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**POSTPRANDIAL STUDIES OF MODERATE EXERCISE AND
TRIACYLGLYCEROL METABOLISM**

by

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A Doctoral Thesis

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ABSTRACT

Exaggerated postprandial lipaemia has been implicated in the development of atherosclerosis. Thus, by reducing postprandial TAG concentrations, exercise may play a role in delaying atherogenic progression. This thesis sought to explore the qualitative nature of, and the mechanisms behind, the moderate exercise-induced attenuation to postprandial lipaemia.


Before the experimental studies commenced, a reproducibility study was undertaken. This showed that in a group of eight middle-aged men, the postprandial plasma TAG response differed by only 1.9 ± 5.1 % (mean \pm standard error) on a test-retest basis, indicating that the oral fat tolerance test had enough precision to detect the effect of exercise on TAG metabolism.

Previous work suggested that the exercise-induced reduction to lipaemia was linked to the energy expended by exercise. As the attenuation may have been mediated by energy deficit, rather than exercise *per se*, a study comparing the effect of a 90-minute moderate exercise session with an equivalent dietary-induced energy deficit on postprandial lipid metabolism was undertaken, in a group of eleven postmenopausal women. This showed that the reduction in postprandial lipaemia elicited by exercise was far greater than that elicited by intake-restriction (20 % vs. 7 %).

The second experimental study aimed to establish the effect of a 90-minute moderate exercise session on postprandial chylomicron- and very-low-density lipoprotein (VLDL)-TAG concentrations, and its effect on exogenous (through use of a ^{13}C -labelled lipid) and endogenous fat oxidation, in a group of twelve middle-aged men. Exercise reduced postprandial lipaemia by 23 %, and over three-quarters of this reduction was due to lower VLDL-TAG concentrations. Increases in endogenous fat oxidation accounted for over half of the increase in postprandial fat oxidation.

In the third experimental study, the effect of a 90-minutes moderate exercise session on Intralipid clearance, and postprandial lipaemia, was determined in a group of eight middle-aged men. Exercise attenuated postprandial lipaemia by 18 %, but did not increase Intralipid clearance.

Taken together, these data imply that moderate exercise predominantly reduced postprandial TAG concentrations by reducing hepatic VLDL secretion, rather than increasing TAG clearance, and this effect is not mediated by whole-body energy deficit. In addition, this work has shown that moderate exercise is effective at attenuating postprandial lipaemia in middle-aged men and postmenopausal women.

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To my parents, my brother and my sister

For every complex problem, there is a simple,
easy to understand, incorrect answer.

PREFACE

Unless otherwise indicated by acknowledgement or reference to published literature the work contained herein is that of the author.

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CHAPTER 1

INTRODUCTION

Atherosclerosis is a disease of the muscular arteries which is characterised by the development, over decades, of fibrous and fatty lesions on the arterial intima (Griffin, 1999). Arteries in the coronary circulation are particularly susceptible to this process and atherosclerosis is the principal determinant of coronary heart disease (CHD) – a major cause of morbidity and mortality in the developed world. In England and Wales, CHD was responsible for the deaths of over 70 000 men and 58 000 women in 1996, making it the nation's number one cause of death, accounting for 26 % of all male and 20 % of all female fatalities (Office for National Statistics, 1998). More years of working life and total life are lost to CHD than to any other disease (Office for National Statistics, 1998). The aetiology of atherosclerosis is complex and many heritable and lifestyle factors contribute to its progression. However, it is probable that derangements to lipid and lipoprotein metabolism play an important role in the advancement of atherosclerosis.

The strong link between high concentrations of plasma cholesterol, particularly low density lipoprotein (LDL) cholesterol, and CHD is clear (Levine et al. 1995), especially when the findings of cholesterol-lowering drug intervention trials are considered (Scandinavian Simvastatin Survival Study Group, 1994; Shepherd et al. 1995). However, it has become increasingly evident that serum total or LDL cholesterol concentration does not adequately predict CHD risk on a population basis, with many CHD patients possessing serum cholesterol concentrations in the desirable range (Griffin, 1999). The relationship between lipoprotein metabolism and CHD is complex and in recent years there has been increasing interest in the role of triacylglycerol (TAG) and TAG-rich lipoproteins in the development of atherosclerosis. The link between TAG and CHD was first established in the 1950s by Gofman et al. (1953) (*op cit* Hokanson and Austin (1996)). Subsequently, a large number of studies have shown a relationship between fasting TAG concentrations and CHD but in multivariate analysis TAG tends to be eliminated as an independent CHD risk factor when high density lipoprotein (HDL) cholesterol is included (Austin, 1991). Indeed, HDL is considered to be one of the most important markers of CHD

risk (Griffin, 1999). The weakness of the association between TAG and CHD appears paradoxical, given the strength of the disease's relationship with HDL cholesterol, as the latter concentration is, in fact, determined by the metabolism of TAG-rich lipoproteins. However, the fasting TAG concentration provides a poor marker of TAG metabolism (Patsch et al. 1992; Miesenböck and Patsch, 1992), being subject to considerable biological variation which reduces its predictive power in multivariate analysis (Durrington, 1998). In addition and perhaps more importantly, the fasting TAG concentration could be considered somewhat artifactual in the context of 24-hour TAG metabolism as it considers an unstressed, equilibrated state which is not representative of the dynamic metabolic state present for most of the day. As human beings consume meals regularly during the waking hours, plasma TAG concentrations are above fasting levels for perhaps three-quarters of the day (Williams, 1997). Furthermore, these postprandial TAG concentrations are not necessarily reflected by the fasting TAG concentration, with individuals possessing similar fasting TAG concentrations potentially exhibiting markedly varying plasma TAG responses to an oral fat load (Patsch, 1987; Merrill et al. 1989; Schrezenmeir et al. 1992) and the postprandial state potentially revealing subtle abnormalities in TAG metabolism not evident in the fasted state (Tall, 1986). Thus, the historically weak association between TAG concentrations and CHD risk should theoretically be strengthened when TAG concentrations in the postprandial state are considered.

Evidence supporting an association between postprandial TAG metabolism and CHD is growing, with a number of case-control studies reporting that augmented postprandial TAG and TAG-rich lipoprotein concentrations are associated with coronary artery disease (Simons et al. 1987; Simpson et al. 1990; Groot et al. 1991; Patsch et al. 1992; Meyer et al. 1996). In addition, Patsch and co-workers (1992) found that the association between postprandial TAG concentrations and coronary artery disease was independent of, and stronger than, that for HDL cholesterol. Prospective studies evaluating the link between postprandial TAG concentrations and CHD are limited at present and the author is only aware of two published studies. Stensvold and co-workers (1993) reported that non-fasting (i.e. postprandial) TAG concentration was an independent risk factor for CHD mortality in women and, similarly, Stampfer et al. (1996) found that non-fasting TAG concentration was a

significant predictor of myocardial infarction (MI). Interestingly, in the latter study HDL cholesterol was eliminated as a risk factor for MI after multivariate analysis, implying that non-fasting TAG was a stronger risk predictor than HDL cholesterol – a reversal of the relationship usually observed when TAG concentrations in the fasted state are considered.

The mechanisms via which the metabolism of TAG-rich lipoproteins affects the pathogenesis and progression of CHD have not been fully elucidated and are currently a matter of debate. Twenty years ago, Zilversmit published a landmark paper in which he proposed that atherogenesis was a postprandial phenomenon, with the remnants of cholesterol-loaded chylomicron particles penetrating the arterial wall and contributing to atheroma (Zilversmit, 1979). This hypothesis has received some independent support (Mamo et al. 1998) but the direct atherogenicity of postprandial lipoproteins has been questioned by other authors who have suggested that chylomicrons and chylomicron remnants are removed from the plasma long before they reach a size that will allow them to penetrate the vascular endothelium (Karpe and Hamsten, 1995; Nordestgaard and Nilsson, 1994). The more recent 'TAG intolerance hypothesis' suggests that an overall impairment in TAG transport leads to increased susceptibility for atherosclerosis (Miesenböck and Patsch, 1992). These workers coined the term 'TAG metabolic capacity' to describe the magnitude of hypertriglyceridaemia following an oral fat challenge. Thus, individuals with low TAG metabolic capacity, and therefore exaggerated postprandial lipaemia, are prone to increased neutral lipid exchange between the TAG-rich lipoproteins and HDL and LDL particles. This results in a preponderance of small dense LDL and small HDL particles, together with low concentrations of HDL₂ particles (Ebenbichler et al. 1995) – a lipoprotein profile associated with increased CHD risk known as the 'atherogenic lipoprotein phenotype' (Griffin and Zampelas, 1995). In addition, the cholesteryl ester (CE)-enrichment of TAG-rich lipoprotein remnants is increased, disturbing their passage through the lipolytic cascade and potentially increasing their atherogenicity (Miesenböck and Patsch, 1992). In fact, postprandial lipaemia affects virtually every lipoprotein class (Ebenbichler et al. 1995) and the link between postprandial TAG-rich lipoprotein metabolism and CHD might owe more to its

influence in remodeling the overall lipoprotein phenotype, rather than the specific atherogenicity of chylomicrons and their remnants.

Thus, it appears that the metabolism of TAG-rich lipoproteins in the postprandial state plays a key role in the progression of atherosclerosis whether these postprandial lipoproteins are directly implicated in the formation of atheromic plaques or not. This, taken with the growing body of evidence statistically associating elevated concentrations of postprandial TAG and TAG-rich lipoproteins with CHD, suggests that interventions – whether pharmaceutical or lifestyle – which can attenuate postprandial lipaemia might play an important role in slowing the progression of atherogenesis.

The link between physical activity and CHD was first established in the early 1950s (Morris et al. 1953a; Morris et al. 1953b) and since this time population studies have consistently found high levels of physical activity are associated with reduced risk of CHD morbidity and mortality. Reports evaluating the results of almost 50 populations studies have concluded that inactive individuals are about twice as likely to develop CHD as their active counterparts (Powell et al. 1987; Berlin and Colditz, 1990). This level of risk is comparable to that conferred by hypertension, smoking or high serum cholesterol concentrations and on the basis of this evidence the American Heart Association acknowledged physical inactivity as a major risk factor for cardiovascular disease (American Heart Association, 1992). Reports examining the association between physical fitness – a characteristic conferred by regular physical activity – and disease risk have similarly reported an inverse relationship between fitness level and CHD morbidity and mortality (for review see Whaley and Blair (1995)) and, importantly, increases in levels of physical activity or fitness are associated with reductions in CHD risk (for review see Whaley and Blair (1995)), suggesting that unfit or sedentary individuals can improve their risk profile by starting an exercise programme.

The mechanisms via which physical activity/physical fitness attenuate CHD risk have not been fully elucidated but are likely to involve changes in lipid and lipoprotein metabolism. Regular exercisers possess lipoprotein profiles consistent with a low risk

of CHD, typically having HDL cholesterol concentrations that are 20-30 % higher than untrained individuals as well as lower TAG concentrations in the fasted state (Durstine and Haskell, 1994). In particular, there appears to be a dose-response relationship between the amount of exercise performed and HDL cholesterol concentration (Durstine et al. 1987; Kokkinos et al. 1995), and longitudinal training interventions often report increases in HDL cholesterol (for review see Durstine and Haskell (1994)). Given the relationship between TAG metabolism and HDL cholesterol concentrations (Miesenböck and Patsch, 1992), it is probable that these elevated HDL cholesterol concentrations are a consequence of efficient metabolism of TAG-rich lipoproteins. Thus, while these studies reveal the potential for exercise to influence lipoprotein metabolism, they provide no information about the mechanisms responsible for these changes and studies of postprandial TAG metabolism might help elucidate how exercise influences the lipoprotein profile.

A number of studies have indicated that endurance trained individuals have an enhanced metabolism of TAG-rich lipoproteins, reporting lower postprandial lipaemia (Cohen et al. 1989; Merrill et al. 1989; Hartung et al. 1993) and/or increased TAG clearance (Ericsson et al. 1982; Sady et al. 1988; Cohen et al. 1989; Podl et al. 1994) in regular exercisers compared with sedentary controls. Similar findings have been reported by some training intervention studies (Altekruse and Wilmore, 1973; Thompson et al. 1988; Weintraub et al. 1989; Drexel et al. 1992; Zmuda et al. 1998), but there is compelling evidence suggesting that the enhancement of TAG metabolic capacity elicited by exercise is predominantly due to the influence of a recent exercise session, rather than to adaptations to training (Aldred et al. 1995; Hardman et al. 1998; Herd et al. 1998).

This is in line with suggestions that many of the health-related changes elicited by exercise are a consequence of 'acute' metabolic responses to a single exercise session rather than a long-term training effect (Haskell, 1994). In addition, there is accumulating evidence that modest amounts of exercise, performed at low and moderate intensities, which might not be sufficient to improve cardiovascular fitness in normally-active adults, can induce metabolic changes consistent with reduced CHD risk (Després and Lamarche, 1994; Haskell, 1994). This is supported by data from

population studies which have indicated that clinical risk decreases steeply between the least fit or active individuals and those who are moderately fit or active (Leon et al. 1987; Blair et al. 1989). Moreover, many individuals who experience orthopaedic contraindications to vigorous exercise can participate in moderate exercise, such as walking, and the risks of cardiovascular (Vuori, 1985) and immunological (Nieman, 1994) complications increase with exercise intensity. Inactivity is endemic in the UK population, with seven out of ten men and eight out of ten women living a sedentary lifestyle (Sports Council and Health Education Authority, 1992). For these people moderate exercise is probably less daunting than vigorous activity and this is acknowledged in current exercise guidelines, which recommend that all adults should participate in exercise of moderate intensity (Pate et al. 1995). Given the potential for moderate intensity exercise to improve 'metabolic fitness', and the new exercise recommendations, it is necessary to increase understanding of the nature and magnitude of the positive metabolic changes elicited by this type of exercise. Indeed, the importance of basic research to define the mechanisms via which exercise influences the development of cardiovascular disease was highlighted in the American National Institutes of Health Consensus Statement on physical activity and cardiovascular health (National Institutes of Health, 1997).

The effects of a recent moderate intensity exercise session on postprandial TAG metabolism have been investigated, with an exercise-induced attenuation in postprandial lipaemia usually being reported (Aldred et al. 1994; Tsetsonis and Hardman, 1996a; Tsetsonis and Hardman, 1996b; Tsetsonis et al. 1997; Herd et al. 1997; Herd, 1997; Zhang et al. 1998; Gill et al. 1998; Malkova et al. 1999). However, the mechanisms behind these decreases are unclear and a detailed description of the changes to postprandial TAG metabolism elicited by moderate exercise is lacking. In addition, there are few data about the effects of this type of exercise on TAG metabolic capacity in adults of middle-age or older. Therefore the studies described in this thesis aimed to reveal the qualitative nature of the changes to postprandial TAG metabolism elicited by a moderate exercise session and to help clarify the mechanisms evoking these changes. Middle-aged men and post-menopausal women were chosen for study to increase the clinical relevance of the

findings as these groups are at higher risk of CHD than the subjects previously studied (Dawber, 1980; Keys, 1980; Wood et al. 1998).

CHAPTER 2

REVIEW OF LITERATURE

2.1 Introduction

This chapter aims to establish a scientific rationale for the experimental studies described later in this thesis. There is a brief overview of lipoprotein metabolism, with particular emphasis on the transport and metabolism of TAG in the circulation. A section reviewing methods of assessing the metabolism of TAG and TAG-rich lipoproteins follows this. The next section considers some relevant factors influencing TAG metabolic capacity and the final section reviews the literature concerning the influence of exercise, particularly moderate exercise, on TAG metabolism and explores possible mechanisms for these effects.

2.2 Lipoprotein metabolism

2.2.1 Classification and properties of the lipoproteins

Lipids are a chemically heterogeneous group of substances which are insoluble in water but soluble in non-polar solvents (Gurr and Harwood, 1991). Due to their hydrophobic nature, lipids are transported in the predominantly aqueous bloodstream in the form of lipoproteins. These are spherical particles with a pseudomicellar structure, incorporating a hydrophobic core of TAG and cholesteryl esters encased in a hydrophilic monolayer shell of phospholipids, unesterified cholesterol and apolipoproteins (Patsch and Patsch, 1984) and form four major classes; chylomicrons, very-low-density lipoproteins (VLDL), LDL and HDL. The composition and some properties of the lipoprotein classes are presented in Table 2.1. Also included in this classification are chylomicron remnants and intermediate-density lipoproteins (IDL), which are formed by the degradation of chylomicrons and VLDL particles, respectively and are denser and less TAG-rich than their parent particles.

Chylomicron remnants have similar physical properties to VLDL particles and tend to fall into the VLDL density and floatation rate range. IDL particles occupy the 1.006-1.019 g.ml⁻¹ density range and have a floatation rate range of Sf 12-20 (Durrington, 1989).

It should be noted that, although the lipoprotein classes are operationally defined on the basis of density or Svedberg floatation rate (Sf) for analytical separation purposes,

lipoproteins do not form discrete classes on this basis and it is not possible to obtain homogenous lipoprotein classes from ultracentrifugation alone.

	Chylomicrons	VLDL	LDL	HDL
Density Range (g.ml ⁻¹)	<0.95	0.95-1.006	1.019-1.063	1.063-1.210
Svedberg Floatation rate (Sf)	>400	20-400	0-12	-
Diameter (nm)	>70	30-90	18-22	5-12
Particle Mass (daltons)	0.4-30x10 ⁶	10-100x10 ⁶	2-3.5x10 ⁶	1.75-3.6x10 ⁶
Apolipoproteins	A-I, B-48, C-I, C-II, C-III, E	B-100, C, E	B-100	A-I, A-II, C, E
Site of Synthesis	Gut	Liver	Peripheral tissue capillaries	Gut/Liver
Major Functions	Transport of dietary fat	Transport of endogenous fat	Transport of cholesterol to periphery	Reverse transport of cholesterol
Protein (% particle mass)	2	7	20	50
TAG (% particle mass)	83	50	10	8
Cholesterol (free and esterified) (% particle mass)	8	22	48	20
Phospholipids (% particle mass)	7	20	22	22

Table 2.1 Properties and functions of the lipoproteins. Modified from Gurr and Harwood (1991), Durrington (1989) and Frayn (1996).

