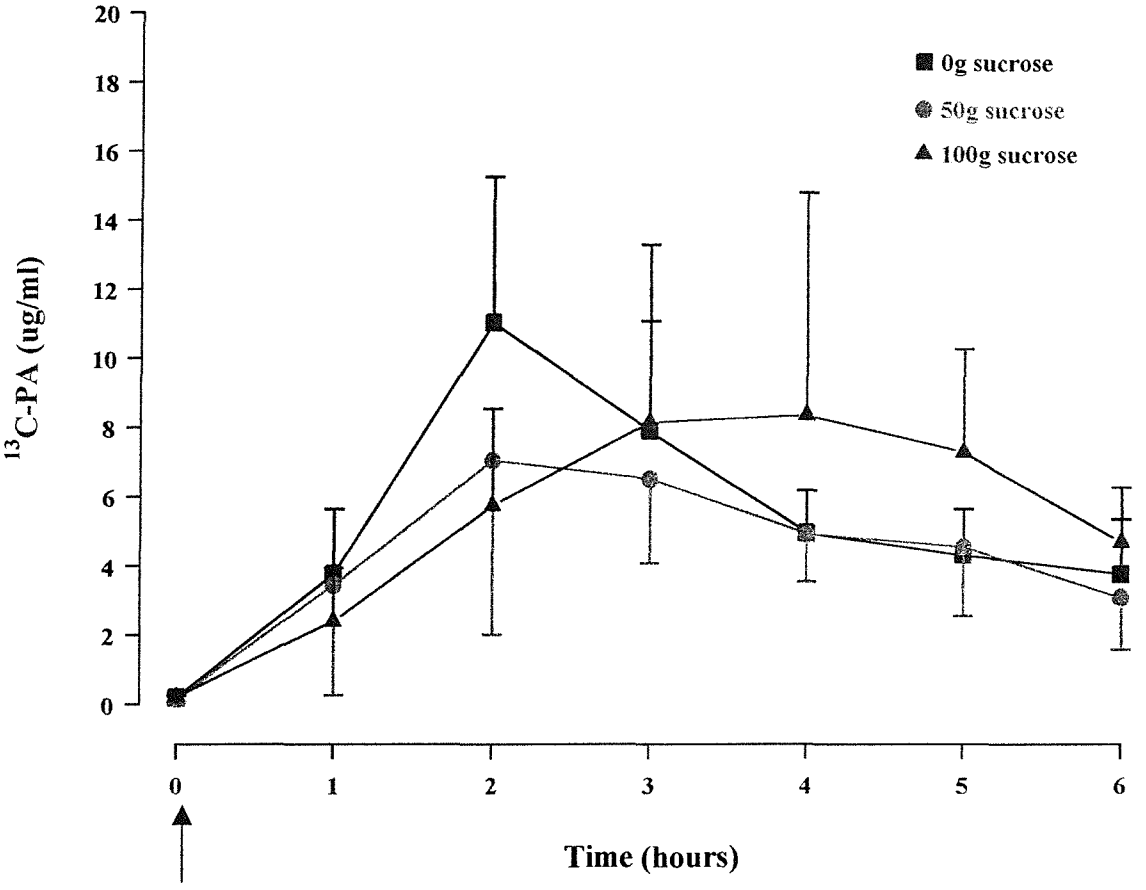
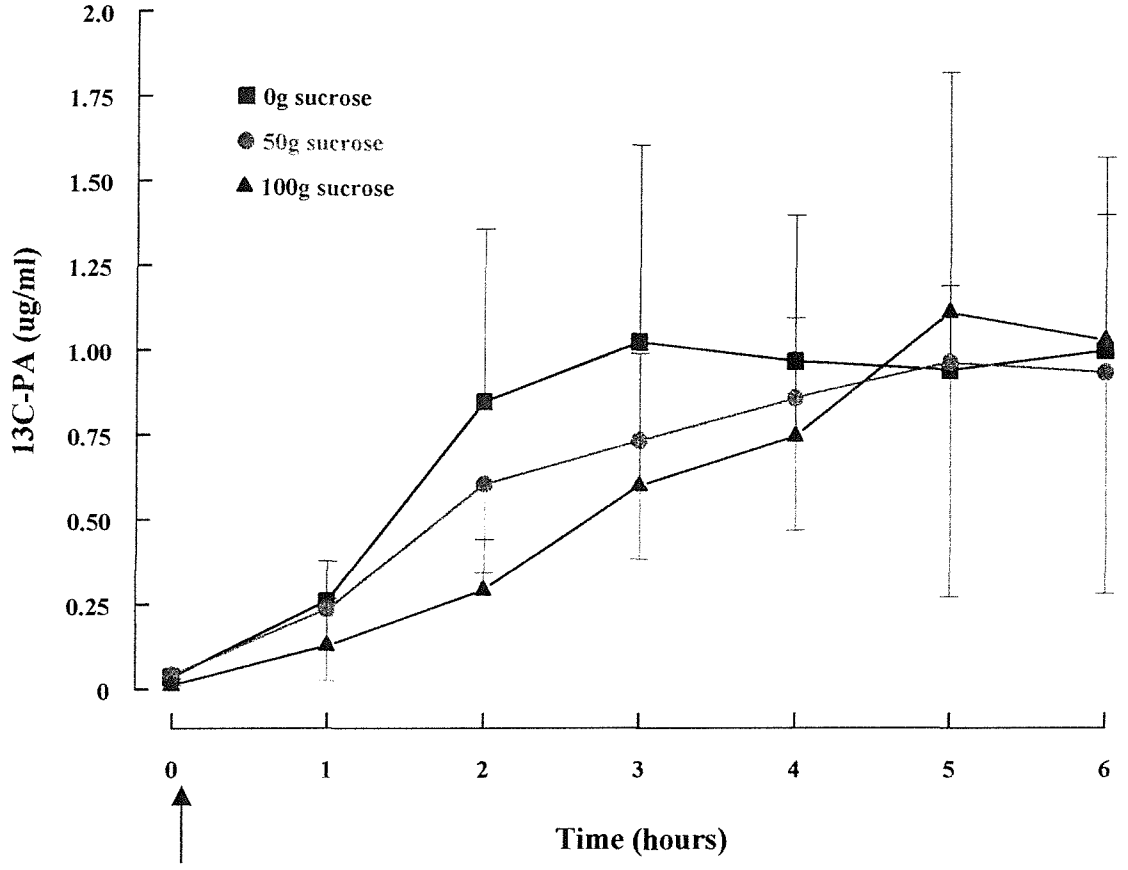


Figure 6.11 **^{13}C-Palmitic acid concentration (ug/ml plasma) in NEFA fraction of plasma over the initial six-hours of a ten hour study period, in a group of six subjects, following consumption of a reference testmeal with varying sucrose loads (group mean ± SD)**



6.6 Discussion

The aim of this present study was to examine the dose-response interaction between the addition of sucrose to a reference testmeal, and postprandial lipid metabolism. Several previous studies have attempted to describe the mechanisms underlying this process but none have reached similar conclusions possibly due to the use of single macronutrient meals, such as cream and sugar. This study differed because it was carried out using mixed meals with stable isotopes to differentiate between meal and endogenous lipid in terms of oxidation, and appearance in the circulation. Results show that the addition of sucrose to a testmeal has a profound effect on postprandial lipid metabolism.

6.6.1 Metabolic disposal of [1,1,1-^{13}C]tripalmitin

The appearance of $^{13}CO_2$ on the breath following ingestion of a ^{13}C-labelled lipid is used as a marker of the oxidation of the lipid, and therefore assumptions can be made regarding the partitioning of the lipid from the diet. In the study described in the present chapter there was a decrease of $^{13}CO_2$ on the breath as the amount of sucrose in the meal increased, suggesting a decrease in the oxidation of dietary lipid. This was expected, primarily due to the increase in carbohydrate oxidation, and the suppression of lipid oxidation by the increasing insulin concentrations.

In addition to a decrease in total $^{13}CO_2$ on the breath, the peak $^{13}CO_2$ excretion did not occur until 8 hours postprandially with the 100g sucrose meal, a phenomenon not seen with the other testmeals., which suggests that the label is either inhibited from entering the circulation due to effects on gastric emptying, or the labelled TAG is not hydrolysed in the circulation releasing fatty acids to be oxidised. It is unlikely that there is a delay in the gastric emptying of the dietary lipid as plasma TAG concentrations exhibit similar initial responses to a meal irrespective of the amount of sucrose. As this is this first known study to use stable isotopes to investigate the effect of meal carbohydrates on lipid metabolism, no direct comparisons can be made. However, glucose and insulin are known to inhibit lipid oxidation [Sidossis *et al.* 1996], and as meal lipid oxidation accounts for a large proportion of total oxidation, on the high

carbohydrate trial when insulin is at high concentrations the oxidation of the ^{13}C-labelled lipid is directly inhibited. Hyperinsulinaemic clamp techniques show a decrease in the oxidation of total lipid and tracer oxidation, [1-^{13}C]oleate when administered intravenously [Sidossis *et al.* 1996], which is in agreement with the present trial using oral administration of the label. There were clear and marked differences between individuals, suggesting metabolic limits for the oxidation of lipid. In some subjects oxidation was raised and remained at a high level, whereas in others this is not the case. There is a known variation in ^{13}C-oxidation between subject groups [Jones *et al.* 1998] yet such large inter-individual variation was not expected in this subject group.

Significant effects of the 100g sucrose meal were observed only when the total breath $^{13}CO_2$ excretion was measured over the initial 10 hours of the study day. The significant effect of the 100g sucrose meal was diminished when the whole 24 hour period was taken into account, suggesting that when subjects are not under supervision it is more likely that the results for the 10 to 24 hour period will not be a clear indication of the metabolism of the [1,1,1-^{13}C]tripalmitin. Subjects may have consumed food or taken part in physical activity which may have affected the results. As there is no method to prevent subjects not adhering to the study protocol once leaving the unit, it is better to measure breath $^{13}CO_2$ over the initial 10 hour period to give a clear indication of the effect of the sucrose alone rather than other foods which may have been consumed after leaving the unit.

6.6.2. Substrate oxidation

Few studies have examined the effects of carbohydrate on lipid oxidation, with the majority of published literature concentrating on postprandial responses in the circulation [Cohen *et al* 1988; Grant *et al* 1994]. A previous study showed that ingesting a combination of lipids and carbohydrates stimulates the action of lipoprotein lipase on chylomicron TAG leading to direct release of fatty acids into the plasma and increased fat oxidation [Griffiths *et al* 1994]. However in our study the opposite was found and an increase in the amount of carbohydrate in a meal led to a decrease in fat oxidation.

Oxidation of different substrates appears to take place in a hierarchical order, some substrates being more readily oxidised than others, with carbohydrate more

oxidisable than lipid due to the size of the body stores. The consequence of the 'oxidative hierarchy' is that when any macronutrient is in excess, fat tends to be stored [Frayn 1995]. This suggests that the decrease in lipid oxidation seen may be merely due to the excess of carbohydrate in the system that must be oxidised as the capacity of the carbohydrate stores is small. Another explanation for the decrease in lipid oxidation is that the increase in glucose and insulin determines fatty acid oxidation by controlling the rate of long-chain fatty acid entrance into the mitochondria [Sidossis *et al* 1996]. In a study examining different levels of carbohydrate in a meal, high fat meals (low CHO) elicited an enhanced production of NEFA accompanied by greater fat oxidation and less carbohydrate oxidation [Whiteley *et al* 1997]. The glucose-fatty acid cycle proposed by Randle *et al* [1963] supports this finding suggesting a reciprocal relationship between carbohydrate and lipid oxidation. Therefore it could be suggested that the increase in the carbohydrate content of the meal is in fact leading to increased fat storage, and an increased risk of obesity. However none of the previous studies have been able to differentiate between meal lipid and endogenous lipid.

It appears that although total lipid oxidation decreases with increasing carbohydrate, exogenous lipid oxidation remains relatively constant. This is shown as exogenous oxidation decreases from 13.6g/10 hours to 9.7g/10 hours, a relatively small decrease when compared to the increase in carbohydrate. Exogenous oxidation remains partially suppressed until 7 hours postprandially when normal values ensue. In contrast, endogenous lipid oxidation is greatly suppressed, and levels fall from 13.4g/10 hours on the 0g sucrose trial to only 1.4g/10 hours following the 100g sucrose trial. This suggests that oxidation of endogenous lipid is suppressed, either at the level of oxidation or due to suppression of release of NEFA from adipose tissue. The presence of insulin inhibits the release of endogenous fatty acids from adipose tissue by the inhibition of hormone sensitive lipase, and also inhibits the oxidation of fatty acids [Sidossis *et al.*1996]. The insulin-mediated suppression of NEFA release from adipose tissue is a normal response following a mixed meal [Coppack *et al.*1990]. It would appear from the data that the release of fatty acids from adipose tissue is the most important factor here as plasma NEFA concentrations fall with the 100g sucrose meal. From ^{13}C-NEFA data, the 'overshoot' of NEFA seen on the low carbohydrate trial can be attributed to a lack of suppression of HSL NEFA release. There are no differences in ^{13}C-NEFA concentration

at 6 hours postprandially between the trials, but substantial differences in total NEFA concentrations. The difference is attributable to endogenous NEFA, that is, as the carbohydrate content of the meal increases, insulin-mediated inhibition of HSL increases and therefore the release of endogenous NEFA decreases. Suppression must be almost complete, to allow such a large decrease in oxidation. In contrast, suppression of oxidation by insulin may not be as effective, as a large proportion of exogenous lipid remains oxidised. In conclusion, therefore, it appears that total lipid oxidation is decreased at the expense of endogenous lipids rather than lipid from the meal, due to the insulin mediated suppression of NEFA release from adipose stores.

Total carbohydrate oxidation was expected to increase with increasing intake of carbohydrate due to the observations of Schutz *et al* [1989], that whilst lipid intake does not appear to promote oxidation, carbohydrate intake is proportional to its oxidation. Therefore as carbohydrate intake increases, as does its metabolic partitioning towards oxidation. This is probably due to the decreased capacity to store carbohydrate as opposed to lipid. High carbohydrate meals have been shown to increase the overall respiratory exchange ratio (RER) over a study period [Whiteley *et al* 1997] suggesting an increase in the amount of carbohydrate being oxidised. In the present study, increasing carbohydrate intake was found to lead to increased oxidation. However, oxidation was not directly related to the intake with 102.1g oxidised per 10 hours on the 0g sucrose trial, 124.4g on the 50g sucrose trial and 159.7g on the 100g sucrose trial. In some subjects there appeared to be a limited capacity to increase oxidation with intake, whilst in others oxidation was more proportional to intake. This highlights the large inter-individual differences suggested previously, although differences between individuals are not as marked as for some of the other measures. Insulin mediated uptake of glucose to the tissues appears to decrease the levels of glucose in the circulation, and also appears to increase the storage of carbohydrate as glycogen. When a large amount of carbohydrate is ingested, glycogen stores could be saturated leading to increased glucose in the circulation and therefore an increased need for clearance, perhaps by oxidation.

6.6.3 Plasma Glucose concentrations

Traditionally, dietary carbohydrates were assigned glycaemic indices, calculated from the measured glycaemic response to a portion of test food that contains 50g of available carbohydrate from a standard food eaten by the same subject [Jenkins *et al.* 1981]. Simple sugars are known to elicit a greater glycaemic response than complex carbohydrates, with more advanced *in vitro* methods of measuring the glycaemic responses of foods available [Englyst *et al.* 1999]. Therefore with the addition of 50g and 100g of sucrose to a testmeal, a greater glycaemic response was expected to occur than with the 0g sucrose trial, although this was not the case. Following the first meal (after 12h without food), there was no significant change in plasma glucose concentration on either testmeal suggesting a rapid clearance of glucose from the circulation in this subject group. In NIDDM patients, the clearance of sugar from the circulation is not as effective resulting in hyperglycaemia [Abraha *et al.* 1998]. Following the second meal, there was an increase in plasma glucose concentration with all meals, possibly reflecting the subjects postprandial state from the first meal (6h postprandially). It has been suggested since completing the study that the glycaemic response to the meal could have occurred during the first hour after the meal was consumed, and have fallen back to baseline by the time the 1h blood specimen was taken in accordance with previous studies [Griffiths *et al* 1994; Whiteley *et al* 1997], however this needs further investigation. In addition, the testmeal contained lipid, carbohydrate and protein, which may lead to a decreased glycaemic response.

There were large differences between individuals, with some subjects showing a marked glycaemic response to the carbohydrate in the meal, and others showing little response, suggesting that there are individual metabolic limits in the control of glucose concentration in the circulation, or perhaps varying levels of insulin resistance and sensitivity within the subject group. Good glycaemic control was expected in this subject group as they were all young and healthy, so the decrease in plasma glucose at 1 hour could reflect this. Such well-controlled glycaemic responses may not occur if a similar testmeal was fed to a group of diabetic patients, in agreement with Abraha *et al.*[1999]. The increase in glucose concentration in the plasma following the second meal suggests

that uptake of glucose to cells was not complete as stores are full from the first meal, six hours previously, resulting in an overspill of glucose into the circulation.

In conclusion, glucose concentration in the plasma appears to be relatively constant for all intakes of carbohydrate, although it appears that individuals have different limits for the control of glucose homeostasis, which may reflect differences between subjects in levels of insulin sensitivity and resistance.

6.6.4 Plasma Insulin

In the present study the consumption of increasing sucrose loads resulted in a subsequent rise in plasma insulin concentration. Insulin is secreted due to a rise in plasma glucose levels, which may have been missed in this study by not blood sampling at 15-30 minutes postprandially. Similar findings were seen in a study by Abraha *et al.*[1998] in which subjects showed little glycaemic response but a greater insulinaemic response to a meal. Our findings are in agreement with other studies examining the effect of carbohydrates on lipid metabolism. Shishehbor *et al* [1998] found a significant increase in plasma insulin with a high carbohydrate meal (1.9g/kg body weight) compared to a low carbohydrate meal (0.3g/kg body weight). In introducing three different levels of carbohydrate into the trial a dose-response relationship between the increase in carbohydrate and insulin can be seen. Insulin rose from 262.5 uU/l/10 hours with no added sucrose to 288.3uU/l/10 hours with 50g of sucrose, which increased to 415.7uU/l/10 hours for the 100g sucrose trial. Between the 0g and 50g trials, there was only a 9.5% increase in insulin concentration for the group as a whole, however, with the addition of 100g, an overall increase in plasma insulin concentration of over 40% was observed. Following the second meal, there appeared to be an upregulation in insulin secretion with a greater magnitude in plasma insulin responses shown.

The concentration of insulin in the circulation is known to be a potent stimulator of lipoprotein lipase [Karpe, 1997] and inhibitor of hormone sensitive lipase [Frayn, 1998] even a low levels. Therefore with insulin concentrations increasing in a stepwise fashion with increasing carbohydrate load, it would be expected that both TAG and NEFA concentrations would also appear in the circulation with varying concentrations. However this does not appear to be the case as plasma TAG concentrations increase with

the addition of 100g of sucrose, suggesting that it is not merely insulin which predicts the concentration of TAG in the circulation.

6.6.5 Plasma Triglyceride Concentration

The present study has shown that the addition of 50g of sucrose to a mixed meal resulted in a decrease in the magnitude of plasma TAG, whilst the addition of 100g of sucrose resulted in an increase in the duration and magnitude of plasma TAG concentrations. It was obvious from the results that more than one explanation of the mechanisms behind the results is needed to fully understand the processes involved, as 50g of sucrose leads to a decrease in PPL and 100g leads to an increase.

Traditional diet based studies examining the effect of low-fat, high-carbohydrate feeding have found conflicting results on plasma triglyceride concentrations based on fasting values [Cominacini *et al* 1988]. Chen *et al* [1993] found the magnitude of postprandial responses was significantly increased following a low-fat, high-CHO, 2-week feeding pattern. However there has been much debate as to whether the rise in postprandial TAG seen was due to chylomicron TAG, from the meal, or from VLDL-TAG, from endogenous sources. Chen *et al* [1995] measured the postprandial response to a fat load following a low fat, high CHO feeding regimen, and demonstrated significantly greater accumulation of chylomicrons and chylomicron remnants than after an average 45% energy from fat feeding regimen. In addition endogenous VLDL-TAG was increased due to a decrease in catabolism and an increase in secretion with a high CHO diet. However it appears that the effect of carbohydrate on fasting lipid parameters depends upon the type of carbohydrate consumed in the diet.

The acute effect of carbohydrate on postprandial lipaemia has been examined in many studies [Mann *et al* 1971; Cohen *et al* 1988; Grant *et al* 1994], yet the results have been conflicting because of the use of differing fat and carbohydrate sources. Cohen & Schall [1988] described a significant increase in lipaemia following ingestion of 100g of sucrose with 40g fat, compared to a fat only meal. The increase in plasma TAG following ingestion of sucrose may be due to a decreased clearance of the meal TAG [Grant *et al* 1994], but this would appear to be unlikely as insulin is known to be a potent stimulator of lipoprotein lipase. There may be three other possible mechanisms

involved; 1) increased intestinal lipid absorption, 2) stimulation of endogenous TAG synthesis, or 3) decreased TAG rich lipoprotein hydrolysis [Mann *et al* 1971]. The majority of such studies used a combination of fat and carbohydrate sources but did not use mixed meal, however in one study an increase in plasma TAG levels were seen with the addition of sucrose to a mixed meal [Hayford *et al* 1979], yet the meals were consumed in a liquid formula. In contrast, Whiteley *et al* [1997] found a decrease in lipaemia with increasing carbohydrate in isoenergetic testmeals. It has been suggested that when 'normal' foods are consumed as part of a testmeal, this reflects a typical food consumption in the individual, and therefore typical gastric emptying and secretion rates in the body.

Earlier results have shown an increase in plasma insulin levels with increasing carbohydrate in the testmeal, which is known to stimulate LPL on the endothelial surface [Olivecrona *et al* 1993]. Therefore clearance of TAG from the circulation would be maximized with the addition of sucrose, by LPL TAG hydrolysis in the capillaries. A suppression of the release of TAG from the liver would also be facilitated by insulin, with increased uptake of chylomicron remnants to the liver, which would result in a net storage of lipid allowing the oxidation and clearance of carbohydrate from the system. With a further increase in carbohydrate, it is possible that the system has become saturated with insulin, therefore preventing further suppression of release of VLDL-TAG from the liver, and perhaps decreased clearance from the circulation. Increased re-esterification of fatty acids and de-novo lipogenesis may result in an increase in the secretion of VLDL-TAG, leading to a competition for the clearance of chylomicron-TAG and VLDL-TAG. Therefore the rise in plasma TAG seen may well be due to a decreased clearance of chylomicron TAG secondary to an increase in secretion [Hollenbeck 1993]. Jepessen *et al* [1994] suggest that whether the primary effect of carbohydrates are to enhance production or decrease removal of endogenous TAG, the consequent increase in TAG pool size would act to decrease the removal of all TAG rich lipoproteins. Therefore there would be an increase the postprandial concentration of TAG-rich lipoproteins of intestinal origin.

The type of carbohydrate used in testmeals is also of great importance. Sucrose has been thought to be hypertriglyceridemic due to its fructose content [Hollenbeck, 1993; Grant *et al* 1994]. This has been supported through work examining the effects of

fructose alone on lipaemia. Jepessen *et al* [1994] found a significant increase in postprandial TAG concentrations, particularly chylomicron TAG with the addition of fructose. Similar results were found both in normal and diabetic subjects [Abraha *et al.* 1998]. The ability of fructose to accentuate postprandial lipaemia would result from a fructose induced increase in hepatic TAG synthesis and secretion leading to competition between triglyceride rich lipoproteins (TRL) of exogenous and endogenous origin. The net effect would be an increase in postprandial concentrations of TRL [Jeppesen *et al.*1995]. However this still does not explain the differences seen between the 50g and 100g trials. If the fructose moiety in sucrose is thought be causing an exaggerated lipaemia, why is this not the case for the 50g trial? This could be explained, at least in part by the observation of Nuttall *et al.*[1992] who suggest that although the dose response relationship for the effect of fructose on PPL has not been adequately tested, there is a marked effect of 50g of fructose on the glycaemic response with a much lesser effect of 35g of fructose. This suggests that 50g of sucrose does not contain a sufficient amount of fructose to see the potentiation effects described in previous studies, whereas the addition of 100g of sucrose presents sufficient fructose to elicit such responses.

There are two explanations as to the mechanism by which fructose may lead to a potentiation of PPL. Firstly fructose induced re-esterification of free fatty acids and de novo lipogenesis may result in an increase in the liver synthesis and secretion of VLDL-TAG [Mayes, 1993; Hollenbeck, 1993]. Secondly, fructose may directly impair the clearance of TRL by inducing physical and chemical changes in the VLDL-TAG particles such that they are removed less effectively from plasma [Mamo *et al.*1979; Hirano *et al.*1989]. This mechanism may also contribute to the fructose induced postprandial accumulation of TAG and TRL of intestinal origin. To summarise, it is clear from the present study that the addition of 50g of sucrose to a mixed meal results in an increased clearance of TAG from the circulation. With the addition of 100g of sucrose, the fructose moiety may cause a stimulation of VLDL-TAG synthesis and secretion resulting in a competition between exogenous and endogenous TAG for clearance by LPL. The net result is a prolongation of lipaemia.

In summary from measurements of total plasma TAG, there are some differences of opinion over the effect of carbohydrates depending upon the testmeal involved. In addition the confusion is enhanced by the lack of differentiation between intestinally and

endogenous derived TAG-rich lipoproteins. Our study is unique as ^{13}C-labelled lipids in the circulation can be used to begin to understand the role of exogenous and endogenous TAG in the postprandial response. The appearance of ^{13}C-palmitic acid in the TAG fraction of plasma signifies the concentration of meal TAG in the circulation at the time of blood sampling. It denotes the product of secretion of TAG from the intestine and the removal by hydrolysis. The highest concentration of meal TAG was seen when no sucrose was added to the testmeal, suggesting an increased secretion or decreased clearance of TAG. In this instance it is suggested that the raised lipaemia is due to a decreased clearance due to the lack of insulin in the circulation. When 50g of sucrose is added, a decrease in the magnitude of lipaemia is seen, reflecting the total plasma TAG data. It is possible that this is due to an upregulation of LPL by the additional insulin, therefore increasing clearance. However, when 100g of sucrose is added, a prolongation of lipaemia is seen. As peak concentration is not seen until 3 hours postprandially, it may be that the secretion of chylomicron TAG is delayed. The prolongation of TAG seen with the total plasma TAG data was suggested to occur due to an increase in VLDL-TAG secretion, possibly due to the fructose moiety in the sucrose, which may lead to a competition between exogenous and endogenous derived TAG for clearance. Therefore we would expect to see a prolongation of ^{13}C-TAG in the circulation as it competes with VLDL-TAG for clearance on the endothelial surface, in agreement with Hollenbeck [1994]. With the increase in insulin, and subsequent stimulation of LPL, we would expect a greater hydrolysis of TAG and perhaps an overspill of NEFA taken up by the liver and re-secreted as VLDL-TAG. However, without direct measures of VLDL-TAG concentrations, it is impossible to make clear deductions regarding the mechanisms involved in this study. The role of a raised magnitude or prolongation of postprandial lipaemia in disease will be discussed in detail in chapter 7 (general discussion).

6.6.6 Plasma Non Esterified Fatty Acid Concentration

It is thought that elevated NEFA concentrations are central in the insulin resistance syndrome [Frayn, 1998], and for this reason may also play a role in other diseases such as CVD and obesity. The concentration of NEFA within the circulation is thought to be a major contributor to the risk of disease, and therefore the clearance of

fatty acids is a very important task. The release of NEFA into the circulation is a carefully controlled, insulin-mediated process. As lipid in the form of TAG enters in circulation, it is broken down to its constituent fatty acids by LPL, and uptake into the adipose tissue is facilitated by insulin. In normal circumstances an individual with a moderate carbohydrate intake would have an almost complete uptake of NEFA from the circulation, with the concentration of NEFA decreasing to near undetectable levels after a meal. In addition to this, insulin suppresses the breakdown of TAG in the adipose tissue to fatty acids by inhibiting HSL. This leads to a strong suppression of the release of endogenous NEFA into the circulation following a meal. As the insulin concentration produced in response to the meal decreases, inhibition is switched off and NEFA release begins to provide energy for oxidation.

This theory is supported by the data collected in the present study. NEFA concentration in the plasma decreased with increasing concentration of carbohydrate for the group as a whole. When no added sucrose was consumed, clearance of lipid to adipose tissue appeared to be complete, however, there was a marked overspill of NEFA into the circulation from 4 hours postprandially. The concentration of plasma NEFA varies markedly depending upon the type and amount of carbohydrate consumed [Wolever *et al* 1995]. Whiteley *et al* [1997] found similar results with a suppression of plasma NEFA with the addition of carbohydrate to a meal, with a relative elevation above baseline levels when no carbohydrate was added. This may reflect the large contribution of NEFA from circulating chylomicron TAG, NEFA spillover of fatty acids arising from LPL action on chylomicron TAG [Coppack *et al* 1990; Fielding *et al* 1996], or that the breakdown of TAG within adipocytes is not fully suppressed. This is supported because increasing the amount of sucrose in the meal results in an eradication of the overshoot past baseline, therefore suggesting that as the amount of insulin rises, inhibition of HSL becomes greater and the release of NEFA is greatly suppressed. It has been suggested that the increased suppression of NEFA with the addition of fructose could be due to a fructose induced acute increase in the sensitivity of lipolysis to suppression by insulin [Abraha *et al* 1998]. If this is the case, the fructose moiety in sucrose could be introducing a similar effect in the present study. In addition insulin is known to stimulate directly either fatty acid transport, or one or more of the enzymes involved in fatty acid esterification [Frayn, 1998]. A combination of increased uptake of

NEFA, use of NEFA for TAG re-synthesis and an inhibition of NEFA release could contribute to the decrease in NEFA concentration, which is seen with the addition of carbohydrate.

Once more the mechanisms underlying the effect of carbohydrates on plasma NEFA metabolism are constrained due to a lack of understanding of the differences between fatty acids released from the chylomicron TAG, and those released either from adipose tissue or VLDL-TAG. The present study is unique as the meal TAG is labelled with ^{13}C, allowing the fate of the meal fatty acids to be followed in the circulation. With no added sucrose, the highest concentration of meal fatty acids are seen in the circulation suggesting that although the chylomicron TAG has been hydrolysed, the resultant fatty acids have not been entrapped to tissues. In contrast, the results could show the increased hydrolysis of TAG, which is met by a similar rate of entrapment. In addition, this could merely signify a competition between exogenous and endogenous NEFA for clearance from the circulation. At 5-6 hours postprandially, ^{13}C-NEFA levels appear to plateau whereas total plasma NEFA rise past baseline levels. This suggests that this large overspill is due to release of fatty acids from adipose tissue when the insulin concentrations fall. With 50g of sucrose, ^{13}C-NEFA concentrations are reduced suggesting a decreased hydrolysis of TAG, or an increased entrapment. Total plasma NEFA concentrations are suppressed possibly due to the increase in insulin, and therefore a increased suppression of HSL. With 100g of sucrose, the concentration of ^{13}C-NEFA is decreased further, in agreement with total NEFA. Insulin may enhance the efficiency of fatty acid removal by the tissues, reflected by an increase in NEFA clearance. On the contrary, insulin is believed to play a role in the liver, where it channels NEFA to reesterification. Whether the increased clearance represents a 'true' stimulation of these pathways or simply reflects the decline in plasma NEFA concentration secondary to the inhibition of lipolysis remains to be determined [Bonadonna *et al* 1990]. Also what role does fatty acid oxidation play in the regulation of plasma NEFA concentration?

Large between individual differences were seen in plasma NEFA concentrations, and in ^{13}C-NEFA concentrations. It has been suggested that those subjects with raised plasma NEFA concentrations may be at an increased risk of disease in later life and that subjects with diagnosed diabetes have raised levels of plasma NEFA [Abraha *et al*

1998]. As such large differences are seen in this subject group, greater differences would be expected if the study was repeated in diabetics or those with dyslipidaemia. The association of raised NEFA concentrations and disease will be discussed further in chapter 7 (general discussion).