2.2.2 Chylomicron and chylomicron remnant metabolism

Chylomicrons are the least dense and most TAG rich of the lipoprotein classes and serve to transport TAG of exogenous origin around the body. They are formed in the enterocyte after the digestion and re-esterification of dietary fat and then transported in the lymphatics before entering the circulation via the subclavian vein (Patsch, 1987). Chylomicrons first appear in the circulation within an hour of eating a meal and the last chylomicrons are removed from the bloodstream 5-8 hours after meal ingestion (Patsch, 1987). Nascent chylomicrons contain the apolipoproteins (apo) B-48 and A-I and once in the circulation acquire apos C-I, C-II, C-III and E from VLDL and HDL, and transfer some apo A to HDL (Patsch, 1987). Much recent interest has focused on apo B-48. This is the truncated form of apo B formed in the intestinal cells and is unique to chylomicrons and their remnants. It remains with these particles throughout their time in the circulation (Patsch, 1987) and only one apo B molecule is present per lipoprotein particle (Young, 1990). This stoichiometric relationship between apo B-48 and chylomicrons allows apo B-48 quantification to be used as a surrogate of the number of intestinally derived lipoproteins in the circulation. Once chylomicrons have acquired apo C-II – a cofactor for lipoprotein lipase (LPL) – their TAG core is able to undergo hydrolysis into fatty acids and 2-monoacylglycerols by the action of this enzyme. LPL resides on the capillary endothelium of the lactating mammary gland, cardiac muscle, lungs, hypothalamus, skeletal muscle and adipose tissue but its presence in the latter two tissues is probably of greatest importance for the clearance of lipoprotein-TAG from the circulation (Taskinen and Kuusi, 1987). Hydrolysis continues until chylomicrons have lost 70-90 % of their TAG content and during this catabolism the surface apolipoproteins A and C are released and transferred to HDL along with much of the chylomicron's phospholipid content (Chen and Reaven, 1991). In addition, some TAG is transferred to HDL and LDL in exchange for CE, catalysed by cholesteryl ester transfer protein (CETP) (Miesenböck and Patsch, 1992).

The chylomicron remnant particles retain apos B-48 and E from the chylomicron, but the loss of apo C-II makes them unable to compete for the action LPL. However, they may undergo further hydrolysis by the action of hepatic lipase in the liver (Shafi et al. 1994). These remnant particles are taken up and internalised by the liver via an interaction between apo E with a specific remnant receptor in the hepatic cells, which

may or may not be independent of the LDL receptor (van Berkel et al. 1994), although some non-receptor mediated uptake may occur (Havel, 1998). In addition a small number of large ($S_f > 400$) chylomicron remnant particles are removed from the circulation by peripheral tissues implying that some removal of TAG-rich lipoproteins can occur via whole-lipoprotein particle removal as well as delipidation via the LPL pathway (Karpe et al. 1997).

2.2.3 VLDL, IDL and LDL metabolism

VLDL are the lipoproteins which carry TAG of endogenous origin to the peripheral tissues and, teleologically, serve the function of transporting TAG in the fasting as well as non-fasting state (Durrington, 1989). These are smaller and less TAG-rich than chylomicrons and are mainly synthesised in the liver, although a small number may be produced intestinally (Karpe and Hamsten, 1995a). The lipid components of VLDL are derived from a number of sources. Non-esterified fatty acids (NEFA) liberated from adipose tissue through lipolysis by hormone sensitive lipase provide a major source of substrate for VLDL secretion (Karpe, 1997), with NEFA 'spilt-over' into the circulation from the LPL-mediated hydrolysis of TAG-rich lipoproteins also contributing to the hepatic NEFA flux, particularly in the postprandial state (Frayn et al. 1994). Poorly lipolysed chylomicron and VLDL remnants can also provide TAG substrate for VLDL production and, particularly when carbohydrate overfeeding is evident, *de novo* synthesis of TAG can occur in the liver (Karpe, 1997). The VLDL secretion rate depends on the contribution of all these TAG-NEFA fluxes and the balance between NEFA oxidation and re-esterification within the liver (Frayn, 1996; Karpe, 1997). Each VLDL particle contains one molecule of the longer apo B isoform – apo B-100 (Durrington, 1989) – but, in contrast to chylomicrons, VLDL obtain all their constituent apolipoproteins within the hepatocyte prior to secretion (Gurr and Harwood, 1991), although they acquire additional apo C and E from HDL once they enter the circulation (Frayn, 1996). The catabolism of VLDL is similar to that of chylomicrons, with the TAG-core undergoing hydrolysis by LPL facilitated by the presence of apo C-II. As for chylomicrons, surface materials are transferred from VLDL to HDL during the catabolism process and VLDL undergo neutral lipid exchange with HDL catalysed by CETP, which enriches the former particle with CE and the latter with TAG (Durrington, 1989). The lipolysis of VLDL particles is much slower than occurs with chylomicrons, even at low VLDL concentrations (Havel,

1997) and their half-life in the circulation is typically two to four hours in normolipidaemic individuals (Durstine and Haskell, 1994).

The VLDL remnant – known as an IDL – retains the apo B-100 and apo E components of the VLDL particle and is CE enriched compared to its parent particle. This can undergo one of two metabolic fates, depending on the size of its parent VLDL particle. Larger IDL particles arising from the delipidation of large VLDL particles appear to be directly removed from the circulation by a receptor mediated process in the liver and other tissues, whereas smaller IDL particles produced by small VLDL are more likely to be further metabolised to LDL prior to uptake (Stalenhoef et al. 1984; Packard et al. 1984). The uptake of IDL particles occurs via the LDL receptor, also known as the B/E receptor as it binds a homologous region in apo B-100 and apo E on the remnant particle (Frayn, 1996).

Catabolism of IDL to LDL requires further hydrolysis of their TAG content by LPL and hepatic lipase (HL) (Taskinen and Kuusi, 1987). During this process the lipoprotein loses most of its surface material and the LDL particle consists of a CE enriched core with a shell containing phospholipid, free cholesterol and apo B-100 (Frayn, 1996). These particles have a half-time in the circulation of approximately 2.5 days (Durstine and Haskell, 1994) and are cleared from the plasma via a number of mechanisms. About half of LDL is taken up by the interaction of the apo B-100 component with the LDL receptor particularly in the liver and other tissues with a specific need of cholesterol for biosynthesis (e.g. adrenal glands, gonads, intestine and spleen) (Shepherd and Packard, 1987). Other LDL is taken up into cells by a low-affinity, receptor-independent process (Spady et al. 1985) and physically and/or chemically modified LDL can be taken up by macrophages via ‘scavenger receptors’ (Kelley, 1991). Unlike the uptake via the LDL receptor, the latter process is not subject to feedback control and consequently macrophages can become excessively cholesterol laden which may promote atherosclerosis (Patsch and Patsch, 1984).

2.2.4 HDL metabolism

HDL are the smallest and most dense of the lipoproteins and unlike the other lipoproteins transport cholesterol away from peripheral tissues back to the liver. This process is termed ‘reverse cholesterol transport’ and is necessary because cholesterol

is transported to the periphery at a rate which exceeds the tissues' requirements (Durrington, 1989). HDL particles are secreted by the liver or intestine in a nascent form – discoidal bilayers of mainly protein and phospholipid containing apo A-I and A-II (Durrington, 1989). The metabolism of HDL is inexorably linked with the metabolism of TAG-rich lipoproteins as the discoidal HDL particles acquire surface materials including unesterified cholesterol from the degradation of chylomicrons and VLDL. Additional cholesterol is obtained from the cells of peripheral tissues and after this is esterified by the action of lecithin-cholesterol acyltransferase (LCAT), activated by apo A-I, mature spherical HDL particles are formed. These lipoproteins continue to interact with TAG-rich lipoproteins. Further transfer of surface materials from lipolysed TAG-rich lipoproteins produces relatively large and buoyant particles known as HDL₂ particles. Conversely, neutral lipid exchange with TAG-rich lipoproteins, catalysed by CETP, results in CE-enriched TAG-rich lipoproteins and TAG-enriched, CE-depleted HDL particles. These TAG-enriched HDL particles are subject to hydrolysis of their TAG and phospholipid content by the action of HL, which results in the formation of small, dense HDL₃ particles (Taskinen and Kuusi, 1987).

Thus, sluggish metabolism of TAG rich lipoproteins results in low HDL concentrations, particularly in the HDL₂ subfraction, and an increased proportion of HDL₃ particles. In addition CE-enrichment of TAG-rich lipoproteins is increased, augmenting their atherogenic potential (Miesenböck and Patsch, 1992). High plasma concentrations of HDL cholesterol, particularly in the HDL₂ fraction are associated with a reduced risk of developing CHD (Patsch et al. 1983), but this might reflect their relationship with the metabolism of TAG-rich lipoproteins – a potentially atherogenic process – rather than an anti-atherogenic effect of the particles themselves *per se* (Miesenböck and Patsch, 1992).

2.2.5 Integration of TAG-rich lipoprotein metabolism

In the fasted state endogenous VLDL are the only TAG-rich lipoprotein and thus their metabolism is not impeded by the presence of chylomicrons in the circulation. After consumption of a meal containing fat, chylomicrons enter the circulation and mix with VLDL in the circulation and these particles compete for the clearance by the same lipolytic pathway (Brunzell et al. 1973). TAG-rich lipoproteins of endogenous

origin accumulate in plasma in the postprandial state (Cohn et al. 1993; Scheeman et al. 1993; Karpe et al. 1993) with 80 % of the postprandial increase in lipoprotein particle number being attributable to increases in apo B-100 containing lipoproteins (Scheeman et al. 1993). In contrast, 80 % of the increase in postprandial TAG concentrations is attributable to apo B-48 containing particles (Cohn et al. 1993), implying that a relatively small number of TAG-rich chylomicrons are responsible for the majority of the postprandial TAG rise. The accumulation of endogenous lipoproteins in postprandial plasma appears to be due to reduced catabolism of VLDL in the postprandial state rather than increased hepatic secretion. The postprandial increase in VLDL particles is restricted to large VLDL (Sf 60-400), with postprandial concentrations of small VLDL (Sf 20-60) decreasing in most individuals (Karpe et al. 1993; Björkegren et al. 1996). As small VLDL are the remnant of the catabolism of large VLDL, these data suggest that VLDL catabolism is delayed by the presence of chylomicrons in the circulation. Indeed, Björkegren and co-workers (1996) (Björkegren et al. 1996) used kinetic modelling with stable isotope tracer techniques to conclude that reduced VLDL catabolism was the most likely explanation for the accumulation of large VLDL in the postprandial state. Thus, there is compelling evidence that chylomicrons are the preferred substrate for LPL *in vivo* (Potts et al. 1991; Karpe and Hultin, 1995b; Björkegren et al. 1996) and that the metabolism of both exogenous and endogenous lipoproteins influences TAG concentrations in the postprandial state.

Lipoprotein lipase is usually considered to be the rate-limiting enzyme in TAG hydrolysis (Taskinen and Kuusi, 1987) (although it has recently been suggested that this might not always be the case (Olivecrona and Olivecrona, 1995)) and therefore plays a key role in the catabolism of TAG-rich lipoproteins. Thus, its activity is a major determinant of postprandial lipaemia under the majority of circumstances. The enzyme is subject to reciprocal regulation in adipose tissue and skeletal muscle with activity increasing in the former tissue, but decreasing in the latter, after ingestion of a meal (Lithell et al. 1978). Thus, the relative contribution of (unexercised) skeletal muscle to lipoprotein-TAG clearance is greater in the fasted state, than the postprandial state. Insulin concentrations increase after ingestion of a meal containing carbohydrate or protein and the hormone facilitates LPL activity in adipose tissue (Sadur and Eckel, 1982) while suppressing LPL activity in skeletal muscle (Kiens et

al. 1989). Therefore, it is likely that insulin plays a major role in the regulation of LPL in the fasted and postprandial states. Adipose tissue LPL activity reaches a peak about four to five hours after ingestion of mixed meals containing 33 g (Coppack et al. 1992) or 80 g (Frayn et al. 1994) of fat, which corresponds with the timing of peak plasma TAG concentrations.

Insulin is an important co-ordinator of lipoprotein metabolism. In addition to regulating LPL activity it suppresses hormone sensitive lipase (HSL) activity and is a potent stimulator of re-esterification of fatty acids in adipose tissue (Coppack et al. 1992). HSL action remains suppressed for about five hours after consumption of high or moderate fat mixed-meals (Coppack et al. 1992; Frayn et al. 1994), inhibiting release of NEFA from adipose tissue in the postprandial state. This leads to low plasma NEFA concentrations early in the postprandial state which acts to reduce hepatic VLDL secretion by reducing the fatty acid flux to the liver (Frayn and Coppack, 1992; Sniderman and Cianflone, 1993). Insulin may also directly inhibit VLDL secretion, possibly by promoting the degradation of apo B (Mason, 1998).

After hydrolysis by LPL, fatty acids derived from the TAG core of chylomicron and VLDL particles are taken up and re-esterified in the peripheral tissues. It appears that entrapment of these fatty acids is high in (forearm) skeletal muscle (Coppack et al. 1990), but in adipose tissue a proportion of these fatty acids are not esterified and 'spill-over' into the plasma NEFA pool (Coppack et al. 1990; Frayn et al. 1994). This appears to be a highly regulated process. There is almost no retention of LPL-derived fatty acids in adipose tissue after an overnight fast and fatty acid entrapment rises to 70-90 % within 90 minutes of ingestion of a mixed-meal containing moderate or large amounts of fat (Frayn et al. 1995) – when insulin concentrations are high. Four to five hours after meal ingestion – when TAG extraction is maximal (Fielding and Frayn, 1998) – less than 50 % of LPL-derived fatty acids are retained in adipose tissue, with the remainder spilling over into the circulation (Frayn et al. 1995). Thus, after the initial suppression, plasma NEFA concentrations rise during the mid-to-late postprandial period, predominately due to the influx of LPL-derived NEFA (Frayn et al. 1996). This increases the fatty acid flux to the liver and therefore, after the initial suppression, VLDL production may be stimulated in the postprandial state.

2.3 Measurement of postprandial TAG metabolism

Precise and accurate quantification of an individual's ability to metabolise TAG is necessary both to compare 'TAG metabolic capacity' in different populations and to assess the efficacy of different interventions on postprandial TAG metabolism.

2.3.1 The oral fat tolerance test

TAG metabolic capacity can be defined as the magnitude of lipaemia occurring after a standardised fat load (Miesenböck and Patsch, 1992) but there is no standard oral fat tolerance test. Various test meals employing either almost pure fat milkshakes e.g. (Cohn et al. 1988; Cohen and Grundy, 1992) or mixed meals containing both fat and carbohydrate e.g. (Schlierf et al. 1987; Merrill et al. 1989; Coppack et al. 1990) have been reported. The magnitude of the postprandial TAG response is related positively to the size of the fat load (Cohen et al. 1988; Murphy et al. 1995; Jeppesen et al. 1995b) and meals containing large amounts of *n*-3 polyunsaturated fatty acids induce a lower lipaemic response than meals containing predominantly saturated, monounsaturated or *n*-6 polyunsaturated fatty acids (for review see (Williams, 1997)). Consumption of carbohydrate with fat, as occurs in real life, augments postprandial lipaemia (Cohen and Schall, 1988; Grant et al. 1994; Jeppesen et al. 1995a; Jeppesen et al. 1995b; Abraha et al. 1998) with fructose having a greater effect than sucrose (Cohen and Schall, 1988) or starch (Abraha et al. 1998). The mechanism by which carbohydrate potentiates lipaemia is unclear and both impaired TAG clearance (Grant et al. 1994; Jeppesen et al. 1995a) and increased VLDL secretion (Jeppesen et al. 1995a) have been proposed as possibilities.

The lipaemic response to an oral fat load can be assessed in a number of ways. Patsch et al. (1983) defined the triglyceridaemic response to a test meal as the area under the postprandial TAG vs. time curve, corrected for the baseline TAG concentration. However, Karpe et al. (1994) considered that the area under the TAG concentration vs. time curve without subtraction of baseline values provided a more meaningful measure of atherogenic risk as all lipoprotein particles in plasma are able to interact with the arterial wall.

Analytical ultracentrifugation of postprandial plasma samples using either gradient density (e.g. Chapman et al. 1981) or sequential flotation (e.g. (Havel et al. 1955;

Potts et al. 1994)) separates lipoprotein classes on the basis of their flotation rates and thus enables their quantification. However, as flotation rates for the different lipoprotein classes overlap it is not possible to obtain pure fractions using this technique. This is particularly true for the Sf 20-400 (VLDL) fraction as both hepatically derived VLDL and intestinally derived chylomicron remnants fall into this range. To overcome this problem, a number of authors have incorporated vitamin A, usually in the form of retinyl palmitate, into the test meal as a marker of intestinally derived lipoproteins (Föger and Patsch, 1993). However, this technique has inherent problems. Studies have shown that labelling of chylomicrons and chylomicron remnants with retinyl esters is not uniform (Karpe et al. 1995) and peak plasma retinyl ester concentration occurs later than the peak for TAG (Krasinski et al. 1990; Karpe et al. 1995) or apo B48 (Karpe et al. 1995). In addition, particularly in the late postprandial period, retinyl esters are present in hepatically-derived particles (Krasinski et al. 1990; Cohn et al. 1993), calling into question the specificity of retinyl esters as markers of chylomicron metabolism. The development of specific assays for apo B48 and apo B100 – the main structural apolipoprotein associated with intestinally- and hepatically-derived lipoprotein particles, respectively – has allowed specific quantification of exogenous and endogenous lipoproteins (Karpe et al. 1995; Karpe et al. 1996). This has provided a valuable tool for the study of postprandial lipoprotein metabolism and the number of laboratories employing these techniques is growing but it will be some time before these methods are widely used on a routine basis.