In summary, increasing the amount of sucrose within a mixed meal appears to have several effects on plasma NEFA concentrations. From this study it is possible to state that whilst increasing sucrose content of the meal decreases total plasma NEFA concentrations, the decrease is now known to be due to a reduction in endogenous NEFA. This could be due to the insulin-mediated suppression of HSL, and therefore a decreased release of NEFA from adipose tissue.

6.7 Summary and Conclusions

The aims of the study described in this chapter were to examine the effect of increasing the sucrose content of a testmeal on the postprandial responses to a mixed meal containing [1,1,1-^{13}C]tripalmitin. The results demonstrate that:-

1) Increasing the sucrose content of a meal led to an increase in carbohydrate oxidation with a decrease in lipid oxidation. The decrease in lipid oxidation was shown to be due to a decrease in endogenous fatty acid oxidation because of an insulin-mediated suppression of HSL derived NEFA release.

2) Glucose concentration in the circulation was closely controlled, reflecting the major role played by insulin in whole body homeostasis, with further studies necessary to determine the measurement of glucose appearance in the plasma prior to the first blood sample.

3) The magnitude of postprandial lipaemia decreased with the addition of 50g of sucrose possibly due to an increased clearance of TAG by an insulin-mediated stimulation of LPL. With the addition of 100g of sucrose, postprandial lipaemia was prolonged suggesting that the fructose moiety in sucrose caused a stimulation of hepatic VLDL-TAG synthesis and secretion. This may have resulted in competition

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CHAPTER 7

General Discussion

The aim of this chapter was to collate the results that have been reported in the previous chapters and to discuss their implications at a general level. Attention will be directed towards the within and between individual variability in measures of lipid metabolism, the first and second meal effects in terms of the appearance of dietary lipids in the circulation, the oxidation of macronutrient substrates and the potential role of the manipulation of lipid metabolism in association with disease.

7.0 Introduction

There is a well-established association between the consumption of dietary lipid and cardiovascular disease, obesity and insulin resistance. In particular this knowledge has been founded from large population based epidemiological studies using postabsorptive measures of circulating lipids as a basis for their observations. More recently the traditional view of cholesterol as the primary risk factor for disease outcome has been disputed, with more and more studies suggesting a role for postabsorptive plasma triacylglycerol levels as an independent risk factor for cardiovascular disease [Hokanson and Austin 1996]. In addition the magnitude of the increase in plasma TAG following a meal is now recognised to be an important marker for CVD [Groot *et al.*1991; Patsch *et al.*1992]. Despite the link between dietary lipid and disease, the mechanisms linking the consumption of dietary lipid with disease are poorly understood.

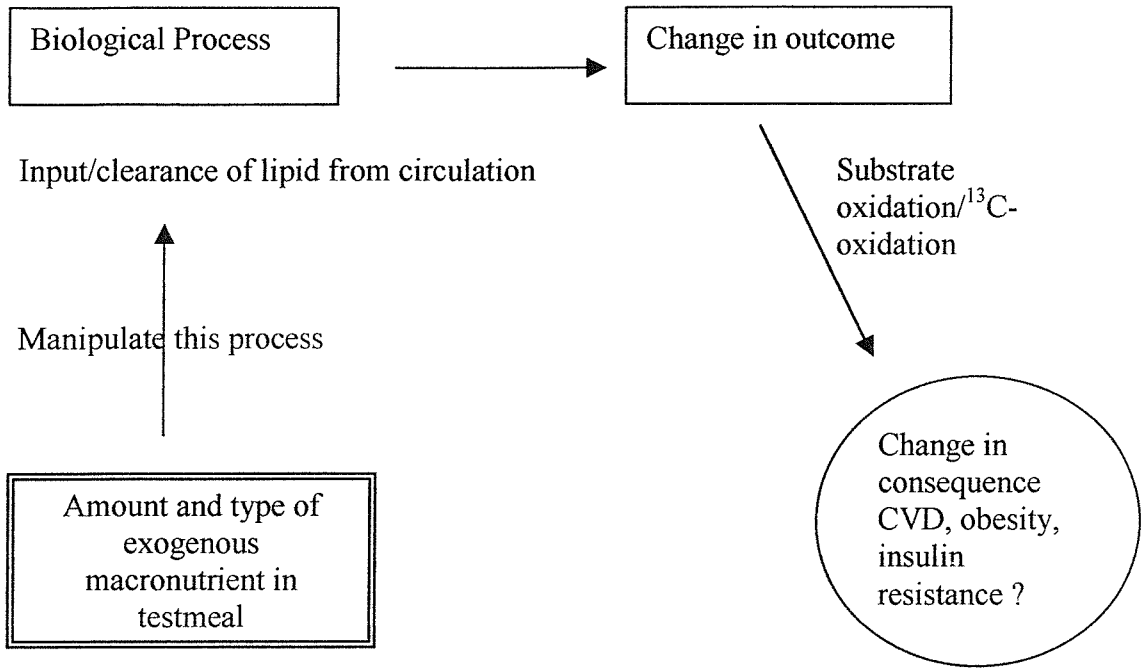
Many of the mechanisms involved in the metabolism of lipid, both from dietary and endogenous sources have been determined by traditional balance studies examining GI handling of lipids and more recently with the introduction of stable isotope tracer methods. In particular, mechanisms have been reported for the digestion and absorption of lipid [Carey, 1983], the transport of lipids in the circulation via lipoproteins [Griffin & Packard 1994], the uptake of fatty acids to cells [Frayn,1998], and the mechanism for the oxidation of substrates [Sidossis *et al.* 1995].

However many questions remain unanswered and of particular interest is the extent to which the way in which an individual will 'handle' a lipid meal can be manipulated, is

it possible to manipulate the appearance and clearance of lipid in the circulation, and the ultimate oxidation of the lipid ? This may lead to a manipulation of the risk promoters for disease such as raised TAG and NEFA in the circulation, and as such manipulate the disease outcome. Historically, the reduction of cardiovascular risk was determined with the use of lipid lowering drug therapy, either by reducing cholesterol or TAG concentrations in the plasma. Several studies in the literature have used a different approach and have examined ways of manipulating the postabsorptive and postprandial responses to lipid by manipulating the macronutrient balance of a diet or meal. Studies have shown that the amount [Murphy *et al.*1995] and type of fat [Knapper *et al.*1996], and the amount and type of carbohydrate [Mann *et al.*1971; Cohen and Berger 1990; Grant *et al.*1994; Jeppessen *et al.*1995] within a meal or diet can profoundly affect lipid metabolism, and lead to an increase or decrease in disease risk factors.

The research reported in this thesis employed traditional measures of lipid metabolism with innovative stable isotope tracer methodologies to examine the effect of manipulating the macronutrient balance of a meal on the postprandial metabolism of dietary lipid. The central hypothesis underlying this research was that the manipulation of the amount and type of macronutrient within a meal will affect the residence time of exogenous and endogenous lipids in the circulation in terms of appearance, clearance and entrapment of fatty acids to tissues. As a consequence, the oxidation of substrates will differ in the manipulation of the ratio of exogenous and endogenous fatty acid oxidation. This information will enable the development of a conceptual framework in which it would be possible to determine how altering the lipid and carbohydrate components of the meal or diet may alter lipid metabolism, and by inference the possible resultant effects on CVD, insulin resistance and obesity can be considered. This is shown in figure 7.1. By manipulating the amount and type of exogenous macronutrient in the testmeal, it may be possible to alter the rates of input and clearance of lipid from the circulation (biological processing of lipid). The concentration of lipid and carbohydrate in the circulation may in turn influence the balance of substrate oxidation, and the proportion of exogenous and endogenous fatty acids oxidised. By manipulating the meal composition, therefore, the risk of developing disease may be increased or decreased, for example, by resulting in raised TAG concentration, or decreased lipid oxidation. The specific aims of the research were stated in section 2.7.

Figure 7.1 Central hypothesis of the present research examining the influence of manipulating meal composition on lipid metabolism

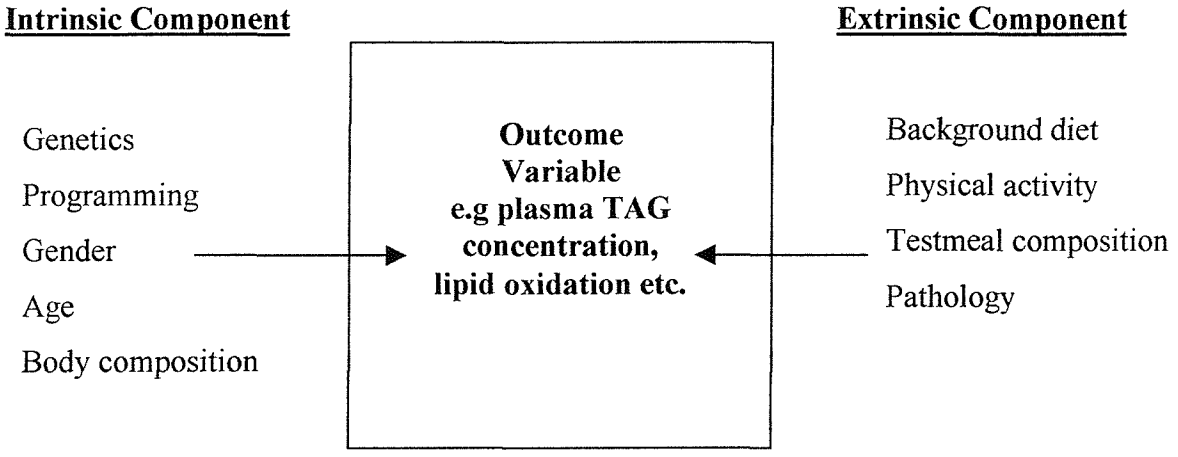


7.1 Within and Between Individual Differences in measures of lipid metabolism

The magnitude of within-individual variability in a measure is rarely measured due to time and cost constraints, despite the importance of knowing what influence variability can have on a particular outcome variable prior to applying an intervention. Within-individual variability may be due to intrinsic factors which cannot be controlled such as genetics, body composition or metabolism, or due to extrinsic factors such as diet, lifestyle or physical activity level. What was unclear was the extent to which the intrinsic factors governed the magnitude of variability, and how by controlling the extrinsic factors the level of variability could be decreased? How would the magnitude of variability differ in a subject population who were not similar? In the research reported in this thesis, diet, lifestyle, physical activity and to an extent body composition were controlled for within the subject group. Subjects were only selected for the studies if they met body composition criteria, i.e. to have a BMI between 20-30 kg/m^2. In addition, subjects were fed a fixed, prescribed diet for a period of days prior to the study (study 1-3 days; study2 -2 days), were asked to continue habitual physical activity and were admitted to the ward for 24 hours over the study period.

Between individual differences in lipid oxidation and the GI handling of lipids described by Jones *et al.*[1998] were dependant upon differences in both intrinsic factors (body composition, age, gender), as well as extrinsic factors (diet, lifestyle, physical activity), whereas in the research reported in this thesis variability was limited to intrinsic factors only. This is illustrated in figure 7.2.

Figure 7.2 Factors governing the magnitude of within and between individual variability in measures of lipid metabolism



Within-individual variability is governed by extrinsic components alone, whereas between individual variability is governed by both extrinsic and intrinsic factors. As the research reported in this thesis is based upon repeated measures in one subject group, differences observed between individuals are less important.

Measurement of the magnitude of intra-individual variability was reported in chapter 4, and a summary table of the results is shown in table 7.1. By knowing the magnitude of within-individual variability it was possible to predict the magnitude of effect required with an intervention to ensure that the effect observed was due to the intervention alone and not merely intra-individual variability.

Table 7.1 Comparison of outcome measures with percentage differences shown when subjects completed two identical trials to examine the postprandial metabolism of dietary lipid

Outcome Measure	Trial 1 (mean)	Trial 2 (mean)	Mean percentage difference
Stool ^{13}C-label (% administered dose)	1.2 %	1.1%	- 73.8 %
Absorption of ^{13}C-label (% administered dose)	98.8 %	98.9%	+ 0.9%
Breath $^{13}CO_2$ (% absorbed dose)	28.2 %	26.8 %	- 6.6 %
Plasma glucose (mmol/l/10 hours)	54.4	62.0	+ 12.9%
Plasma TAG (mmol/l/10 hours) AUC_0	8.6	8.6	+ 6.3 %
Plasma NEFA (mmol/l/10 hours)	2.2	2.2	+ 0.97%
Total lipid oxidation (g/10 hours)	28.2	31.3	+ 9.7 %
Total carbohydrate oxidation (g/10 hours)	107.9	103.8	- 3.4 %
Exogenous lipid oxidation (g/10 hours)	15.0	14.2	- 7.8 %
Endogenous lipid oxidation (g per 10 hours)	13.2	17.2	+ 26.3

The results suggest that with an anticipated effect size of 50% with an intervention that the changes in all outcome variables, except for stool ^{13}C-label excretion, would be due to the intervention alone, with a power of over 80% in all cases. Table 7.2 shows the magnitude of change with the application of a high fat or 100g of sucrose testmeal from the studies described in chapters 5 and 6.

Table 7.2 The magnitude of effect following the consumption of a high-fat or 100g sucrose testmeal compared to the reference testmeal or 0g sucrose testmeal

Outcome Variable	Percentage difference high-fat testmeal (mean± SD)	Percentage difference 100g sucrose study (mean± SD)
Stool ^{13}C-label (% administered dose)	48.2± 38.8	N/A
Breath $^{13}CO_2$ (% absorbed dose)	26.5± 15.0	29.8± 43.9
Plasma glucose (mmol/l/10 hours)	12.6± 14.2	3.2± 10
Plasma TAG (mmol/l/10 hours) AUC_0	34.6± 22.6	12.9± 21.7
Plasma NEFA (mmol/l/10 hours)	43.5± 31.4	55.1± 21.6
Total lipid oxidation (g/10 hours)	16.7± 17.8	83.6± 67.5
Total carbohydrate oxidation (g/10 hours)	6.1± 13.5	44.0± 8.5
Exogenous lipid oxidation (g/10 hours)	36.1± 14.3	33.6± 32.7
Endogenous lipid oxidation (g per 10 hours)	21.2± 64.3	162.5± 540.2

Intra-individual and inter-individual variability are dependant upon both intrinsic and extrinsic factors. By controlling for extrinsic factors such as diet, physical activity and lifestyle it was possible to examine the intra-individual variability. Intra-individual variability varied depending upon the outcome variable being measured. The magnitude of variability was used to calculate the percentage change in a variable required to indicate the change was solely due to the intervention applied. From the results it appeared that whilst concentrations of plasma TAG and glucose appear to be unaffected

by the consumption of a high-fat meal or 100g of sucrose testmeal, substrate oxidation and plasma NEFA concentration are profoundly altered. This suggests that the oxidation of substrates is closely regulated by the composition of the testmeal consumed.

7.2 Lipid in the circulation

Within a given individual the appearance of lipid in the circulation, in the form of both triacylglycerol and non esterified fatty acids, depends upon the composition of the meal consumed and the metabolic capacity of the individual to clear lipid from the circulation. The magnitude and duration of lipaemia was significantly greater when a high fat meal was consumed compared to a reference testmeal. In contrast, postprandial lipaemia decreased with the consumption of 50g of sucrose compared to 0g, but lipaemia was prolonged with the addition of 100g of sucrose. The testmeals were directly comparable as the basis of the testmeal, the emulsion and ham sandwich remained constant throughout all trials. In the repeatability and high fat trials, subjects also consumed 200g of orange juice, which provided 18g of carbohydrate, and on the carbohydrate trials, either 0g, 50g or 100g of added sucrose. The subject groups were different for the two studies, however, the same inclusion criteria were observed for both studies, resulting in similar characteristics between the studies. On the whole, subjects taking part in study one were older (25.5 years compared to 24 years), and had a greater BMI (23.2 kg/m^2 compared to 22.1 kg/m^2), but the differences were minimal.

The changes in plasma TAG concentration observed with the five meals is shown in figure 7.3. The greatest magnitude of plasma TAG was observed with consumption of the high fat meal. Following this, the next greatest concentration of plasma TAG was observed following consumption of the 0g sucrose meal, which suggests that despite the lipid load being consistent with the remaining trials, the lack of carbohydrate within this meal affects the clearance of lipid from the circulation, resulting in a raised magnitude of lipaemia. The remaining testmeals (reference testmeal, 50g sucrose and 100g sucrose) show a similar timecourse of lipaemia, although the duration of raised lipaemia is prolonged with the 100g sucrose testmeal. There was no indication of a biphasic response in plasma TAG appearance in the circulation following any of the testmeals in contrast to biphasic responses reported in a previous study [Shishebor *et al.* 1998]. Even with the introduction of 100g of sucrose a mean monophasic response was observed,

possibly due to the composition of the testmeal which was meal-based rather than in liquid form.

There were large inter-individual differences in postprandial lipaemia depending upon the individual capacity to clear lipids from the circulation. In particular, on the high fat trial, two subjects displayed a raised lipaemia. On the sucrose trials all subjects showed a greater variation in lipaemia between trials. As the subjects all received identical testmeals, the differences between subjects cannot be attributed to the changes in testmeal but may be due to differences in levels of insulin sensitivity, in the metabolic capacity to remove lipids from the circulation, in body composition or in genetic composition. Figure 7.4 show the individual subject plasma TAG responses to the five different testmeals. The between subject variation becomes particularly apparent from these figures and observations can be made regarding the individual capacity of the subjects to cope metabolically with a lipid testmeal. For example on the reference testmeal trial, subject 5 shows a much greater lipaemia than the remainder of the group, yet when the subject consumed the high fat meal there is virtually no change in the lipaemic response, suggesting that the maximal response to a meal was observed with the reference testmeal lipid concentration. What is unclear from the data is whether subject 5 was able to increase TAG clearance with the addition of the high-fat testmeal which allowed the concentration over the ten hour study period to remain similar to the reference testmeal, or if it merely reflects an inability to clear lipid from the circulation below a certain threshold resulting in a greater magnitude and duration of lipaemia. At the other end of the scale is subject 3 who displays a modest lipaemic response upon consumption of either testmeal suggesting a greater capacity for the clearance of lipid from the circulation, suggesting that subject 3 could consume even greater lipid loads and remain able to clear the lipid efficiently from the circulation. What is clear is that a raised magnitude and duration of postprandial lipaemia is known to be associated with an increased risk of disease [Groot *et al.*1991; Patsch *et al.* 1992], therefore it is better for the individual to be able to clear lipids from the circulation efficiently irrelevant of the amount of lipid consumed. In the situation of subject 5, it is less beneficial for the concentration of plasma TAG to remain elevated compared to the maximal clearance observed in subject 3. A similar situation is displayed with the sucrose trials, with a different subject group. Subject 3 displays the greatest lipaemia on the 0g and 100g sucrose trials, but only following the second meal on the 50g sucrose trial. Subject 6

shows an intermediate response on the 0g sucrose meal but this appears to be diminished with the introduction of sucrose. This data suggests that the underlying metabolism of dietary lipid may play an active role in the degree and length of postprandial lipaemia, and that this appears to be not only dependant upon the composition of the meal consumed but also on the individuals lifestyle, dietary and genetic constraints. There are also questions which remain unanswered such as how are the concentrations of circulating lipids and carbohydrate related to, and regulated by, the rate and extent of lipid oxidation? Is the concentration of lipid in the circulation regulated by the rate of fatty acid oxidation or vice versa?

The differences in plasma NEFA concentrations over the timecourse observed with the five meals is shown in figure 7.5. A similar timecourse of plasma NEFA concentration was observed with all the testmeals following the first meal of the day, with varying magnitudes of response. The greatest suppression of NEFA response was observed with the 100g sucrose trial, followed by the 50g sucrose trial, high-fat trial, reference testmeal trial, and the 0g sucrose trial. Following the second meal, a similar pattern was observed except for the high-fat trial. From 6 to 10 hours postprandially, there was an increased magnitude of plasma NEFA concentration which could possibly be due to an increase in CM-TAG hydrolysis but a lack of entrapment of fatty acids, or due to an increase in adipose tissue lipolysis. It is unlikely that the increase in NEFA concentration is due to an increase in adipose tissue lipolysis for two reasons. Firstly, the increase is observed immediately following the meal when insulin concentration would be at its highest, therefore inhibiting the action of HSL. Secondly the increased concentration of fatty acids in the circulation would inhibit the release of additional fatty acids from adipose tissue. Therefore it is probable that the excursion of NEFA following the second meal reflects an inability for the entrapment of fatty acids hydrolysed by LPL due to the increased amount of dietary lipid entering the circulation, but why does this inability to entrap fatty acids occur? Does fatty acid oxidation reach a maximal level above which not further fatty acids can be oxidised and therefore there is negative feedback on fatty acid transport into the muscle, leading to increased NEFA concentration in the circulation?

Therefore the changes in lipaemia observed for the groups as a whole in the various trials can be attributed to the change in macronutrient composition of the testmeal. The manipulation of the meal composition resulted in an increase in the

magnitude of lipaemia (high-fat meal; 0g sucrose meal), a decrease in the magnitude of lipaemia (50g sucrose meal), or an increase in the duration of lipaemia (100g sucrose meal). The possible mechanisms behind the manipulation in plasma lipids will be discussed in turn.

Figure 7.3 Plasma TAG concentration (mmol/l) over a ten hour study period following consumption of five different testmeals (group mean)

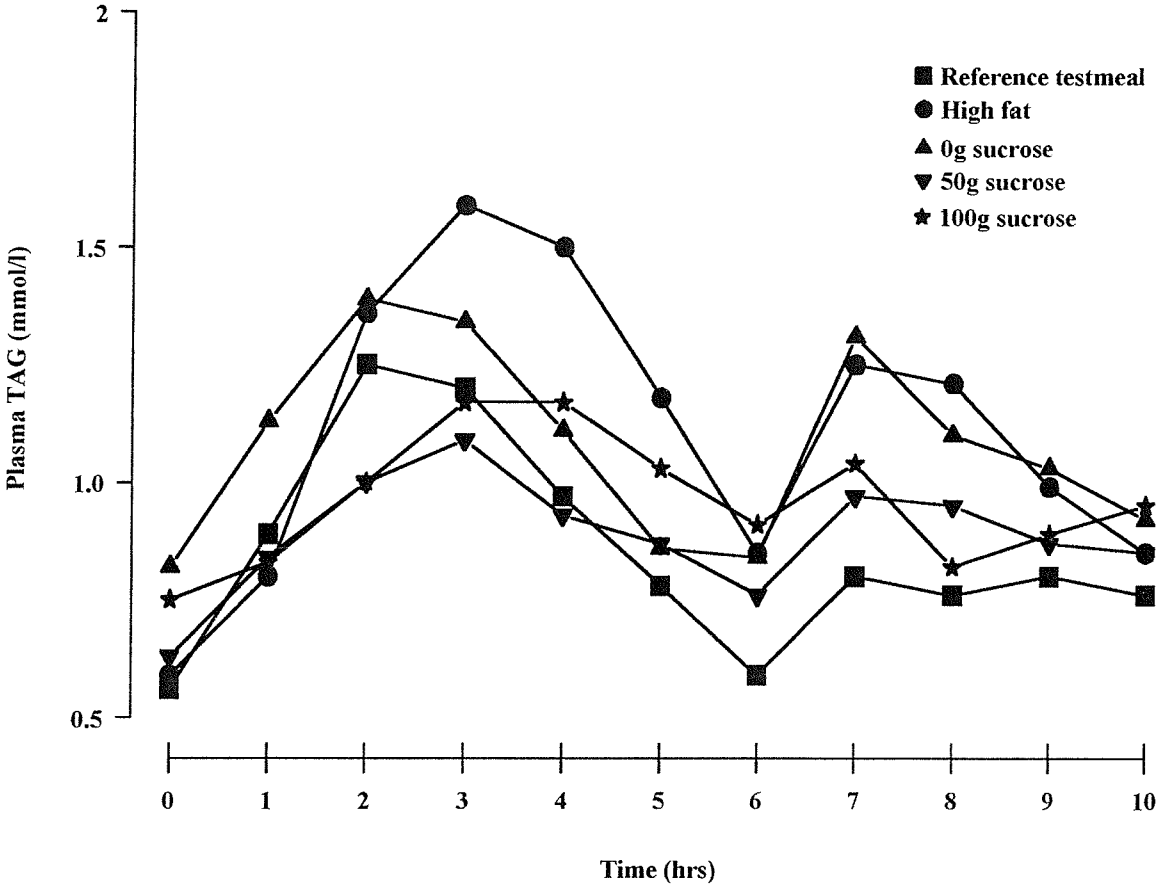
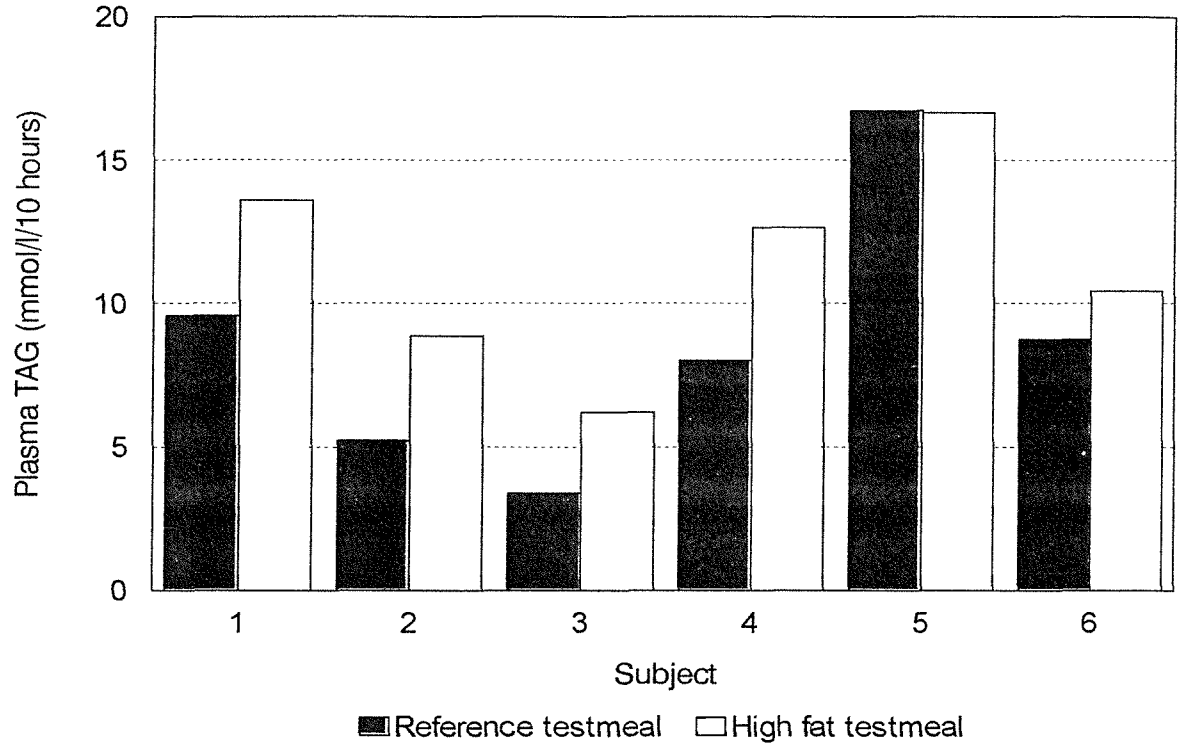


Figure 7.4 Total plasma TAG concentration (mmol/l/10 hours) over a ten hour study period following consumption of five different testmeals (group mean)

Study 1:-



Study 2:-

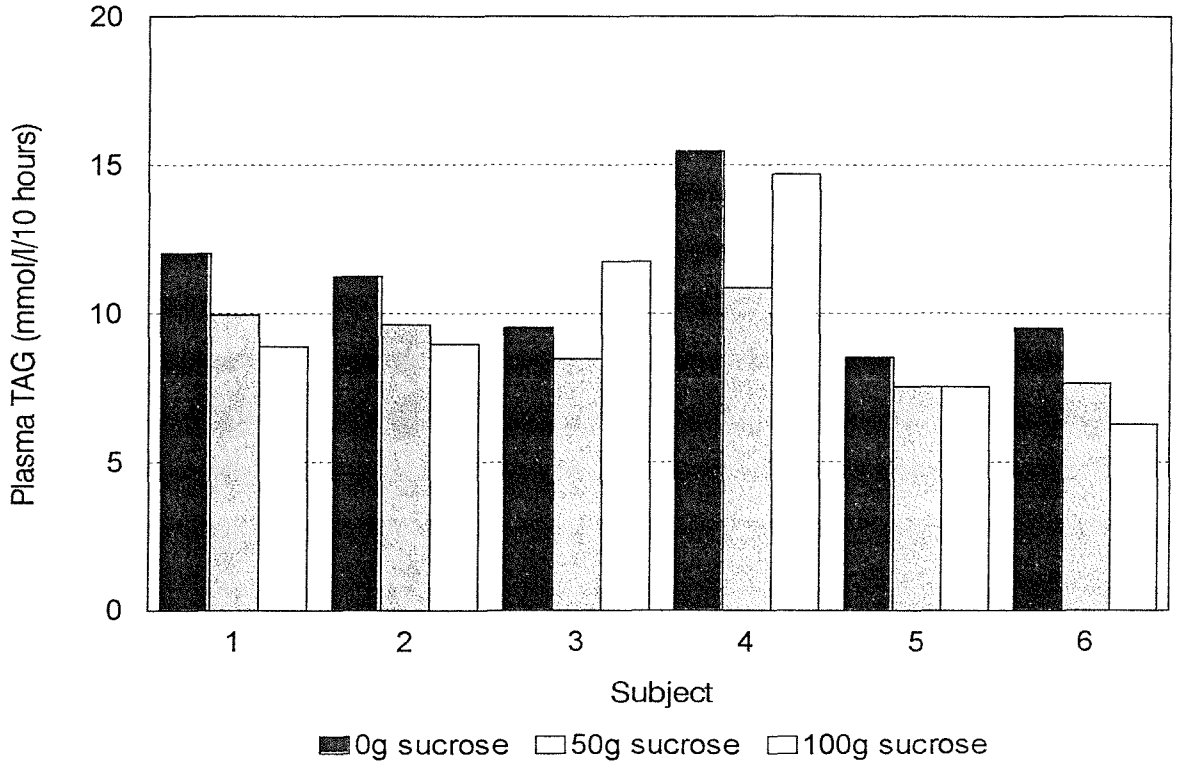
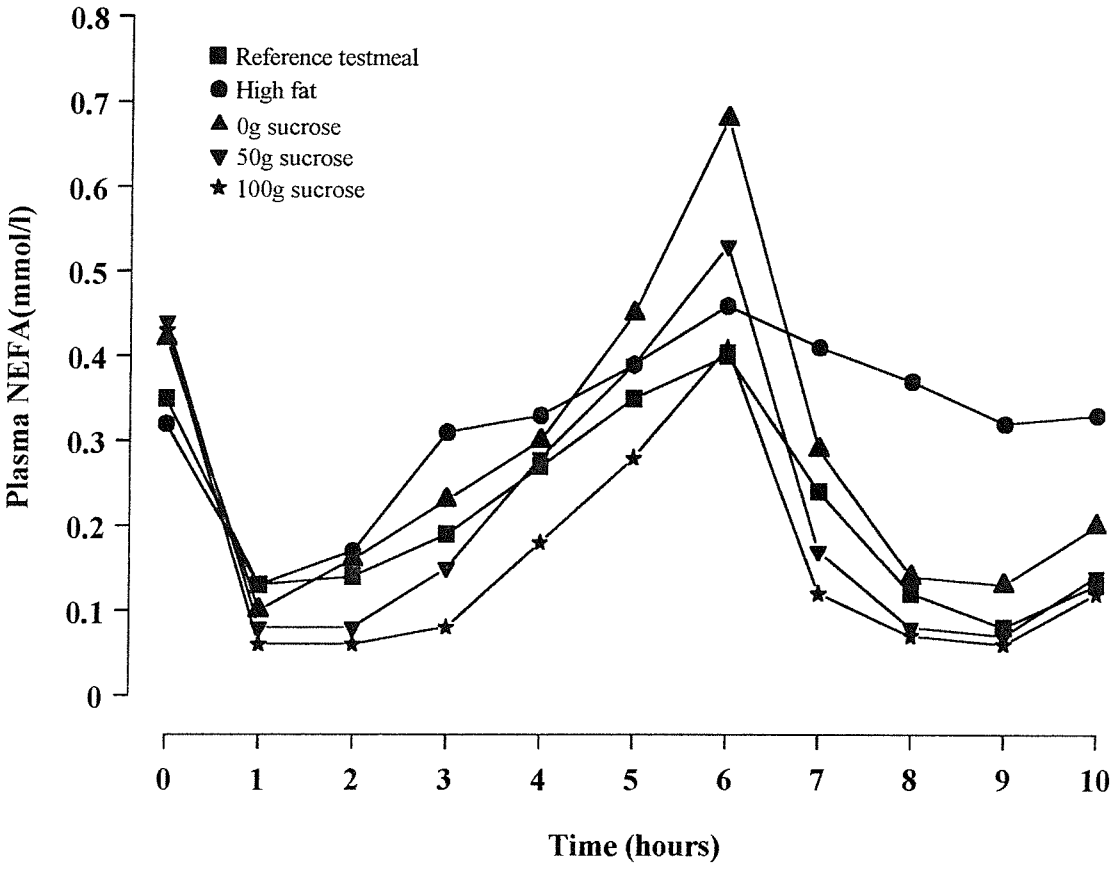


Figure 7.5 Plasma NEFA concentration (mmol/l) over a ten hour study period following consumption of five different testmeals (group mean)



7.2.1 Exaggerated magnitude of postprandial lipaemia

The rise in the magnitude of lipaemia, which is displayed following consumption of the high fat meal and the low carbohydrate meal is in accordance with other studies [Murphy *et al.*1995]. It is suggested that an excessive duration or extent of raised lipaemia may result in: transfer of TAG onto high density lipoproteins (HDL) and low density lipoproteins (LDL) with subsequent hydrolysis by hepatic lipase resulting in the formation of small dense HDL and LDL; accumulation of chylomicron remnant particles which become cholesterol enriched by transfer of cholesterol esters by HDL [Sethi *et al.*1993].