2.3.2 The intravenous fat tolerance test

Clearance of lipoprotein-TAG from the circulation is an important component of TAG metabolic capacity. Postprandial lipoprotein metabolism is complex and interpretation of results from oral fat tolerance tests is often not straightforward, particularly if no quantification of lipoprotein classes is performed. The intravenous fat tolerance test provides a simple measure of TAG clearance, unconfounded by absorption processes in the gastrointestinal tract and the rate of synthesis and influx of chylomicrons into the circulation (Carlson and Rössner, 1972) and was pioneered by Lars Carlson and Dag Hallberg in the early 1960s, initially in dogs (Carlson and Hallberg, 1963) and then in humans (Hallberg, 1965a; Hallberg, 1965b). Carlson and Rössner (1972) reported an intravenous fat tolerance test procedure in which a bolus

dose of Intralipid (0.1 g TAG per kg body mass) – an artificial TAG emulsion – was injected into a forearm vein, with blood being drawn from a contralateral antecubital vein at 5 minute intervals for 40 minutes. Plasma Intralipid concentrations were determined indirectly by measuring plasma turbidity using nephelometry and TAG clearance was observed to follow first order kinetics (i.e. the clearance rate was directly proportional to the concentration). Rössner (1976) later reported that the reproducibility of this procedure was good when repeated tests were performed at intervals between one week and six months (coefficient of variation of 14 % in Intralipid clearance (k_2)). More recently Sady and co-workers (1986) evaluated both nephelometry and enzymatic TAG quantification to assess clearance of an intravenous fat emulsion and reported better reproducibility with the latter method.

If the intravenous fat tolerance test is to be used as a measure of lipoprotein-TAG clearance, the clearance kinetics of artificial TAG emulsions must be similar to those of TAG-rich lipoproteins. This appears to be the case as TAG emulsion particles rapidly acquire apolipoproteins, including apo C-II, from circulating lipoproteins once injected into the circulation (Carlson, 1980; Iriyama et al. 1988; Tonouchi et al. 1990) and, like TAG-rich lipoproteins, are hydrolysed by the action of LPL. Indeed, studies have shown that emulsion-TAG and chylomicron-TAG are cleared from the circulation in a similar manner (Carlson and Hallberg, 1963; Hultin et al. 1995) and there is a high correlation between intravenous fat tolerance and chylomicron-TAG clearance as measured by duodenal perfusion (Cohen, 1989), although catabolism of lipid emulsion particles is not identical to chylomicron catabolism (Hultin et al. 1995). In addition, if the changes in TAG concentration seen during the intravenous fat tolerance test are taken to reflect the TAG clearance rate, it must be assumed that VLDL production and degradation is constant for the duration of the test (Sady et al. 1986).

2.3.3 Measurement of fat oxidation

Oxidation is one of the two major fates for TAG of both exogenous and endogenous origin, the other being storage. Indirect calorimetry allows non-invasive measurement of energy expenditure and substrate utilisation by measuring whole body oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$). This simple technique is based on the principle that knowledge of the stoichiometry for the oxidative reactions of fat,

carbohydrate and protein allows determination of substrate utilisation from $\dot{V}O_2$, $\dot{V}CO_2$ and urinary nitrogen excretion. The respiratory exchange ratio (R or RER) is derived by dividing $\dot{V}CO_2$ by $\dot{V}O_2$ and is normally between 0.7 and 1.0, with the extreme values representing 100 % fat and 100 % carbohydrate oxidation, respectively. The principle relies on a number of assumptions and an understanding of the theoretical and physiological limitations of the technique is necessary for the meaningful interpretation of indirect calorimetry data in a number of circumstances.

Normally one value is taken to represent the volume of oxygen consumed and carbon dioxide produced during the complete oxidation of one gram of each of the substrates. However, in reality, a mixture of different fats, carbohydrates and proteins, with slightly differing values for oxygen consumption and carbon dioxide production are oxidised. Thus, the oxygen and carbon dioxide values employed should reflect the average of the fuels used. Frayn (1983) chose palmitoyl-stearoyl-oleoyl-glycerol (PSOG) as the representative lipid. This reflects the usual composition of human adipose tissue and thus if fat oxidation is expected to be predominantly endogenous or that exogenous lipid oxidised is of a similar composition, this value should be adopted. However, if other exogenous lipids are predominately being oxidised, the indirect calorimetry equations should be adapted accordingly. Similarly, the choice between glucose and glycogen as the assumed carbohydrate fuel should be dependent on the physiological state under study (Ferrannini, 1988), with the contribution of glycogen as a fuel being higher during exercise than at rest and increasing with exercise intensity (Romijn et al. 1993). Protein oxidation is low under normal physiological conditions and a four-fold error in protein oxidation only leads to a 13-15 % error in lipid and carbohydrate oxidation (Ferrannini, 1988). Thus, for most conditions it is possible to eliminate urinary nitrogen collection and assume a fixed protein oxidation rate, or ignore protein oxidation, without introducing large errors into the calculations. Indeed, the use of fixed values for $\dot{V}O_2$ and $\dot{V}CO_2$ in the fat and carbohydrate oxidation equations does not introduce large errors into indirect calorimetry estimations in practice and other limitations of the technique cause greater cause for concern.

The assumption that the body oxygen and carbon dioxide pools (Ferrannini, 1988) remain in equilibrium during the measurement is necessary for indirect calorimetry

estimations to be valid. This does not present a problem for oxygen, as there is virtually no oxygen reserve in the body so oxygen consumption at the mouth reflects whole body oxygen consumption (Ferrannini, 1988). However, the body possesses a large bicarbonate pool and changes to this can markedly affect estimations of fat and carbohydrate oxidation. Hyperventilation – as often occurs when a novice subject uses an unfamiliar mouthpiece – causes carbon dioxide to be eliminated in excess of oxidative metabolism, resulting in an overestimation of carbohydrate oxidation and an underestimation of fat oxidation and on occasion can result in apparently negative fat oxidation rates (Jéquer et al. 1987). However, a period of compensatory hypoventilation usually follows hyperventilation to re-equilibrate the bicarbonate pool and if the measurement period encompasses both these phases, the mean substrate utilisation values obtained will be correct (Jéquer et al. 1987). When lactic acid is produced in excess of its utilisation rate, hydrogen ions may be buffered by bicarbonate displacing CO_2 and thus increasing CO_2 excretion on the breath (Frayn, 1983). Therefore, during intense exercise – where there is an accumulation of lactic acid – substrate utilisation calculations made by indirect calorimetry may not be valid, although the extent of the uncertainty is unclear (Frayn, 1983). However, estimates of energy production are more robust than those for substrate utilisation (Ferrannini, 1988), so valid estimations of metabolic rate during intense exercise can be made, despite possible errors occurring in calculations of substrate utilisation rates.

Indirect calorimetry relies on the principle that intermediate metabolic processes do not influence the overall conclusions. This is true if all the metabolic intermediates are ultimately oxidised and do not accumulate within the body or get excreted in products other than CO_2 or H_2O (Frayn, 1983). However, the processes of lipogenesis, gluconeogenesis and net ketone body formation or utilisation influence $\dot{\text{V}}\text{O}_2$ and $\dot{\text{V}}\text{CO}_2$ and result in calculated rates of fat and carbohydrate oxidation which do not reflect the ‘true’ oxidation rates for these substrates (Frayn, 1983). RER values of less than 0.7 – implying negative carbohydrate oxidation – can be obtained if net gluconeogenesis or ketogenesis are occurring but in the latter situation buffering of ketoacidosis from the bicarbonate pool confounds quantification of the effect (Jéquer et al. 1987). Conversely net lipogenesis or ketone body utilisation have the effect of raising the RER (Frayn, 1983). However, except in extreme situations, the effects of these pathways on calculated rates of carbohydrate and fat oxidation are small and

indirect calorimetry provides 'almost true' values for substrate utilisation (Frayn, 1983).

One limitation of indirect calorimetry is that it cannot distinguish between endogenous and exogenous fat oxidation. This problem can be addressed through use of stable isotope tracer methodologies. One approach is to incorporate ^{13}C -labelled TAG or fatty acids into a test meal and use $^{13}\text{CO}_2$ excretion on the breath and $\dot{V}\text{CO}_2$ to calculate exogenous fat oxidation (Murphy et al. 1995; Bennoson et al. 1999). This approach is straightforward in principle but an understanding of the gastrointestinal and metabolic handling of the ^{13}C label is necessary to interpret the results from this type of study meaningfully.

In order to determine the metabolic disposal of ^{13}C labelled lipid it is vital to know what proportion of the ingested dose of ^{13}C is absorbed, as $^{13}\text{CO}_2$ excretion on the breath is dependent on the amount of labelled lipid which enters the circulation. This often requires measurement of ^{13}C enrichment in faeces, but a recent study by Bennoson et al. (1999) found that, when a $[1,1,1-^{13}\text{C}]$ tripalmitin label was ingested as part of a lipid-casein-glucose-sucrose emulsion, there was almost complete (98 %) absorption of the label, suggesting that faecal ^{13}C measurements would not be necessary if this protocol was adopted.

The assessment of exogenous fat oxidation rates from $^{13}\text{CO}_2$ excretion is further complicated by the fact that some ^{13}C is oxidised but not excreted on the breath. These losses are mainly due to $^{13}\text{CO}_2$ being incorporated and trapped into intermediary metabolic products (Elia, 1990). However, by applying a 'bicarbonate correction' to $^{13}\text{CO}_2$ excretion values to account for these losses, it is possible to obtain a valid estimate of exogenous fat oxidation (Jones et al. 1999).

Thus, by combining indirect calorimetry and stable isotope techniques it is possible to assess the rates of exogenous and endogenous fat oxidation and the extent of exogenous fat storage in the postprandial state.

2.4 Factors affecting postprandial lipid metabolism

2.4.1 Age and gender

Cohn et al. (1988) demonstrated that postprandial lipaemia was greater in older individuals than in their younger counterparts and that lipaemia was lower in females than males at all ages. Lower postprandial lipaemia in women than men has also been reported by other authors (Kashyap et al. 1983; Ohta et al. 1992). The lower lipaemia in women might have been due to increased clearance of lipoprotein-TAG in the females. Tollin and co-workers (1985) reported that TAG clearance was greater in women than men for all age groups, although TAG clearance rates decreased with age for the females but not the males. Other authors using retinyl palmitate as a marker of exogenous lipoprotein metabolism have demonstrated higher postprandial areas of retinyl ester in the chylomicron fraction (Weintraub et al. 1987; Krasinski et al. 1990) and whole plasma (Krasinski et al. 1990; Johnson et al. 1992) in older compared with younger subjects. In addition, Johnson et al (1992) reported higher retinyl ester concentrations in males than females for both the younger and older subjects. Krasinski et al (1990) found that these differences were due to poorer clearance of exogenous TAG-rich lipoproteins in the older subjects; a finding consistent with reports of lower LPL activity in older compared with younger individuals (Weintraub et al. 1987).

2.4.2 Preceding diet

Altering the macronutrient composition of the background diet can substantially influence postprandial TAG metabolism. A moderately high carbohydrate diet (55-60 % of energy from carbohydrate) consumed for two (Chen et al. 1993) to six (Chen et al. 1995; Blades and Garg, 1995) weeks has been shown to promote greater postprandial lipaemia than a preceding diet lower in carbohydrate and higher in fat in patients with non-insulin-dependent diabetes mellitus. This augmented lipaemia was attributed to increased hepatic VLDL secretion (Chen et al. 1995; Blades and Garg, 1995) and/or reduced catabolism of TAG-rich lipoproteins after the high carbohydrate diet (Chen et al. 1993; Chen et al. 1995). Similar data have been reported in healthy postmenopausal women with Jeppesen and co-workers (1997) finding that consumption for three weeks of a diet containing 60 % carbohydrate, 25 % fat and 15 % protein induced augmented postprandial TAG and retinyl palmitate concentrations,

compared with an isoenergetic diet containing 40 % carbohydrate, 45 % fat and 15 % protein.

In addition, studies have shown that the fatty acid composition of the background diet can substantially influence postprandial lipaemia with *n*-3 polyunsaturated fatty acids having a potent hypotriglyceridaemic effect (Harris and Conner, 1980; Weintraub et al. 1988; Harris et al. 1988).

The effects of chronic alcohol consumption on postprandial TAG metabolism are unclear. Superko (1992) reported that postprandial lipaemia was not affected by two weeks of moderate (3 to 6 oz per day) daily alcohol consumption in healthy male subjects, whereas Hartung et al. (1993) found that 20 days of ingestion of ~ 41 g of ethanol per day did not affect postprandial lipaemia in runners but resulted in a 31 % increase in the postprandial TAG response in inactive subjects. The results of these studies suggest that prior nutritional influences can markedly affect postprandial TAG metabolism and highlight the importance of controlling subjects' dietary intakes in the days leading up to an oral fat tolerance test in an intervention study.

2.4.3 Fasting TAG pool

As all TAG-rich lipoproteins are cleared by a common, saturable pathway (Brunzell et al. 1973; Björkegren et al. 1996), postabsorptive TAG-rich lipoproteins influence the magnitude of postprandial lipaemia by competing with postprandial lipoproteins for hydrolysis by LPL. This is evidenced by the positive relationship between fasting TAG concentrations and postprandial lipaemia which has been demonstrated by a number of authors (Patsch et al. 1983; Cohn et al. 1988; O'Meara et al. 1992; Potts et al. 1994). However, although the size of the fasting TAG pool may be the most important determinant of postprandial lipaemia (Chen and Reaven, 1991; O'Meara et al. 1992), fasting TAG concentrations only account for part of the variance in postprandial TAG concentrations. Indeed, individuals with similar fasting TAG concentrations can have postprandial TAG responses that differ markedly (Patsch, 1987; Merrill et al. 1989; Schrezenmeir et al. 1992), highlighting the importance of assessing TAG metabolic capacity in the dynamic postprandial state.

2.4.4 Apolipoprotein E phenotype

Apo E serves as a ligand for receptor-mediated uptake of TAG-rich lipoprotein remnants (Gurr and Harwood, 1991) and thus plays an important role in TAG metabolism. It exists in three major isoforms (E2, E3 and E4) and as each person carries two alleles, there are six apo E phenotypes; E2/2, E3/3, E4/4, E2/3, E2/4 and E3/4, with the E3/3 phenotype being the most common (Weintraub et al. 1987).

It has been reported that normolipidaemic subjects who are homozygous (Brenninkmeijer et al. 1987) for the E2 isoform experience delayed chylomicron remnant clearance compared with subjects possessing other apo E phenotypes, a finding which is consistent with the fact that apo E2 binds poorly to remnant receptors (Davignon et al. 1988). However, findings are less consistent for normolipidaemic heterozygous E2 carriers with studies reporting chylomicron remnant clearance which is similar (Brenninkmeijer et al. 1987; Brown and Roberts, 1991) or delayed (Weintraub et al. 1987) compared with subjects not possessing the E2 isoform.

The influence of the E4 isoform on postprandial TAG metabolism is less clear. Weintraub and co-workers (1987) found that subjects who possessed the E4 isoform experienced faster chylomicron remnant clearance than those who did not possess this isoform, but Brown and Roberts, (1991) found that E4 subjects had higher postprandial TAG and chylomicron retinyl ester concentrations than E2/3 or E3/3 subjects. Similarly, Bergeron and Havel (1996) found the postprandial increase in apo B-48 and apo B-100 was more prolonged in individuals possessing the E4/3 phenotype compared with those possessing the E3/3 phenotype.

The effects of apo E phenotype on plasma TAG concentrations in the fasted state have been comprehensively examined, with a meta-analysis of 45 studies from 17 countries reporting that fasting TAG concentrations were higher in individuals possessing one or more $\epsilon 2$ or $\epsilon 4$ alleles than those who were homozygous for the $\epsilon 3$ allele (Dallongville et al. 1992). Thus, although the mechanisms have not been fully elucidated, it seems clear that the genetic variation in apo E has an influence on the metabolism of TAG-rich lipoproteins and their remnants.

2.5 Exercise and postprandial TAG metabolism

The effects of exercise training and single exercise sessions on plasma lipids and lipoproteins in the fasted state have been well researched and will not be extensively reviewed here as this thesis is primarily concerned with the effects of exercise on postprandial lipid and lipoprotein metabolism. Instead the interested reader is directed to the comprehensive review papers from Pronk (1993) and Durstine and Haskell (1994). However, as TAG concentrations in the fasted state have a large influence on postprandial TAG concentrations because a common saturable pathway clears all TAG-rich lipoproteins (Brunzell et al. 1973), the effect of exercise on TAG concentrations in the fasted state will be briefly mentioned.

Regular exercisers tend to have lower fasting concentrations of TAG than their sedentary counterparts (see Durstine and Haskell (1994) for review) and a period of endurance training can elicit a decrease in TAG concentrations in the fasted state (see Durstine and Haskell (1994) for review). In addition a single exercise session can attenuate TAG concentrations in the fasted state (see Pronk (1993) for review). In particular exercise training (Wirth et al. 1985) (Després et al. 1990) and individual exercise sessions (Dufaux et al. 1981; Baumstark et al. 1993) appear to reduce concentrations of TAG in the VLDL fraction.

2.5.1 Cross-sectional studies

Although there has been considerable research investigating the effects of exercise on fasting lipid and lipoprotein concentrations, less research has focused on the effects of exercise on postprandial lipid metabolism. Cross-sectional studies have shown that postprandial lipaemia is lower (Cohen et al. 1989; Merrill et al. 1989; Hartung et al. 1993) and/or TAG clearance is greater (Ericsson et al. 1982; Sady et al. 1988; Cohen et al. 1989; Podl et al. 1994) in regular exercisers compared with sedentary controls. However, caution is advised in the interpretation of these data as they do not necessarily imply that TAG metabolic capacity is enhanced by exercise training *per se*.

An inherent criticism of all cross-sectional studies is that the direction of causality cannot be determined from the findings. Thus, while it is possible, even probable, that the reduced postprandial lipaemia and/or increased TAG clearance were, at least

in part, due to exercise, self-selection might also have contributed to the improved TAG metabolic capacity seen in the regular exercisers. It is not inconceivable that individuals with high functional capacities or low levels of adiposity would be more likely to exercise regularly and that it is these underlying traits, rather than the regular exercise, which contributed to the exercisers' lower levels of postprandial lipaemia and/or increased rates of TAG clearance. To overcome this intrinsic deficiency evident in cross-sectional studies, a longitudinal study design – in which the effects of an exercise training (or detraining) programme on measures of TAG metabolic capacity are determined – is required.

The above data are additionally confounded by the fact that in all the above studies the trained subjects are likely to have exercised in the days leading up to the fat tolerance test. Regular exercisers will invariably exercise unless they are specifically asked not to as this is part of their lifestyle and the influence of an individual exercise session on postprandial lipaemia is considerable (see section 2.5.3). Thus, as none of the studies cited above specifically required subjects to refrain from exercise for more than one day prior the fat tolerance tests, it is not possible to ascertain whether the high TAG metabolic capacity observed in the regular exercisers was a consequence long-term adaptations to training or simply reflected the acute effect of their last exercise session. Indeed, cross-sectional data from two recent studies suggest that in the absence of an acute exercise effect, well-trained individuals do not enjoy enhanced TAG metabolic capacity. Tsetsonis et al. (1997) conducted oral fat tolerance tests on trained and untrained middle-aged women and found that when subjects had refrained from exercise for three days, there were no significant differences in postprandial lipaemia between the two groups. Similarly, Herd (1997) found no differences in postprandial lipaemia between sprint-trained, endurance-trained and untrained groups when the subjects had not exercised for two days.

2.5.2 Longitudinal studies

It has been known since the 1970s that a period of training could alter postprandial TAG metabolism. Altekruze and Wilmore (1973) reported that after a fairly modest 10-week training programme (a mean of 52 miles covered in 413 minutes over the 10 weeks), postprandial TAG concentrations were on average 24 % lower than pre-training values. Four years later Zauner and Benson (1977) found that after an eight-

week training programme, in men selected for an abnormally high response to an oral fat tolerance test, serum optical density (a surrogate for lipaemia) was reduced by 24 %, although this reduction was not statistically significant. However, the modest nature of the training programme in the latter study, with subjects building up to a maximum of just 600 yd of swimming, 25 minutes of cycling or half to one mile of running per exercise session, might well have contributed to the null finding.

Patsch et al. (1983) evaluated postprandial responses to a fat-load procedure for two subjects over a four-year training period. Data were only presented for one subject in whom postprandial lipaemia decreased by 95 % with training, compared with pre-training values.

Since the mid 1980s, more comprehensive studies reporting the effects of exercise training on postprandial lipaemia and TAG clearance, and of detraining on postprandial lipaemia, have been published. Aldred and co-workers (1995) observed no changes in postprandial lipaemia, compared with a control group, after a 12 week brisk walking programme in middle-aged female subjects despite clear increases in endurance fitness and an indication that insulin sensitivity was enhanced. However, in that study the post-training oral fat tolerance test was performed after two days of inactivity to eliminate the confounding influence of acute exercise on the plasma TAG response. Thus the findings support the hypothesis that in the absence of a recent exercise session, exercise training does not improve TAG metabolic capacity.

Conversely, Drexel et al. (1992) found that after a 12-week training programme both the plasma TAG and chylomicron (retinyl palmitate) responses to an oral fat tolerance test were reduced in overweight women, the latter response by 74 %. The post-training oral fat tolerance test was conducted four days after the cessation of exercise to eliminate possible acute effects of exercise on lipoprotein metabolism so, at first glance, these data appear to conflict with the findings of Aldred et al (1995).

However in Drexel and co-worker's (1992) study, subjects lost 4.3 kg of body mass during the training period and this, rather than fitness changes, may have mediated the improvement in TAG metabolic capacity as weight loss can mediate reductions in plasma TAG concentrations (Andersen et al. 1995).

Weintraub et al. (1989) found that after a seven-week training programme that elicited a 43 % improvement in maximal oxygen uptake, the area under retinyl palmitate curve for the chylomicron fraction after vitamin A-fat loading was 37 % lower than before training. There was also a non-significant 28 % reduction in the area under the non-chylomicron (i.e. chylomicron remnant) retinyl palmitate curve. Although the authors ascribed these reductions to changes in fitness, the fact that the post-training fat tolerance test was performed only 36 hours after the last training session means that the acute influence of the last exercise session cannot be ignored when interpreting the findings of this study.

Fewer studies have evaluated the effect of exercise training on responses to intravenous, rather than oral, fat tolerance tests. Wirth et al. (1985) found that after four months of training (three one-hour sessions per week consisting of jogging, handball and soccer), in patients with primary hypertriglyceridaemia, TAG clearance was increased by 8 % but this did not differ significantly from clearance rates measured in control subjects. The post-training fat tolerance test was conducted 48 to 72 hours after the last exercise session to exclude the acute effects of exercise but this, in itself, might not adequately explain the null result as fasting TAG concentrations were 27 % lower after training. The exercising group lost body fat and changed their diet over the training period and these confounding factors might have contributed to the reduction in fasting TAG. However, the possibility exists that exercise training *per se* reduced TAG concentrations by mechanisms other than increased TAG clearance. Thompson and co-workers (1988) similarly found no significant increase in TAG clearance after 14 weeks of exercise training (five one-hour cycling sessions per week at 80 % of maximum heart rate) despite a 26 % improvement in maximal oxygen uptake. Continuation of training (four one-hour cycling sessions per week) for a further 18 to 34 weeks produced no further increase in aerobic power, but at this time TAG clearance was 49 % greater than pre-training values. The intravenous fat tolerance tests were performed just 10 hours after the subjects' last exercise session, so acute exercise effects would have undoubtedly affected the results. Thus, even in the presence of an acute exercise effect, TAG clearance was not significantly enhanced by 14 weeks of training, despite a 16 % reduction in plasma TAG concentrations in the fasted state. A recent study by Zmuda and colleagues (1998) reported that 12 months of exercise training (four 50-minute sessions per week at 60-

80 % of maximal heart rate) increased TAG clearance 14 % in men with HDL cholesterol concentrations initially above 1.13 mmol.l^{-1} but had no effect on TAG clearance in men with initial HDL cholesterol concentrations below 1.03 mmol.l^{-1} . However, as in many studies, subjects continued their exercise regimens during the post-training testing period, so these data are confounded by the effect of the subjects' last exercise session.

Detraining studies can reveal much about the nature of exercise training-induced changes to lipoprotein metabolism. Mankowitz et al. (1992) found that after a 14-22 day detraining period in well-trained runners, the area under the retinyl palmitate curve for the chylomicron fraction increased by 41 %, with an almost significant ($p = 0.058$) 37 % increase in the retinyl palmitate chylomicron remnant curve, suggesting that the beneficial effects of training on lipoprotein metabolism might be short lived. Indeed, two recent studies from this laboratory suggest that the exercise-induced changes to TAG metabolism might be even less persistent. Herd and co-workers (1998) reported a study in which subjects underwent a 13-week running training programme, followed by a 9-day detraining period. Postprandial lipaemia was 30 % lower 15 hours after the last exercise session compared with pre-training but this reduction was not statistically significant. However, postprandial lipaemia increased by 37 % between 15 and 60 hours after the cessation of training and by 46 % between 15 hours and 9 days of detraining. These differences were significantly different from those observed in the control group and suggest that postprandial lipaemia rapidly increases after the withdrawal of an exercise stimulus. The most convincing data that the low postprandial lipaemia enjoyed by well-trained individuals is transient come from another study by Hardman and co-workers (1998). Ten (nine male) athletes (seven distance runners, two triathletes and one cyclist), five of whom competed at international or regional level, were asked to refrain from training for a week and oral fat tolerance tests were performed 15 hours, 60 hours and 6.5 days after the subjects' last exercise session. Compared with values measured 15 hours after exercise, postprandial lipaemia was 35 % higher 60 hours after the cessation of exercise and 42 % higher after 6.5 days of detraining. These data imply that, even in very well trained athletes, postprandial lipaemia increases rapidly after the removal of an exercise stimulus, with the majority of the enhancement to TAG metabolic capacity being lost within two and a half days of detraining.

Thus, although some studies have shown that postprandial lipaemia is reduced following a period of exercise training, the weight of evidence suggests that in the absence of an acute exercise effect, exercise training does not attenuate postprandial TAG concentrations. The transient nature of the training-induced attenuation to postprandial lipaemia argues against long-term structural adaptations to training mediating these changes as these adaptations persist for much longer than the effects on TAG metabolism. Instead, it appears that short-term changes to metabolism might be predominantly responsible for the exercise-induced changes in TAG metabolic capacity, although exercise training might augment this acute effect. This is in line with the reasoning forwarded by Haskell (1994) who proposed that some of the major health-related changes elicited by exercise might be due more to a 'last bout effect' rather than a training response. These data suggest that, perhaps, future research examining the effects of exercise on postprandial TAG metabolism should focus on the acute influence of individual exercise sessions, rather than on prolonged exercise training programmes. The effects of exercise training on TAG clearance are less clear than its effects on postprandial lipaemia. This may be a consequence of the paucity of information in this area, but the possibility that mechanisms other than increased TAG clearance might contribute to the exercise-induced attenuation in plasma TAG concentrations cannot be discounted.

2.5.3 Effects of individual exercise sessions on postprandial lipoprotein metabolism

It has been known for nearly 40 years that exercise performed during the postprandial period could influence TAG metabolism. In 1960, Cohen and Goldberg (1960) found that walking 6 miles after consumption of a high-fat meal attenuated the postprandial increase in plasma turbidity, suggesting reduced lipaemia. Since this pioneering study, a number of investigators have examined the effect of postprandial exercise on TAG responses to a test meal (Nikkilä and Kontinen, 1962; Maruhama et al. 1977; Welle, 1984; Schlierf et al. 1987; Schlierf et al. 1988; Klein et al. 1992; Hardman and Aldred, 1995), the most recent of these finding that 90 minutes of walking at 40 % of maximal oxygen uptake ($\dot{V}O_2$ max), starting 90 minutes after ingestion of a high-fat meal, reduced the lipaemic response by 24 %.

The effects of an exercise session on TAG metabolism persist for a number of hours after the cessation of exercise, long after many of the perturbations to metabolism occurring during and immediately after an exercise session (e.g. increase in muscle blood flow, decrease in liver and gut blood flow, suppression of the insulin response (Åstrand and Rodahl, 1986)) have subsided. Thus, if an exercise intervention is undertaken after ingestion of a test meal, the substantial shifts in metabolism occurring during exercise might obscure the possibly subtle mechanisms eliciting the more prolonged changes to TAG metabolism. Thus, the study of prior exercise on subsequent TAG metabolism might provide a clearer picture of the exercise-induced changes to postprandial TAG metabolism.

It has only been in the last decade that studies investigating the effects of prior exercise on postprandial lipaemia have been published. Cohen et al. (1989) found that postprandial lipaemia was not reduced by one hour of moderate intensity exercise performed 12 hours before an oral fat tolerance test, in sedentary men. These findings contrast with other reports which suggest that prior moderate-intensity exercise can attenuate postprandial lipaemia (Aldred et al. 1994; Tsetsonis and Hardman, 1996a; Tsetsonis and Hardman, 1996b; Tsetsonis et al. 1997; Herd et al. 1997; Herd, 1997; Zhang et al. 1998; Gill et al. 1998; Malkova et al. 1999) and perhaps suggest that one hour of moderate exercise did not provide a great enough stimulus to substantially effect postprandial TAG metabolism. Indeed the finding of Cohen et al (1989) is in agreement with data presented by Herd (1997) who found that postprandial lipaemia was significantly attenuated by two hours, but not one hour, of moderate exercise at 50 % of $\dot{V}O_2$ max performed on the prior afternoon.

Aldred and co-workers (1994) were perhaps the first authors to show that a single moderate intensity exercise session performed some hours prior to ingestion of a test meal could attenuate postprandial lipaemia. These authors found that two hours of brisk walking at 30 % $\dot{V}O_2$ max on an afternoon reduced the lipaemic response to a test meal consumed the following morning by 31 %, in a group of young normolipidaemic men and women. The results of subsequent studies suggest that the exercise-induced attenuation to postprandial lipaemia is linked to the energy expended during the exercise session. Tsetsonis and Hardman (1996a) found that 90 minutes of walking at an intensity of 60 % $\dot{V}O_2$ max attenuated subsequent postprandial lipaemia

by 26 %, whereas the same duration of exercise performed at 30 % $\dot{V}O_2$ max produced a non-significant reduction in lipaemia of 16 %. A further study published by the same authors (Tsetsonis and Hardman, 1996b) found that three hours of walking at 30 % $\dot{V}O_2$ max produced an identical attenuation in postprandial lipaemia as 90 minutes of walking at 60 % $\dot{V}O_2$ max, implying that differences in energy expenditure, rather than exercise intensity, were mediating the different effects of the two exercise interventions in the previous study. The association between the energy expended during exercise and the subsequent attenuation in postprandial TAG concentrations was also evident in the previously cited study by Herd (1997) – the TAG attenuation elicited by two hours of walking at 50 % $\dot{V}O_2$ max was approximately twice as great as that elicited by one hour of walking at the same intensity (although the reduction in the latter case was not significant). More recent research has shown that when a total of 90 minutes of exercise at 60 % $\dot{V}O_2$ max was performed in one session, or split into three 30-minute sessions, the reduction in postprandial lipaemia was the same (Gill et al. 1998).

The relative importance of muscle TAG and glycogen as substrates is much greater during moderate intensity exercise than low intensity exercise, when plasma NEFAs are the main fuel source and the reliance on fat as a substrate for exercise increases with the duration of an exercise session (Romijn et al. 1993). Thus, the fact that similar attenuations in postprandial lipaemia were elicited when the same amount of energy was expended by exercise performed at two different intensities (Tsetsonis and Hardman, 1996), or in two different formats (i.e. continuous or discontinuous) (Gill et al. 1998) suggests these TAG reductions did not depend on pattern of substrate utilisation during exercise. This was further evidenced by a study from Malkova et al. (1999) who manipulated substrate utilisation during exercise by administering acipimox (a nicotinic acid analogue) prior to exercise in one of two exercise trials (each 90 minutes of running at 60 % $\dot{V}O_2$ max). Acipimox is a powerful inhibitor of adipose tissue lipolysis and, indeed, the rise in plasma NEFA seen during exercise in the placebo trial was abolished during the exercise session with acipimox administration. Consequentially, fat utilisation was reduced by 43 % and carbohydrate utilisation increased by 16 % during exercise in the acipimox trial compared with the placebo trial, although the total energy expenditure was the same in the two trials. However, on the morning after exercise both the acipimox and

placebo exercise trials reduced lipaemia by the same amount (18 % placebo, 19 % acipimox), compared with a non-exercise control trial.

In a recent study, Zhang et al. (1998) found that one hour of exercise at an intensity of 60 % $\dot{V}O_2$ max performed 12 hours prior to consumption of a fat-rich milkshake reduced postprandial lipaemia to a greater extent than the same exercise session performed immediately before the fat tolerance test, and that this exercise performed after ingestion of the test meal did not significantly attenuate lipaemia. These data suggest that the attenuation in lipaemia elicited by exercise might be delayed and this could prove important when considering the mechanisms for this reduction. It should be noted however, that the test meal used in this study was almost exclusively fat (100 g fat, 17 g carbohydrate, 3 g protein), in contrast to all the other studies cited which used mixed test meals including a substantial amount of carbohydrate (with the exception of Cohen et al. (1989)) and thus direct comparison of these results with the others might not be possible.

The studies reviewed above suggest that a single prior exercise session can markedly reduce postprandial lipaemia even in untrained subjects, reinforcing the notion that it is acute changes occurring during and after an exercise session which mediate the attenuation in TAG. However, data from Tsetsonis et al. (1997) suggest that the acute effect of an individual exercise session on postprandial lipaemia may be greater in trained than untrained individuals. Ninety minutes of walking at an intensity of 60 % $\dot{V}O_2$ max on the afternoon prior to consumption of a high-fat mixed breakfast attenuated postprandial lipaemia by 16 % in untrained middle-aged female subjects but by 30 % in their trained counterparts. This might reflect a synergistic interaction between exercise training and acute exercise on TAG metabolism, but equally could reflect the fact that the trained women expended 41 % more energy during the exercise session than the untrained subjects. Thus, exercise training appears to be beneficial to TAG metabolism, even if this is only because trained individuals are able to expend more energy during an exercise session than their untrained peers.

Data examining the qualitative changes to postprandial lipoprotein metabolism elicited by an exercise session are sparse. Herd et al. (1997) found that the morning after a 90 minute exercise session at 60 % $\dot{V}O_2$ max, postprandial TAG concentrations

were reduced by 24 %, with a 53 % reduction in the 'chylomicron-rich' fraction accounting for just over half of this attenuation. It should be noted though, that the lipoprotein separation in this study was performed in a bench-top centrifuge and not an ultracentrifuge as is common practice; this might have affected the purity of the fractions obtained.

Few studies have evaluated the effect of a single exercise session on TAG clearance. Dufaux et al. (1981) found that, one day after a 3 hour running test during which the (untrained) subjects covered between 29 and 45 km, Intralipid clearance was 22 % faster than it was on the day before the run. Similarly, Sady et al. (1986) found that TAG clearance was increased by 76 %, compared with pre-race values, on the day after trained subjects completed a 42 km marathon race. In a study using exercise of a more moderate intensity, Annuzzi et al. (1987) found that 3 hours, but not 90 minutes, of exercise at 50 % of maximal work load (70 to 85 % of maximum heart rate) increased the clearance rate of an intravenous fat emulsion on the day after exercise (by 26 %).

Thus, the data investigating single exercise sessions, taken together with the longitudinal and cross-sectional studies, are strongly suggestive that exercise predominantly influences TAG metabolism in an acute manner, although it is not possible to discount the possibility of a interaction between long-term training and the 'last-bout' effect. Although the research has convincingly demonstrated that moderate and low intensity exercise can attenuate postprandial lipaemia and the magnitude of this reduction is linked to the energy expended during exercise, there are clear areas where data are limited. Apart from Tsetsonis and co-worker's (1997) study of middle-aged women, there are no data, to the author's knowledge, looking at the effects of individual exercise sessions on postprandial TAG metabolism in middle-aged and older adults, and these individuals are at a higher risk of CHD than the younger subjects previously studied (Dawber, 1980; Keys, 1980; Wood et al. 1998). In addition, data examining the effect of a prior exercise session on different lipoprotein classes in the postprandial state are extremely limited and further research in both these areas is warranted.