The implications of this are an increased risk of disease due to the atherogenic properties of chylomicron remnants [Zilversmit,1979]. Therefore the increased magnitude of lipaemia which is demonstrated following the high fat meal may indicate an increased risk of disease development. A raised lipaemia can result from a number of processes including an increased input of lipid from the GI tract, a decreased clearance of dietary lipid by LPL and uptake to tissues for fatty acid oxidation or storage, or an upregulation of TAG synthesis by the liver. In these studies, a combination of all three mechanisms could be the case as there is a known increase in the presentation of lipid to the circulation from the GI tract (high fat trial only), clearance may be compromised (lack of insulin stimulus), and the presence of a large quantity of NEFA could result in an upregulation of TAG synthesis.

Despite the doubling of fat intake in the high fat trial there was not a doubling in the amount of TAG in the circulation (8.62 mmol/l/10 hours to 11.40 mmol/l/10 hours). This suggests that as fat intake increases there is a marked increase in clearance, which maintains circulating concentrations between defined limits [Murphy *et al.*1995]. This homeostatic mechanism may partly operate through regulation of LPL activity observed in other studies with increasing fat intake. LPL activity increases with the addition of a lipid rich meal [Karpe *et al.*1992], but it is not known whether this activity will increase further with the addition of increasing fat loads. In addition LPL activity is known to be related to the actions of insulin [Eckel *et al.*1989; Ong and Kern, 1989; Raynolds *et al.*1990]. Insulin data was not available for the high fat study but it would be expected that insulin concentration would not differ with the consumption of the high fat testmeal, as carbohydrate content of the meal remained unchanged, in accordance with Murphy *et*

al.[1995] who displayed no differences in insulin concentrations despite increases in fat intake of 80g. It is possible, however that the increase in fat intake resulted in an increase in gastric inhibitory polypeptide [Morgan *et al.*1988] which has been shown to be a potent stimulator of LPL [Knapper *et al.*1993]. On the sucrose trials, insulin was at its lowest concentration when no sucrose was added to the testmeal, and it was on this trial that the greatest magnitude of plasma TAG was observed. The decrease in insulin concentration may have resulted in a decrease in LPL stimulation, resulting in a delayed clearance of TAG from the circulation.

In addition to chylomicron TAG appearing in the circulation, there may also be an input of triglyceride rich lipoproteins from the liver in the form of VLDL-TAG. In the high fat trial, it may be unlikely that the TAG measured in the circulation would be a high proportion of VLDL-TAG as it has been shown that the smaller the fat load, the greater the number of particles in the VLDL fraction and the less in the chylomicron fraction [Chait *et al.*1973; Jeppesen *et al.*1995]. Therefore the greater the fat load, the less the number of VLDL-TAG, but as the TRL fractions were not measured in the present study it is not possible to say whether this is the case. The raised lipaemia shown with the low carbohydrate meal is probably in part due to a delayed clearance of TAG particles from the circulation, but may also suggest an increased endogenous secretion of TAG due to the increased NEFA concentration in the circulation.

The addition of ^{13}C-TAG and ^{13}C-NEFA data for the carbohydrate study has allowed further examination of the role of exogenous and endogenous lipids in the postprandial period. ^{13}C-TAG data for the group showed a mirror pattern of the total plasma TAG data, in that the 0g sucrose trial showed the greatest magnitude of lipaemia, with a decrease in magnitude with 50g of sucrose. With the addition of 100g of sucrose a prolongation in lipaemia was seen. As an exaggerated lipaemia was seen with the 0g sucrose trial this suggests that clearance of dietary TAG is delayed due to a lack of insulin response, or that there is a competition occurring between dietary and endogenous TAG for hydrolysis by LPL, perhaps because of a stimulation of endogenous TAG synthesis by elevated NEFA concentrations.

On both studies, raised concentrations of NEFA have been shown. Plasma NEFA concentrations also appeared to be dependant upon the composition of the testmeal and upon the individual. For the group as a whole total plasma NEFA concentrations increased with the introduction of a high fat meal. As the high fat meal TAG was

hydrolysed by LPL the resultant NEFA were not entirely removed by tissue due to the increased magnitude and as such were retained in the circulation. NEFA concentrations decreased in a stepwise fashion with the introduction of three different amounts of sucrose. The NEFA response to all testmeals is shown in figure 7.5.

The most interesting observation from this data is the lack of suppression of NEFA in the circulation following the second high fat meal. In all the other meals a similar pattern emerged with only the magnitude of the response altering. It appears that the high fat meal caused the normal postprandial NEFA response in the circulation to be completely abolished in all subjects. The concentration of NEFA in the circulation is a product of fatty acids from two sources, from chylomicron-or VLDL-TAG hydrolysis, or from hydrolysis of adipose tissue TAG. The accumulation of NEFA in the circulation may affect TAG clearance by LPL as there is evidence that fatty acids can dissociate LPL from its binding sites [Saxena *et al.*1989; Peterson *et al.*1990], therefore disrupting the capability of LPL to hydrolyse TAG and release further fatty acids. Elevated NEFA concentrations will also impair glucose uptake in skeletal muscle by mechanisms which are part of the glucose-fatty acid cycle [Randle *et al.*1963; Boden *et al.*1994]. The net effect will be decrease in the sensitivity of glucose metabolism to insulin. This will be reinforced by the marked stimulatory effect of NEFA on hepatic glucose output, and a reduction in hepatic insulin clearance [Svedberg *et al.*1990]. Elevated NEFA concentrations may also lead to an increased secretion of VLDL-TAG from the liver, which is strongly dependent upon the rate of supply of NEFA. Inappropriate postprandial stimulation of VLDL-TAG secretion may lead to increased competition with chylomicron TAG for clearance by LPL and therefore increase postprandial lipaemia [Frayn, 1998]. In addition elevated NEFA concentrations stimulate the activity of cholesteryl-ester transfer protein therefore potentially increasing adverse lipid depletion of HDL and LDL particles [Lagrost *et al.*1995]. What remains unclear is the role of lipid oxidation on NEFA concentrations –is lipid oxidation increased because of the increased NEFA concentration? Or is the increase in lipid oxidation merely due to a lack of glucose within the testmeal?

Following the high fat meal the majority of NEFA probably resulted from the hydrolysis of the chylomicron-TAG but it is unclear how HSL was affected by the rise in concentration of NEFA in the circulation. Did the increase in NEFA concentration result in an inhibition of HSL in adipose tissue? What proportion of the fatty acids in the

circulation were from endogenous and exogenous sources? For the 0g sucrose study, however, the additional ^{13}C-NEFA data allows mechanisms to be suggested regarding the origin of the NEFA in the circulation. At 6 hours postprandially there were no differences between the trials in the ^{13}C-NEFA concentration suggesting that changing the sucrose content of the meal had no adverse effects on exogenous NEFA metabolism. What is interesting is that the total plasma NEFA data is very different between trials at 6 hours postprandially suggesting that the differences seen are due to release of endogenous fatty acids. As the 0g sucrose meal elicited the lowest insulin response, it would appear logical that the inhibitory effect of insulin would deplete more rapidly in this trial resulting in a release of the inhibition of HSL and an increased hydrolysis of adipose tissue TAG.

In summary, the high fat and 0g sucrose trials appear to elicit similar responses in terms of an elevated magnitude of postprandial lipaemia. From the data collected from the study and what has been shown from previous studies it is possible to make some conclusions regarding the mechanisms underlying this process. An increased fat load or decreased carbohydrate load with a fat meal resulted in exaggerated TAG and NEFA responses possibly due to:

1) a delayed clearance of chylomicron-TAG due to a lack of insulin-mediated stimulation of LPL, or a dissociation of LPL from endothelium by elevated NEFA concentrations;
2) An increase in endogenous TAG synthesis due to stimulation by an elevated NEFA response, leading to a competition for TAG hydrolysis by LPL;
3) An exaggerated NEFA response from hydrolysis of adipose tissue TAG or chylomicron TAG;
4) An exaggerated NEFA response resulting in stimulation of CETP leading to perturbations in atherogenic lipoproteins.

From the research reported in this thesis it is possible to make further assumptions regarding the mechanisms underlying the exaggerated TAG and NEFA response observed. It is possible that the increased magnitude of TAG is due to:

1) A decrease in LPL activity due to the decrease in insulin concentration on the 0g sucrose trial, but a dissociation of LPL from the endothelium by elevated NEFA concentrations on the high-fat trial. This hypothesis could be tested by measuring the activity of LPL in the plasma, and ascertaining whether the low insulin concentration results in a decrease in activity of LPL, or an increase in NEFA concentration results in an increase of free LPL in the plasma;
2) As VLDL-TAG concentration was not measured directly in the studies it is not possible to make statements regarding the contribution of VLDL-TAG to the raised lipaemia observed. To test whether this was the case, the concentration of VLDL-TAG would need to be measured throughout the study day;
3) It is clear from the sucrose study that the exaggerated NEFA response is due to a lack of inhibition of HSL, resulting in an increased lipolysis of endogenous TAG. This is the first time that differentiation between endogenous and exogenous lipids has allowed a clear understanding of the effect of insulin on HSL activity;
4) An exaggerated NEFA response has been indicated to result in a stimulation of CETP resulting in perturbations in atherogenic lipoproteins but it is impossible to suggest that this could occur following the testmeals. Perturbations in lipoprotein metabolism may occur over a longer period of time, rather than meal to meal. Therefore to examine the association between exaggerated NEFA response and disease, it would be necessary to prolong the feeding of a high fat or low sucrose diet for a period of time.

7.2.2 Suppression of postprandial lipaemia

The decrease in the magnitude of postprandial lipaemia observed with the introduction of 50g of sucrose is similar to some studies in the literature [Cohen and Berger 1990], but is contradictory to many others [Grant *et al.* 1994; Jeppesen *et al.* 1995]. In long term low fat, high carbohydrate feeding regimens, several studies have reported variable increases in fasting blood TAG concentrations [Nestel *et al.* 1979; Gonen *et al.* 1981; Hollenbeck *et al.* 1985], whereas others [Anderson *et al.* 1980; Jenkins *et al.* 1985; Cominacini *et al.* 1988], have in contrast demonstrated a significant decrease in plasma TAG concentrations. The reason for the conflicting results was the type and amount of carbohydrate used in each case. Those studies which demonstrated an increase

in TAG concentrations generally used diets consisting of a lower intake of starch and an increased sucrose content. In contrast those with a hypotriglyceridemic effect used a greater proportion of starch (85-90% of total carbohydrate). In addition, in accordance with the reference testmeal trial, the introduction of a lower fat meal also decreased the production of chylomicrons therefore decreasing the amount of TAG entering the circulation, and decreasing postprandial lipaemia. This however would not apply to the series of sucrose studies as the amount of lipid within the testmeal remained constant throughout the trials.

The risks of an increased magnitude or duration of lipaemia are highlighted in section 7.3.1, suggesting that increased postprandial lipaemia resulted in increased cholesteryl-ester transfer [Sethi *et al.*1993]. Therefore manipulation of a testmeal composition to achieve a decrease in lipaemia would be assumed to lead to a decrease in cholesterol transfer, and therefore a decreased risk of disease. There are few studies in the literature to have examined the effect of lowering the lipaemic response on the circulating lipid and lipoprotein responses, and the focus has been on understanding raised lipaemia in disease states and high fat diets. The majority of studies using carbohydrate to elicit a response in lipid metabolism have demonstrated an increase in postprandial lipaemia dependant upon the amount and type of carbohydrate used.

Recently the treatment of hypertriglyceridemia has been drug based with the addition of fibric acid derivatives, which act either to increase LDL receptor number or to decrease the production of VLDL-TAG in the liver [O'Connor *et al.*1990]. Fibrates have been shown to decrease TAG concentration by 30% over a 3 month period [Brown *et al.*1986], by acting to limit the production and promote the catabolism of VLDL by activating LPL. In the present study the addition of 50g of sucrose resulted in a 20% reduction in plasma TAG concentration (11.06 mmol/l/10 hours to 9.02 mmol/l/10 hours), in normolipidemic subjects. Without conducting the experiment in subjects with raised lipaemic responses there is no way of ascertaining the effectiveness of the addition of sucrose to a meal, and in this subject group could in fact result in an amplification of lipaemia, depending upon their insulin sensitivity. In addition, the addition of sucrose to a meal will act in an entirely different way to the addition of fibrates; sucrose appears to increase the activity of LPL via insulin resulting in increased clearance of CM-TAG in the postprandial state, whereas fibrates act to reduce VLDL-TAG in the post-absorptive state. The action of sucrose has been observed in a single day of feeding whereas fibrates

act over a longer period of time. What would be interesting would be to observe the effects of continuous sucrose feeding on plasma TAG concentration over a period of time and then to compare the dietary effects with those of drug therapy. Although it appeared from the present study that a 20% reduction in plasma TAG was achievable, how effective would such a feeding pattern be in reducing plasma TAG concentration over a period of time?

The results from the present study were surprising in light of previous studies examining the effect of sucrose on lipid metabolism, which observed an increase in plasma TAG concentration with the addition of sucrose to a meal. In the study presented in this thesis, the introduction of 50g of sucrose to a mixed meal resulted in a 20% decrease in postprandial lipaemia. Although glucose ingestion has been shown to diminish postprandial lipaemia [Nikilla and Pelkonen 1966; Mann *et al.*1971; Cohen and Berger, 1990], sucrose ingestion leads to an augmentation of lipaemia [Grant *et al.*1994]. Therefore the question which remains unanswered is why does the addition of 50g of sucrose to a mixed meal result in a reduced lipaemia?, and why does this differ from previous studies?.

The reduction in postprandial lipaemia (TAG and NEFA concentrations) with the addition of 50g of sucrose could be due to a number of mechanisms. Firstly, this could reflect an increased clearance of chylomicron TAG by insulin-mediated upregulation of LPL, as LPL activity is known to be stimulated by the presence of insulin [Eckel *et al.*1989; Ong and Kern, 1989; Raynolds *et al.*1990]. Secondly the decrease in lipaemia could reflect decreased hepatic synthesis of TAG due to a decrease in NEFA as substrates [Frayn, 1998], or thirdly the reduction in postprandial lipaemia may be due to a combination of both processes. Figure 7.6 shows the interaction between plasma insulin concentration and plasma TAG concentration for the six subjects following consumption of all three sucrose loads, and figure 7.7 the association between ^{13}C-TAG and plasma insulin. There appears to be an association between the insulin response to the meal and the concentration of plasma TAG over the ten hour study period, with an increase in plasma insulin concentration resulting in a decrease in plasma TAG concentration, which is particularly apparent in the 50g sucrose trial. With the ^{13}C-TAG data further assumptions can be made regarding TAG metabolism. The addition of 50g of sucrose to the meal led to a decrease in the magnitude of ^{13}C-TAG, suggesting that there is increased clearance of meal TAG from the circulation, and a decreased release of

VLDL-TAG from the liver. Once again the concentration of ^{13}C-TAG appears to be related to the concentration of insulin, which is apparent for the 50g and 100g sucrose trials. The ^{13}C-data suggests that the concentration of exogenous TAG is dependant upon the concentration of insulin, due to insulin mediated stimulation of LPL activity. If insulin concentration is increased, LPL activity is enhanced and clearance of exogenous TAG is increased.

The reasons why the 50g sucrose study appears to show conflicting effects of sucrose compared to other studies is possibly due to the composition of the meal, and the amount of sucrose included. Other studies, which have shown an amplification of lipaemia, have used amounts of sucrose ranging from 60g [Mann *et al.*1971] to 1.5g/kg BW [Grant *et al.*1994]. This equates to approximately 105g of sucrose in an average 70kg subject, a similar quantity consumed on our high carbohydrate trial, where a prolongation of lipaemia was seen. In addition, the oral fat tolerance tests consisted of a fat source (sunflower oil or cream) with an additional flavouring but no testmeal. It is possible that gastric emptying times of the stock meals could have varied greatly. In our testmeal, subjects received an additional ham sandwich and the sucrose load in a squash drink to reflect a typical meal consumption. Therefore the results from the present study may better reflect postprandial lipid metabolism in healthy man on a day to day basis.

The addition of 50g of sucrose to the testmeal, and the consumption of the reference testmeal resulted in a decrease in the NEFA concentration in the plasma. There was a 25% decrease in NEFA concentration with the addition of 50g of sucrose compared to 0g sucrose, and a 100% decrease in NEFA concentration with the reference testmeal compared to the high fat meal. From this it can be concluded that the manipulation of meal composition can profoundly affect plasma NEFA concentration. Plasma NEFA concentration is the product of hydrolysis of CM-TAG and VLDL-TAG by LPL, and the release of NEFA from adipose tissue TAG. The reduction in NEFA concentration with the reference testmeal was primarily due to a decrease in substrate for hydrolysis by LPL, therefore resulting in decreased NEFA. In addition, the blunted NEFA response would not have caused an overproduction of VLDL-TAG for hydrolysis by the liver. It is possible that a higher proportion of NEFA in the circulation following the reference testmeal were from adipose tissue hydrolysis but as this was not measured directly, it is not possible to be clear on this issue. ^{13}C-analysis of plasma NEFA during the study period would permit the examination of the source of the circulating NEFA,

and would allow assumptions to be made regarding the clearance and appearance of dietary and endogenous NEFA to the circulation. The addition of 50g of sucrose would have had two main effects on the NEFA concentration. Primarily, the enhanced insulin response following this meal would have led to increased hydrolysis of TAG with adequate NEFA entrapment, and at the same time, an inhibition of HSL mediated TAG hydrolysis in adipose tissue.

With the additional ^{13}C-NEFA data, it can be confirmed that the changes in total plasma NEFA were due to changes in endogenous secretion of NEFA as there are no differences in ^{13}C-NEFA between the trials. Therefore the decrease in total NEFA upon consumption of 50g of sucrose was probably due to an inhibition of HSL by insulin, inhibiting the hydrolysis of adipose tissue TAG, and resulting in a decrease in circulating NEFA concentration. Therefore a decreased lipid load or increased sucrose load (50g) resulted in a diminished postprandial lipaemia and NEFA response to a testmeal, which may be due to:

1) an enhanced clearance of CM-TAG from the circulation, due to an insulin-mediated stimulation of LPL;
2) a decrease in the total amount of fat consumed, and therefore a decrease in the total chylomicron secretion by the GI tract;
3) a decrease in the production of VLDL-TAG due to a decrease in NEFA as substrate;
4) a decrease in the release of NEFA from adipose tissue TAG, due to an insulin-mediated inhibition of HSL;
5) an overall decrease in activity of CETP, resulting in a decrease in cholesterol transfer and a decreased risk of disease.

Figure 7.6 Relationship between plasma insulin concentration and plasma TAG concentration

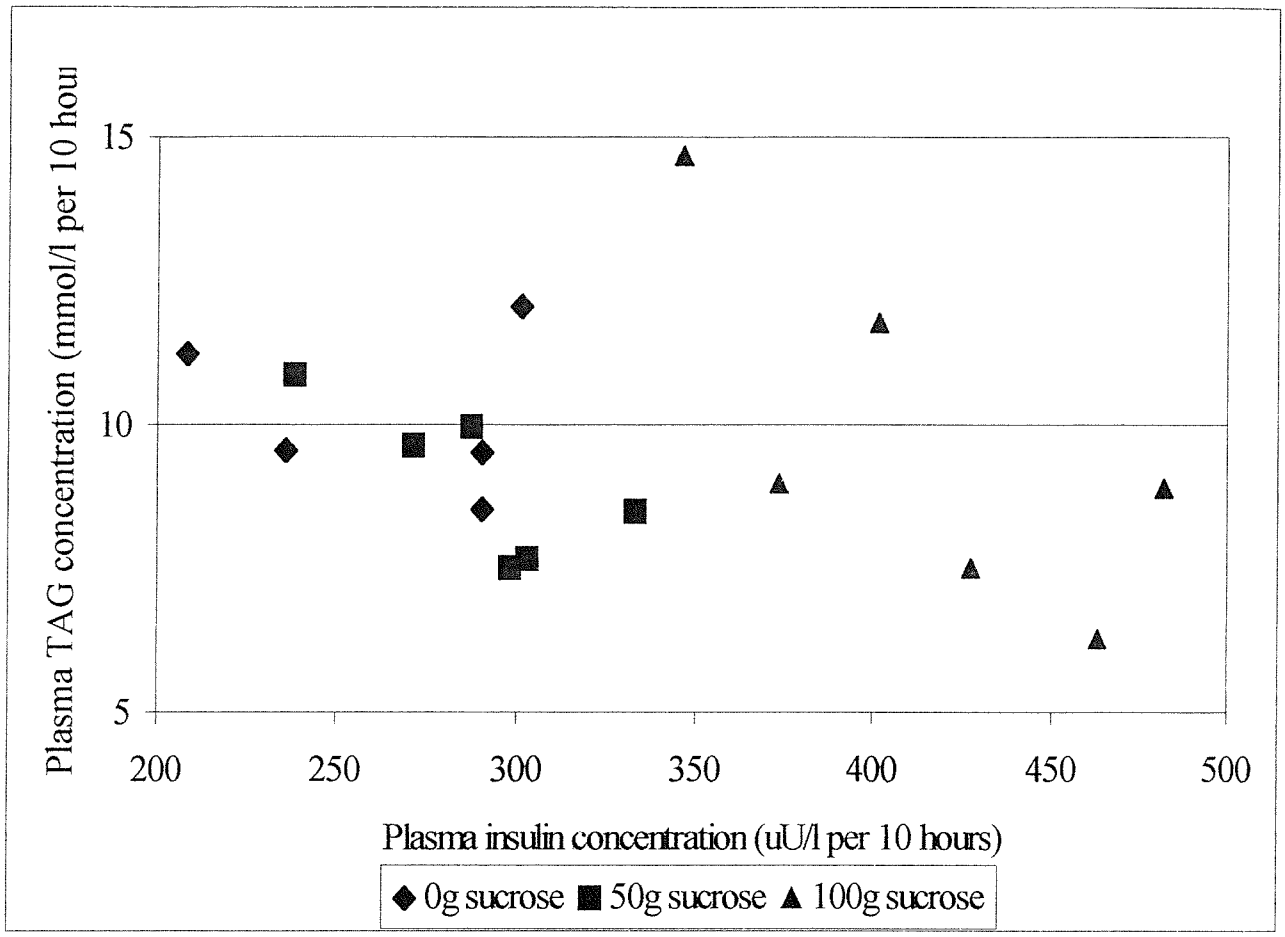
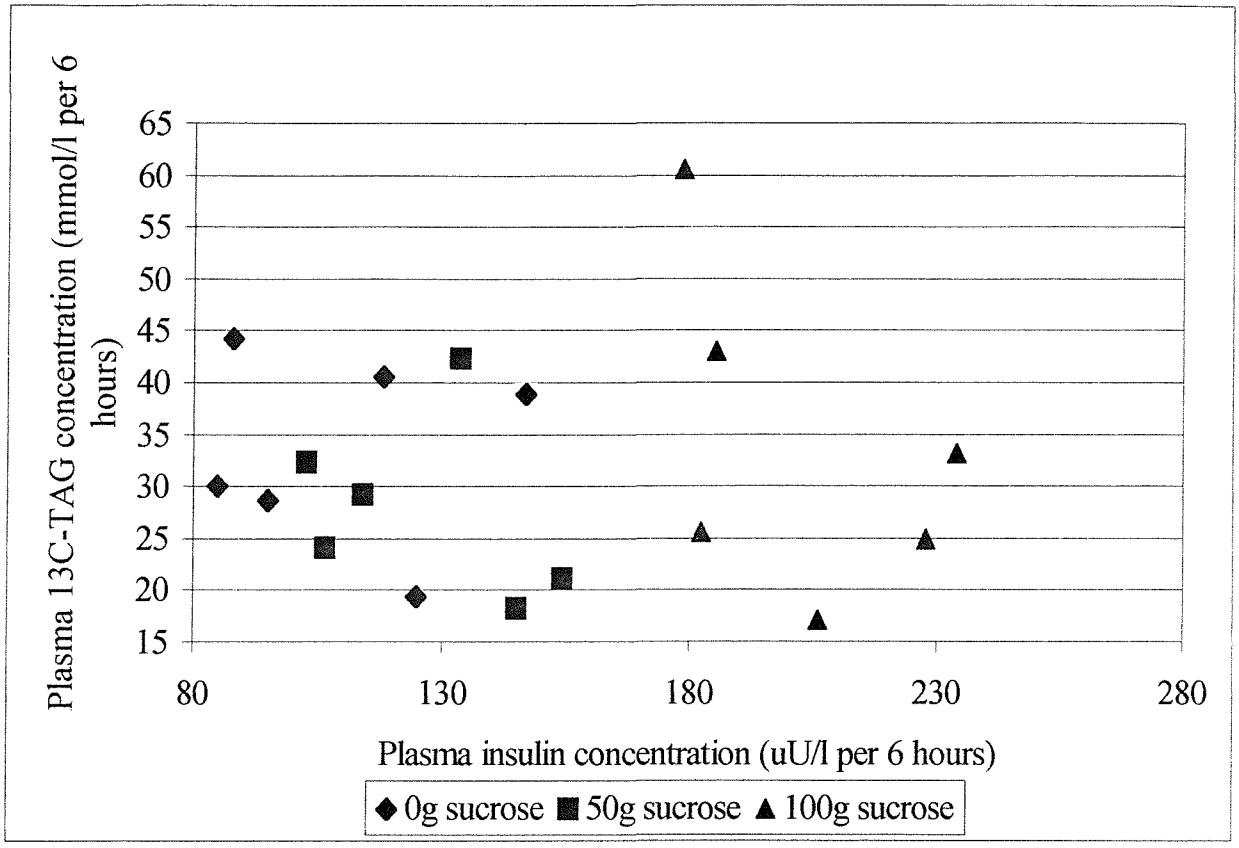


Figure 7.7 Relationship between plasma ^{13}C-TAG concentration and plasma insulin concentration



From the research presented in this thesis, it is possible to make further assumptions regarding the underlying mechanisms related to the decrease in circulating lipid concentration observed with the reference testmeal and the 50g sucrose meal. It appears that the decrease in lipaemia may be due to different mechanisms depending upon the composition of the meal consumed. For example, the addition of 50g of sucrose resulted in an inhibition of the lipolysis of adipose tissue TAG, resulting in a decrease in NEFA concentration. In addition, the ^{13}C-TAG concentration was found to decrease with the addition of 50g of sucrose, suggesting an increase in the activity of insulin-mediated LPL. In terms of the reference testmeal, there was an overall decrease in the availability of lipid as compared to the high-fat meal, therefore a decreased magnitude of lipaemia. In both cases, there was a decreased availability of circulating NEFA suggesting that there was less substrate available for the secretion of VLDL-TAG from liver. Therefore there was less competition for hydrolysis by VLDL-TAG and CM-TAG, and therefore clearance of CM-TAG was maximal. What remains unclear is the extent to which increasing the sucrose load or decreasing the lipid load affects the activity of LPL? How does decreasing the amount of lipid in a meal affect the concentration of circulating endogenous and exogenous TAG? How does decreasing the lipid content and increasing the carbohydrate content of a meal affect the balance of substrate oxidation?

7.2.3 Prolongation of postprandial lipaemia

The addition of 100g of sucrose to a mixed meal resulted in an initial decrease in postprandial lipaemia up to 2.5 to 3 hours postprandially, followed by a increased magnitude of lipaemia which remained elevated for several hours of the study day. Total plasma TAG concentrations did not differ significantly between the 50g and 100g of sucrose trial suggesting that the addition of sucrose had little or no effect on TAG metabolism. However the differences between the trials were apparent in the timecourse of plasma TAG concentration over the study day. The prolongation of lipaemia with 100g of sucrose was not expected following the decreased magnitude of lipaemia observed following the 50g sucrose trial. It was expected that as lipaemia was diminished with 50g, it would be further diminished with 100g sucrose, however this was not the case, and it appears, that this is not due merely to alterations in TAG clearance from the circulation but to the production of VLDL-TAG in the liver. Several

studies have reported an augmentation of lipaemia with the consumption of sucrose, which appears to be similar to the rise in lipaemia observed with the addition of fructose to a meal. Cohen and Schall [1988] demonstrated a similar lipaemic response with the addition of 50g of fructose and 100g of sucrose to a testmeal, suggesting that the effect of sucrose on postprandial lipaemia was due to its fructose component. Increasing the fructose component of the diet is also known to increase fasting plasma TAG values and to increase the VLDL-TAG component of plasma [Hollenbeck, 1993]. In the present study it is possible that the fructose component of sucrose resulted in an increase in hepatic VLDL-TAG synthesis and secretion leading to competition between TRL of exogenous and endogenous origin for removal from plasma [Jeppesen *et al.*1995]. In addition, the increase in sucrose in the meal may have increased the synthesis of glycerol via α-glycerophosphate from the absorbed fructose in the intestinal mucosa [Nikkila and Pelkonen, 1966], but this is unlikely as sucrose feeding alone does not directly increase postprandial lipaemia [Grant *et al.*1994].

Therefore, it is probable that the clearance of CM-TAG *per se* is unlikely to be decreased due to the pronounced insulin responses seen and that the production of glycerol and *de novo* lipogenesis is unlikely due to evidence from previous studies. *De novo* lipogenesis may be more likely to occur in a period of carbohydrate overfeeding but this depends upon the supply of carbohydrate which exceeds NEFA oxidation requirements [Roche, 1999]. Carbohydrate feeding stimulates *de novo* lipogenesis by increasing the flux of glucose or fructose along the glycolytic pathway, thereby increasing the availability of acetyl-CoA and stimulating fatty acid synthesis [Frayn and Kingham, 1995]. The prolongation of lipaemia following the 100g of sucrose meal is probably due to a fructose-induced stimulation of VLDL-TAG synthesis and secretion resulting in a competition for clearance between exogenous and endogenous TAG, in accordance with Jeppesen *et al.*[1995]. Why does the 50g sucrose meal not lead to similar effects? It appears that the amount of fructose in the 50g sucrose meal is not sufficient to cause a stimulation of endogenous lipid secretion, and therefore the magnitude of lipaemia is decreased. The ^{13}C-TAG data supports this theory as a similar prolongation of lipaemia is seen, which could either reflect a decreased clearance of CM-TAG because of a decreased action of LPL but this is unlikely as stated previously, or a competition for clearance with VLDL-TAG produced by the liver in response to fructose overfeeding.