2.5.4 Exercise-induced changes to postprandial substrate utilisation and metabolic rate

Studies have reported that whole body fat oxidation in the fasted state is increased on the day after an exercise session (Weststrate et al. 1990; Calles-Escandón et al. 1996), implying a shift in the partitioning of lipid away from storage. This finding has been replicated in the postprandial state by Tsetsonis and Hardman (1996) who estimated that whole body postprandial fat oxidation was increased 30 % by both 90 minutes of walking at 60 % $\dot{V}O_2$ max and 3 hours of walking at 30 % $\dot{V}O_2$ max on the previous afternoon. Tsetsonis et al (1997) (Tsetsonis et al. 1997) similarly found that postprandial fat oxidation was 41 % higher in trained women and 28 % higher in untrained women on the day after 90 minutes of walking at 60 % $\dot{V}O_2$ max. In addition, Herd (1997) found that postprandial fat oxidation was increased by 30 % on the day after a 90 minute moderate intensity (60 % $\dot{V}O_2$ max) exercise session, but this was not statistically significant. Postprandial oxygen uptake was not significantly increased following exercise in any of these studies, suggesting that the effect of the exercise sessions on metabolic rate was short lived and did not persist to the following day. However, although it appears that a prior exercise session can increase whole body fat oxidation, it is not known whether this increase reflects increased oxidation of adipose tissue TAG, skeletal muscle TAG or TAG from exogenous sources.

2.5.5 Possible mechanisms for the exercise-induced reduction in postprandial lipaemia

The weight of evidence from the studies reported in the literature suggest that the attenuating effect of exercise on postprandial lipaemia is predominantly due to 'last bout effect', rather than long-term adaptations to training. In addition it has been shown that prior moderate intensity exercise – i.e. exercise of the intensity recommended in current exercise guidelines (Pate, 1995) – can markedly reduce postprandial TAG concentrations. Thus, this section of the review will focus on mechanisms via which a single moderate intensity exercise session might attenuate postprandial lipaemia.

Most authors discussing this have alluded to the fact that exercise might be reducing TAG concentrations by accelerating the rate of clearance of TAG-rich lipoproteins from the circulation. Indeed, studies reported earlier in this review have shown that

TAG clearance can be enhanced by a single exercise session (Dufaux et al. 1981; Sady et al. 1986; Annuzzi et al. 1987). However, it should be noted that the exercise interventions in the two former studies were both prolonged (≥ 3 hours) and intense (race pace) (Dufaux et al. 1981; Sady et al. 1986), and in the latter study, which used a more moderate exercise intervention, 90 minutes of exercise did not affect TAG clearance (Annuzzi et al. 1987).

Lipoprotein lipase is the enzyme responsible for the hydrolysis of lipoprotein-TAG and consequently increases in this enzyme's activity may play a key role in mediating exercise-induced reductions in postprandial lipaemia. Increases in post-heparin plasma LPL activity of 74 % (Kantor et al. 1984) and 46 % (Sady et al. 1986) have been reported 18 hours after a 42 km marathon race and a more modest 11 % increase in LPL activity has been reported 24 hours after one hour of cycle ergometer exercise (Kantor et al. 1987). Gordon et al. (1994) found that an exercise energy expenditure of 800 kcal performed at 60 % or 75 % $\dot{V}O_2$ max elicited increases in post-heparin LPL activity 24-hours after exercise of 13 % and 27 % respectively (after correction for differences in plasma volume) but these rises did not become significant until the results of the two exercise trials were combined. Herd (1997) performed two separate studies to elucidate the time-course of LPL activation following one or two hours of moderate exercise (50 % $\dot{V}O_2$ max) in pre-menopausal women. In the first study, post-heparin LPL activity measured one hour post-exercise was increased by 60 % following one hour of exercise and by 35 % following two hours of exercise. Twenty-four hours after exercise LPL activity was 27 % higher than control in the one-hour exercise trial and 32 % higher in the two-hour exercise trial. In the second study, post-heparin LPL activity measured 18 hours after exercise was increased by 54 % following 2 hours of moderate exercise. However, none of these increases were statistically significant; a finding the author attributed to the large variation in enzyme activity between subjects and the conservative nature of the statistical analyses used.

Increases in LPL activity following exercise may reflect increases in both skeletal muscle and adipose tissue LPL activity, although the results of a study by Taskinen et al (1980) who found that LPL activity increased by 112 % in skeletal muscle and 20 % in adipose tissue immediately after a 20 km run, suggest that increases in the former may be more important. Prolonged and intense exercise can yield remarkable

increases in skeletal muscle LPL activity. After an 85 km cross-country skiing race (Lithell et al. 1979) or many hours of military exercises (Lithell et al. 1981; Lithell et al. 1984) a trebling of skeletal muscle LPL activity has been reported. However, the morning after a moderate intensity (60 % $\dot{V}O_2$ max) exercise session lasting 90 minutes, skeletal muscle LPL activity was not increased (Herd et al. 1997), despite exercise eliciting a 53 % reduction in postprandial 'chylomicron-rich' TAG concentrations – a result consistent with increased TAG clearance given that chylomicrons are the preferred substrate for LPL (Potts et al. 1991; Björkegren et al. 1996). Kiens and Lithell (1989) found no increases in skeletal muscle LPL activity immediately after two hours of one-legged knee extension exercise at 65 % of maximal work capacity in either trained or untrained legs, but this might have been a consequence of the timing of the post-exercise biopsy as the increase in muscle LPL protein mass following exercise is delayed (Seip et al. 1997). Indeed Kiens et al. (1989) found that, although muscle LPL activity was not higher immediately after one hour of one-legged knee extension exercise at 75 % of maximal work capacity, LPL activity was 62 % higher in the exercised muscle four hours after exercise, compared with the non-exercised contralateral thigh, although this difference was not evident eight hours after exercise. Thus, there is considerable evidence suggesting that prolonged, intense exercise can markedly increase plasma post-heparin and skeletal muscle LPL activity. However, it is not clear from the literature whether LPL activity and/or TAG clearance are increased following exercise of a more moderate nature and further research is needed to determine whether the reduction in postprandial lipaemia evident after moderate exercise is, in fact, attributable to increased TAG clearance.

The fact that the attenuation in postprandial lipaemia seen following low and moderate intensity exercise is linked to the energy expended during exercise and independent of exercise intensity raises the suggestion that the attenuation in postprandial TAG concentrations seen after exercise might be mediated by energy deficit and not exercise *per se*. To the author's knowledge, no studies have evaluated the effect of an acute energy deficit, of the magnitude elicited by a single prolonged exercise session, on postprandial lipaemia, TAG clearance or LPL activity. However, data from Taskinen and Nikkilä (1979) have indicated that after two days on a 400 kcal diet skeletal muscle LPL activity rose by 24 %, although adipose tissue LPL activity fell by 62 % during this period. Consumption of a high-fat test meal on the

morning after a prolonged exercise session is, in effect, refeeding after an energy deficit. Thus, studies examining the effects of refeeding following energy intake restriction might provide clues to the mechanisms via which moderate exercise attenuates postprandial lipaemia. Taskinen and Nikkilä (1987) measured adipose tissue LPL activity in the fasted state and one hour after an 800 kcal (45 % carbohydrate, 35 % fat) lunch which was preceded by a 500 kcal breakfast, in pre-menopausal women. The postprandial increase in LPL activity was 21 % when subjects were previously fed an isocaloric diet but was 69 % after the subjects consumed a 400-kcal diet for 10 days. Fried et al. (1983) found that, in rats, postprandial epididymal adipose tissue LPL activity was ~70 % higher after rats were refed for 3 days following fasting, compared with the pre-fasting value. Similarly, Cruz and Williamson (1992) found that in meal-fed rats, who consumed 42 % of the food intake of rats fed *ad libitum*, postprandial LPL activity was almost twice as high in both epididymal and subcutaneous adipose tissue as that measured in their *ad libitum* fed litter mates. Thus, it seems possible that the reductions in postprandial lipaemia seen after an exercise session might be mediated by energy deficit and further investigation into this mechanism is warranted.

2.6 Summary

This chapter attempted to briefly review current knowledge of lipid and lipoprotein metabolism, particularly in the postprandial state, highlight the factors affecting this and examine methods for assessing 'TAG metabolic capacity'. Particular emphasis was placed on the effects of exercise on postprandial TAG metabolism. While the manner in which exercise, particularly moderate exercise, affects TAG metabolism is much better understood now than it was even five years ago, there are clear gaps in current knowledge. A detailed description of the changes to postprandial lipid and lipoprotein metabolism elicited by a moderate exercise session is lacking. Such information could help to further elucidate the mechanisms via which moderate exercise is attenuating postprandial lipaemia. Indeed, understanding of these mechanisms is limited at present. Although many authors have alluded that exercise can increase lipoprotein-TAG clearance, the effect of moderate exercise on this is unclear. In addition, it is not clear whether some of the 'exercise-induced' changes to TAG metabolism might, in fact, be mediated by energy deficit. Thus, further investigation is required to increase understanding of the manner in which moderate

exercise attenuates postprandial lipaemia. Such data would contribute to the scientific rationale for exercise guidelines, as well as aiding understanding of the transport and utilisation of TAG in man. In particular, studies should focus on subjects of middle-age and older as moderate intensity exercise is especially applicable for these age groups but data for these populations are lacking.

CHAPTER 3

GENERAL METHODS

This chapter is divided into two main parts. The first part describes the experimental procedures employed during the experimentation, many of which are common to several studies, including a correction that needed to be made to some expired air data. The second section reports on a study conducted to examine the reproducibility of responses to an oral fat tolerance test. All studies were conducted under the approval of Loughborough University Ethical Advisory Committee.

3.1 Subject recruitment

Subjects were recruited from within Loughborough University and the Loughborough area by local advertising. Volunteers were provided with written information about the study including possible risks and discomforts (an example of this information is given in Appendix A1) and were encouraged to ask questions and discuss the study before signing a statement of informed consent (Appendix A2). Exclusion criteria were employed to minimise risk to subjects and avoid confounding data. Subjects were only included in the studies if they:

- a) were free of known cardiovascular disease or abnormalities, acute illness or active, chronic systemic disease.
- b) had resting arterial blood pressure (mean of three readings) $< 160/95$ mm Hg.
- c) were not taking any medication known to influence carbohydrate or lipid metabolism.
- d) were not regular smokers
- e) had no coagulation disorders
- f) had no orthopaedic contra-indications to prolonged walking
- g) were not completely sedentary
- h) were amenorrhoeic for > 2 years following the menopause (chapter 4 only)
- i) had plasma total cholesterol concentration < 7.8 mmol.l⁻¹, fasting plasma TAG concentration < 2.3 mmol.l⁻¹; as measured in a blood sample at screening (chapters 5 and 6 only)
- j) were free of existing CHD as defined by examination of a 12-lead ECG obtained during a clinical exercise stress test (chapters 5 and 6 only).

At screening, volunteers completed confidential health history (Appendix A3) and physical activity questionnaires (Appendix A4) with the experimenter, had their arterial blood pressure measured and had a screening blood sample taken in the fasted state (chapters 5 and 6 only) to ensure they met the inclusion criteria for the study.

3.2 Clinical exercise stress test

The middle-aged men recruited for the studies described in chapters 5 and 6 all underwent a clinical exercise stress test with the Department of Cardiology (chapter 5) or the Department of Respiratory Medicine (chapter 6) at Glenfield hospital to ensure they were free from existing cardiovascular disease and had no cardiovascular contraindications to prolonged exercise. Twelve lead electrocardiographic (ECG) and arterial blood pressure monitoring was conducted at rest and during a Bruce exercise test protocol (Bruce et al. 1973). One volunteer was excluded from the study due to an abnormal exercise ECG and his case was referred to the Glenfield hospital Department of Cardiology.

3.3 Anthropometry

Anthropometric measurements were made in all the studies described in this thesis.

3.3.1 Height

Height was measured using a Holtain fixed-wall stadiometer (Seca, Germany). Subjects stood barefoot with their heels together against a metal back plate. The head was placed in the Frankfort Plane, with the line between the lower orbits of the eyes and the external auditory meatii perpendicular to the vertical board. Subjects were instructed to inhale deeply and a movable headboard was lowered onto the top of the head with enough pressure to compress the hair. The experimenter applied vertical traction under the mastoid process and height then was measured to the nearest 0.1 cm.

3.3.2 Body mass

Body mass was measured, to the nearest 0.05 kg, using a level platform beam balance (Avery Industrial Ltd., Leicester, UK), with subjects barefoot and wearing light clothing. This measurement was conducted for anthropometric calculations (body

mass index (BMI), fat free body mass) and prior to each exercise test for calculation of oxygen uptake per kg of body mass.

3.3.3 Skinfold thickness

Measurements of skinfold thicknesses were made in private, by a same sex experimenter, using Holtain Tanner/Whitehouse skinfold calipers (Holtain Ltd., Crymych, UK). Skinfolds were measured at the following sites on the left side of the body with the subject standing:

- a) Biceps – With the elbow flexed at an angle of 90° , the mid point between the lateral projection of the acromial process and the inferior margin of the olecranon process was marked. The arm was allowed to hang loosely with the palm facing forward and the skinfold measurement was made at the level of the mark over the belly of the biceps muscle above the centre of the cubital fossa.
- b) Triceps – This measurement was at the same level as the biceps measurement on the midline of the posterior aspect of the arm.
- c) Subscapular – This measurement was made along the natural cleavage line of the skin just inferior to the inferior angle of the scapula with the subject's arms hanging loosely by the sides of the body.
- d) Suprailiac – This skinfold was measured vertically in the mid-axillary line, half way between the costal margin and the superior iliac crest.

Each skinfold was lifted between the thumb and index finger of the experimenter's left hand, 1 cm above the site of measurement. The skinfold calipers were applied and the measurement was taken after 5-8 seconds of caliper pressure. The sum of skinfold measurements was used to estimate body fatness and fat-free body mass using the predictive equations derived by Durnin and Womersley (1974).

3.3.4 Waist and hip circumferences

Waist and hip circumferences were measured using a flexible, inelastic Fibron tape measure (CMS Weighing Equipment, London, UK). The waist circumference was measured in the horizontal plane midway between the costal margin and iliac crest with the abdominal muscles relaxed and the subject breathing shallowly. The hip measurement was made horizontally around the maximum circumference of the buttocks. For both measurements subjects stood with their feet together and were

dressed in underclothes. As for skinfold measurements, these circumferences were measured in private by a same sex experimenter.

3.4 Measurement of oxygen uptake and carbon dioxide production

Oxygen uptake and carbon dioxide production were determined during exercise in chapters 4, 5 and 6, at rest before and after exercise in chapter 4 and at rest in the fasted and postprandial states in chapter 5.

Samples of expired air were collected into 50 litre (for resting samples and exercise samples when volumes of expired air were low) or 150 litre (for large volume samples) Douglas bags (Plysu protection Systems, Milton Keynes, UK). Subjects, whilst wearing a nose clip, breathed through a mouthpiece (Harvard Apparatus, Edenbridge, UK) fitted to a lightweight one-way respiratory valve (Jakeman and Davies, 1979), which in turn was connected to a 1.5 metre length of 30 mm bore lightweight tubing (Falconia flexible ducting, Baxter, Woodhouse and Taylor Ltd., Macclesfield, UK). The tubing terminated at a two-way valve which opened and closed the Douglas bag.

An aliquot of expired air (measured using a flow meter) was removed from each Douglas bag to determine the fraction of oxygen in each sample using a paramagnetic oxygen analyser (chapter 4: Taylor-Servomex, Model 570A, Crowborough, UK, chapters 5 and 6: Servomex, Series 1400, Crowborough, UK). The fraction of carbon dioxide was similarly determined using an infra-red carbon dioxide analyser (chapter 4: Lira, Model 3250, Mines Safety Appliances Ltd., chapters 5 and 6: Servomex, Series 1400, Crowborough, UK). The analysers were calibrated before each use with certified reference gases (Cryoservice Ltd., Worcester, UK or Air Products, Crewe, UK). The reference gases were calibrated against a 'gold standard' reference gas to ensure consistency of results.

The remaining volume of expired air in each Douglas bag was measured by evacuation through a dry gas meter (Harvard Apparatus, Edenbridge, UK). The dry gas meter had been previously calibrated against a 600-litre Tissot spirometer (Warren E Collins Inc., Massachusetts, USA). The temperature of air in the Douglas

bag was measured during evacuation using a thermister (Edale, type 2984, Model C, Cambridge, UK) placed in the air outlet tubing of the dry gas meter.

Barometric pressure was measured using a Fortin barometer (F.D. and Company, Watford, UK) and the measured expired gas volumes were corrected to standard temperature and pressure for a dry gas (STPD) using the universal gas equation. Inspired gas volumes were derived using the Haldane transformation (Consolazio et al. 1963) and oxygen uptake, carbon dioxide production, minute ventilation, respiratory exchange ratio and the ventilatory equivalent for oxygen were calculated. Rates of substrate utilisation expenditures were calculated via indirect calorimetry using the equations described by Frayn (1983) and energy expenditures were determined by multiplying the mass of substrates used by their respective energy densities.

3.4.1 Correction of $\dot{V}O_2$ and $\dot{V}CO_2$ measurements

3.4.1.1 Discovery of the problem

Towards the end of data collection for the study described in chapter 5, it became apparent that resting $\dot{V}O_2$ and $\dot{V}CO_2$ values were lower than expected, and that respiratory exchange ratios seemed too high for measurements obtained at rest. However, $\dot{V}O_2$ and $\dot{V}CO_2$ values obtained during exercise appeared to be normal, as did the expired air volumes at rest and during exercise. As the oxygen and carbon dioxide analysers were calibrated prior to each measurement and the volumes and temperatures of expired air were measured through a calibrated dry gas meter and a calibrated thermistor, it was unlikely that large errors would have been introduced at these stages. Indeed, if errors occurred at any of the above stages, obvious errors in expired air volumes and in exercise $\dot{V}O_2$ and $\dot{V}CO_2$ values would have been seen, and these did not occur. Instead, the low values for the resting $\dot{V}O_2$ and $\dot{V}CO_2$ values appeared to be caused by unexpectedly high and low values for F_{EO_2} and F_{ECO_2} , respectively.

The pieces of Falconia tubing used to transport the expired air sample from the mouthpiece to the Douglas bag in chapters 4 and 5 were rather old and it was possible that their 'air-proofing' coating had decayed, rendering the tubing permeable to oxygen and carbon dioxide, resulting in diffusion of oxygen into and carbon dioxide

out of the tubing. This would account for the errors in $\dot{V}O_2$ and $\dot{V}CO_2$ being greater at rest (i.e. low flow rates) than during exercise (i.e. high flow rates) as transit times for air in the tube are higher at low flow rates allowing more time for diffusion to occur. This would also explain the elevated respiratory exchange ratios, as O_2 molecules are smaller and lighter than CO_2 molecules so would diffuse through the tubing at a faster rate, resulting in a greater underestimation in $\dot{V}O_2$ than $\dot{V}CO_2$.

New pieces of Falconia tubing were obtained and expired air measurements were made at rest and during low and high intensity exercise with new and old pieces of tubing. Volumes of expired air obtained using the two tubes were similar and the differences between F_{EO_2} and F_{ECO_2} values obtained using the old and new tubing were greatest at rest, with the differences reducing as the flow rate increased with increasing exercise intensity. In addition, respiratory exchange ratios were higher with the old tubing. This confirmed the suspicion that the errors observed in the expired air data were due to gases diffusing through the Falconia tubing.

Once the nature of the problem was identified, an attempt retrospectively to correct the expired air data was made. Initially an attempt to solve the problem theoretically, determining permeability constant for the Falconia tubing, was considered but would have been difficult to implement in practice, thus this was discounted in favour of an empirical approach.

3.4.1.2 Methods for quantifying the error

After a number of attempts, an approach using a ventilator was adopted to quantify, and correct for, the errors incurred from the use of the old Falconia tubing. A schematic diagram outlining the apparatus used is shown in Figure 3.1, below. Douglas bag 1 was filled with a reference gas containing between 15 % and 20.5 % O_2 and 0.5 to 5 % CO_2 , providing gas mixtures within the physiological range for expired air composition. This gas was then pumped into the ventilator (Engström Respirator System 300, LKB Medical AB, London, UK), at a rate of 18 breaths per minute (the lowest breath rate available) via impermeable plastic Harvard tubing (Harvard Apparatus, Edenbridge, UK) and then out of the ventilator either through the Harvard tubing or a piece of Falconia tubing into Douglas bag 2. Before Douglas bag 2 was opened to receive the gas from the ventilator, the gas was allowed to flow for

between 1 and 2 minutes (depending on flow rate) to flush atmospheric air from the system. The gas was then collected into Douglas bag 2 for between 1½ and 6 minutes (depending on flow rate) and the composition and volume of this gas (under STPD conditions) was then determined. The flow rate was calculated by dividing the volume by the collection time. This procedure was conducted for both the Harvard and Falconia tubing in duplicate for a range of flow rates and gas compositions. The $F_{E}O_2$ and $F_{E}CO_2$ values obtained via the Harvard and Falconia tubings were compared to assess the error incurred in using the Falconia tubing. Two different pieces of old Falconia tubing were used in data collection. This procedure was carried out for both pieces as the permeability of the two tubes may have differed.

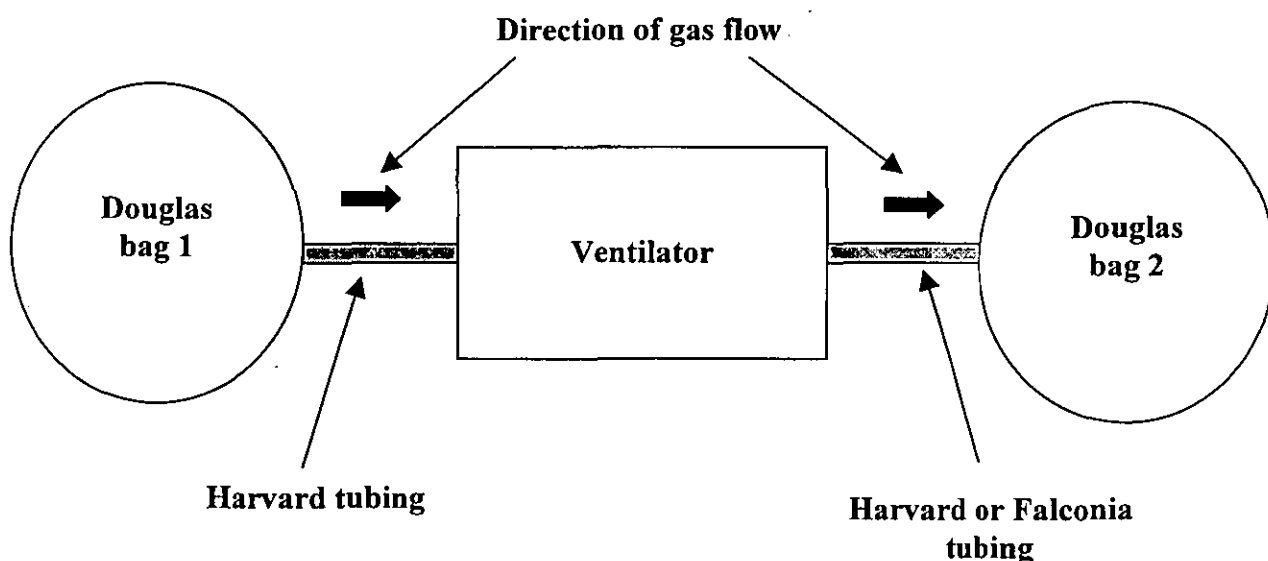


Figure 3.1 Schematic representation of apparatus used to determine permeability of Falconia tubing. Douglas bag 1 contained a reference gas. Composition and volume of gas in Douglas bag 2 were measured to determine the permeability of the Falconia tubing.

3.4.1.3 Quantification of the error and data correction

The $F_{E}O_2$ and $F_{E}CO_2$ values obtained from gases of different compositions passing through the Harvard tubing and Falconia tubing at various flow rates are shown in Tables 3.1 and 3.2 for the two pieces of Falconia tubing used. These flow rates ($\sim 5 \text{ l.min}^{-1}$ to $\sim 20 \text{ l.min}^{-1}$) covered the range achievable by the ventilator. The volumes of gas collected into the Douglas bags did not differ between the Harvard tubing and the

Falconia tubing, indicating that air was not leaking out through the latter. When a new piece of Falconia tubing was tested, the $F_{E}O_2$ and $F_{E}CO_2$ values did not differ from the Harvard tubing indicating that the new tubing was not permeable to oxygen and carbon dioxide.

Flow rate (l.min ⁻¹)	Harvard tubing		Falconia tubing (Tube 1)	
	$F_{E}O_2$ %	$F_{E}CO_2$ %	$F_{E}O_2$ %	$F_{E}CO_2$ %
4.5	16.33	3.27	19.15	1.49
4.5	16.90	2.89	19.35	1.31
4.5	17.55	2.43	19.63	1.10
9.3	16.20	3.73	18.10	2.45
9.3	17.00	3.10	18.65	1.99
9.3	18.00	2.02	19.20	1.31
18.7	16.20	3.84	17.30	3.13
18.7	16.50	3.46	17.53	2.83
18.7	18.30	2.07	18.95	1.70

Table 3.1 $F_{E}O_2$ and $F_{E}CO_2$ of different gas compositions after passing through Harvard and Falconia tubing for Falconia tube 1. Value in each cell is the mean of duplicate values.

Flow rate (l.min ⁻¹)	Haldane tubing		Falconia tubing (Tube 2)	
	F _E O ₂	F _E CO ₂	F _E O ₂	F _E CO ₂
4.6	16.10	3.75	18.85	1.86
4.6	17.55	2.63	19.50	1.28
9.4	16.13	3.75	17.75	2.69
9.4	17.50	2.76	18.70	1.92
20.0	16.05	3.90	17.00	3.25
20.0	17.70	2.58	18.30	2.18

Table 3.2 F_EO₂ and F_ECO₂ of different gas compositions after passing through Harvard and Falconia tubing for Falconia tube 2. Value in each cell is the mean of duplicate values.

These data were then used to establish a method for correcting the expired air data collected via the old Falconia tubing. The gas compositions measured after the gas had passed through the Harvard tubing were taken to represent the gas composition that entered the Falconia tubing. The correction method is described below using the data collected from Falconia tube 1. The same procedure was conducted for Falconia tube 2 but, as the data for Falconia tube 2 were collected after the correction procedure was established, the correction could be made using fewer observation points.

Plots of the difference between input and output concentrations against the input concentration for a given flow rate revealed a linear relationship for both oxygen (Figure 3.2) and carbon dioxide (Figure 3.3). This suggested that the rates of diffusion of oxygen and carbon dioxide molecules were directly proportional to the diffusion gradients, in accordance with Fick's law of diffusion. This was confirmed by including the points where no diffusion would be expected (i.e. (20.93, 0) for oxygen and (0.03, 0) for carbon dioxide) on the graphs and showing that these points fit the linear relationship.

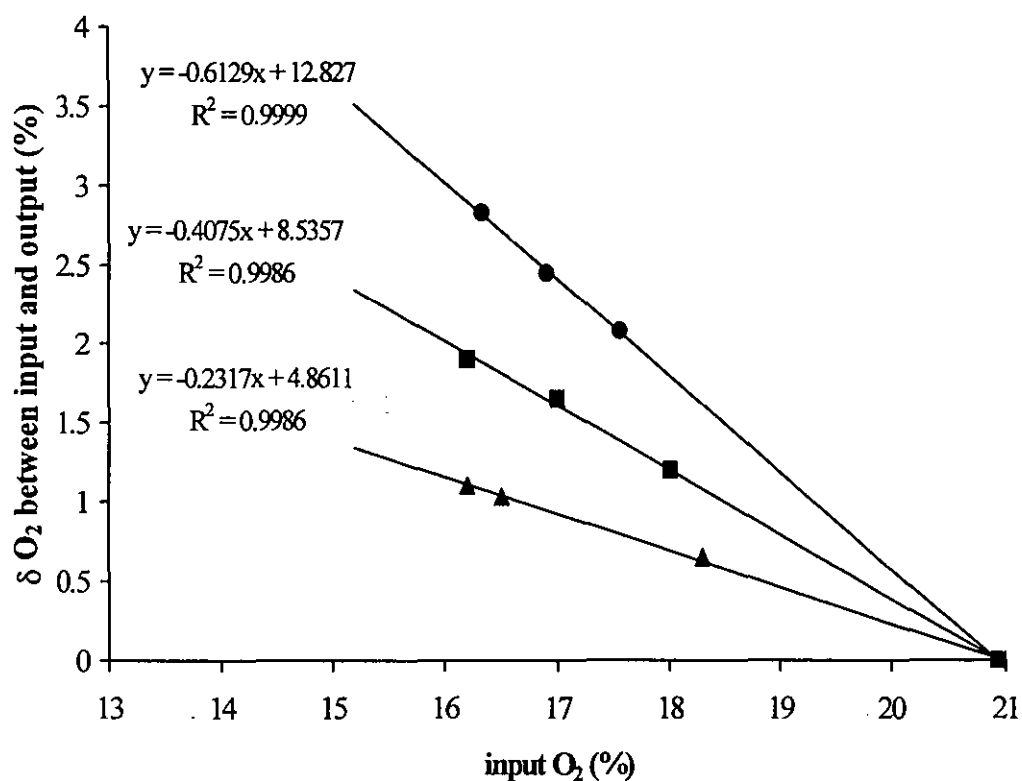


Figure 3.2 Difference in oxygen concentration between gas entering and leaving the Falconia tubing against the oxygen concentration of the gas entering the tube at flow rates of 4.5 l.min⁻¹ (circles), 9.3 l.min⁻¹ (squares) and 18.7 l.min⁻¹ (triangles) with equations of lines and R^2 values displayed.

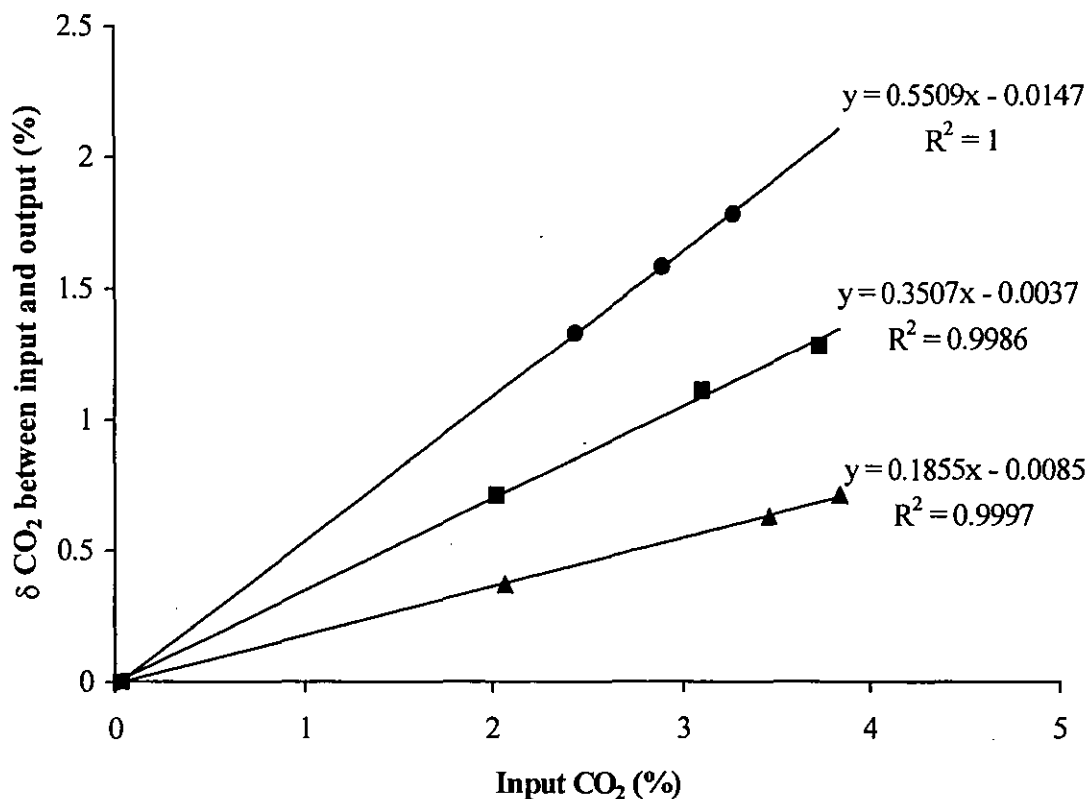


Figure 3.3 Difference in carbon dioxide concentration between gas entering and leaving the Falconia tubing against the carbon concentration of the gas entering the tube at flow rates of 4.5 l.min^{-1} (circles), 9.3 l.min^{-1} (squares) and 18.7 l.min^{-1} (triangles) with equations of lines and R^2 values displayed.

Once it was established that the error caused by passing the gas through the Falconia tubing was proportional to the diffusion gradient, calculating the input gas concentration when the output concentration was known became straightforward, for a measured flow rate, as this relationship would also be linear. Table 3.3 shows the equations for the relationships between input and output concentrations at the three measured flow rates for oxygen and carbon dioxide. From these equations it was possible to generate a spreadsheet calculating input oxygen and carbon dioxide productions across the range of output values for the three measured flow rates.

Flow rate (l.min ⁻¹)	Gas	Equation of line	R ²
4.5	O ₂	$y = 2.5743x - 32.953$	0.9998
4.5	CO ₂	$y = 2.2265x - 0.0325$	0.9999
9.3	O ₂	$y = 1.6866x - 14.385$	0.9993
9.3	CO ₂	$y = 1.5396x - 0.0047$	0.9996
18.7	O ₂	$y = 1.3014x - 6.3236$	0.9999
18.7	CO ₂	$y = 1.2278x - 0.0104$	1.0000

Table 3.3 The equation of the lines and R² values for relationships between oxygen and carbon dioxide concentrations of gas entering and leaving the Falconia tubing at flow rates of 4.5 l.min⁻¹, 9.3 l.min⁻¹ and 18.7 l.min⁻¹, where x is the output gas concentration, y is the input gas concentration.

Next, input percentages of oxygen and carbon dioxide, for flow rates not directly measured, were determined. Plots of the difference between input and output concentrations (determined from the spreadsheet generated from the equations in Table 3.3) against flow rate for a series of different output concentrations were made. These data fit curves of the form $y = ax^{-b}$ with R² values > 0.99 (see Figures 3.4 and 3.5 for examples of the curves for oxygen and carbon dioxide, respectively). However, it was possible that strong relationships could be generated between *any* three points. If this were the case then the equations generated would have little meaning and it would have been necessary to collect more data at different flow rates to establish the relationships. This possibility was discounted by the fact that changing any one of the three values weakened the relationship, reducing the R² value. Thus, the curve was adequately described from the three points used.

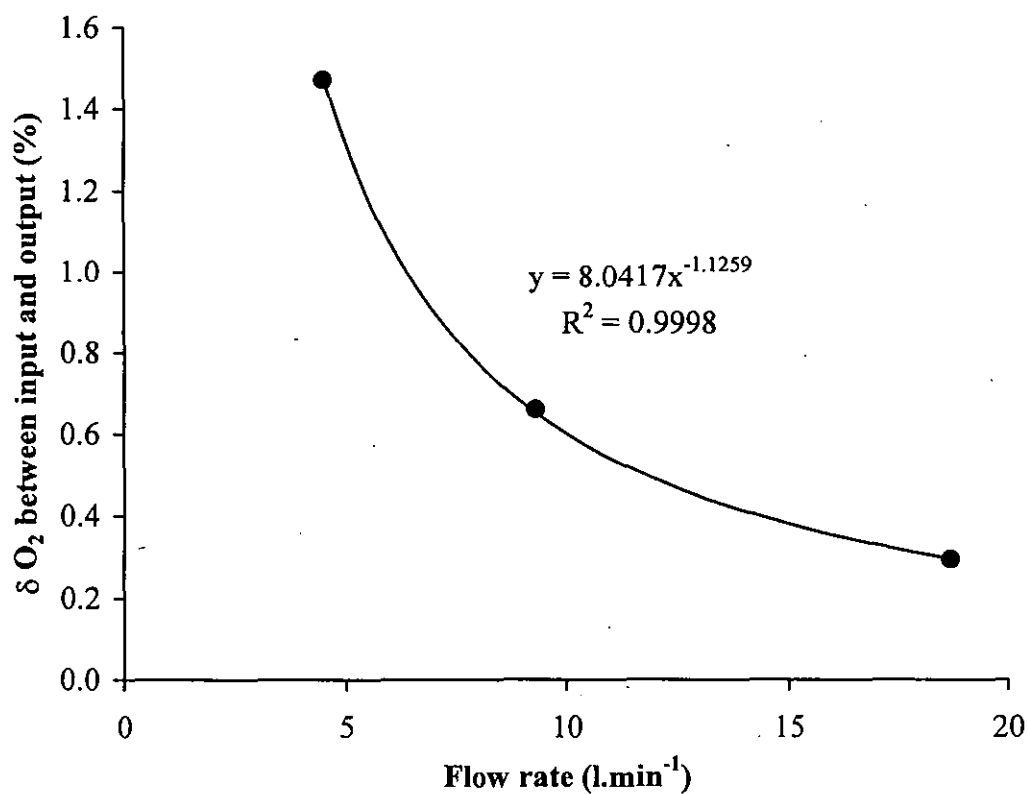


Figure 3.4 Difference in oxygen concentration between gas entering and leaving the Falconia tubing against the flow rate at an output oxygen concentration of 20.0 %, with the equation of the line and R^2 value displayed.