NEFA concentrations decreased further with the introduction of 100g of sucrose suggesting that the hydrolysis of adipose tissue TAG was suppressed further following inhibition of HSL by insulin. This was confirmed with the ^{13}C-NEFA data, which did not differ between the trials suggesting that any variation in NEFA metabolism must be due to changes in endogenous NEFA concentration. Therefore the introduction of a high sucrose load with a mixed meal results in a prolongation of postprandial lipaemia which may be a result of:

1) decreased clearance of CM-TAG;
2) increased production of VLDL-TAG due to a fructose-mediated enhancement of VLDL-TAG synthesis;
3) competition for clearance from the plasma between dietary and endogenous TAG;
4) *de novo* lipogenesis due to an increased flux of acetyl-CoA stimulating fatty acid synthesis.

From the studies presented in this thesis it is possible to further examine the mechanisms underlying the prolongation of postprandial lipaemia observed with 100g of sucrose added to the meal. It appears that the most important contributor to the prolongation is fructose mediated enhanced VLDL-TAG secretion. When 50g of sucrose was added to the testmeal, the prolongation in lipaemia was not observed. In accordance with previous studies, it was not until the fructose concentration of the sucrose load reached a 'critical' threshold (>30g), that the stimulation of VLDL-TAG secretion occurred. It is unlikely that a single 100g sucrose meal would result in *de novo* lipogenesis, as this is more likely to occur following a period of carbohydrate overfeeding. In addition, it has been shown that the 100g sucrose meal resulted in a large insulin response, suggesting that is unlikely that the prolongation in lipaemia was due to decreased clearance. Therefore, it would seem probable that the prolongation of lipaemia observed with the 100g sucrose meal was due to an overstimulation of VLDL-TAG secretion resulting in a competition for clearance between exogenous and endogenous TAG. This was confirmed by ^{13}C-TAG data which also demonstrated a prolongation, suggesting a decrease in clearance due to competition for LPL. In order to test the mechanism fully, it would be necessary to measure the contribution of chylomicron-TAG and VLDL-TAG to the circulating lipids over the complete study day.

7.3 Substrate oxidation

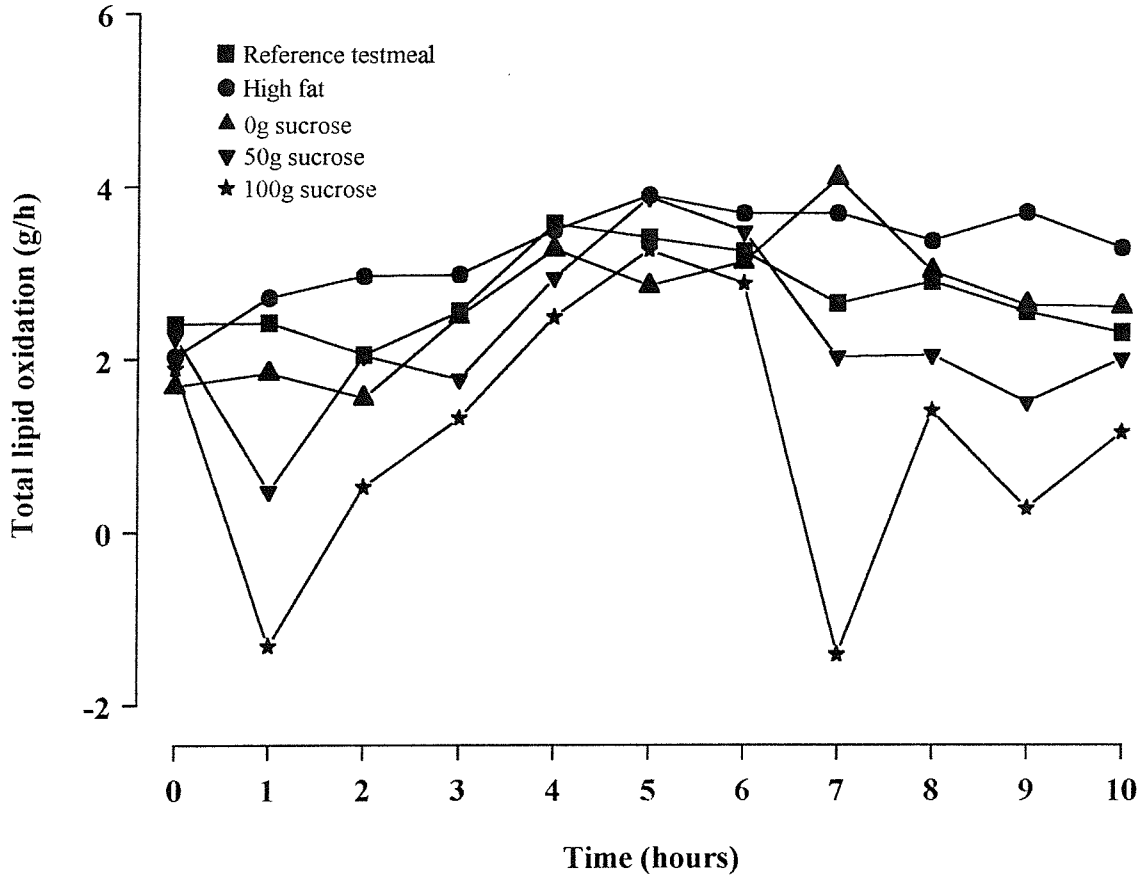
Carbohydrate and lipid oxidation appear to be influenced by the composition of the meal consumed. In addition, as lipid oxidation increases so carbohydrate oxidation decreases and vice versa, suggesting that the overall energy balance remains unchanged during the study period. In power analysis calculations made in chapters 4,5 and 6, it was found that in all studies lipid and carbohydrate oxidation were influenced by the composition of the meal, over and above the variation observed within-individuals. In other words, it appears that substrate oxidation is the single most important outcome variable which is influenced by the meal composition. In addition, plasma NEFA concentration was closely influenced by meal composition, and as plasma NEFA concentration may be highly important in regulating fatty acid oxidation, this is clearly important. The use of ^{13}C tracers allows the differentiation of exogenous and endogenous fatty acid oxidation, which suggests that not only is total substrate oxidation altered by meal composition but so is the proportion of fatty acids oxidised. The central hypothesis underlying the work presented in this thesis is that by manipulating the composition of a meal, the appearance and clearance of circulating lipids will be altered and that this may result in altered substrate oxidation. This appears to be the case, however the mechanisms underlying the association between circulating lipids and oxidation has not been extensively studied. Which outcome variable controls the process – is it the concentration of circulating lipids which controls oxidation, or oxidation which controls the concentration of circulating lipids? Also how does altering the composition of a meal affect the balance of oxidation? The following sections will discuss meal composition manipulation resulting in increased lipid oxidation, and in decreased lipid oxidation. Figure 7.8 shows the total lipid oxidation pattern for the group as a whole following the five testmeals.

The reference testmeal and 0g sucrose trials appear to elicit a similar response in terms of total lipid oxidation with a total of 28.2g/10 hours oxidised following the reference testmeal, and 27.0g oxidised following the low carbohydrate meal. The similarity between the two trials was not surprising, however, as the reference testmeal contained an additional 18g of simple sugars compared to the 0g sucrose meal. In comparison to these trials, only the high-fat trial showed an increase in lipid oxidation for the group as a whole. An increase in oxidation is shown in all subjects except 1,

however the amount of lipid oxidised and the degree of the increase depends upon the individual. For example, subject 1 increases oxidation from 20.6g per 10 hours following the reference testmeal to 24.3 g per 10 hours following the high fat meal. In contrast subject 6 increases oxidation from 44.1 g/10 hours to 46.3g/ 10 hours, suggesting that there are different metabolic capacities for individuals to oxidise lipid, and that subjects not displaying an increase in lipid oxidation with a high fat meal may already have reached their personal capacity to oxidise lipid with the reference testmeal.

The high-fat study reported in chapter 5 is one of the first to demonstrate that an increase in lipid intake results in an increase in lipid oxidation. There are very few studies in the literature to examine the effect of a high-fat meal on oxidation, but long term diet based studies have reported no effect of lipid intake on lipid oxidation [Astrup *et al.*1994]. In the present study, consumption of a high fat meal resulted in a maintenance of lipid oxidation throughout the 10-hour study period. In contrast, the addition of 100g of sucrose suppressed lipid oxidation to such an extent that at 1 hour and 7 hours postprandially, negative lipid oxidation was observed. So what is happening to the lipid which is not oxidised? In the high-fat trial, although 32.9g of lipid was oxidised over the study day, a total of 134g was consumed. There was a significant increase in plasma TAG concentration over the study day, but this could not account for the additional 100g of lipid. Therefore the majority of the ingested lipid must have been directed towards storage. Many questions remain unanswered such as how is the manipulation of meal composition related to macronutrient balance? As carbohydrate oxidation increases, lipid oxidation decreases but what are the proportions of exogenous and endogenous fatty acids and why? What controls/ regulates the rate of fatty acid oxidation-is it supply or demand? What controls the flux of glucose and fatty acids following alterations in the composition of a meal?

Figure 7.8 **Total lipid oxidation (g/h) over a ten hour study period following consumption of five different testmeals, shown for six subjects (group mean)**



7.3.1 Increased lipid oxidation

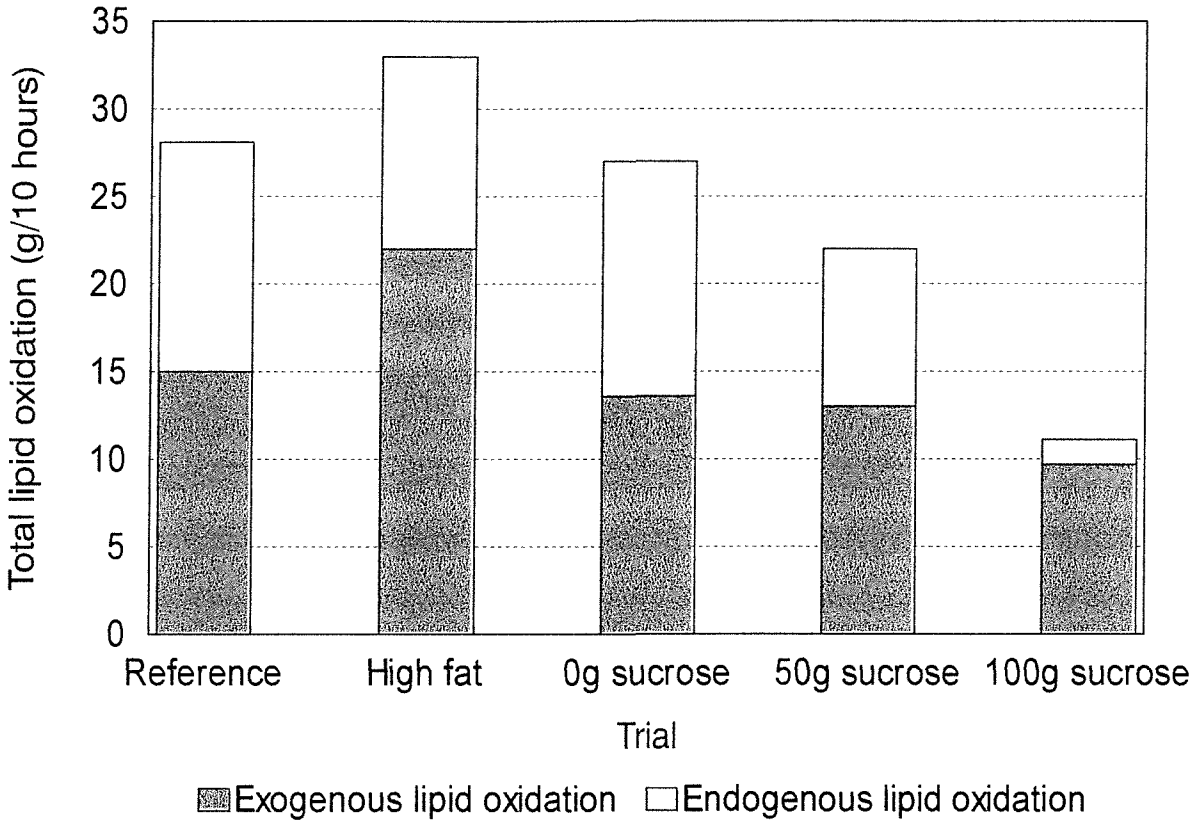
The increase in lipid oxidation was not expected in the high fat trial as previous work with long term diets has shown a failure of fat oxidation to increase with fat intake [Schutz *et al.*1989; Astrup *et al.*1994], which has been suggested as a factor favouring the development of obesity. Although excessive gains in glycogen are prevented by increased carbohydrate oxidation, irrespective of the consequences that this may have on the fat balance, a metabolic response serving to increase fat oxidation in the face of increased fat intake appears to be lacking [Schutz *et al.*1989]. Despite lipid oxidation increasing overall in the present study similar mechanisms could be applied. Following the high fat meal lipid oxidation increased from 28.2g/10 hours to 32.9g/10 hours, an increase of 4.7g of lipid oxidised. However, the amount of lipid in the meal increased from approximately 35g to 70g, a doubling in the amount of lipid ingested. Therefore the failure of the increase in dietary lipid consumption to promote lipid oxidation is apparent, which is in agreement with a previous study when the addition of 80g of fat to a meal led to increased fat oxidation. Nevertheless the addition of this large amount of fat brought about a net fat storage of 70g suggesting that the major effect of added dietary fat was to increase the body's fat store [Griffiths *et al.*1994]. Increased fat oxidation is a consequence of raised NEFA concentrations in the plasma, or a 'spill-over' of excess LPL derived fatty acids in the plasma where they may become a substrate for oxidation [Frayn *et al.*1994; Frayn, 1995]. In the high-fat study, there was a large 'spill-over' of fatty acids which is particularly apparent following the second meal, suggesting that the fatty acids are not being cleared for oxidative purposes and remaining in the circulation prior to uptake by adipose tissue or the liver. In contrast to the increase in lipid oxidation, there was a decrease in net carbohydrate oxidation from 107.9g/10 hours to 101.1g/10 hours. Once again the change between the testmeals for the group remains small but differences within individuals display more variation. Increased carbohydrate availability is known to increase carbohydrate oxidation [Abbot *et al.*1988], as is the type of carbohydrate [Ritz *et al.*1991]. Whiteley *et al.*[1997] showed similar results with the amount of fat and carbohydrate altering in isoenergetic testmeals. As the fat content of the meal increased, fat oxidation was shown to increase, however, this study did not use stable isotopes and therefore were unable to differentiate between meal derived and endogenous fatty acids.

In the present study the use of ^{13}C-tracers allowed the determination of the proportion of exogenous and endogenous fatty acids oxidised over the study period. Figure 7.9 shows the contribution of exogenous and endogenous lipid oxidation to net lipid oxidation for all five testmeals. The individual responses to the meals can be seen in the relevant results chapters. With consumption of the high fat testmeal, net lipid oxidation increased from 28.2g/10 hours to 32.9g/10 hours. Of this, 53% was exogenous lipid following the reference testmeal, and 67% was accounted for by exogenous lipid following the high fat meal, which suggests that the doubling in fat intake resulted in a 14% increase in the proportion of lipid oxidation resulting from exogenous lipid sources. Although the effect of increasing meal fat content on the exogenous and endogenous lipid oxidation ratio has not been studied previously, the result was expected. If an increased amount of fat is entering the system from exogenous sources, the oxidation of exogenous sources would be expected to increase. In addition, a large proportion of the ingested lipid is entering the adipose tissue to be stored, and the ingestion of a large amount of exogenous lipid is preventing the use of endogenous fatty acids from adipose stores for oxidation. However it remains unclear what controls the rate and extent of fatty acid oxidation, and is this dependant upon an individuals metabolic competency?
The between individual differences in both total lipid oxidation and the proportions of exogenous and endogenous oxidation were substantial. For example, subject 4 oxidised 15.9g lipid/10 hours (86% exogenous lipid) on the reference testmeal trial, and 23.4g lipid/10 hours (80% exogenous lipid) on the high fat trial. In contrast subject 6 oxidised 44.0g lipid/ 10 hours (34% exogenous lipid) on the reference testmeal, and 46.3g lipid/10 hours (62% exogenous lipid) on the high fat trial. This suggests that on the reference testmeal trial subject 4 has reached a maximum capacity for the oxidation of exogenous substrates which could not be increased with the introduction of the high fat meal, whereas subject 6 is able to upregulate exogenous lipid oxidation to 'cope' with the additional fat load. It is possible that these differences between individuals are an indication of their predisposition to later disease, and that if the same experiment was repeated in obese or insulin resistant subjects comparisons could be made. What is interesting is the association between lipid oxidation and circulating lipid concentrations. For example subject 3 demonstrates a substantial increase in lipid oxidation with the high-fat meal, coupled with a modest increase in plasma TAG concentration, and a large elevation of plasma NEFA concentration. In contrast in subject 6, there were no

differences in plasma TAG, lipid oxidation, carbohydrate oxidation or plasma glucose concentration between the trial, yet a massive increase in plasma NEFA concentration suggesting that this subject has the ability to hydrolyse exogenous TAG, but is unable to 'upregulate' lipid oxidation to compensate which results in elevated NEFA concentration which has been associated with disease [Frayn, 1998].

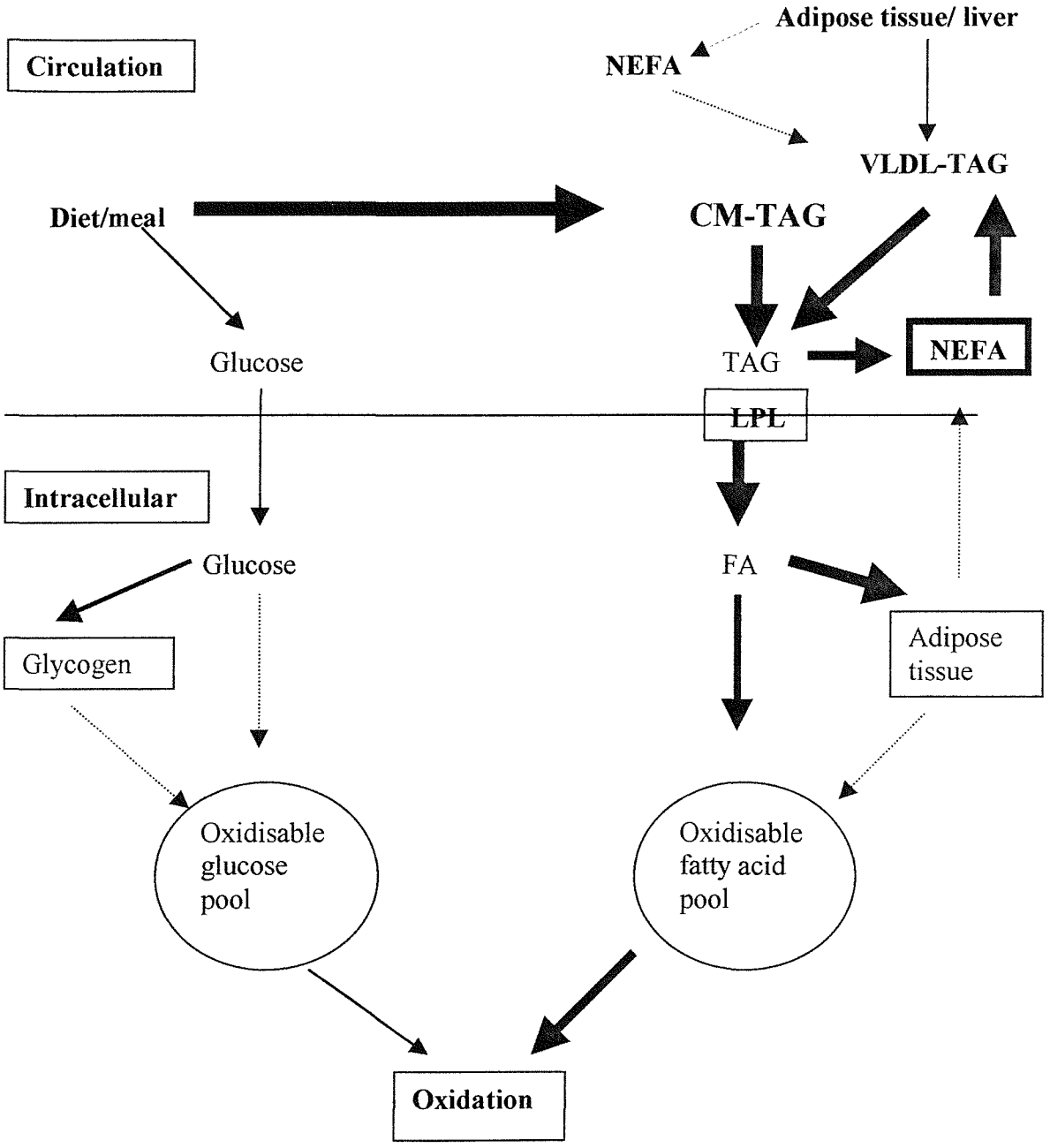
How does increasing the consumption of lipid result in an increase in exogenous lipid oxidation whilst endogenous lipid oxidation appears to remain constant? Figure 7.10 shows a schematic diagram of the metabolic processes occurring during the postprandial period following a high-fat meal. The results from the present research have demonstrated that an increase in dietary lipid results in an increase in plasma TAG concentration, however it is not clear whether the increase is due to CM-TAG or VLDL-TAG. At the same time, there was an increase in exogenous lipid oxidation, and a decrease in glucose oxidation despite an increase in plasma glucose concentration. NEFA concentration in the plasma was also increased following the high-fat meal, suggesting that the increase in lipid oxidation was stimulated by the increase in circulating NEFA. It was probable that hydrolysis of adipose tissue TAG was inhibited by the presence of insulin and the raised NEFA concentration although there is no data from the study to support this. Therefore hydrolysis of TAG would result in a greater release of NEFA to the circulation than the rate of entrapment leading to an 'overspill' of NEFA in the circulation. NEFA could have been taken up by the liver and re-secreted as VLDL-TAG, therefore increasing the plasma TAG concentration further, and adding to the elevated NEFA pool following hydrolysis. NEFA entrapment to the muscle would have occurred preferentially to glucose as NEFA concentration remained elevated resulting in an increase in exogenous fatty acid oxidation.

Figure 7.9 **Proportion of total lipid oxidation accountable for by exogenous and endogenous lipid sources following consumption of five different testmeals (group mean)**



From the research presented in this thesis, it is apparent that the process of substrate oxidation and the concentration of circulating lipids appears to be 'driven' by the availability of the exogenous macronutrient. In the high-fat study, it seems that the rate limiting factor preventing the clearance of lipid from the circulation was not LPL, but the rate of entrapment of fatty acids and fatty acid oxidation. If fatty acid oxidation cannot be 'upregulated' to accommodate the increased influx of lipid, both TAG and NEFA concentrations in the plasma increase resulting in hypertriglyceridemia. The majority of studies in the literature have concentrated on the effect of meals on postprandial lipaemia alone, and have not considered the impact of fatty acid oxidation or a lack of fatty acid oxidation. The present research provides evidence that the whole process of postprandial lipaemia and circulating NEFA concentration, is actually governed by the capacity of the individual to increase or decrease fatty acid oxidation.

Figure 7.10 Regulation of lipid oxidation following consumption of a high-fat meal



7.3.2 Decreased lipid oxidation

Total lipid oxidation decreased as the sucrose content of the meal increased, which was expected as when glucose availability increases, fatty acid oxidation is known to decrease in response to an insulin mediated inhibition of lipolysis [Wolfe and Peters, 1987; Wolfe *et al.*1988]. Total lipid oxidation decreased from 27g/10 hours on the 0g sucrose meal to 22g/10 hours (50g sucrose) and 11.1g/10 hours (100g sucrose). At the same time carbohydrate oxidation increased in a step wise fashion in agreement with several studies which have suggested that carbohydrate oxidation is proportional to intake [Abbot *et al.*1988; Flatt,1995]. Despite all three testmeals containing an identical amount of lipid, lipid oxidation decreased by over 80% on consumption of the 100g sucrose meal. Several mechanisms have been proposed for the decrease in lipid oxidation seen with the addition of carbohydrate to a meal. From work with rats, it was proposed that increased pyruvate availability in the carbohydrate fed state results in an increased formation of malonyl-CoA. Malonyl-CoA is known to be a potent inhibitor of carnitine o-palmitoyl transferase 1 (CPT-1), an enzyme which catalyses the binding of LCFA to carnitine to gain access to the mitochondria [McGarry *et al.*1989]. Therefore accelerated glucose metabolism may inhibit fatty acid oxidation by limiting LCFA uptake by the mitochondria. It has also been proposed that insulin activates acetyl-CoA carboxylase [Mabrouk *et al.*1990], which is the enzyme that catalyses malonyl-CoA formation. Malonyl-CoA in turn decreases fatty acid oxidation. However glucose needs to be present for insulin to decrease fatty acid oxidation, because insulin alone cannot alter malonyl-CoA concentration [Duan and Winder, 1993]. Sidossis and Wolfe [1996] suggest that the intracellular availability of glucose, rather than FFA, determines the nature of substrate oxidation in human subjects, but what determines this? From the present research it is clear that the concentration of circulating glucose was tightly regulated with only minimal increases in plasma glucose concentration following 100g of sucrose in a testmeal, which suggests that glucose was cleared from the circulation almost immediately following absorption. Glucose rapidly diffuses across membranes by a process of passive diffusion along a concentration gradient, with phosphorylation of glucose to glucose-6-phosphate and ultimately glycogen synthesis stimulated by the presence of insulin. Therefore in the post-absorptive state, it would be expected that glycogen stores would be empty resulting in a rapid phosphorylation of glucose. In

addition an increase in glucose and insulin in the circulation results in a stimulation of glucose uptake in skeletal muscle and glucose oxidation. At the same time insulin activates muscle glycogen synthase resulting in the replenishment of muscle glycogen stores. Following the second meal, glycogen stores would be full from the first meal, and therefore the concentration gradient observed in the post-absorptive state is not operational, therefore there is an increase in plasma glucose concentration. The influence of glucose uptake and oxidation on fatty acid entrapment and oxidation following a high sucrose meal is shown in figure 7.11

The availability of glucose and insulin in the present study may explain the reduction in total lipid oxidation that occurs but how can the availability of carbohydrate explain the reduction in endogenous lipid oxidation that is seen?. Figure 7.9 shows the proportion of exogenous and endogenous fatty acids oxidised over the study period with consumption of the three carbohydrate meals. As lipid oxidation decreases, exogenous lipid oxidation remains relatively constant whereas endogenous lipid oxidation declines rapidly. In some subjects, no endogenous lipid oxidation occurred at any point during the high carbohydrate trial (see chapter 6). The lack of effect of carbohydrate on exogenous lipid oxidation suggests that glucose is not exerting an effect on the entry of LCFA to the mitochondria as previously suggested [McGarry *et al.* 1989], but that the raised insulin concentration is acting at the level of HSL to prevent the release of endogenous fatty acids from adipose tissue [Frayn *et al.* 1995], and therefore preventing their oxidation. This theory is supported by the ^{13}C-NEFA data which suggests that the changes in plasma NEFA concentrations with the addition of carbohydrate is due to a decrease in release of endogenous fatty acids. What is important is how does the reduction in lipid oxidation following a sucrose meal affect the balance between 'fat burning' and fat storing' and does this matter? Will some individuals have a lower 'critical limit' where exogenous lipid oxidation is suppressed which may be a causal factor in, for example, obesity? Will middle-aged subjects begin to suppress exogenous lipid oxidation with a 50g sucrose load compared to a 100g sucrose load in young, healthy subjects?

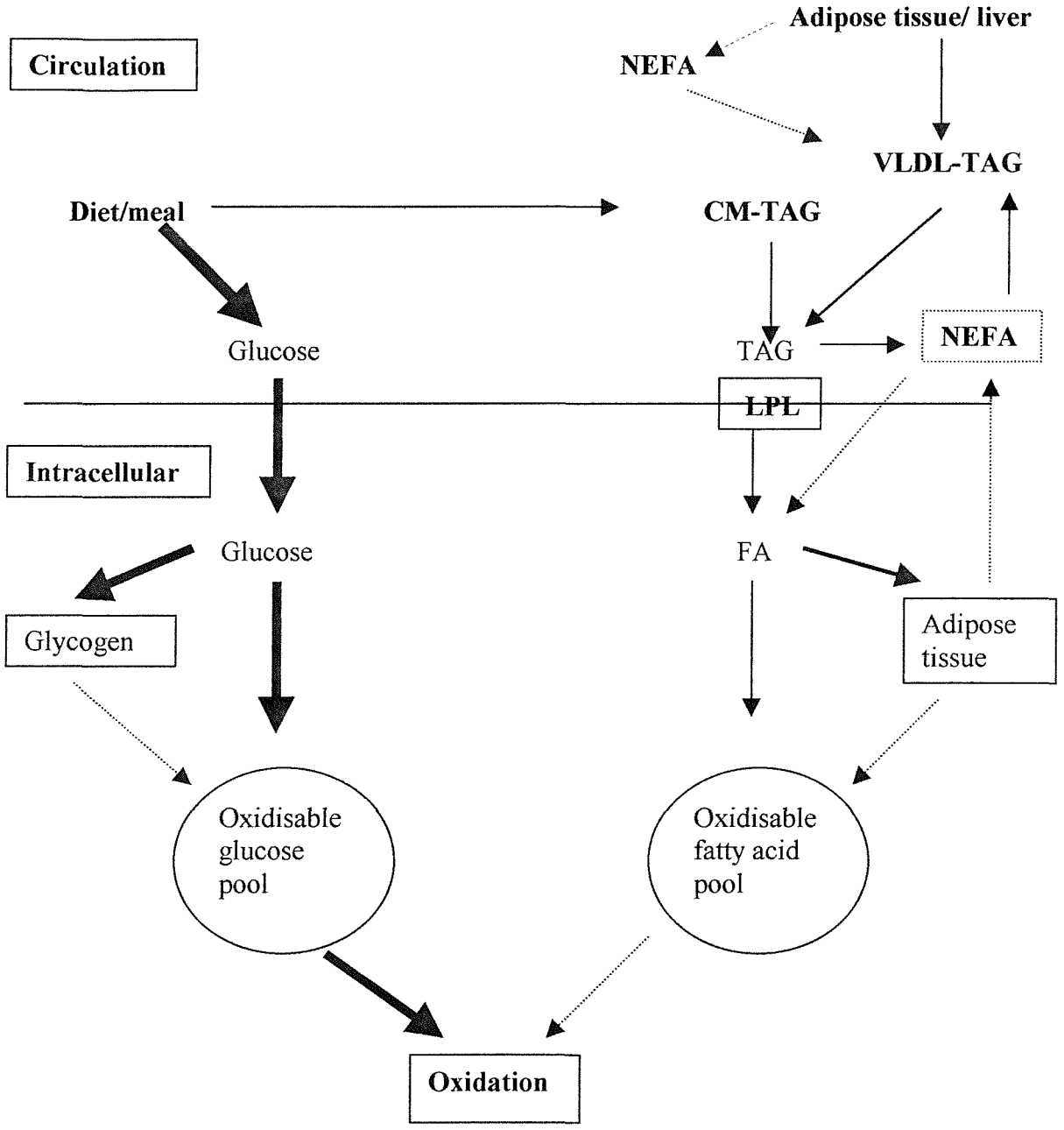
Could obesity occur because of a perturbation in lipid oxidation which allows a greater proportion of lipid to enter adipose stores? In NIDDM, does insulin resistance preventing uptake of glucose to the cells have a negative feedback effect on fatty acid oxidation leading to obesity, or does excess adipose stores from obesity prevent glucose oxidation and therefore a negative feedback to affect insulin action? It is obviously not

possible for these questions to be answered from the results of the present study, and the studies would have to be repeated with different subject groups, and with additional testmeals to examine critical limits in the macronutrient balance.

The present research has demonstrated that whilst total lipid and carbohydrate oxidation appear to be influenced by the manipulation of the macronutrient composition of the meal, the most important findings are the way in which changing the proportions of macronutrients in a meal can affect the balance of fatty acid oxidation. For example, increasing the amount of lipid in a meal results in an increase in exogenous lipid substrate whilst endogenous lipid oxidation remains constant or decreases, suggesting that the release of endogenous fatty acids is inhibited to allow preferential clearance of exogenous fatty acids from the circulation. It appears that the raised concentration of NEFA in the circulation, results in an increased flux of fatty acids into oxidative tissue, and an increase in fatty acid oxidation. It is known that the delivery of fatty acids influences the rate of fatty acid oxidation, which is dependant upon the concentration of fatty acids and blood flow. At the same time, an increase in plasma glucose concentration suggests that glucose oxidation is inhibited by the presence of high NEFA concentration resulting in a poor concentration gradient for the uptake of glucose into the cell, and an increase in plasma glucose concentration.