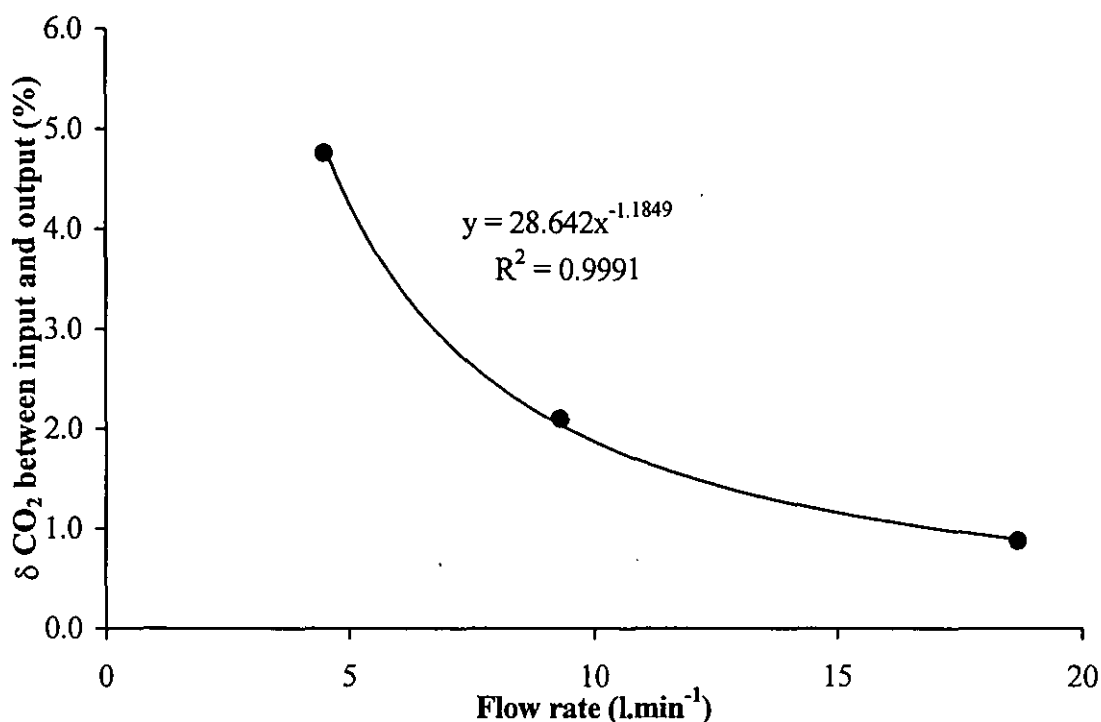


Figure 3.5 Difference in carbon dioxide concentration between gas entering and leaving the Falconia tubing against the flow rate at an output carbon dioxide concentration of 3.9 %, with the equation of the line and R^2 value displayed.

The equations from these curves were used to generate a spreadsheet so that, given a flow rate and output oxygen and carbon dioxide concentrations, input oxygen and carbon dioxide concentrations could be calculated. These equations were then validated by collecting more data at flow rates and gas compositions not directly measured and comparing the results obtained with those predicted from the spreadsheet equations (Table 3.4). The validation of the correction spreadsheet showed that, on the whole, the precision of the correction was within ± 0.1 % across the range of flow rates seen at rest – this was considered to be acceptable.

Flow rate l.min ⁻¹	Input oxygen (%)		Input carbon dioxide (%)	
	Predicted	Actual	Predicted	Actual
4.88	18.7	18.6	1.88	1.85
4.89	16.2	16.2	3.82	3.86
6.79	16.1	16.1	3.86	3.96
7.06	18.5	18.4	1.94	1.98
8.45	16.1	16.1	3.88	4.00
8.59	18.3	18.3	2.08	2.21
9.75	16.1	16.1	3.93	3.97
9.80	18.1	18.2	2.23	2.21
10.44	17.75	17.8	2.58	2.56
11.38	16.1	16.2	3.95	3.88
11.95	15.9	16.1	4.04	3.98
12.28	18.55	18.6	1.89	1.88
12.45	16.35	16.4	3.73	3.64
13.73	16.7	16.8	3.40	3.35

Table 3.4 Validation of the spreadsheet to predict input oxygen and carbon dioxide concentrations by comparing actual input values with those predicted from the spreadsheet.

Once the correction spreadsheets were completed for both pieces of Falconia tubing, it was possible to correct the $F_{E}O_2$ and $F_{E}CO_2$ values obtained in the studies described in chapters 4 and 5 from the uncorrected $F_{E}O_2$ and $F_{E}CO_2$ values and the \dot{V}_{ESTPD} value and therefore correct the $\dot{V}O_2$ and $\dot{V}CO_2$ data.

3.4.1.4 Limitations to the correction

While it was possible to correct the error in $F_{E}O_2$ and $F_{E}CO_2$ seen after the gas passed through permeable Falconia tubing with an acceptable degree of precision, there are limitations to the data. It was not possible to take into account variations in breath rate in the correction, which might have influenced the results. For the data collection the ventilator was set to a breath rate of 18 breaths per minute, which although higher than the usual resting breath rate, was its lowest setting. A small experiment in which

the gas was pumped through the Falconia tubing at 18, 24, and 30 breaths per minute at a given flow rate resulted in a variation in $F_{E}O_2$ and $F_{E}CO_2$ of $\sim 0.1\%$, suggesting that the extra error incurred by not accounting for breath rate would have been small. Another potential source of error was the fact that the gases used in this correction experiment were dry and expired air is saturated with water vapour. However, this error would have been systematic, so while it might affect the absolute values for $F_{E}O_2$ and $F_{E}CO_2$, the data values would be correct relative to each other. The correction factors necessary for the two pieces of Falconia tubing differed slightly, which confounded the corrections when it was not known which tube was used. Implications of this and other specific factors arising from the correction of the expired air data are discussed in the chapters in which the corrections were used.

3.5 Monitoring of heart Rate

Heart rate was monitored continuously during exercise and the recovery period by either modified Lead I electrocardiography (Rigel Cardiac Monitor 302, Rigel Research UK, Ltd, Morden, UK) (chapter 4), or by short range telemetry (Polar PE 3000 Sport Tester or Polar Favor, Polar Electroky, Kempele, Finland) (chapters 5 and 6).

3.6 Exercise tests

Several exercise tests were conducted in the studies described in this thesis. All involved walking on a calibrated motorised treadmill (Quinton Q65 Series 90, Quinton Instrument Company, Seattle USA). All subjects were familiarised with treadmill walking prior to testing. During the familiarisation session, a comfortable brisk walking speed was established for each subject (Range of walking speeds: chapter 4; 1.34 to $1.61\text{ m}\cdot\text{s}^{-1}$, chapter 5; 1.56 to $2.01\text{ m}\cdot\text{s}^{-1}$, chapter 6; 1.56 to $2.01\text{ m}\cdot\text{s}^{-1}$) and this speed was employed in all subsequent testing.

3.6.1 Submaximal exercise testing

In the studies described in chapters 4, 5 and 6 subjects undertook a four-stage submaximal exercise test to determine the relationship between increasing treadmill gradient and $\dot{V}O_2$ at their self-selected walking speed. In chapter 4 – where directly measured $\dot{V}O_2$ max values were not obtained – this test was also used to estimate $\dot{V}O_2$ max by extrapolation of the oxygen uptake/heart rate relationship up to the subject's

predicted maximum heart rate (Maritz et al. 1961) (predicted maximum heart rate: mean of $[220 - \text{age}] \text{ beat} \cdot \text{min}^{-1}$ and $[205 - 0.5 \times \text{age}] \text{ beat} \cdot \text{min}^{-1}$). In chapters 5 and 6, in which the subjects were male, each stage was four minutes long with expired air samples being taken during the last minute of each stage. In chapter 4, the subjects were female and their ventilation rates were relatively low. Therefore, a two-minute expired air collection was taken at the end of each stage increasing the stage duration to five minutes. Heart rates were recorded during the expired air collections as were perceived rates of exertion using the Borg scale (Borg, 1973). The first stage of each test was on a level treadmill with gradient increasing by 1.5 % to 3.0 % in each subsequent stage. The increase in gradient for each stage was established on an individual basis based on the subject's heart rate response in the familiarisation session. Each individual's relationship between $\dot{V}\text{O}_2$ and treadmill gradient was determined and, together with the estimated (chapter 5) or measured $\dot{V}\text{O}_2 \text{ max}$ (chapters 5 and 6, see section 3.6.2), the gradient necessary to elicit an intensity corresponding to 60% $\dot{V}\text{O}_2 \text{ max}$ was calculated.

3.6.2 Maximal exercise testing

In chapters 5 and 6, where subjects had undertaken a clinical exercise stress test to ensure no contraindications to maximal exercise, $\dot{V}\text{O}_2 \text{ max}$ was directly determined using a modified version of the protocol devised by Taylor et al. (1955). This involved an incremental walking test divided into three-minute stages all at the subject's self-selected walking speed. The starting gradient (range 4.0 % to 8.0 %) and the increase in gradient with each stage (range 3.0 % to 4.0 %) were determined individually, based on each subject's ability, to elicit a test duration of between seven and twelve minutes. An expired air collection was taken from 1:45 to 2:45 of each stage and during these collections heart rate and perceived rate of exertion were recorded. The test was open-ended and concluded with a final expired air collection when the subject signalled that they could only manage one more minute. The highest $\dot{V}\text{O}_2$ achieved during the test (usually the final collection) was accepted to be the subject's $\dot{V}\text{O}_2 \text{ max}$. On one occasion, where the author felt that the subject had not achieved a true maximum, the test was repeated at a later date.

3.6.3 Prolonged treadmill walking

On one occasion in chapter 4 and 5, and on two occasions in chapter 6, subjects performed a 90-minute walk on the treadmill on the day prior to a fat tolerance test. In chapter 4, an additional 90-minute walk was conducted on a prior occasion to determine the net energy expenditure of the walk. These prolonged walks were carried out at an intensity of about 60 % $\dot{V}O_2$ max. Two-minute expired air samples were obtained starting 13, 28, 58 and 88 minutes into the walk in chapter 4 and one-minute expired air samples were obtained every 15 minutes in chapters 5 and 6. Perceived rates of exertion and heart rates were recorded during each expired air collection. Water was provided *ad libitum* during and in the recovery from these walks.

3.7 Fat tolerance tests

Two types of fat tolerance test were carried out in the studies described in this thesis. In all the experimental studies, as well as the reproducibility study described in section 3.11 of this thesis, subjects undertook at least two oral fat tolerance tests and in chapter 6 subjects also underwent two intravenous fat tolerance tests.

3.7.1 Oral fat tolerance tests

Subjects reported to the laboratory after an overnight fast of at least twelve hours, having travelled by car to ensure that they were in a rested state. A cannula was introduced into an antecubital or forearm vein under local anaesthesia and then subjects were allowed to rest on a couch for ten minutes before any measurements were made. A blood sample was obtained in the fasted state and then subjects ingested a high fat test meal (see section 3.7.1.1). Further blood samples were obtained 15 (chapters 5 and 6 only), 30, 45 (chapters 5 and 6 only), 60, 90 (chapters 5 and 6 only), 120, 180, 240, 300, 360 and 480 (chapter 5 only) minutes after completion of the test meal. All samples were obtained with the subjects seated or lying on a couch and the cannula was kept patent by flushing with non-heparinised saline solution (0.9%). Subjects rested or performed sedentary activities (watching television, reading, listening to music etc) during the six or eight-hour postprandial observation period and no food or drink other than water was consumed during this time. Water intake was *ad libitum* for the first trial, this intake was recorded and replicated in subsequent trials.

3.7.1.1 The high-fat test meals

The test meals employed in the studies in this thesis were all modifications of the meal described by Schlierf et al. (1987) and were administered according to fat-free body mass (reproducibility study and chapter 4) or body mass (chapters 5 and 6). A study to determine the reproducibility of lipaemic, insulinaemic and glycaemic responses to this test meal was conducted and is reported in section 3.11 of this thesis. For the reproducibility study, chapter 4 and chapter 6 the meal comprised cereal, fruit, nuts, chocolate and whipping cream, the nutritional information and composition being shown in Tables 3.5 and Table 3.6 respectively.

In chapter 5 the test meal comprised two parts; a heated lipid-casein-glucose-sucrose emulsion containing [1, 1, 1-¹³C] tri-palmitin to assess the metabolism of exogenous lipid, and a modification of the cereal based meal used in the other studies. The ¹³C label was administered in a heated emulsion to maximise its absorption into the body – this is greater than 98 % when the label is ingested in this form (Bennoson et al. 1999). The emulsion was prepared by weighing the [1, 1, 1-¹³C] tri-palmitin powder into a beaker using a high precision electronic balance (Mettler Toledo, model AG245, Switzerland), adding whipping cream (see Table 3.7a for quantities), then heating in a water bath at 85 °C until the label melted. Glucose and beet sugar were dissolved in 60 g of mineral water and casein was mixed into this solution using a hand blender (Braun). The sugar/casein mix was then added to the heated lipids and mixed continuously for five minutes with the beaker immersed in a bowl of hot water. Drinking chocolate powder (Nesquick, Nestlé, York, UK) was then mixed into the emulsion. The blender was rinsed into the beaker with 40 g of heated mineral water and the emulsion was consumed immediately. After consumption of the emulsion, the beaker was rinsed with 50 g of heated mineral water and this water was consumed. The proportions of the cereal portion of the test meal were altered in this study to make the macronutrient composition of the complete test meal similar to meals ingested in the other studies. Tables 3.7a and 3.7b give the composition of the emulsion and cereal portions of the test meal and Table 3.5 the nutritional information for the complete test meal.

In the reproducibility study, chapter 4 and chapter 5, a fairly large fat load was administered in the test meal in order to provide a large to stress lipoprotein metabolism. This, it was hoped, would increase the sensitivity of the test by maximising potential differences between the control and intervention conditions. The meal size was reduced in chapter 6 in an attempt to make the fat load more applicable to daily living. In chapters 5 and 6, 1.5 g paracetamol was ingested with the test meal as a marker of gastric emptying (Heading et al. 1973; Clements et al. 1978).

	Reproducibility study and chapter 4 (per kg fat-free mass)	Chapter 5 (per kg body mass)	Chapter 6 (per kg body mass)
E (kJ)	98.9	79.9	58.2
Fat (g)	1.70	1.32	1.00
CHO (g)	1.65	1.36	0.97
Pro (g)	0.25	0.30	0.15
E from fat (%)	67.4	64.7	67.4
E from CHO (%)	28.3	28.9	28.3
E from pro (%)	4.3	6.4	4.3

Table 3.5 Macronutrient composition of the test meal in the reproducibility study and chapters 4, 5 and 6. (E = energy, CHO = carbohydrate, pro = protein)

Ingredient	Reproducibility study and chapter 4 (g per kg fat-free mass)	Chapter 6 (g per kg body mass)
Whipping cream	3.59	2.11
Apple	0.95	0.56
Banana	1.59	0.94
Milk chocolate	0.19	0.11
Sultanas	0.19	0.11
Brazil nuts	0.21	0.12
Oats	1.06	0.625
Desiccated coconut	0.10	0.06

Table 3.6 Quantities of ingredients comprising the test meal, administered per kg fat-free body mass or per kg body mass for the reproducibility study, chapter 4 and chapter 6.

Emulsion ingredient	Chapter 5 (g per kg body mass)
[1, 1, 1- ¹³ C] tri-palmitin	0.010
Whipping cream	0.429
Casein	0.171
Glucose	0.129
Beet sugar	0.064
Nesquick	0.143

Table 3.7a Emulsion ingredients for the test meal in chapter 5, prescribed per kg body mass.

Cereal ingredient	Chapter 5 (g per kg body mass)
Whipping cream	2.27
Apple	0.71
Banana	1.20
Milk chocolate	0.14
Sultanas	0.14
Brazil nuts	0.16
Oats	0.57
Desiccated coconut	0.07

Table 3.7b Cereal ingredients for the test meal in chapter 5, prescribed per kg body mass.

3.7.2 Intravenous fat tolerance tests

As in the oral fat tolerance tests subjects reported to the laboratory after an overnight fast of at least twelve hours, having travelled by car to ensure that they were in a rested state. A cannula was introduced into an antecubital vein under local anaesthesia and subjects rested on a couch for ten minutes before any measurements were made. Two baseline blood samples were obtained at an interval of 10 minutes. A bolus dose of 20 % Intralipid (0.5 ml (0.1 g fat) per kg body mass) was then

injected over a 2 minute period through the cannula extension. After flushing the cannula with 0.9 % saline, the cannula extension was changed to avoid contamination of blood samples with Intralipid which had adhered to the walls of the extension. (A pilot study (described in section 6.2) indicated that it was acceptable to sample blood from the Intralipid injection site if the cannula extension was changed prior to blood sampling.) Blood samples were then obtained 2.5, 5, 7.5, 10, 15, 20, 25, 30, 40 and 60 minutes after completion of the Intralipid injection. During this period subjects lay on a couch and consumed no food or drink.