In contrast, increasing the amount of carbohydrate in a meal has an opposite effect on lipid oxidation. Increasing the availability of glucose results in a stimulation of glucose oxidation and glycogen formation by insulin, which therefore increases the subsequent concentration gradient resulting in greater uptake of glucose. At the same time the presence of insulin suppresses the lipolysis of adipose tissue TAG resulting in a decrease in endogenous NEFA concentration in the circulation. In addition the entry of LCFA into the mitochondria for oxidation is inhibited resulting in a decrease in fatty acid oxidation.

Figure 7.11 Regulation of lipid oxidation following consumption of a 100g sucrose meal



The most interesting finding from this research presented in this thesis is that there appears to be an individual based 'metabolic capacity' to 'metabolise' dietary and endogenous lipid. It appears that in some individuals there is an upper limit to oxidise fatty acids which resulted in an elevation of plasma TAG and NEFA concentrations following the high-fat meal. When the high-fat meal was consumed such subjects were unable to increase lipid oxidation to accommodate the influx of lipid. The addition of sucrose to a meal encompasses glucose metabolism into the equation, and the results from this study were more difficult to interpret. It is possible that some subjects exhibited an insulin resistance, although this was not directly studied. More interestingly perhaps would be the effect of increasing the amount of sucrose in NIDDM patients or the obese in order to understand to role of glucose and insulin in influencing fatty acid metabolism. However, several questions remain unanswered such as what controls the rate of entrapment of fatty acids into the cells? Is entrapment governed by concentration alone, or is this dependant upon the fatty acid binding proteins and fatty acid transporters? If so, what is controlling the proteins, and how can this be manipulated? What determines an individuals metabolic capacity to 'cope' with dietary lipid? How can the ability to metabolise lipid effectively or ineffectively affect the balance between dietary lipid and disease?

7.4 Dietary lipid and disease – Potential consequences of manipulating the macronutrient composition of meals

The present research to date has provided evidence that manipulating the macronutrient composition of meals can have a profound effect on postprandial lipid metabolism. From the research it is known that by altering the amount of lipid or carbohydrate within a meal, circulating lipids and the ratio of exogenous and endogenous lipid oxidation can be manipulated. The question that remains unanswered, however, is how the manipulation of meal composition may affect disease outcome, and how the results from this research can be utilised to develop a conceptual framework to make statements regarding the use of dietary manipulation in the treatment of disease.

A large proportion of postprandial lipid metabolism research has focused on perturbations in lipid metabolism in individuals with cardiovascular disease, obesity and insulin resistance, prompting the collation of several review articles on the subject

[Patsch, 1987; Austin, 1990; Patsch *et al.*1992; Havel, 1994; Karpe and Hamsten, 1995]. Raised postprandial lipaemia has been shown in CVD following a moderate lipid meal [Patsch *et al.*1992; Shepherd and Packard, 1996; Braun *et al.*1997; Karpe, 1997]. The mechanisms for the role of raised TAG concentrations in CVD remain unclear, however, it has been suggested that an increased magnitude and duration of lipaemia results in enhanced cholesteryl ester transfer from HDL-cholesterol to TRL [Tall *et al.*1986], as discussed in section 2.2.3. Raised postprandial lipaemia has also been demonstrated in patients with NIDDM [Coppack, 1997]. NIDDM [Yki-Jarvinen *et al.*1988], and obesity [Bjorntorp, 1994; Frayn *et al.* 1996] are marked by insulin resistance and a failure to suppress plasma NEFA concentrations. Obese patients display raised endogenous lipid levels with higher plasma TAG and VLDL-TAG in the fasted state, whilst postprandial chylomicron-TAG clearance by adipose tissue was suppressed in the obese [Potts *et al.*1995]. In general, substrate oxidation has not been as extensively studied in disease groups as plasma lipid measurements, however, as has been demonstrated in the research reported in this thesis fatty acid oxidation may be key in determining fatty acid uptake and plasma lipid concentrations. A failure to increase fat oxidation in response to increasing dietary fat content was shown in formerly obese women [Astrup *et al.*1994]. The failure of dietary fat to promote fat oxidation was suggested to be a factor favouring the development of obesity [Schutz *et al.*1989]. The inability of the individual to adjust metabolism so as to attenuate the effect of changes in fat intake on fat balance provides a metabolic explanation for epidemiological findings that obesity is enhanced by dietary fat intake [Schutz *et al.*1989]. Indirect calorimetry data suggests that obese individuals are less able to regulate lipid oxidation to intake than lean individuals [Thomas *et al.*1992; Volschenk *et al.*1993]. Several questions remain unanswered such as how does a failure to increase in fat oxidation result in obesity? How can obesity therefore be explained when lipid oxidation has been shown to be increased in obese subjects [Jones,1996]? How can the results from the present research be associated with disease and in what ways could manipulation of the macronutrient composition of meals be useful in the treatment of such disease?

The research reported in this thesis has demonstrated that the balance of macronutrient oxidation, and concentration of circulating lipids can be profoundly affected by the manipulation of the macronutrient composition of meals in young, healthy subjects. A doubling in the amount of lipid in a meal resulted in an overall

increase in total lipid oxidation, which was found to be due to an increase in exogenous lipid oxidation. Some individuals, however, appeared to have an upper metabolic capacity to oxidise lipid, and that this had a 'negative feedback' effect on circulating lipid concentrations. It is possible that such individuals may have an increased risk of developing disease in later life, although the subjects would have to be studied over several years to validate this theory. Also what determines the 'metabolic capacity? Is this dependant on genetics, foetal programming, lifestyle, diet, body composition or other factors? In addition there appears to be individual metabolic capacities in response to a meal with added sucrose. Some individuals show little glycaemic and insulinogenic response, yet also have a modest circulating lipid concentration, whilst others demonstrate large insulinogenic responses with raised concentrations of plasma lipids. Is this related to insulin resistance, or that some individuals have a better control over glucose and fatty acid metabolism? What is clear is that consumption of a high sucrose meal results in an almost complete suppression of endogenous lipid oxidation, preventing the release of adipose tissue stores which could be inferred as promoting obesity. However, at the same time, exogenous lipid oxidation remains relatively constant with 50g of sucrose and only begins to decrease with 100g of sucrose suggesting a mechanism by which glucose and fatty acid oxidation are occurring simultaneously.

Cardiovascular risk, NIDDM and obesity are all related to an increase in the magnitude and duration of postprandial lipaemia, and alterations in macronutrient balance although this has not been extensively studied. An increase in postprandial lipaemia was observed following the 0g of sucrose meal and the high fat meal, with the highest levels of lipid oxidation (exogenous and endogenous), suggesting that these meals may result in an increased risk of CVD and possibly obesity. The addition of 50g of sucrose led to a decrease in postprandial lipaemia, with a reduction in NEFA concentration, and this could be inferred to be a decrease in 'atherogenicity'. At the same time, adipose tissue TAG hydrolysis was decreased and endogenous lipid oxidation was suppressed suggesting a possible increase in the risk of obesity. With the addition of 100g of sucrose, a prolongation of lipaemia over a longer time period was observed, possibly increasing the retention of TRL in the circulation resulting in enhanced cholesteryl ester transfer, and cholesterol enriched remnant particles. In addition, 100g of

sucrose completely suppressed endogenous lipid oxidation, a factor possibly favouring obesity.

Obviously there are several confounding factors in utilising the data from the present research to offer dietary advice to reduce the risk of developing such disease or to reduce the symptoms of disease. The studies were all carried out in young, healthy subjects in order to understand how manipulating the composition of a meal affects the subtle balance between glucose and fatty acid oxidation, and circulating lipid concentrations under circumstances of 'normal' metabolism. All the results were based on studies examining the effects of two sequential meals on outcome-what would be the outcome if identical meals were consumed for a period of time? How would consumption of a high-fat diet affect lipid oxidation? Would there be changes in the role of exogenous and endogenous lipid in oxidation over time? Would the flux of lipid following a meal (described in figure 7.11) alter with time? How would continuous sucrose overfeeding affect postprandial lipaemia? Would there be a cumulative effect resulting in a raised post-absorptive magnitude of plasma TAG? Would lipid oxidation be continuously suppressed resulting in weight gain and obesity? Would insulin responses be able to 'cope' with such increased sucrose quantities or would this eventually result in the onset of insulin resistance? The only method available to test the questions relating to the effect of diet on lipid metabolism would be to carry out a longitudinal study with lipid or sucrose overfeeding coupled to examination of the postprandial response to a meal of similar composition at regular intervals throughout the study period.

7.5 Conclusions and further work

The research reported in this thesis used a combination of traditional measures of lipid metabolism, such as indirect calorimetry and enzymatic methods, with novel ^{13}C-labelling techniques to determine the effect of manipulating the macronutrient composition of a meal on postprandial lipid metabolism. The magnitude of within-individual variability was examined when extrinsic factors such as background diet, physical activity and lifestyle were controlled. There was little within-individual variation in measures between the trials suggesting the protocol was acceptable for use in further studies when manipulating macronutrient compositions. In contrast to previous

studies [Murphy *et al.*1995; Jones *et al.*1998] absorption of the labelled lipid was between 97% and 100% complete irrespective of the testmeal composition. This suggested that by following the study protocol and administering the labelled lipid via an emulsion, further stool collections were unnecessary. This study was the first time that low and repeatable stool excretion of ^{13}C had been accomplished.

The role of fatty acid oxidation as a regulator in controlling not only the rate and extent of lipid and carbohydrate oxidation, but also as a possible regulator in circulating lipid concentrations was examined. Between individual differences in the ability to oxidise lipid were observed in all trials, with some subjects displaying an upper capacity to oxidise lipid dependant upon the amount of exogenous macronutrient in the meal. It is possible that the metabolic capacity to oxidise lipid may have a 'negative feedback' effect on the entrapment of fatty acids into tissue, and as a consequence the concentration of circulating NEFA and TAG. By altering the composition of meals, it was possible to manipulate the macronutrient oxidation balance, and to alter the ratio of exogenous and endogenous fatty acid oxidation. By doing this, the concentrations of circulating TAG and NEFA were manipulated and by inference the association of dietary lipid with disease was also altered. By applying the results of this research to other subject groups such as the obese or hypertriglyceridemic it may be possible to provide a conceptual framework whereby the progression and symptoms of such disease may be manipulated by an alteration in the macronutrient composition of a meal or diet.

In order to develop and extend our understanding of lipid metabolism, and in particular the dietary manipulation of lipid metabolism further work is necessary. Studies examining the effect of different types of carbohydrate on the lipaemic and metabolic responses to meals could improve the existing knowledge of the effect of carbohydrates with varying glycaemic indices. How does the consumption of starch affect the magnitude of postprandial lipaemia, compared to simple sugars such as glucose or fructose? How would an alteration in plasma lipid concentration be related to the glucose/ fatty acid regulation of substrate oxidation? What effect would changing the digestibility of carbohydrate have on gastric emptying and the appearance of lipids in the circulation? In order to develop understanding of the regulation of the process of lipid oxidation, and what influence this may have on postprandial lipaemia, measures of the flux of fatty acid and glucose in the circulation and across membranes could provide an insight into rate limiting steps and negative feedback loops. This particular research

would probably involve invasive techniques and may not be possible on human subjects, however the use of animal models may provide an insight into the effect of meal composition on fatty acid flux. On a more general level, it may be possible to develop theories of amino acid flux used to calculate the rates of protein synthesis and degradation to provide a mathematical answer to the question.

It would be beneficial to repeat the trials presented in this thesis in different subject groups such as the obese, insulin resistant or hypertriglyceridemic patients to understand how disease may confer a metabolic capacity for an individual to metabolise lipid, or how the metabolic capacity may actually result in disease; is this cause or effect? Finally, the effect of the meal composition on fatty acid oxidation and circulating lipids over a long period of time would enable the development of specific dietary guidelines to reduce the risk of CVD, insulin resistance and obesity.

CHAPTER 8

Appendices

Appendices 1: Chapter 3

Appendix 1.1 Preceding diet composition consumed by all subjects prior to study 1 (reference testmeal and high fat trials)

Day 1

Food	Weight (g)	Energy (kJ)	Protein (g)	Carbohydrate (g)	Lipid (g)
Breakfast					
Branflakes	50	676	5.1	34.7	1.0
S.S.milk	250	505	8.5	12.5	4.3
Orange juice	200	310	1.2	18.6	0.0
White bread	38	386	3.1	17.1	1.2
Jam	15	164	0.1	10.4	0.0
Clover margarine	10	285	0.1	0.1	7.5
Lunch					
BLT sandwich	174	1594	18.4	28.2	21.6
Crisps	40	888	2.3	19.2	14.2
Orange	70	100	0.6	5.6	0.0
Evening meal					
Chicken curry and rice	340	2377	32.3	45.9	29.9
Naan bread	75	881	6.2	36.9	4.1
Sticky toffee pudding	110	1275	3.9	50.3	12.4
Single cream	30	233	0.8	1.2	5.4
Snacks					
Kit Kat	24	502	2.0	14.5	6.4
Total		**10176**	**84.6**	**295.2**	**108.0**
% total energy			**14.1%**	**46.4%**	**39.3%**

Day 2

Food	Weight (g)	Energy (kJ)	Protein (g)	Carbohydrate (g)	Lipid (g)
Breakfast					
Rice krispies	45	707	2.7	40.4	0.4
S.S.milk	250	505	8.5	12.5	4.3
Orange juice	200	310	1.2	18.6	0.0
White bread	38	386	3.1	17.1	1.2
Jam	15	164	0.1	10.4	0.0
Clover margarine	10	285	0.1	0.1	7.5
Lunch					
Turkey & coleslaw sandwich	170	1020	17.5	29.9	5.8
Crisps	40	888	2.3	19.2	14.2
Banana	100	337	1.1	19.2	0.3
Evening meal					
Sausages in onion gravy	285	1625	21.9	24.5	22.5
Potato croquettes	250	1330	6.4	42.6	13.4
Vegetables	100	83	1.8	6.1	0.0
Chocolate mousse	100	1344	3.1	19.2	26.0
Snacks					
Chocolate cake	72	1340	4.6	40.0	16.8
Total		**10324**	**74.4**	**299.6**	**112.4**
% total energy			**12.3%**	**46.4%**	**40.3%**

Day 3

Food	Weight (g)	Energy (kJ)	Protein (g)	Carbohydrate (g)	Lipid (g)
Breakfast					
Shreddies	50	706	5.0	37.0	0.8
S.S.milk	250	505	8.5	12.5	4.3
Orange juice	200	310	1.2	18.6	0.0
White bread	38	386	3.1	17.1	1.2
Jam	15	164	0.1	10.4	0.0
Clover margarine	10	285	0.1	0.1	7.5
Lunch					
Cheese sandwich	273	3016	28.1	55.9	42.9
Crisps	40	888	2.3	19.2	14.2
Apple	100	196	0.3	11.9	0.0
Evening meal					
Chicken & pasta bake	283	1485	26.3	23.5	17.3
Mixed salad	100	93	1.0	4.0	0.0
Lemon cheesecake	90	1051	8.2	28.7	11.5
Snacks					
Chocolate biscuits	34	740	2.2	22.4	8.8
Apple pie	66	928	2.3	35.9	7.5
Total		**10753**	**88.6**	**297.2**	**116.0**
% total energy			**14.0%**	**44.2%**	**39.9%**

Appendix 1.2 Preceding diet composition consumed by all subjects for two days prior to study 2 (sucrose trials)

Day 1

Food	Weight (g)	Energy (kJ)	Protein (g)	Carbohydrate (g)	Lipid (g)
Breakfast					
Rice krispies	45	713	2.7	39.6	0.9
S.S.milk	250	505	8.5	12.5	4.3
Orange juice	200	310	1.2	18.6	0.0
White bread	38	386	3.1	17.1	1.2
Jam	15	164	0.1	10.4	0.0
Clover margarine	10	285	0.1	0.1	7.5
Lunch					
BLT sandwich	174	1594	18.4	28.2	21.6
Crisps	40	888	2.3	19.2	14.2
Banana	100	337	1.1	19.2	0.3
Evening meal					
Chicken curry and rice	450	2540	35.6	76.5	17.6
Chocolate mousse	100	1344	3.1	19.2	26.0
Snacks					
Apple pie	55	766.2	1.6	31.7	6.4
Kit Kat	24	502	2.0	14.5	6.4
Total		**10336**	**79.7**	**299.5**	**106.6**
% total energy			**13.1%**	**46.4%**	**38.2%**

Day 2

Food	Weight (g)	Energy (kJ)	Protein (g)	Carbohydrate (g)	Lipid (g)
Breakfast					
Shreddies	50	706	5.0	37.0	0.4
S.S.milk	250	505	8.5	12.5	4.3
Orange juice	200	310	1.2	18.6	0.0
White bread	38	386	3.1	17.1	1.2
Jam	15	164	0.1	10.4	0.0
Clover margarine	10	285	0.1	0.1	7.5
Lunch					
Cheese sandwich	273	3016.7	28.1	56.0	42.9
Crisps	40	888	2.3	19.2	14.2
Orange	70	100	0.6	5.6	0.0
Evening meal					
Chicken & pasta bake	283	1570.6	27.5	25.2	19.8
Mixed salad	100	93	1.0	4.0	0.0
Lemon cheesecake	125	1736.3	5.6	26.8	32.5
Snacks					
Chocolate biscuits	34	739.8	2.2	22.3	8.7
Total		**10503**	**85.3**	**253.7**	**131.5**
% total energy			**13.8%**	**38.6%**	**46.3%**

Appendix 1.3 Methods used for the analysis of plasma glucose, TAG and NEFA concentrations using enzymatic reagent kits

Glucose Analysis

Principle:
Glucose is phosphorylated in the presence of ATP to glucose-6-phosphate by hexokinase. The G-6-P is oxidised in the presence of NAD to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. In this reaction an equimolar amount of NAD is reduced to NADH which is measured at 340nm.

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NAD} \xrightarrow{\text{Glucose-6-phosphate dehydrogenase}} \text{6-PG} + \text{NADH}$$

Reagent kit:
Glucose (HK) enzymatic determination of glucose supplied by Sigma

Reagent preparation:
Reconstitute with 10ml of distilled water and store in fridge for up to 4 weeks. Bring to room temperature before use.

Standard:
100mg/dL = 5.56mmol/lt glucose standard

Sample preparation:
Samples and standards can be used without further preparation

Analysis:
5µl blank x 2
5µl standard x 3
5µl QC x 2
5µl sample x 2
Add 0.5ml of reagent, mix by gentle inversion and leave at room temperature for 5 minutes. Measure absorbance of blank and set to zero. Read absorbance at 340nm

Sample concentration (mmol/l) = Abs sample x standard concentration / abs standard
QCs: Precipath = 13.8 mmol/l (11.8 - 16.0 mmol/l)
Precinorm = 6.66 mmol/l (5.66 - 7.66 mmol/l)

Triacylglycerol Analysis

Principle:
TAG is hydrolysed by lipase to glycerol and NEFA. Total glycerol is quantified by a Trinder type peroxidase reaction following generation of hydrogen peroxide sequential action of glycerol kinase and glycerol phosphate oxidase.

$$\text{TAG} \xrightarrow{\textit{Lipoprotein lipase}} \text{Glycerol + NEFA}$$

$$\text{Glycerol + ATP} \xrightarrow{\textit{Glycerol kinase}} \text{G-1-P + ADP}$$

$$\text{G-1-P + } O_2 \xrightarrow{\textit{Glycerol phosphate oxidase}} \text{Dihydroxyacetone phosphate + H2O2}$$

$$\text{H2O2 + 4-aminoantipyrine + ESPA} \xrightarrow{\textit{Horseradish peroxidase}} \text{Quinonimine dye + } H_2O$$

Reagent kit : Triglyceride enzymatic determination of glycerol, true TAG and total TAG

Reagent preparation: Reconstitute 1 bottle of reagent A with 40ml of distilled water and 1 bottle of reagent B with 10ml of distilled water. Bring to room temperature before use.

Standard: 2.29 mmol/l glycerol standard

Sample preparation: Samples and standards are used without further preparation.

Analysis:
Mix reagents A & B in ratio 4:1 to volume required for assay size
5µl blank x 2
5µl standard x 3
5µl QC x 2
5µl sample x 2
Add 0.4ml reagent mixture, mix and incubate at room temperature for 15 minutes
Measure absorbance of blank and set to zero
Read absorbance at 540nm

NEFA Analysis

Principle : Trinder type peroxidase reaction following generation of hydrogen peroxide from NEFA by sequential action of Acyl-CoA synthetase, Acyl-CoA oxidase and added peroxidase

$$\text{RCOOH (NEFA)} + \text{ATP} + \text{CoA} \xrightarrow{\textit{Acyl CoA Synthetase}} \text{Acyl-CoA} + \text{AMP} + \text{Ppi}$$

$$\text{ACYL-CoA} + O_2 \xrightarrow{\textit{Acyl-CoA Oxidase}} \text{2,3-trans-Enoyl-CoA} + \text{H2O2}$$

$$\text{2H2O2} + \text{3-methyl-N-ethyl-N-(β-hydroxy-ethyl)-aniline} + \text{4-aminoantipyrine} \xrightarrow{\textit{Peroxidase}} \text{Quinonimine dye} + \text{4H2O}$$

Reagent kit: WAKO NEFA kit

Reagent preparation: Reconstitute 1 bottle of reagent 1 with 10ml of buffer from bottle 1a, and 1 bottle of reagent 2 with 20ml of buffer from bottle 2a. Bring to room temperature before use

Standard: 1.0mmol/l NEFA standard

Sample preparation: Samples and standards are used without further preparation

Analysis:
10µl blank x 2
10µl standard x 2
10µl sample x 2
Sample blank x 2
Add 100µl reagent 1, mix and incubate at 37 degrees for 10 minutes
Add 200µl reagent 2, mix and incubate at 37 degrees for 10 minutes
Add 10µl sample to sample blanks, mix
Measure absorbance of blank and set to zero
Read absorbance at 550nm

Sample concentration (mmol/l) = Abs sample - abs sample blank) x
standard concentration/ abs standard

Appendix 1.4 Final method used to prepare plasma specimens to determine the ^{13}C-enrichment using GC-IRMS

Stage 1- Lipid Extraction

1) Pipette plasma sample (1ml) into labelled glass tubes with lids
2) Add 60µl TAG standard (C17:0) + 30 µl NEFA standard (C21:0) surrogate standard to each tube (concentration 1µg/µl chloroform methanol 2.1 (w/v).
3) Add 5ml [chloroform:methanol (2:1) =50mg BHT] to each tube.
4) Vortex for 30 seconds
5) Shake for 15 minutes
6) Add 1ml of 1M NaCl in distilled water to each tube and vortex for 30 seconds.
7) Spin @ 2000rpm for 10 minutes at 14°C.
8) Remove aqueous layer with glass pipette and discard.
9) Pass glass pipette through white disk to solvent layer, and remove solvent layer to clean glass tube.
10) Repeat steps 3-9 on remaining white disk. Combine solvent layers
11) Seal solvent layer under nitrogen and store at -20°C.

Stage 2- TLC

1) Remove solvent gently under nitrogen.
2) Add 100µl chloroform to each sample and swirl gently.
3) Tale up approx. 10µl with Hamilton syringe, spot onto TLC plate in 4 adjacent spots. Repeat with remaining solvent extract.
4) Add 100µl chloroform to rinse tube and pipette onto TLC plate. Repeat with additional 50µl of chloroform.
5) Pipette 15µl (TAG) or 30µl (NEFA) surrogate standard to middle of plate
6) Place TLC plate in solvent tank for approx. 30-40 minutes
7) Remove and leave in fume cupboard to dry.
8) Spray with Fluorescein (0.25% in ethanol); use appropriate standard bands to locate sample bands and mark spots under UV light.
9) Scrape off spots into marked glass tubes.
10) Add 1ml of toluene and 2ml 2% sulphuric acid in methanol (v/v) to TLC scrapings.
11) Seal tubes and incubate overnight at 50°C.
12) Remove and allow to cool.
13) Add 2ml of neutralising reagent and 2ml hexane.
14) Vortex for 30 seconds and shake for 15 minutes.
15) Centrifuge for 10 minutes at 2000rpm.
16) Remove solvent layer into round-bottomed tubes (RBT) and dry gently under nitrogen.
17) Add 2ml hexane to original scrapings tube and repeat steps 14 to 16.
18) Dry solvent under nitrogen completely.
19) Wash RBT with 200µl dry-hexane, vortex and transfer to mini-vial. Repeat with 3 x 200µl dry hexane, transferring to mini-vial. Dry solvent completely in min-vial.
20) Add internal standard (C23:0 100µg/100µl dry hexane); 60µl (TAG) or 30µl (NEFA) to mini-vial.
21) Dry completely and seal sample under nitrogen. Store at -20°C prior to analysis

Appendix 1.5 Calculation of the total ^{13}C-enrichment of stool samples following ingestion of [1,1,1-^{13}C]tripalmitin within a standard meal, using the predictive equations of Schoeller *et al* [1981]

Stage 1
To calculate the isotope ratio value for the labelled substrate

Isotope ratio = (RPDB/ 1000) x (M/P x n) x 100

RPDB = ^{13}C:^{12}C ratio of reference standard (0.0112372)
M = Molecular weight of substrate
P = Isotopic purity of substrate
n= number of carbon atoms labelled per molecule of substrate

Stage 2
To calculate the mmol of carbon in the sample

The amount of carbon in the sample is calculated as μg of carbon in the sample. This figure should be divided by 1000 to give the mg of carbon in the sample, and then divided by 12 (atomic weight of carbon) to give the mmol of carbon in the sample.

Stage 3
To calculate the mmol of carbon in the whole stool sample

Total dry stool (g) = ? mg dry stool
In xmg of dry stool (from ANCA analysis), there is xmmol of carbon. Therefore in the whole stool sample there is xmmol of carbon.
e.g 61g of dry stool
61g = 61100mg of dry stool
1.77mg = 0.06313 mmol of carbon
61100mg = x mmol of carbon

Stage 4

To calculate the total faecal excretion of ^{13}C-label as a proportion of administered dose per 24 hours

$$\text{Faecal ex/24 hours} = \frac{\text{IR x } (\delta^{13}Ct - \delta^{13}Ct = o) \text{ x mmol C/24 hours x 100}}{\text{mg administered}}$$

IR = isotope ratio (stage 1)
$\delta^{13}Ct$ = ^{13}C-enrichment of sample
$\delta^{13}Ct = o$ = ^{13}C-enrichment of baseline sample
mmol C = as calculated in stage 3
mg administered = measured during emulsion preparation

Worked Example for total ^{13}C-enrichment of stool samples:-

Subject 1 – Standard-fat trial

Stage 1:-

Isotope ratio = (RPDB/1000) x (M/P x n) x 100
IR= (0.0112372/ 1000) x (810.3/99 x 3) x 100

= 3.066×10^{-3} (constant for all subjects)

Stage 2:-

mmol carbon in the sample analysed:-
757.59 ug carbon in 1.77 mg of stool
Therefore, 0.75759 mg carbon in 1.77 mg stool (divide by 1000)
Therefore, 0.06313 mmol carbon in 1.77 mg stool (divide by 12 atomic weight of carbon)

Stage 3:-

mmol carbon in whole stool sample:-
whole sample = 61.12g = 61120 mg stool

1.77mg = 0.06313 mmol carbon
61120 mg = x
therefore x = 61120 x 0.06313/ 1.77
= **2179.95 mmol carbon**

Stage 4:-

Faecal excretion/24 hours = $\frac{\text{IR x } (\delta^{13}Ct - \delta^{13}Ct = o) \text{ x mmol C/24hours x 100}}{\text{mg administered}}$

= $\frac{3.066 \text{ x } 10\text{-}3 \text{ (-26.37—26.43) x } 2179.95 \text{ x } 100}{670}$

= 0.06% of administered dose

Appendix 1.6 The recovery of $^{13}CO_2$ on the breath calculated as a percentage of administered dose of [1,1,1-^{13}C]tripalmitin within a standard meal, using the predictive equations of Watkins *et al* [1982]

Stage 1: -
How much ^{13}C-label has been administered to the subject?

$$\text{mmol } ^{13}\text{C administered} = \frac{\text{mg substrate} \times (\text{P} \times \text{n}/100)}{\text{M}}$$

M = molecular weight of [1,1,1-^{13}C]tripalmitin
P = Purity of substrate (99 atom % excess)
n = Number of carbons which are 13c

Stage 2: -
Conversion of VCO_2 (ml/min) to VCO_2 (mmol/hour)
1 mol of gas = 22.4 litres (Avogadros constant), therefore VCO_2 (ml/min) divided by 22.4 x 60 = VCO_2 (mmol/hr)

Stage 3: -
Calculate the mmol excess of ^{13}C per mmol CO_2

$$\text{mmol excess } ^{13}\text{C/mmol } CO_2 = (\delta^{13}Ct - \delta^{13}Ct{=}o) \times \text{RPDB} \times 1/1000$$

$\delta^{13}Ct$ = ^{13}C-enrichment of breath at timepoint
$\delta^{13}Ct = o$ = ^{13}C-abundance of baseline sample
RPDB = ^{13}C to 12C ratio of reference standard (0.0112372)

Stage 4
Calculation of the percentage of administered dose excreted as $^{13}CO_2$ per hour

$$\text{\% administered dose/ hour} = \frac{\text{mmol excess } ^{13}\text{C/mmol } CO_2 \times VCO_2 \times 100}{\text{mmol } ^{13}\text{C administered}}$$

VCO_2 = Rate of CO_2 excretion in mmol/hour

mmol ^{13}C administered = as calculated in stage 1

Worked example for calculation of $^{13}CO_2$ on breath:-

Subject 1- Standard fat trial

Stage 1:-

mmol ^{13}C administered = mg substrate x (P x n/100)
M

= 670 x (99 x 3/100)
810.3
= **2.456 mmol ^{13}C administered**

Stage 2:-

Time	VCO_2 (ml/min)	VCO_2 (mmol/hour
Base	209	559.8
1	274	733.9
2	285	763.4
3	247	661.6
4	209	559.8
5	196	525.0
6	207	554.5
7	236	632.1
8	252	675.0
9	240	642.9
10	230	616.1
15	209	559.8
24	209	559.8

Stage 3:-

1 hour specimen only:-

mmol excess ^{13}C/mmol CO_2 = ($\delta^{13}Ct$ - $\delta^{13}Ct=o$) x RPDB x 1/1000

= (-23.37—25.13) x 0.0112372 x 1/1000

= 1.76 x 0.0112372 x 1/1000

= 1.977 x 10-5

Stage 4:-

% administered dose/ hour = $\frac{\text{mmol excess } ^{13}C/\text{mmol } CO_2 \times VCO_2 \times 100}{\text{mmol } ^{13}C \text{ administered}}$

$$= \frac{1.9777 \times 10\text{-}5 \times 733.9 \times 100}{2.456}$$

= 0.59% of administered dose

The process was repeated for the remaining timepoints and from a graph the area under the timecourse curve was calculated to determine total $^{13}CO_2$ excretion over the study period

Appendix 1.7 Equations used to determine the recovery of ^{13}C-label from plasma samples, expressed as the ug ^{13}C per ml of plasma

Stage 1:-

What is the concentration of palmitic acid in the sample?

Calculation of area under the palmitic acid peak from GC-IRMS chromatogram using Fig.P for Windows, and comparison to standard peak of known concentration. Therefore have the concentration of palmitic acid in the sample.

Stage 2:-

What proportion of the total palmitic acid in the sample is enriched with ^{13}C?

Proportion of ^{13}C-FA/FA= (^{13}C ($^{0}/_{00}$) + 31.57)/ 53.8

Where:-

^{13}C ($^{0}/_{00}$) = enrichment of palmitic acid peak in delta units
(Equation is derived from a series of standard curves)

Stage 3:-

What amount of palmitic acid is labelled with ^{13}C?

Labelled palmitic acid (ug/ml) = Concentration of palmitic acid/ml (stage 1) x proportion of ^{13}C-PA/PA (stage 2)

Stage 4:-

What is the total amount of ^{13}C-labelled palmitic acid recovered over the study period?

Calculation of the total area under the time versus concentration of labelled fatty acid curve over the study period, expressed as the ug labelled fatty acid/ ml plasma per six hours

Worked example to calculate the total amount of ^{13}C-palmitic acid recovered over the study period:-

Sample	Area surrogate	Area FA	Amount surrogate (ug)	Concentration of FA (ug/sample)	Concentration of FA (ug/ml)	Enrichment ($^0/_{00}$)	*FA/FA ($^0/_{00}$)	***FA (ug/ml)**
Base	0.6393	0.4906	31.93	24.50	24.50	-11.67	0.37	**0.09**
1 hour	1.0210	0.3876	31.93	12.12	12.12	140.79	3.20	**0.39**
2 hour	1.3549	0.5866	31.93	13.82	13.82	339.88	6.90	**0.95**
3 hour	2.7992	1.5483	31.93	17.66	17.66	215.73	4.60	**0.81**
4 hour	1.2020	0.8800	31.93	23.38	23.38	85.388	2.17	**0.51**
5 hour	3.0086	1.3437	31.93	14.26	14.26	49.838	1.51	**0.22**
6 hour	1.8627	1.4277	31.93	24.47	24.47	25.083	1.05	**0.26**

Appendix 1.8 Method used for lipid extraction, separation and derivatisation of plasma samples with surrogate standards added at the points shown in italics

STAGE 1 - LIPID EXTRACTION

1) Pipette plasma sample into labelled glass tubes with lids (if lipoprotein sample use present tube).

Sample 1

2) Add 60µl TAG standard (C:17) + 30µl NEFA standard (C:21) surrogate standard to each tube (Concentration 1µg/µl chloroform methanol 2:1, SO make up 1mg/ml etc.)

3) Add 5ml of [chloroform:methanol (2:1) + 50mg/l BHT] to each tube (5 x 1ml microman).

Sample 2

4) Vortex

5) Shake for 15 minutes

6) Add 1ml of 1M NaCl in distilled water to each tube and vortex

Sample 3

7) Spin @ 2000rpm for 10 minutes @ 14°C

8) Remove aqueous layer (top layer)with glass pipette and white disc with spatula, and discard

Sample 4

9) Seal solvent layer under nitrogen and then store @ -20°C

STAGE 2 - TLC

1) Remove solvent gently under nitrogen

Sample 5

2) Add 100µl chloroform to each sample and swirl gently

3) Take up approx. 10µl with TLC syringe, spot onto TLC plate in 4 adjacent spots. Repeat with remaining solvent extract

4) Add 100µl chloroform to rinse tube and pipette onto TLC plate. Repeat with additional 50µl of chloroform, and pipette onto TLC plate

5) Pipette 15µl standard to middle of the plate

6) Place TLC plate in solvent tank for approx. 30 minutes until solvent has reached line B

7) Remove and leave in fume cupboard to dry

8) Spray with Rhodamine B (0.25% in ethanol-250mg in 200ml ethanol); use appropriate standard bands to locate sample bands and mark spots under UV light

9) Scrape off spots into marked glass tubes

10) Wrap TLC plate in foil and file

Sample 6

11) Add 1ml toluene and 2ml 2% sulphuric acid in methanol to TLC scrapings

12) Place in heating block overnight @ 50°C

13) Remove and allow to cool

Sample 7

14) Add 2ml of neutralising reagent and 2ml hexane

15) Vortex for 30 seconds and shake for 15 minutes

16) Centrifuge for 10 minutes @ 2000rpm

17) Either parafilm and leave in fridge, or remove solvent layer into round bottomed glass tubes and blow down gently under nitrogen

18) Add 2ml hexane to original scrapings tube and repeat steps 15 to 17

19) Dry solvent under nitrogen from round bottomed tubes, and remove remaining solvent to mini-vials

20) Wash round-bottomed tubes with 3x 200µl hexane and repeat step 19

Sample 8

21) When solvent is completely dry add C:23 internal standard to each tube (concentration 1µg/µl hexane). Add 30µl to NEFA and 60µl to TAG samples

22) Wash out round bottomed tubes with acetone and soak in Decon overnight

Appendix 1.9 Four methods of lipid extraction examined to validate existing methods used for the extraction of lipid from plasma

METHOD 1- Traditional Method

1) Pipette plasma sample into labelled glass tubes with lids (if lipoprotein sample use present tube).

2) Add 60µl TAG standard (C:17) + 30µl NEFA standard (C:21) surrogate standard to each tube (Concentration 1µg/µl chloroform methanol 2:1, SO make up 1mg/ml etc.)

3) Add 5ml of [chloroform:methanol (2:1) + 50mg/l BHT] to each tube (5 x 1ml microman).

4) Vortex

5) Shake for 15 minutes

6) Add 1ml of 1M NaCl in distilled water to each tube and vortex

7) Spin @ 2000rpm for 10 minutes @ 14°C

8) Remove aqueous layer (top layer)with glass pipette and white disc with spatula, and discard

9) Seal solvent layer under nitrogen and then store @ -20°C

METHOD 2- Modified Method

1) Pipette plasma sample into labelled glass tubes with lids (if lipoprotein sample use present tube).

2) Add 60µl TAG standard (C:17) + 30µl NEFA standard (C:21) surrogate standard to each tube (Concentration 1µg/µl chloroform methanol 2:1, SO make up 1mg/ml etc.)

3) Add 5ml of [chloroform:methanol (2:1) + 50mg/l BHT] to each tube (5 x 1ml microman).

4) Vortex

5) Shake for 15 minutes

6) Add 1ml of 1M NaCl in distilled water to each tube and vortex

7) Spin @ 2000rpm for 10 minutes @ 14°C

8) Remove aqueous layer (top layer) with glass pipette and discard

9) Pass through white disk to solvent layer and remove to clean tube

10) Repeat steps 3-9 on white disk.

11) Seal solvent layer under nitrogen and then store @ -20°C

METHOD 3- Modified Folch

1) Add 2ml of ice cold methanol to a clean glass tube and add 30μg of surrogate standard

2) Gradually add 1ml of plasma to the methanol by dripping from a Gilson. Vortex methanol continuously during this process.

3) Add 3ml chloroform and vortex for 30 seconds to 1 minute

4) Shake for 15 minutes

5) Add 1ml of 1M NaCl in distilled water to each tube and vortex

6) Spin @ 2000rpm for 10 minutes @ 14°C

7) Remove aqueous layer (top layer) with glass pipette and discard

8) Pass through white disk to solvent layer and remove to clean tube

9) Repeat steps 3-9 on white disk.

10) Seal solvent layer under nitrogen and then store @ -20°C

METHOD 4- Folch method

1) Add 2ml of ice cold methanol to a clean glass tube. Add 30μg of surrogate standard (C21:0 1μg/μl chloroform:methanol.

2) Add 1ml of plasma gradually to the methanol and vortex continuously.

3) Add 3 ml of chloroform and an additional 2 ml of methanol.

4) Vortex and shake for 15 minutes.

5) Spin @ 2000rpm for 10 minutes to form a pellet of protein at the base of the tube.

6) Decant off solvent into clean glass tube.

7) Resuspend pellet in 4ml methanol and 3 ml chloroform.

8) Repeat steps 4 to 6.

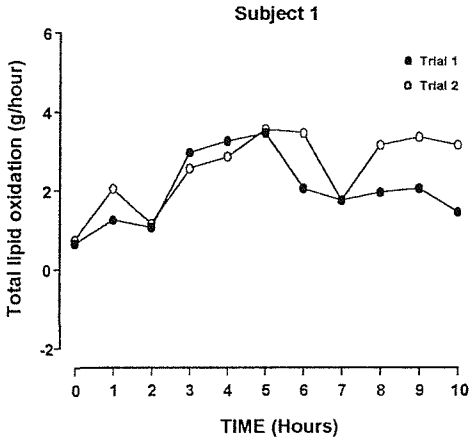
9) Add 2 ml distilled water to solvent and spin @ 2000rpm for 10 minutes.

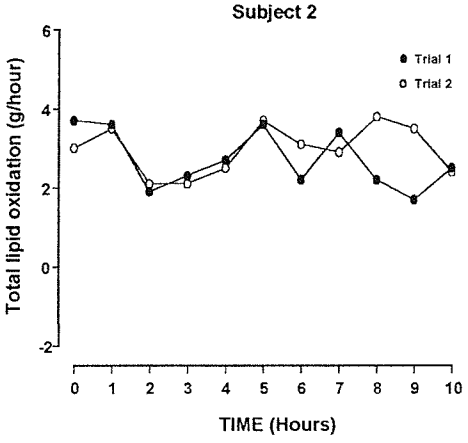
10) Remove solvent layer (bottom) to clean glass tube.

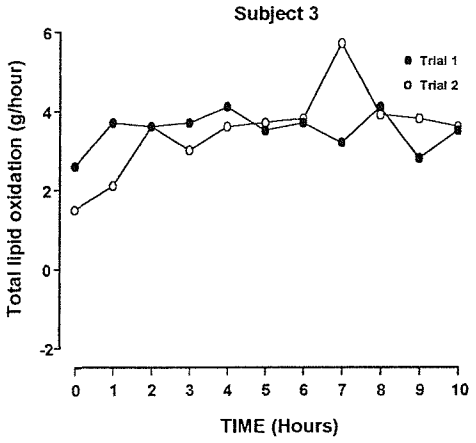
11) Continue with TLC method as before.

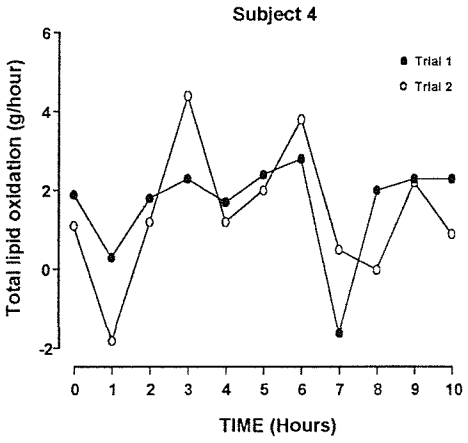
Appendices 2: Chapter 4

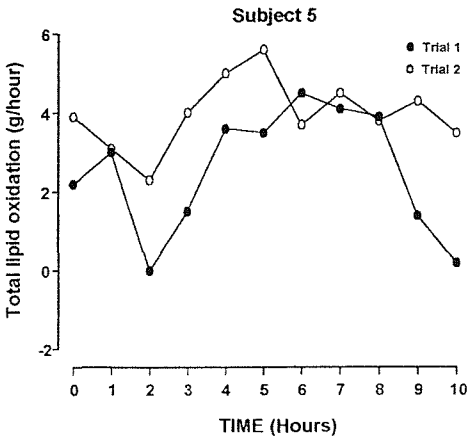
Appendix 2.1 **Total lipid oxidation (g/hour) over a ten hour study period in a following consumption of two identical reference testmeals, in six subjects.**

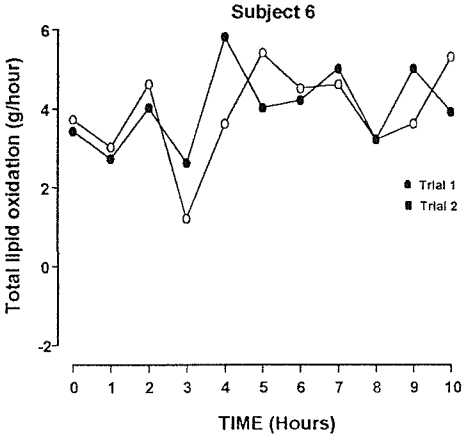




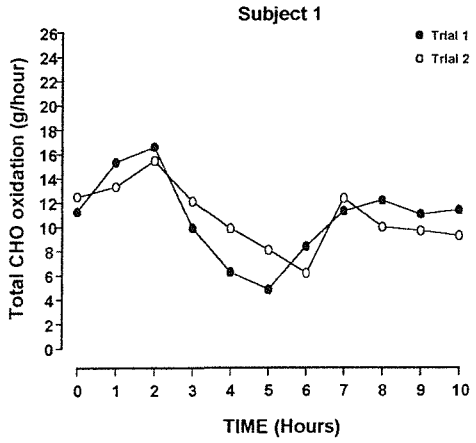


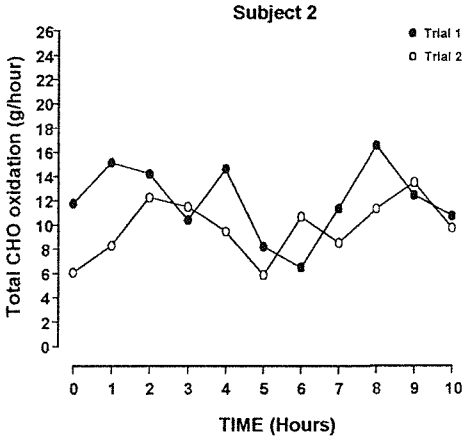


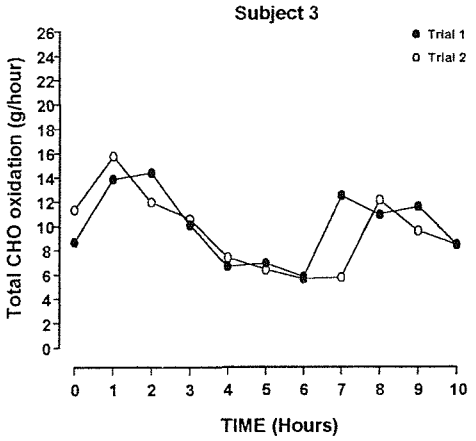


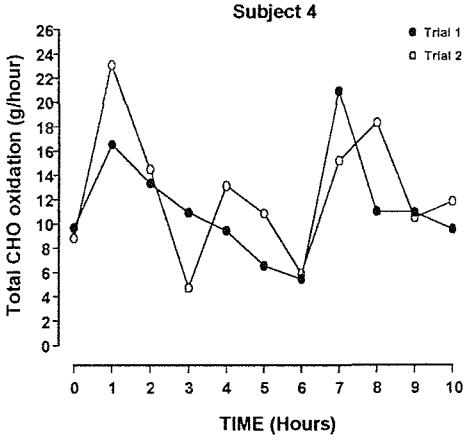


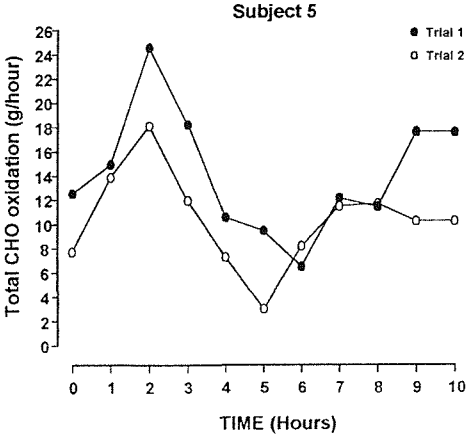
Appendix 2.2 **Total carbohydrate oxidation (g/hr) over a ten hour study period following consumption of two identical reference testmeals, in six subjects.**

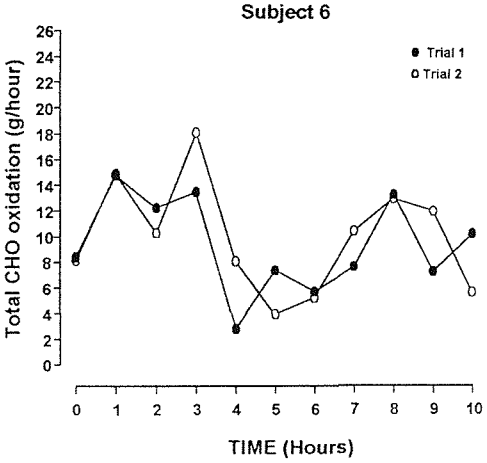




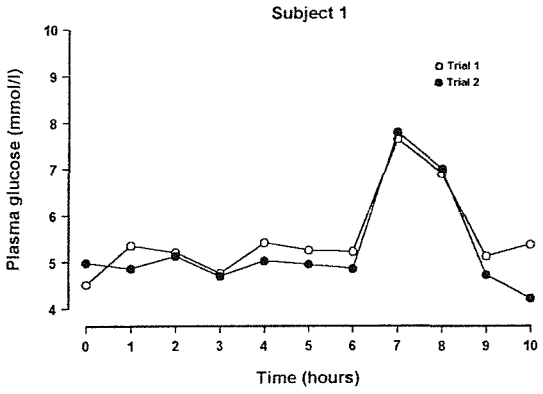


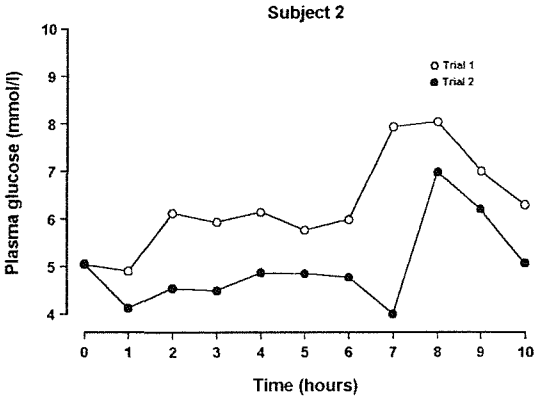


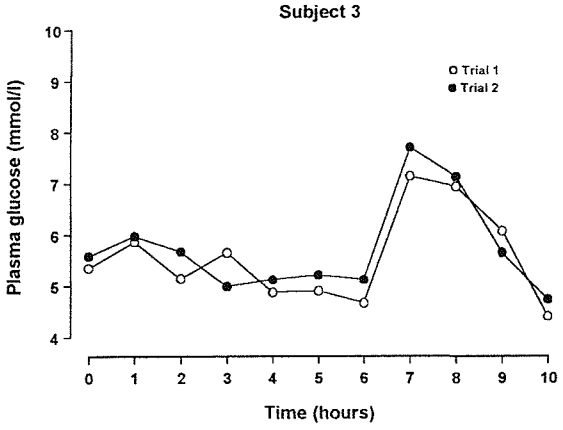


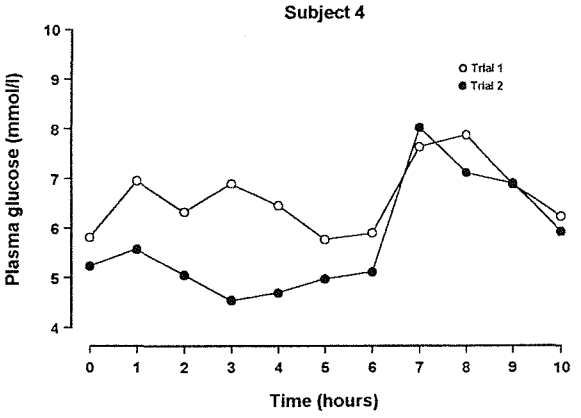


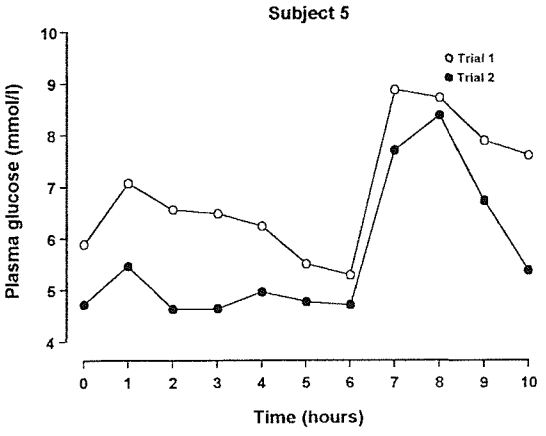
Appendix 2.3 **Plasma glucose concentration (mmol/l) over a ten hour study period following consumption of two identical reference testmeals in six subjects.**

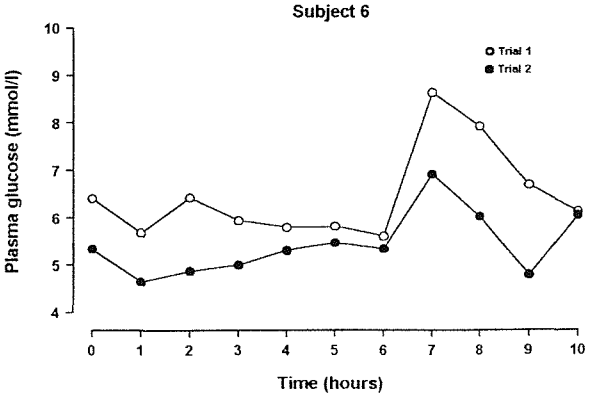




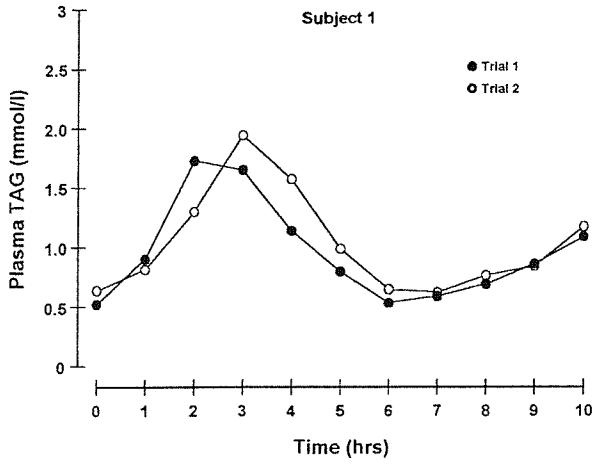


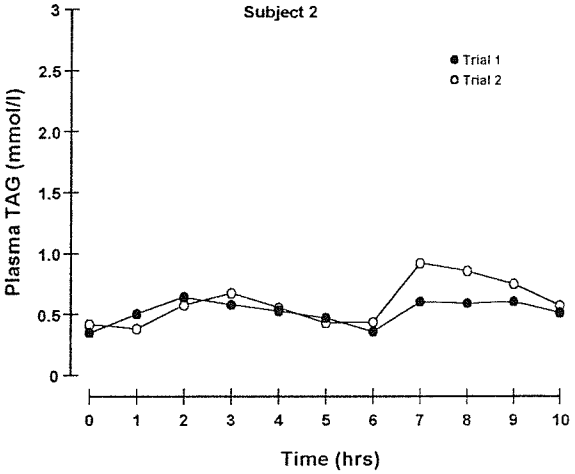


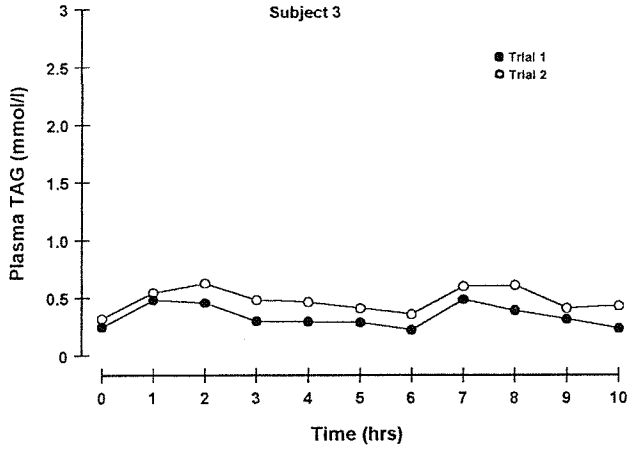


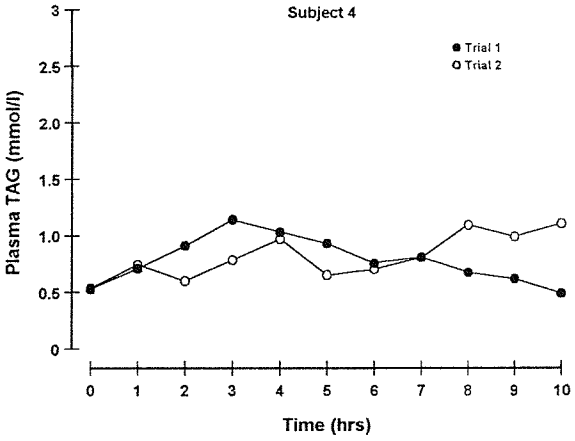


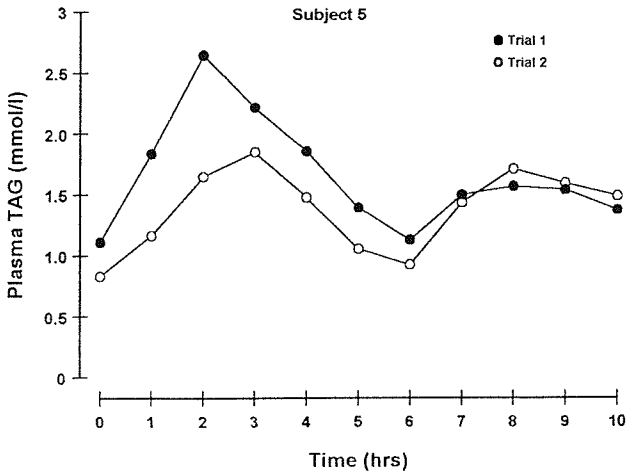
Appendix 2.4 **Plasma TAG concentrations (mmol/l) over a ten hour study period following consumption of two identical reference testmeals, shown in six subjects.**

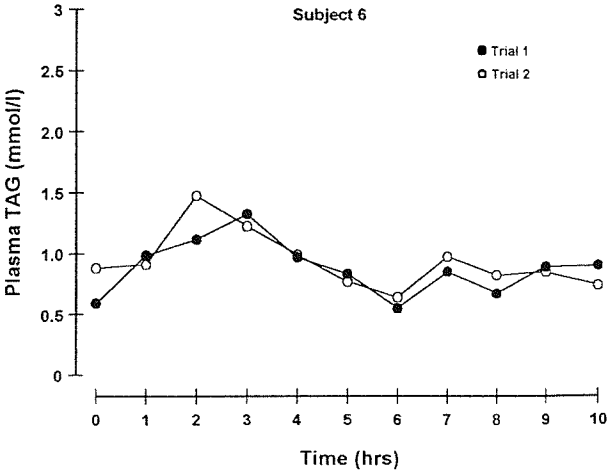




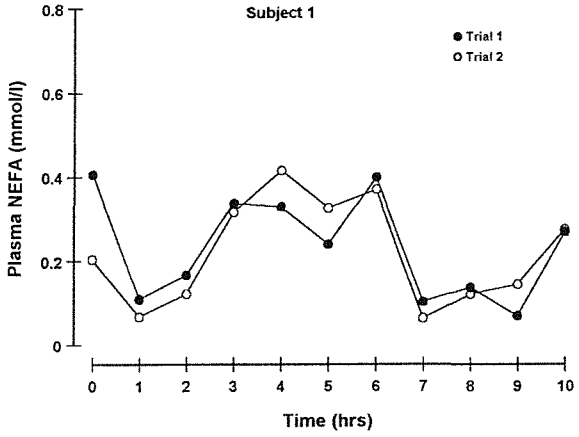


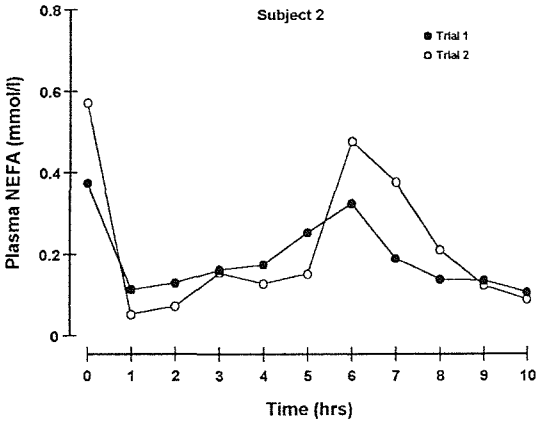


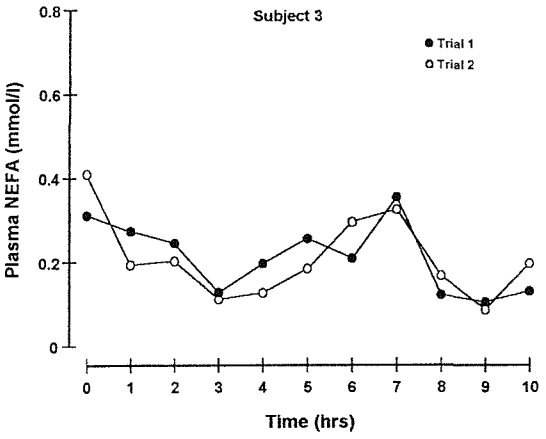


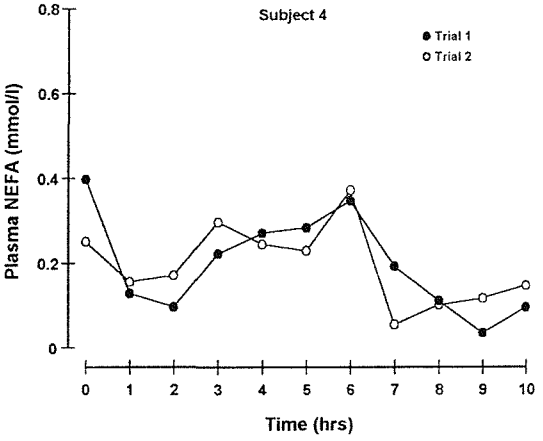


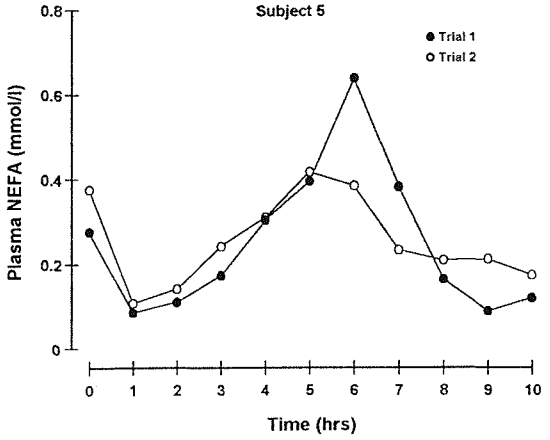
Appendix 2.5 Plasma NEFA coneentration (mmol/l) over a ten hour study period following consumption of two identical reference testmeals, in six subjects.

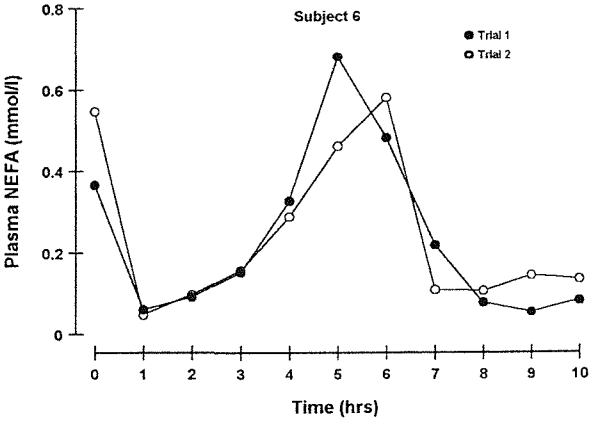






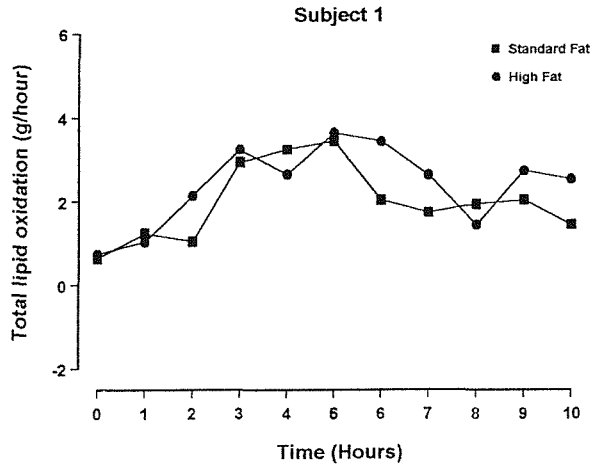


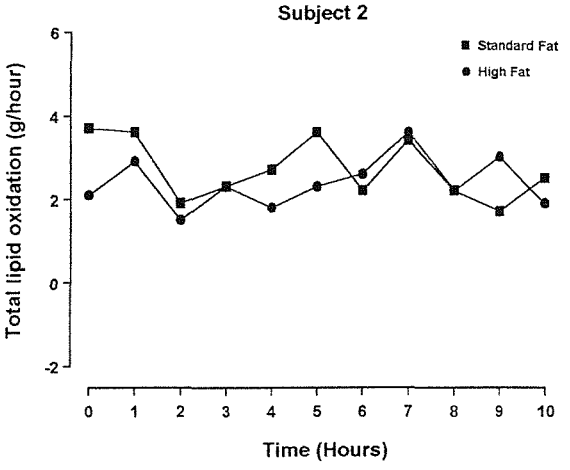


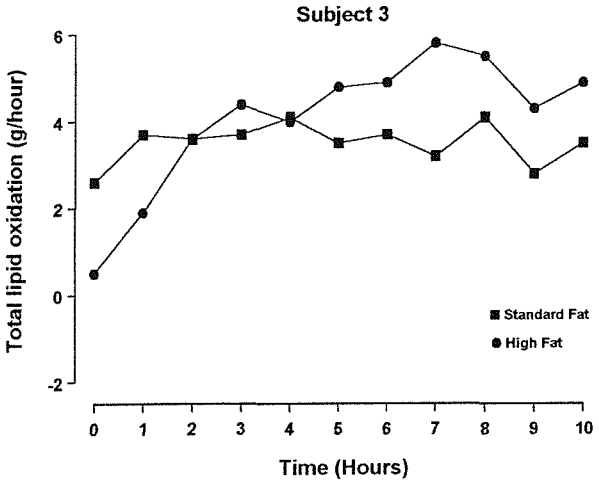


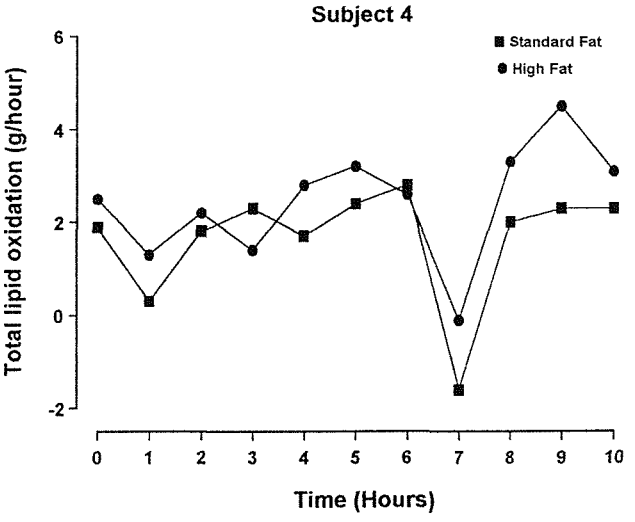
Appendices 3: Chapter 5

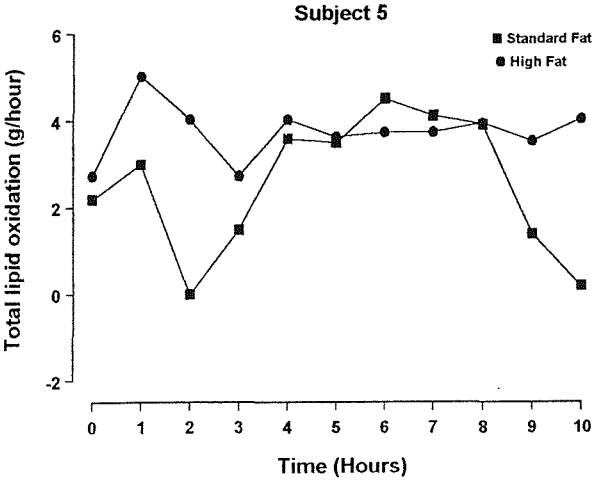
Appendix 3.1 Total lipid oxidation (g/hr) over a ten-hour study period, following consumption of a reference testmeal (standard fat) and a high fat testmeal in six subjects

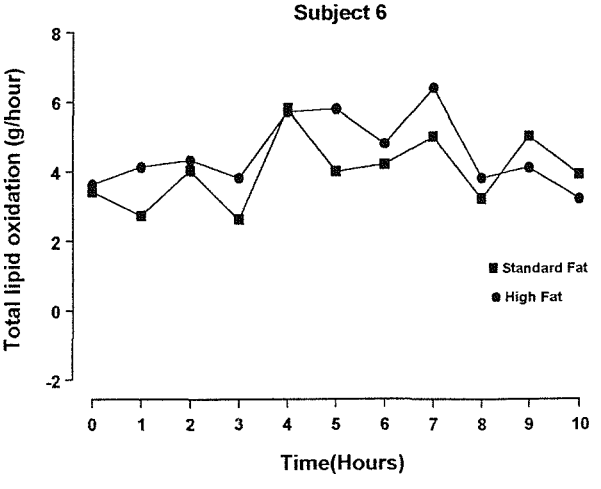




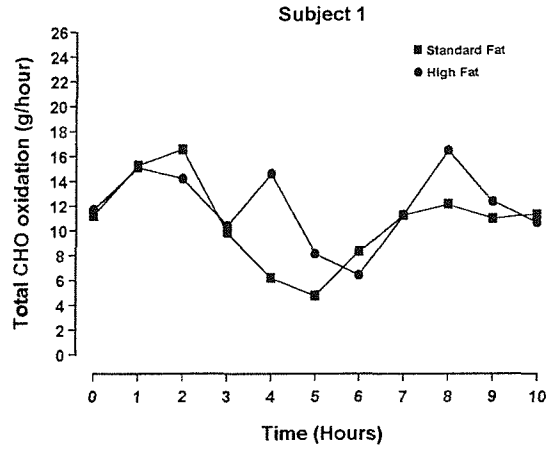


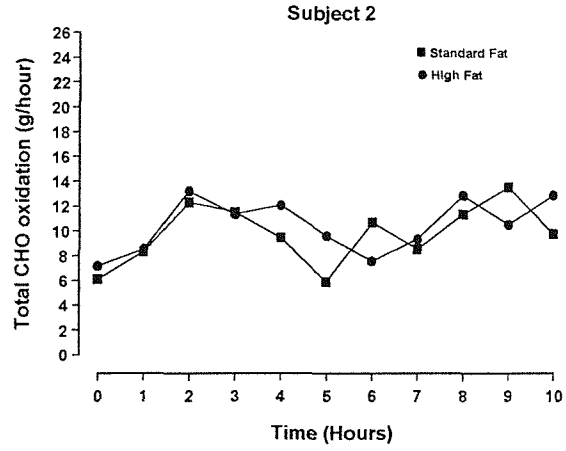


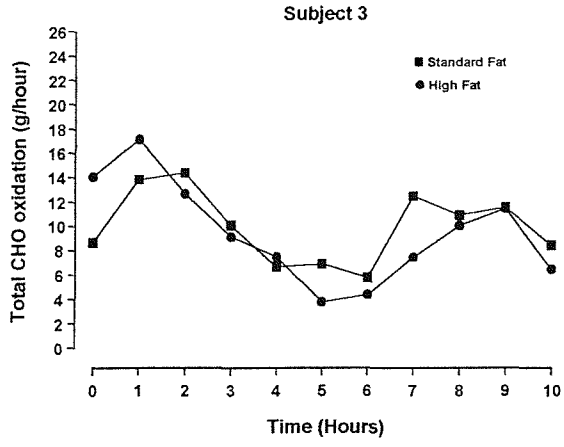


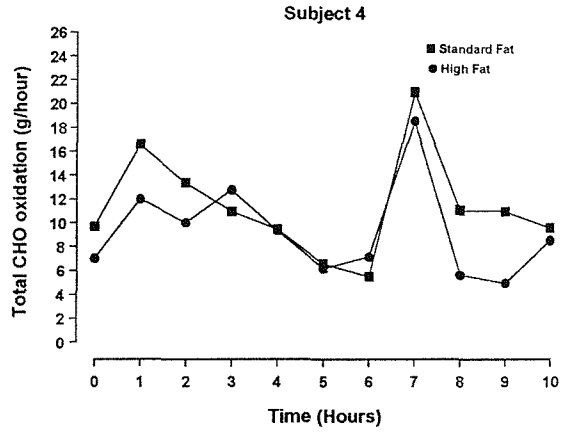


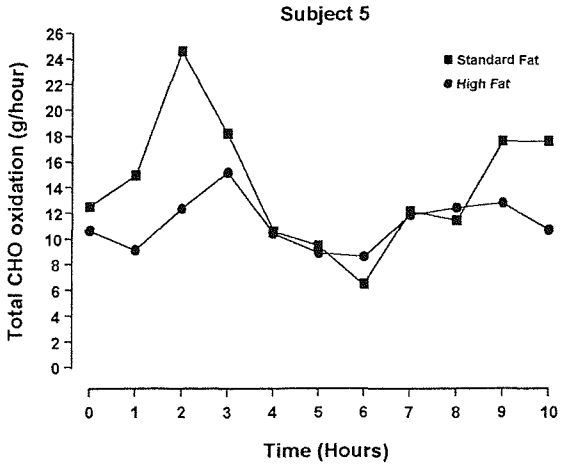
Appendix 3.2 Total carbohydrate oxidation (g/hr) over a ten-hour study period, following consumption of a reference testmeal (standard fat) and a high fat testmeal in six subjects

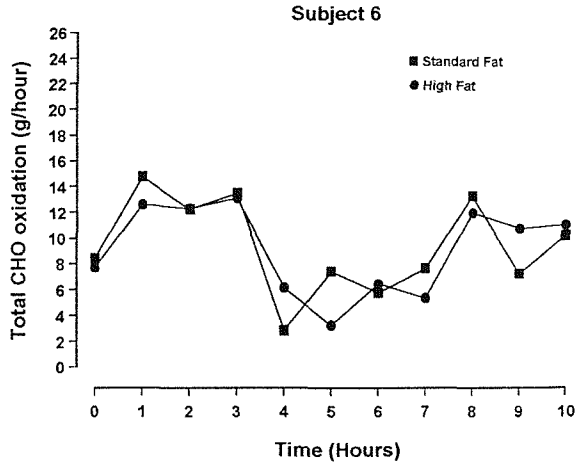




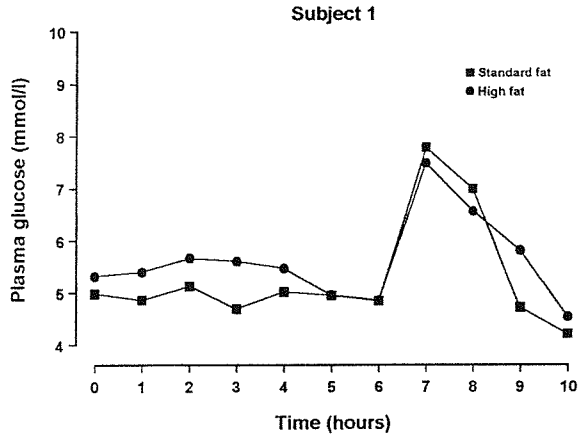


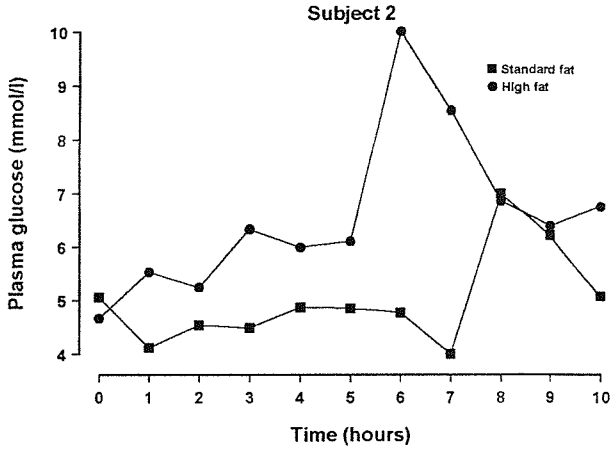


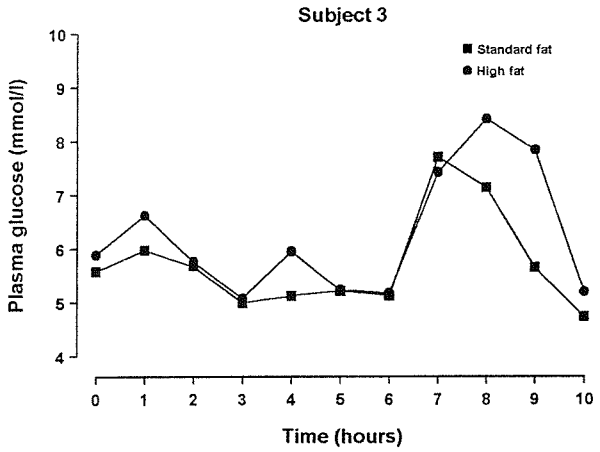


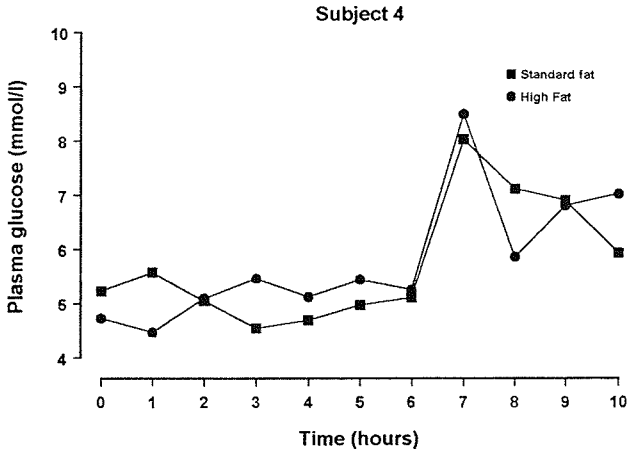


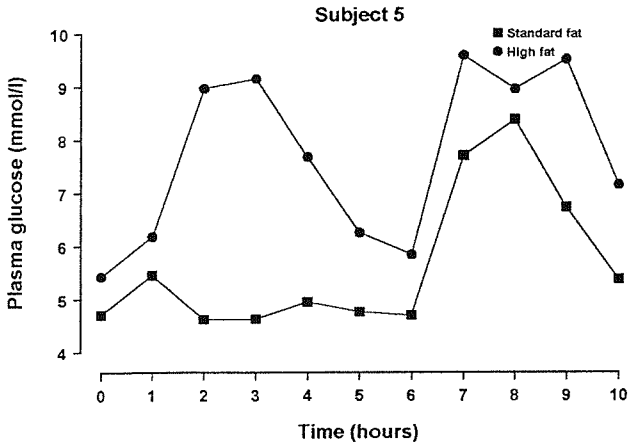
Appendix 3.3 **Plasma glucose concentration (mmol/l) over a ten hour study period following consumption of a reference testmeal (standard-fat) and a high fat testmeal, shown in six subjects.**

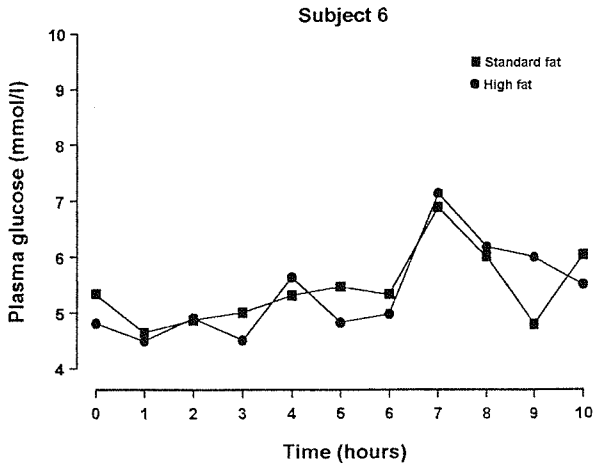




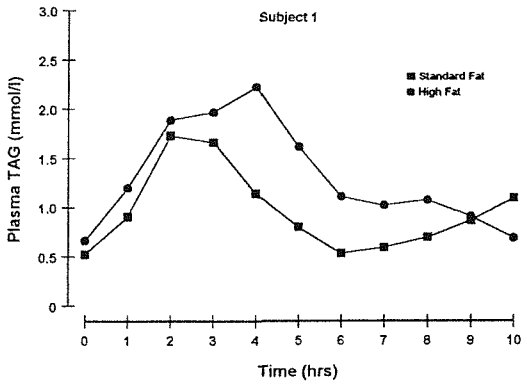


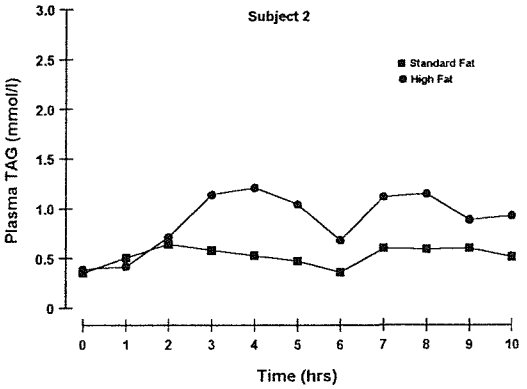


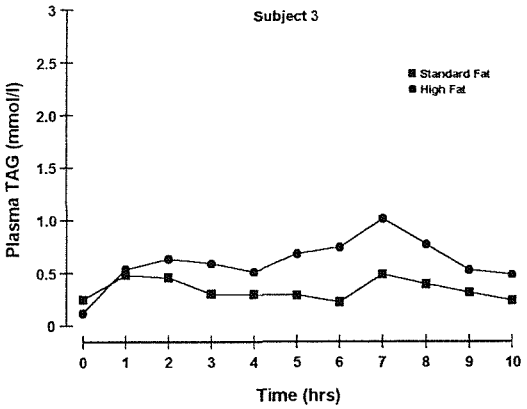


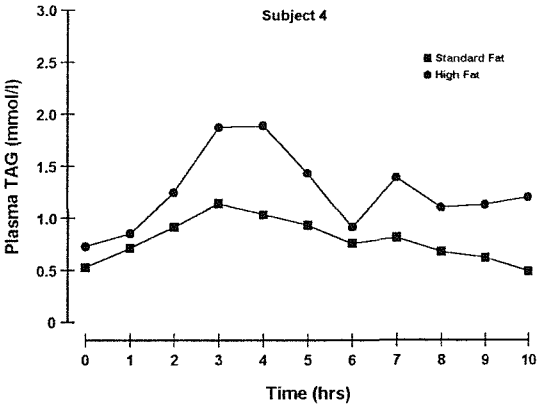


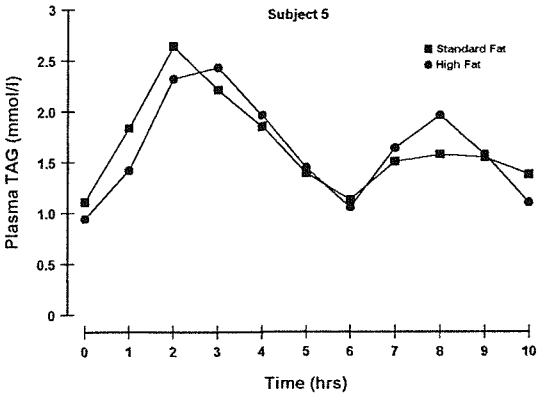
Appendix 3.4 Plasma TAG concentration (mmol/l) over a ten hours study period following consumption of a reference testmeal (standard fat) and a high fat testmeal, shown in six subjects.

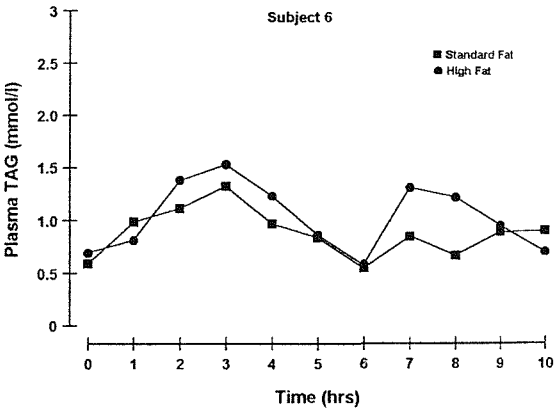




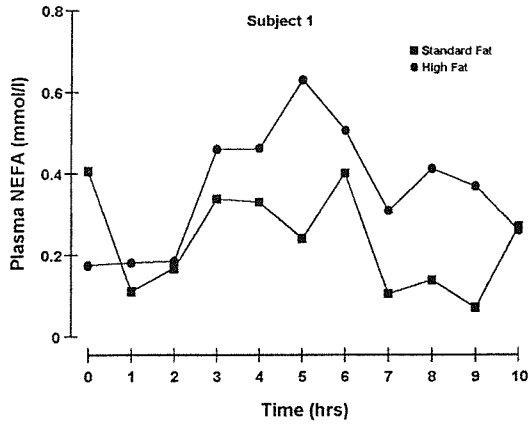


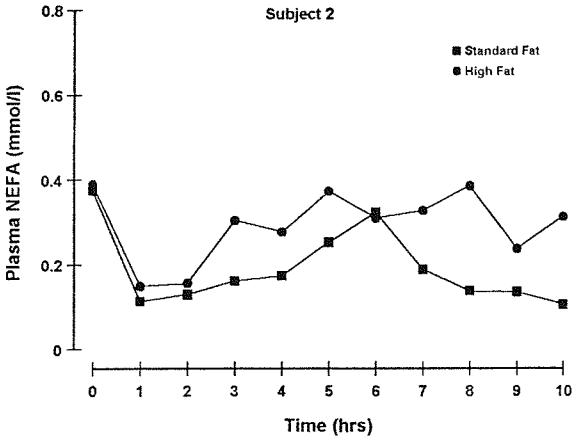


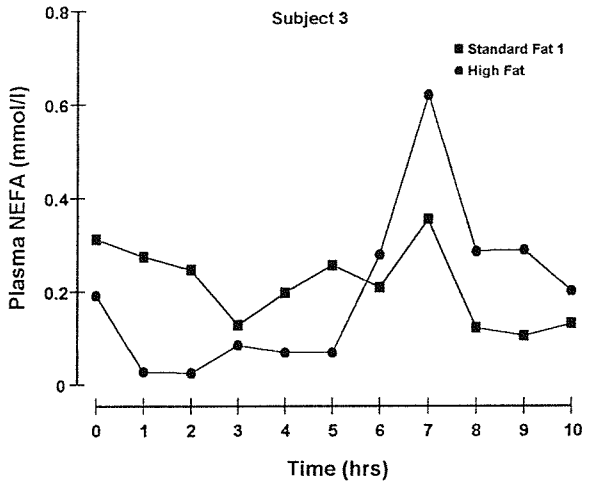


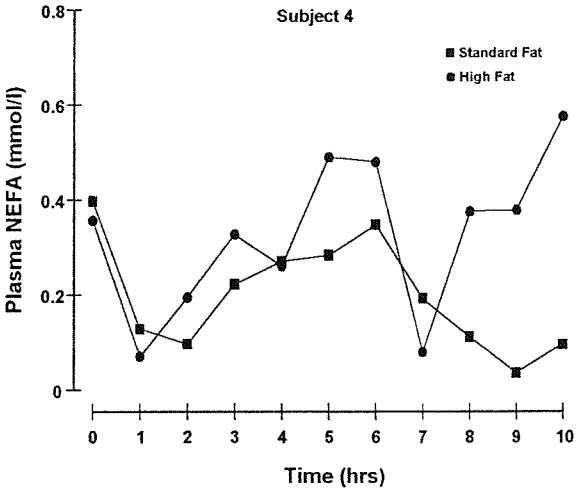


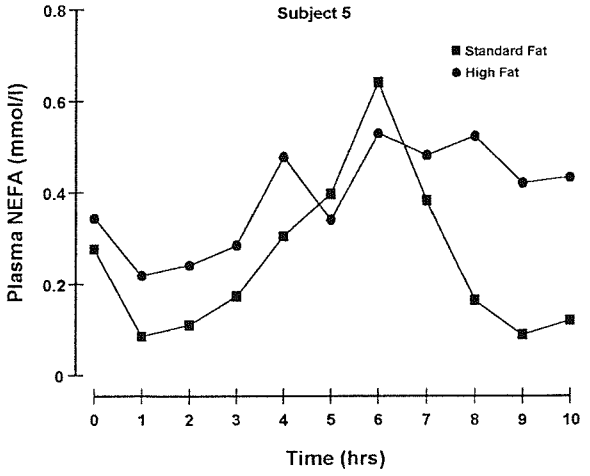
Appendix 3.5 **Plasma NEFA concentration (mmol/l) over a ten hour study period following consumption of a reference testmeal (standard fat) and a high fat testmeal, shown in six subjects.**

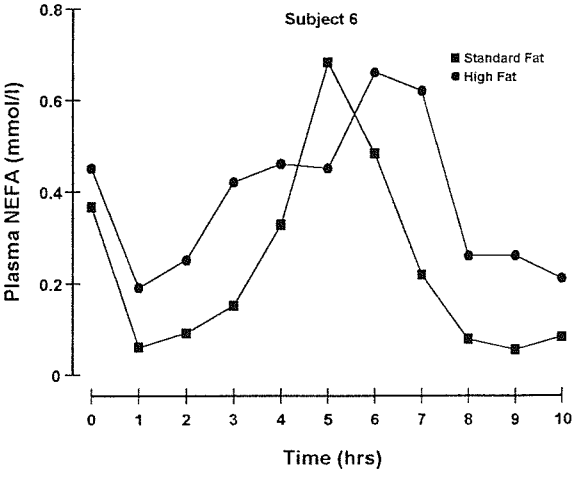






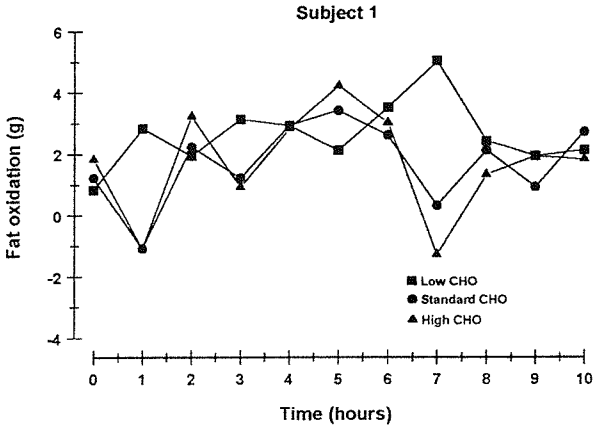


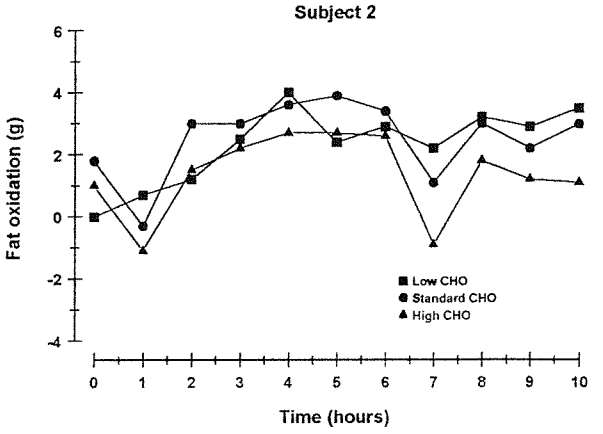


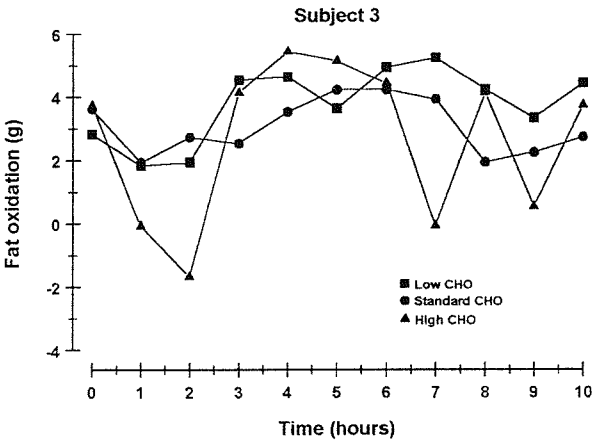


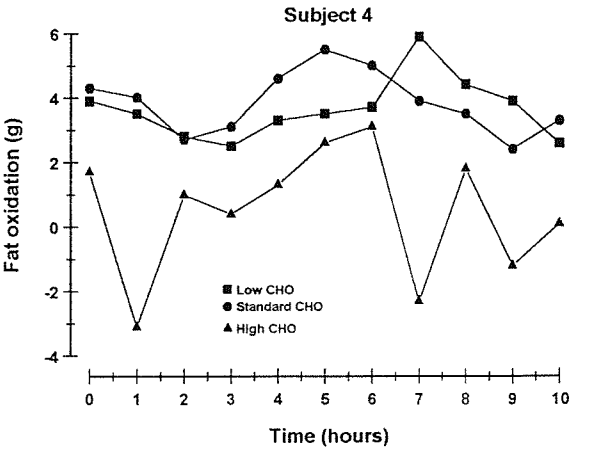
Appendices 4: Chapter 6

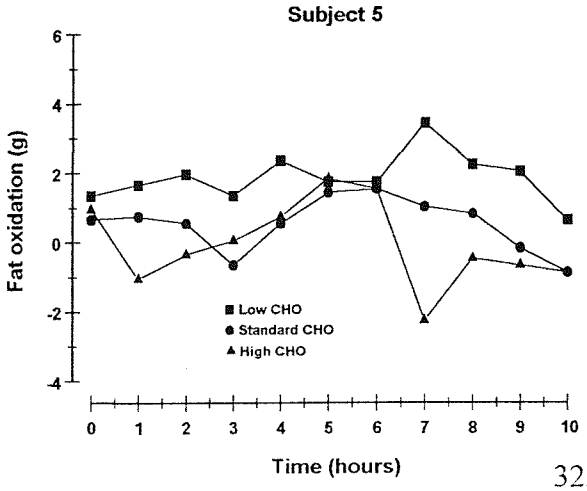
Appendix 4.1 Total lipid oxidation (g/hr) over a ten hour study period, following consumption of a reference testmeal with 0g sucrose (low CHO), 50g sucrose (standard CHO) or 100g sucrose (high CHO), in six subjects

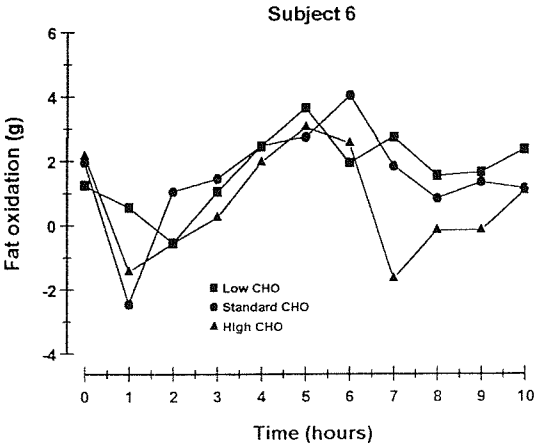




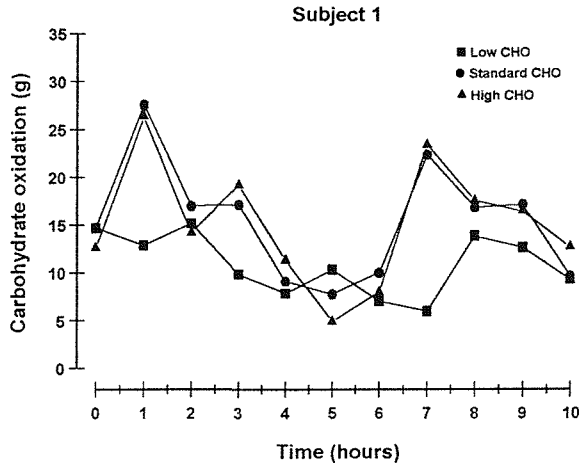


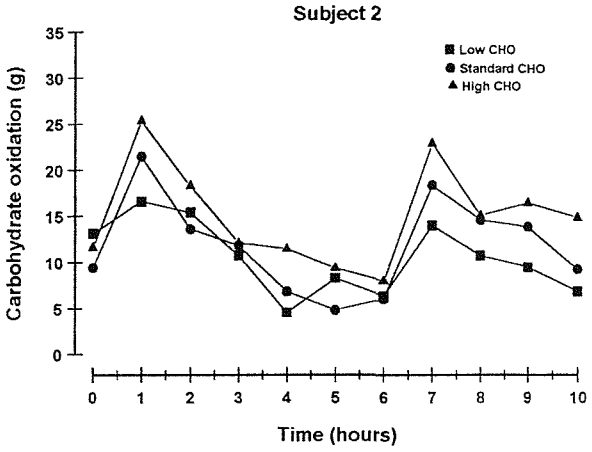


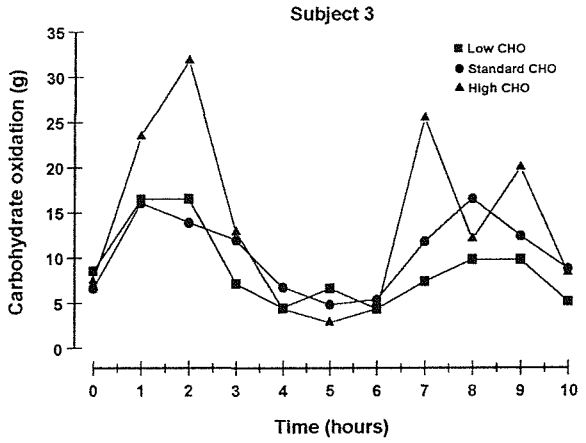


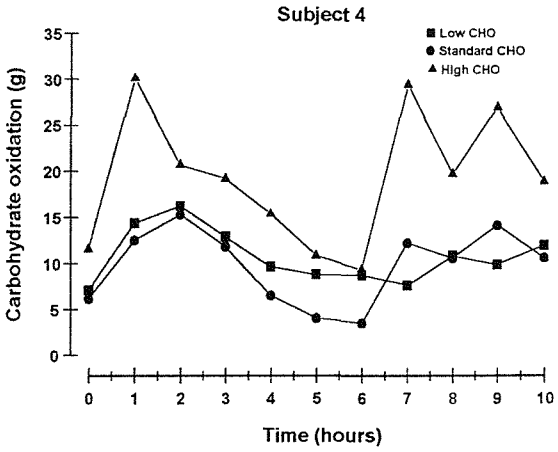


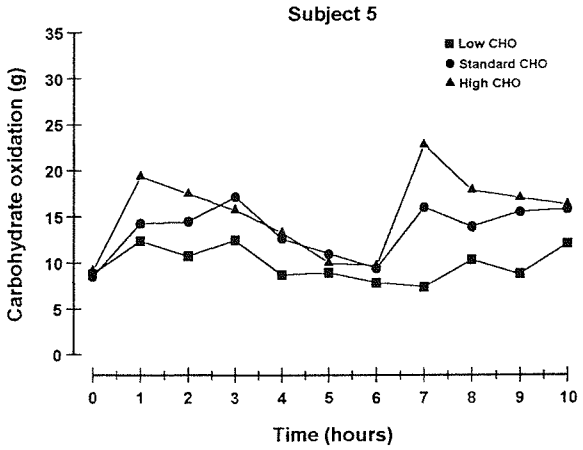
Appendix 4.2 Total carbohydrate oxidation (g/hr) over a ten hour study period, following consumption of a reference testmeal with varying sucrose loads, in six subjects

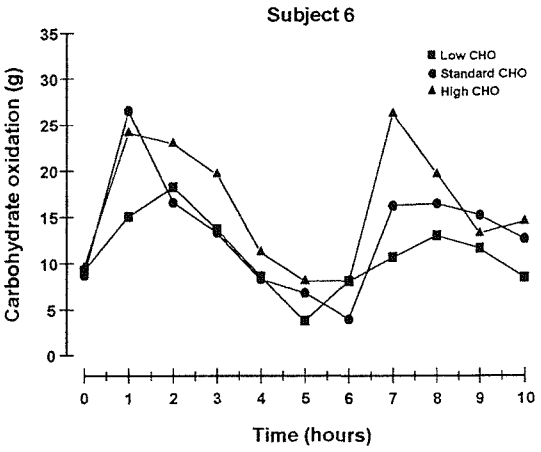




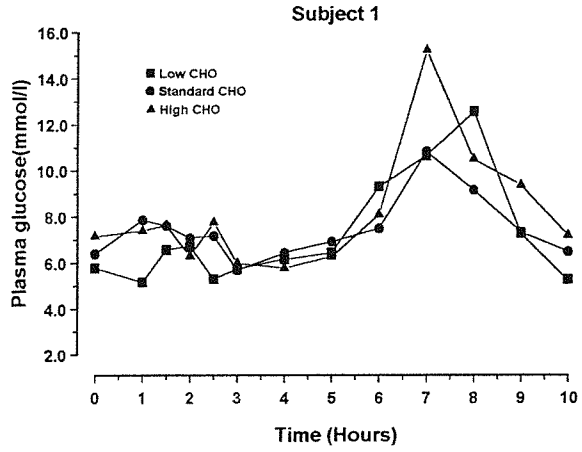


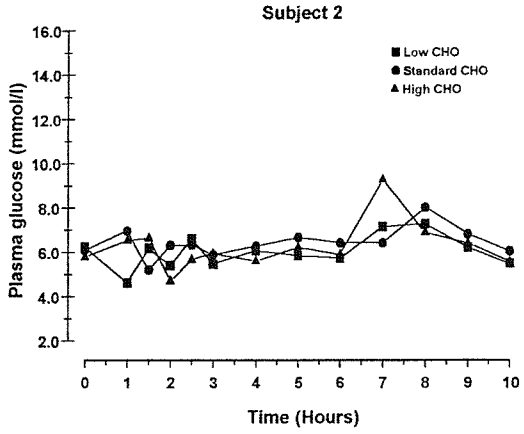


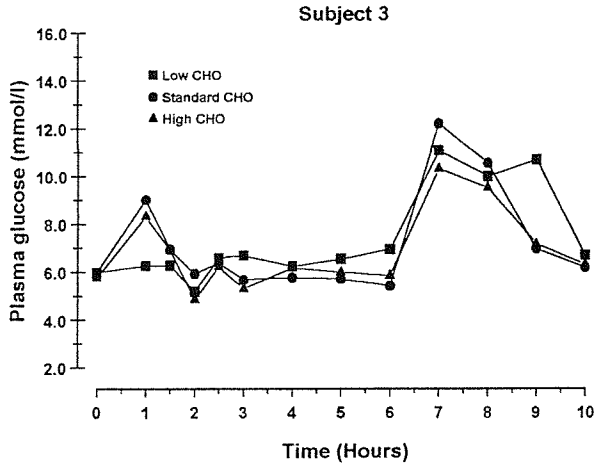


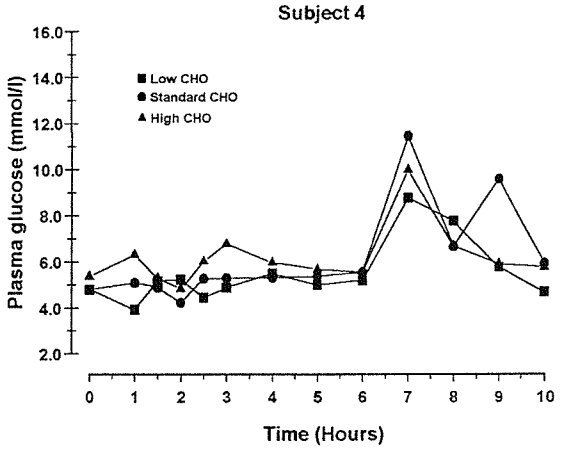


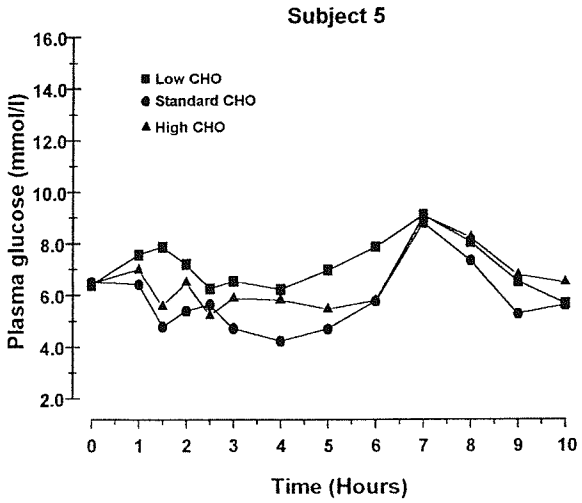
Appendix 4.3 **Plasma glucose concentration (mmol/l) over a ten hour study period following consumption of a reference testmeal with varying sucrose loads, shown in six subjects.**

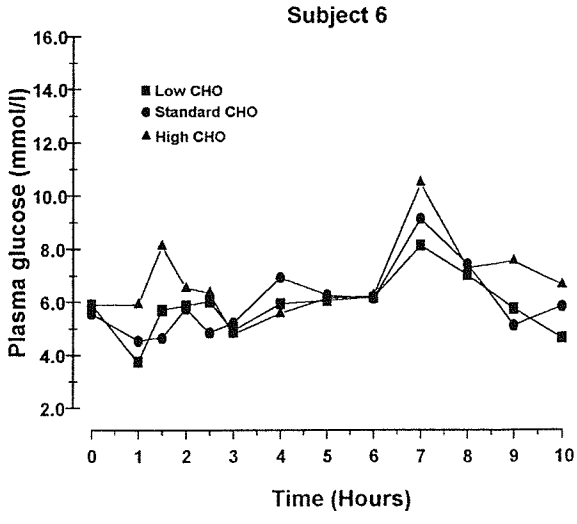




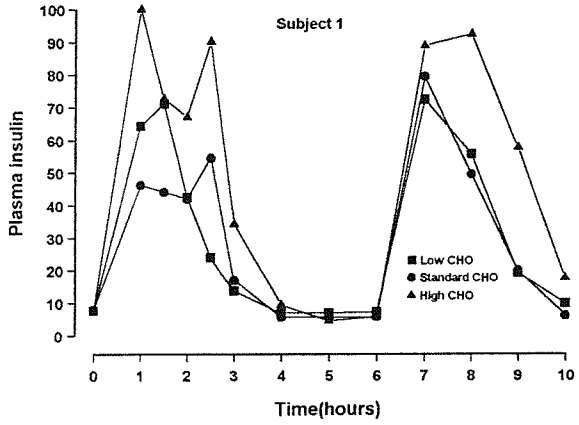


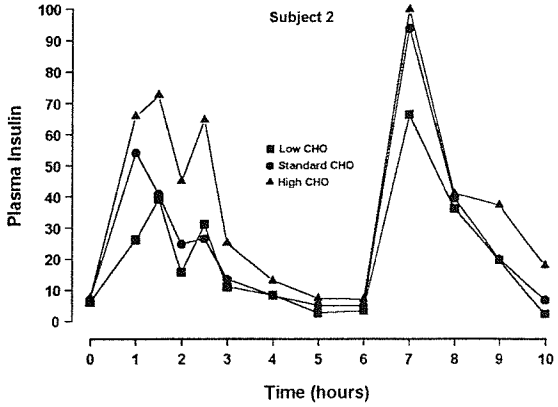


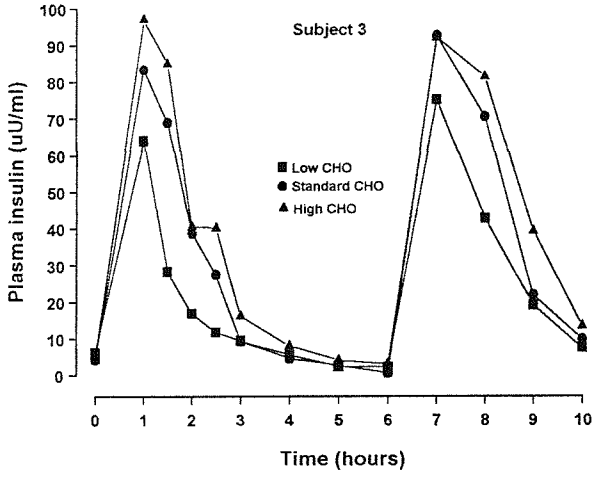


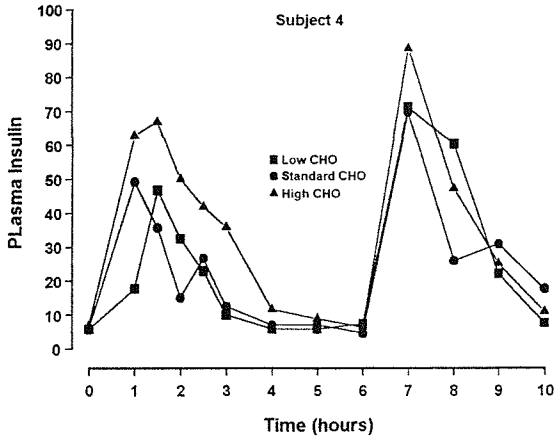


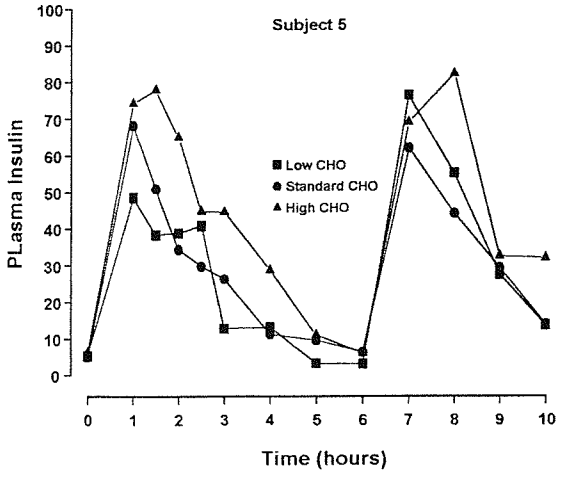
Appendix 4.4 Plasma insulin concentration (uU/l) over a ten hour study period following consumption of a reference testmeal with varying sucrose loads

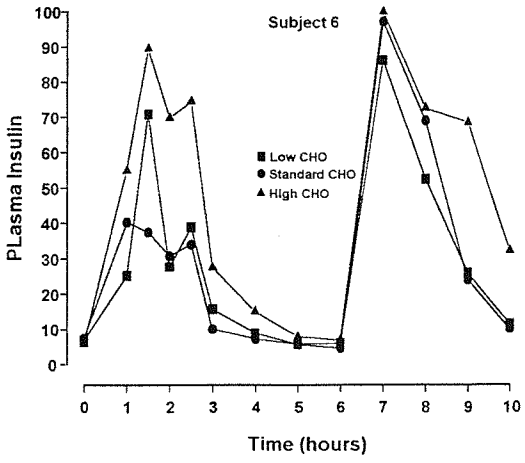




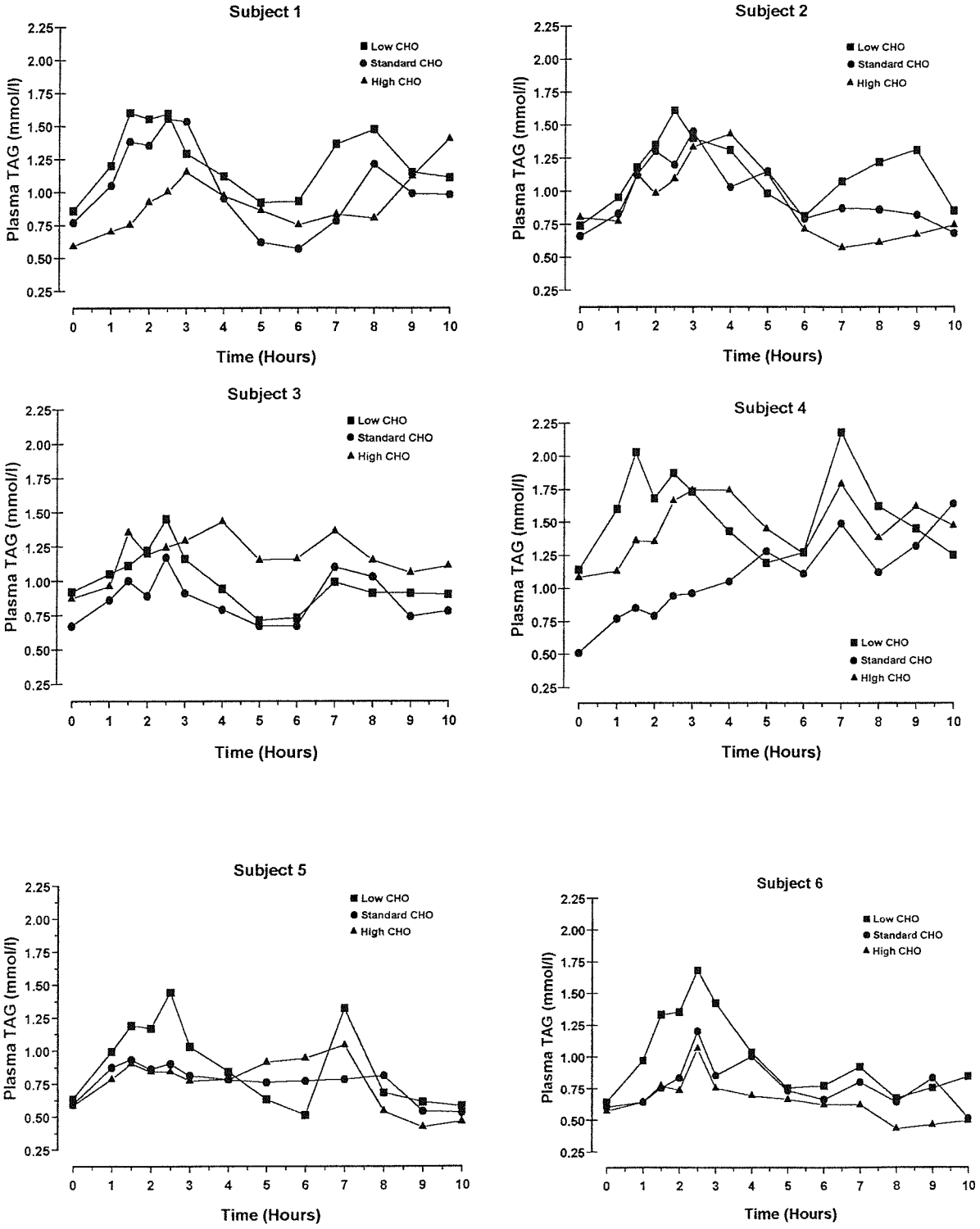




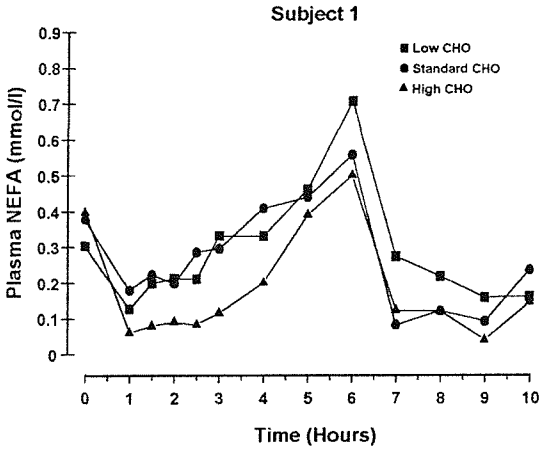


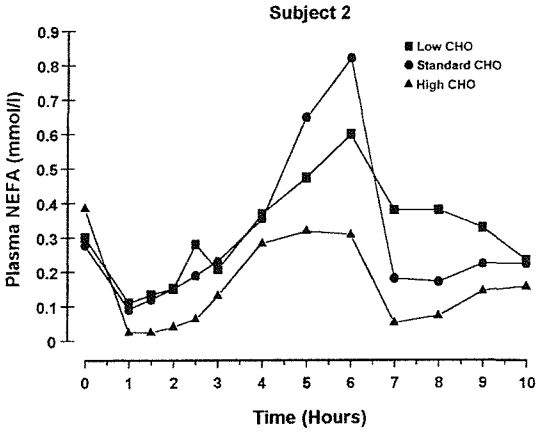


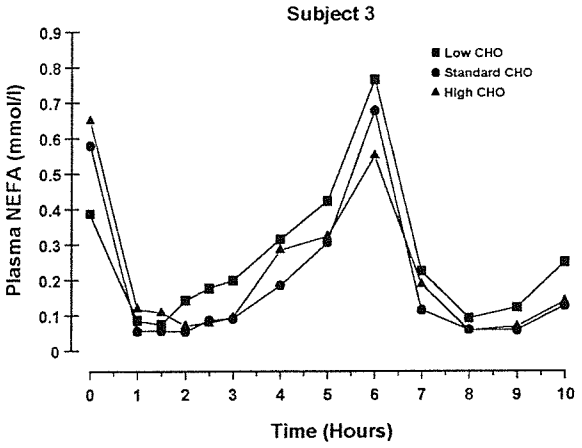
Appendix 4.5 Plasma TAG concentration (mmol/l) over a ten hour study period following consumption of a reference testmeal with varying sucrose loads, shown in six subjects.

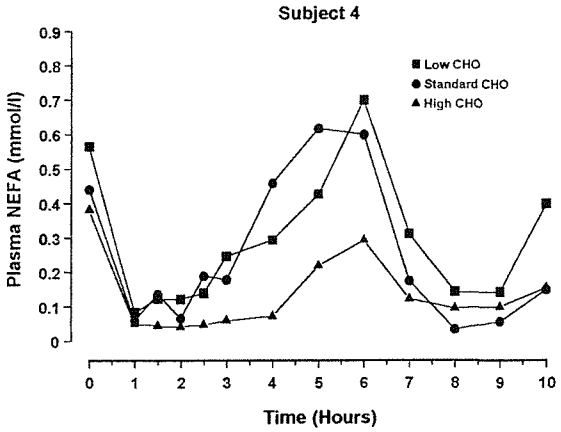


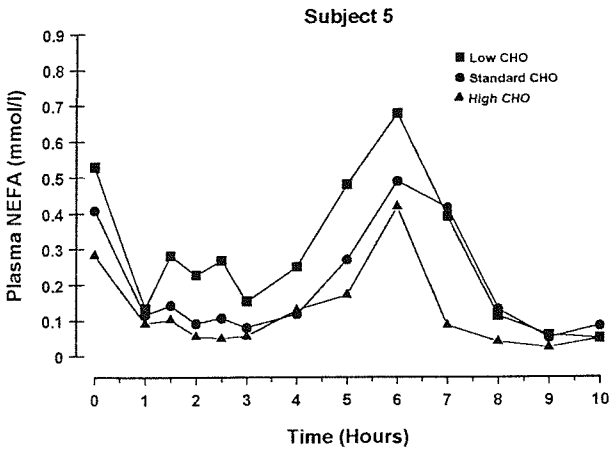
Appendix 4.6 **Plasma NEFA concentration (mmol/l) over a ten hour study period following consumption of a reference testmeal with varying sucrose loads, shown in six subjects.**

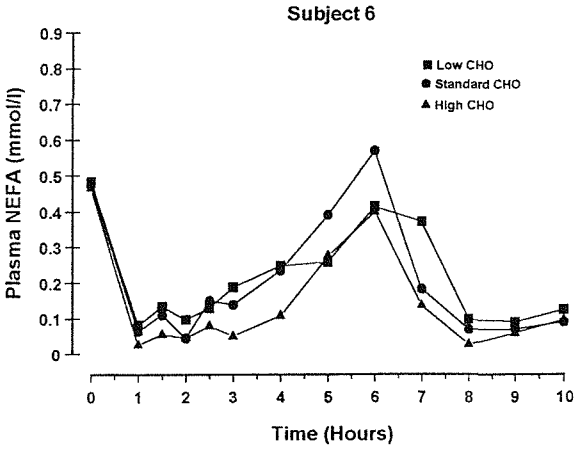




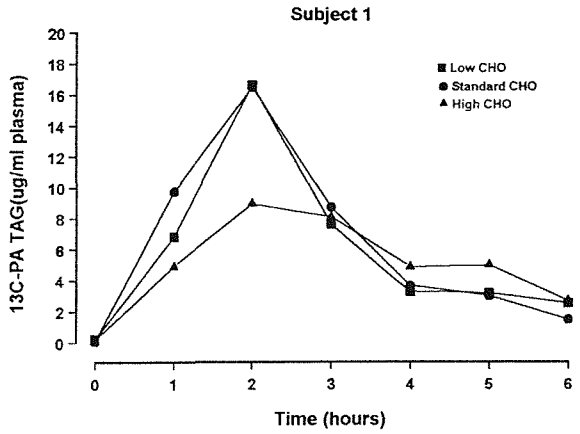


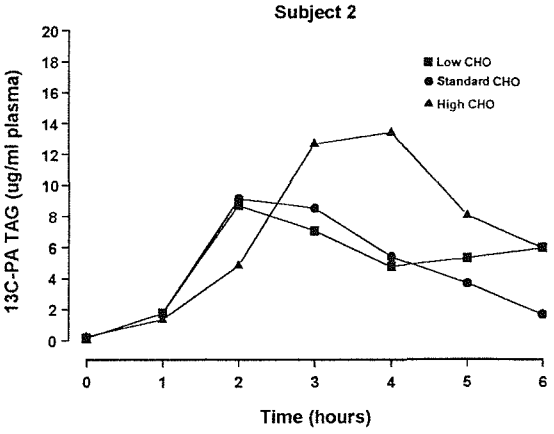


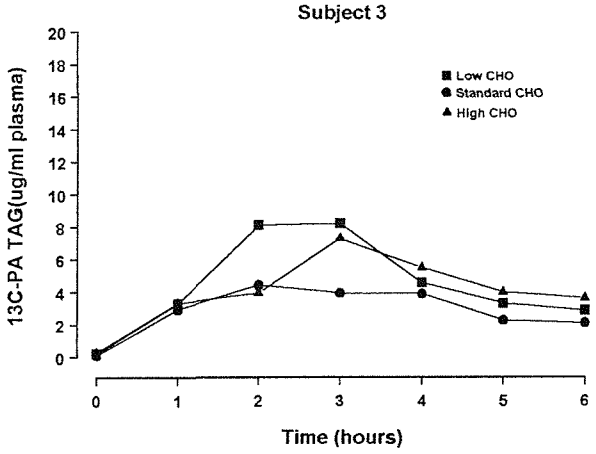


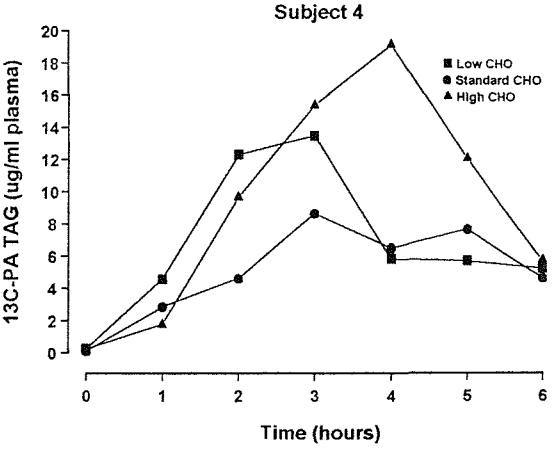


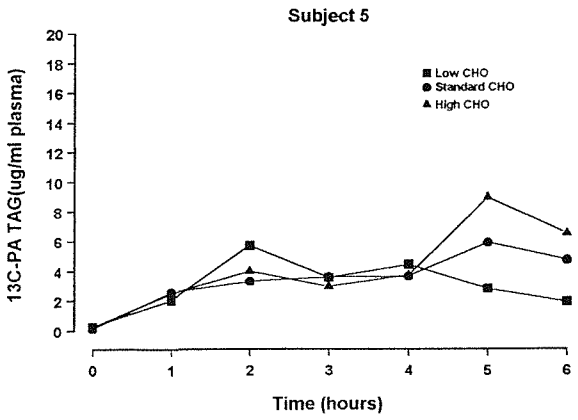
Appendix 4.7 Plasma ^{13}C-TAG concentration (ug/ml) over a six hour study period following consumption of a reference testmeal with varying sucrose loads, shown in six subjects.

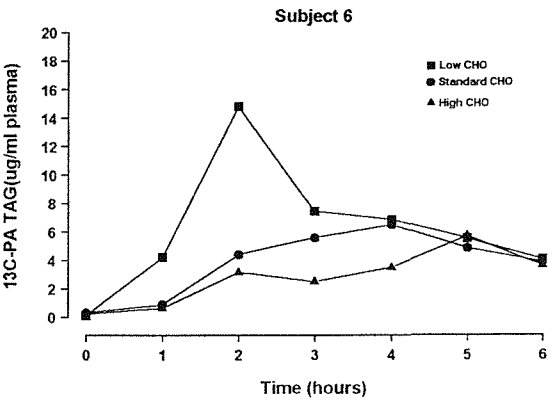




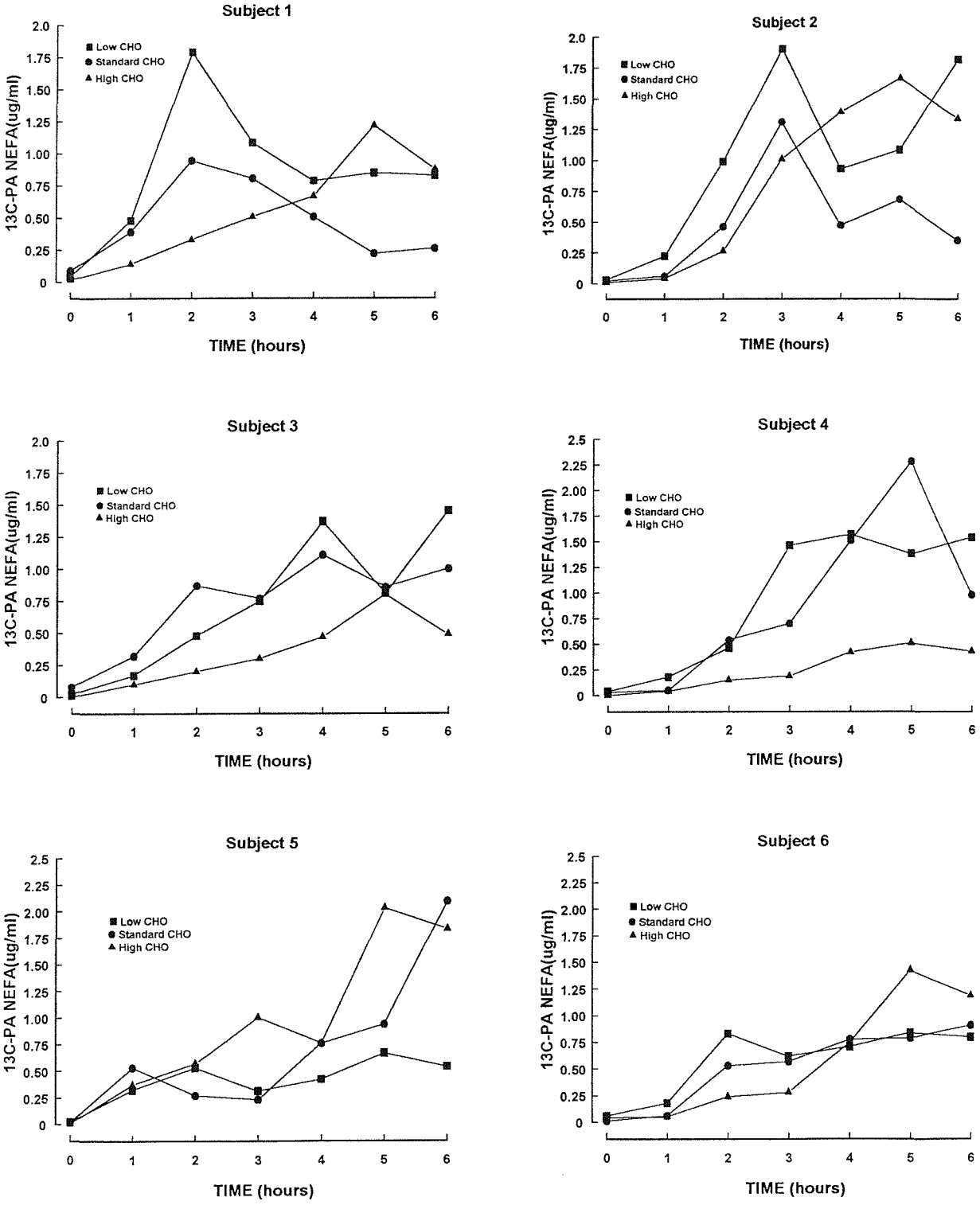








Appendix 4.8 **Plasma ^{13}C-NEFA concentration (ug/ml) over a six hour study period following consumption of a reference testmeal with varying sucrose loads, shown in six subjects.**



CHAPTER 9

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