3.7.3 Preparation for the fat tolerance tests

Preceding each fat tolerance subjects' diets were standardised. In the reproducibility study, chapter 5 and chapter 6 subjects weighed and recorded their dietary intake for two days prior to their first fat tolerance test, using electronic kitchen scales and dietary record sheets, and replicated this diet prior to subsequent trials. In chapter 4, in which a dietary intervention was employed, subjects consumed a diet provided by the experimenters on the day prior to each fat tolerance test. In this study, subjects weighed and recorded their dietary intake on the day prior to the first intervention and replicated this for subsequent trials. In chapter 5, subjects avoided foods naturally enriched with ^{13}C (i.e. any corn products (e.g. cornflakes, sweetcorn, popcorn, cornchips etc.), cane sugar and prepared or processed foods containing cane sugar (e.g. breakfast cereals, cakes, biscuits), tropical fruits (e.g. mango and pineapple), sports drinks (e.g. Gatorade, Lucozade Sport, Isostar) for three days before each fat tolerance test. In all studies, subjects refrained exercise for the three days preceding each fat tolerance test, except for the planned treadmill walking in the exercise trials.

Diets were analysed using a computerised version (Comp-Eat 4 or Comp-Eat 5, Nutrition Systems, London, UK) of food composition tables (Holland et al. 1991).

3.8 Blood sampling

Venous blood samples were obtained via an indwelling cannula (Venflon 2, 18G/45 mm: BOC Ohmeda AB, Helsingborg, Sweden) placed in an antecubital or forearm vein or occasionally by venopuncture of an antecubital vein. For cannulation, subjects lay on an examination couch and a small amount of local anaesthetic (1 % w/v lignocaine hydrochloride; Antigen Pharmaceuticals Ltd., Roscrea, Ireland) was

injected subcutaneously at the cannula's site of injection. The cannula was then introduced and a 10 cm three-way stopcock (Connecta, BOC Ohmeda AB, Helsingborg, Sweden) was attached for blood sampling. The cannula was kept patent by flushing with non-heparinised saline solution (0.9 %; B. Braun Medical Ltd, Buckinghamshire, UK). All blood samples were obtained with subjects in the same posture (either sitting or lying on a couch) as shifts in plasma volume may occur with changes in posture (Rowell, 1993). Blood samples were collected directly into Monovettes (Sarstedt Ltd, Leicester, UK) via a 'multi-adaptor' (Sarstedt Ltd, Leicester, UK) or into syringes (Plastipak, Becton Dickinson, Oxford, UK) and dispensed into blood collection tubes (Sarstedt Ltd, Leicester, UK). Different types of blood collection tubes and Monovettes were used depending on the subsequent analysis. Serum Monovettes containing no anticoagulant were used to collect samples for analysis for insulin and 3-hydroxybutyrate. Samples to be analysed for glucagon were collected into pre-chilled potassium ethylenediamine tetra-acetic acid (EDTA) coated blood collection tubes with 50µl per ml blood Trasylol added and kept chilled until centrifugation. All other blood samples were collected into pre-chilled EDTA Monovettes or blood collection tubes which were kept chilled until centrifugation. In each study small aliquots of blood were sampled from the EDTA tube for determination of haemoglobin and haematocrit (see section 3.9.1).

Blood collected into EDTA Monovettes was centrifuged (Koolspin; Burkard Scientific Ltd, Uxbridge, UK) within 15 minutes of collection for 15 minutes at 6000 rpm and 4°C. Blood collected into serum Monovettes was allowed to clot at room temperature for 60 minutes, before centrifugation for 15 minutes at 6000 rpm and 4°C. Plasma and serum was pooled and mixed where necessary and the majority was pipetted into aliquots of not less than 0.5 ml (to minimise any freeze drying effect) and stored at -20°C (EDTA plasma) or -70°C (serum) for later analysis. In study 2, 3.6 ml aliquots of EDTA plasma were dispensed into tubes containing 90 µl of a preservative cocktail (44 g EDTA, 2.93 g chloramphenicol, 3.67 g sodium azide, 2.93 g gentamicin sulphate, 366 670 units Trasylol per litre) and refrigerated for lipoprotein fractionation on the following day.

3.9 Analysis of blood samples

These analyses were conducted in the Biochemistry Laboratory in the Department of Physical Education, Sports Science and Recreation Management unless otherwise stated. Full details of the blood biochemistry are provided in the appendices, but a brief overview is provided in this section.

3.9.1 Estimation of changes in plasma volume

Haemoglobin concentration (Appendix B9) was determined spectrophotometrically (Digital Grating Spectrophotometer Series 2, Model CE393; Cecil Instruments Ltd, Cambridge, UK) in duplicate aliquots (20 μ l) of EDTA blood by a cyanmethemoglobin method using a commercially available kit (Test-Combination Haemoglobin, Boehringer Mannheim UK Ltd, Lewes, UK). Haematocrit was determined in triplicate from aliquots of EDTA blood dispensed into heparinised micro-haematocrit tubes (Scientific Industries, Loughborough, UK). These tubes were centrifuged for 15 minutes (micro-haematocrit centrifuge, Hawksley and Sons Ltd, Lancing, UK) and haematocrit values were determined using a micro-haematocrit reader (Hawksley and Sons Ltd, Lancing, UK). Changes in plasma volume were estimated from changes in haemoglobin and haematocrit using the method described by Dill and Costill (1974).

3.9.2 Spectrophotometric assays

Plasma total cholesterol concentration (Appendix B3) was determined by an enzymatic method using a commercially available kit (Boehringer Mannheim GmbH, U.K. Ltd.). A magnesium chloride/phosphotungstic acid precipitation method (Boehringer Mannheim GmbH, U.K. Ltd.) was used to isolate an HDL supernatant from plasma (Appendix B2) which was subsequently analysed for cholesterol.

TAG concentrations were determined in frozen plasma in the reproducibility study, chapter 4 and chapter 6 by an enzymatic method (Boehringer Mannheim GmbH, U.K. Ltd.) (Appendix B1). In chapter 5, TAG concentrations were determined in fresh whole plasma and lipoprotein fractions (see section 3.9.5) using the same method. Plasma glucose (Appendix B5), NEFA (Appendix B4), paracetamol (Appendix B6) and serum 3-hydroxybutyrate (Appendix B7) concentrations were determined by

enzymatic methods (Boehringer Mannheim GmbH, U.K. Ltd., Wako Chemicals GmbH, U.K. Ltd. Cambridge Life Sciences plc, Cambridge, UK and Sigma Diagnostics, Poole, UK respectively).

All spectrophotometric assays were performed on an automated centrifugal analyser (Cobas Bio, Version 8326 (for the reproducibility study, chapter 4 and analysis on fresh plasma samples in chapter 5) or Cobas Mira Plus (for chapter 6 and analysis on frozen plasma in chapter 5); Roche Diagnostic Systems, Hertfordshire, UK).

3.9.3 Radioimmunoassays

Concentrations of serum insulin were determined using a solid-phase ¹²⁵Iodine radioimmunoassay (COAT-A-COUNT Insulin, EURO/DPC Ltd., Caernarfon, UK) (Appendix B8). Radioactivity was measured using an automated gamma counting system (Cobra II, Packard Instrument Company Inc, USA).

Plasma glucagon concentrations were determined by Ms ML Clark in the laboratories of the Oxford Lipid Metabolism Group at the Radcliffe Infirmary, Oxford by double-antibody radioimmunoassay.

3.9.4 Apolipoprotein E phenotyping

Apolipoprotein E phenotype was determined for each subject in chapters 4, 5 and 6 by Dr SJ Mastana and Mrs A Pacynko in the Human Genetics Laboratory (Department of Human Sciences, Loughborough University), by isoelectric focussing using Western blot techniques, as described by Eichner et al. (1991).

3.9.5 Separation of chylomicrons and VLDL from whole plasma

Chylomicrons (Sf ~ > 400) and VLDL (Sf ~ 20-400) fractions were separated from whole plasma by sequential floatation in an ultracentrifuge. This procedure was based on the method described by Havel et al. (1955) and introduced to the author by researchers in the Oxford Lipid Metabolism Group. The technique was then established, with some small modifications in the aspiration and rinsing of the samples to improve the precision and recovery, in the laboratory at Loughborough. The technique as performed in Loughborough is described below. In a methodological study, plasma was stained with Sudan Black (0.1 g Sudan Black

dissolved in 100 ml of ethylene glycol at 65°C and then mixed in the ratio 1:10 with plasma) to elucidate the exact locations of the chylomicron and VLDL fractions after ultracentrifugation (Terpstra et al. 1981). These data were used to determine where to slice the ultracentrifuge tubes to recover the respective lipoprotein fractions. The recovery of TAG from the separated lipoprotein fractions and the coefficients of variation for TAG concentrations in the lipoprotein fractions were assessed during the development of the technique. Before experimentation in chapter 5 commenced, a recovery of TAG from the lipoprotein fractions ($S_f \sim > 400$, $S_f \sim 20-400$ and $S_f \sim < 20$) of $93.9 \pm 0.7\%$ (mean \pm SEM) was achieved; with coefficients of variation for chylomicron-, VLDL- and $S_f < 20$ -TAG of 6.3 %, 4.1 % and 1.6 % respectively.

The lipoprotein separations were conducted on fresh plasma on the day after the samples were collected. The plasma had been mixed with a preservative cocktail and stored overnight at 4°C. During the analyses, the samples were chilled in ice trays.

Chylomicron ($S_f \sim < 400$) fractions were prepared by pipetting 1.25 ml of 1.006 g.ml⁻¹ density solution (which, in turn, was prepared by dissolving 11.0 g NaCl in 995 ml distilled water and adding 0.1 ml of 0.1 mmol.l⁻¹ EDTA, checking density gravimetrically and adjusting if necessary) into open top Beckman ultracentrifuge tubes (Product no: 347357, Beckman Instruments Inc., London, UK) in duplicate. This was carefully underlayered with 750 μ l of plasma and then overlaid with 0.45 ml of density solution. The tubes were gently placed in the buckets of a Beckman TLS 55 swinging bucket rotor (Beckman Instruments Inc., London, UK) and centrifuged at 30 000 rpm (average $g = 59,000$) for 20 minutes at 4°C in Beckman Optima TLX ultracentrifuge (Beckman Instruments Inc., London, UK). The tubes were then placed in a Beckman CentriTube slicer (Beckman Instruments Inc., London, UK) and sliced 2.9 cm from the bottom to isolate the chylomicron fraction. (The methodological study in which plasma was stained with Sudan Black to increase the visibility of the lipoprotein fractions indicated that all visible chylomicrons were contained in the supernatant when the tube was sliced at this level). The chylomicron suspension was then aspirated into a pre-weighed 5 ml polycarbonate tube (Fisher Scientific, Loughborough, UK) using a 200 μ l Gilson pipette (Gilson Pipetman, Gilson Medical Electronics, France). The tube slice and tube slicer blade were then thoroughly rinsed

with density solution three times to dislodge chylomicrons which were stuck to the walls of the tube slicer and this liquid was added to the chylomicron suspension in the polycarbonate tube. The tube was then reweighed to determine the mass of the chylomicron suspension.

The infranatents from the duplicate open top tubes were aspirated into a Beckman Quick-seal ultracentrifuge tube (Product no: 362248, Beckman Instruments Inc., London, UK) topped up with 1.006 g.ml^{-1} density solution then sealed with a Beckman tube topper kit (Beckman Instruments Inc., London, UK). These tubes were then placed in a Beckman 100.4 angle head rotor, in batches of eight and ultracentrifuged for $2 \frac{1}{2}$ hours at 100 000 rpm (average $g = 417,000$) at 4°C . The VLDL (Sf 20-400) fraction was obtained by slicing the tube 4.4 ml from the bottom and aspirating the top layer into a pre-weighed polycarbonate tube, using an 18G needle (Sabre International Products Limited, Reading, UK) attached to a 2 ml syringe (Plastipak, Becton Dickinson, Oxford, UK) (The Sudan Black methodological study indicated that all visible VLDL were contained in the supernatant when the tube was sliced at this level). The tube slice was rinsed thoroughly three times to remove any VLDL particles which had adhered to the tube slice and these rinsings were added to the VLDL suspension in the polycarbonate tube, which was then reweighed. The Sf < 20 fraction was the infranatent left following this spin, this was aspirated into a pre-weighed polycarbonate tube using an 18G needle (Sabre International Products Limited, Reading, UK) attached to a 2 ml syringe (Plastipak, Becton Dickinson, Oxford, UK).

The concentrations of TAG in whole plasma and in each of the lipoprotein fraction suspensions were then determined. The TAG concentrations in the lipoprotein fraction suspensions were multiplied by the mass of the fraction (assuming the density of the fraction to be 1 g.ml^{-1}) to give the molar quantities of TAG in each fraction. These were then divided by the original volume of plasma used to generate the fraction, having taken into account the dilution of the original sample with the preservative, to give the concentrations of TAG in each fraction of the plasma sample.

Recovery of TAG from the lipoprotein fractions in chapter 5 was 91.1 ± 0.8 % in the control trial and 89.4 ± 1.4 % in the exercise trial.

3.9.6 Accuracy and precision of assays

The accuracy and precision of the assays described in the sections above were monitored using quality control sera (Precinorm U and Precipath U, Boehringer Mannheim UK Ltd, Lewes, UK; Control Serum N (Human), Roche Diagnostic Systems, Welwyn Garden City, UK; Seronorm Lipid, Sero AS, Billingstad, Norway; CON6, EURO/DPC Ltd., Caernarfon, UK; β -HBA Control-N (Human), Sigma Diagnostics, Poole, UK). All samples for each subject were performed in the same analyser run for each assay, except for plasma and lipoprotein fraction TAG chapter 5, where analyses were performed on fresh plasma. Within-batch coefficients of variation were 1.3 % for TAG, 0.8 % for glucose, 1.1 % for total cholesterol, 1.8 % for HDL cholesterol, 0.9 % for NEFA, 3.9 % for insulin, 6.9 % for glucagon, 0.8 % for paracetamol and 2.3 % for 3-hydroxybutyrate. The between-batch coefficient of variation was 1.7 % for plasma TAG.

3.10 Determination of ^{13}C enrichment of breath and plasma concentrations of ^{13}C palmitic acid

The ^{13}C enrichment of breath samples in chapter 5 was measured using continuous flow isotope ratio mass spectrometry (CF-IRMS) (Europa Scientific Ltd, Crewe, UK) by researchers at the Institute of Human Nutrition (IHN), Southampton General Hospital. This is described in detail in appendix B10.

In addition, plasma concentrations of ^{13}C palmitic acid were determined in a limited number of samples from chapter 5, to aid the interpretation of the NEFA data obtained in chapters 4, 5 and 6. These procedures were performed by the author under the supervision of researchers at the Institute of Human Nutrition and comprised four stages: total lipid extraction, separation of lipid classes by solid phase extraction (Bond Elut), methylation of the NEFA extract to form fatty-acid-methyl-esters and measurement of total and ^{13}C palmitic acid concentrations by gas chromatography-IRMS (GC-IRMS). These procedures are described in appendix B11.

3.11 The reproducibility of an oral fat tolerance test

3.11.1 Introduction

A quantitative knowledge of the precision or reproducibility of a measurement tool is essential to assess the validity of data obtained from its use. There have been a number published reports investigating the reproducibility of oral fat tolerance test protocols, but few have analysed intra-subject variability in a systematic and controlled manner. The results of previously published reproducibility trials are presented in Table 3.8. These data reveal that plasma TAG responses to oral fat loads have not been consistently reproducible and the range of intra-individual variation in postprandial lipaemia has been wide. One group (Patsch et al. 1983; Patsch, 1987; Brown et al. 1992) concluded that their oral fat tolerance test procedure was highly reproducible; however, this statement was not necessarily supported by their data. The two most recent reproducibility studies (Aldred and Hardman, 1993; Kaminsky et al. 1997) have both expressed reservations about the precision of oral fat tolerance test procedures.

The relatively poor reproducibility of postprandial TAG concentrations in previous studies may have been due in part to inadequate control, in the days preceding as well as during the oral fat tolerance tests, of factors known to affect lipaemia. Research from this and other laboratories has clearly demonstrated the effect of exercise in the hours and days preceding an oral fat tolerance test on subsequent lipaemia (Aldred et al. 1994; Tsetsonis and Hardman, 1996a; Tsetsonis and Hardman, 1996b; Tsetsonis et al. 1997; Zhang et al. 1998; Gill et al. 1998; Malkova et al. 1999), yet many of the previous studies examining the reproducibility of oral fat tolerance tests either did not control or inadequately controlled the subjects' exercise habits in the days prior to the fat tolerance tests. Studies have shown that altering macronutrient (Chen et al. 1993; Chen et al. 1995; Blades and Garg, 1995; Jeppesen et al. 1997) or fatty acid intake (Harris and Conner, 1980; Weintraub et al. 1988; Harris et al. 1988) in the days and weeks prior to an oral fat tolerance test influences postprandial lipaemia. Similarly, alcohol consumption in the days prior to an oral fat tolerance test may or may not influence postprandial lipaemia (Superko, 1992; Hartung et al. 1993). However, in many of the reproducibility studies diet and alcohol consumption in the days preceding the oral fat tolerance tests had not been adequately controlled. In

addition, fluid intake during the oral fat tolerance test may affect plasma TAG concentrations by influencing the rate of gastric emptying, and physical activity during the oral fat tolerance tests may attenuate the lipaemic response (Hardman and Aldred, 1995).

The test meal used in this laboratory contains both fat and carbohydrate as fat is rarely ingested without carbohydrate in daily living. As a consequence, ingestion of the meal elicits an insulin response. Insulin is an important co-ordinator of postprandial lipid metabolism; regulating adipose tissue (Sadur and Eckel, 1982) and skeletal muscle (Kiens et al. 1989) LPL activity, fatty acid esterification and release (Coppack et al. 1992), and VLDL secretion either directly (Mason, 1998) or through its effect on NEFA. Thus, the reproducibility of the insulinaemic response (and glycaemic response as this affects insulin) to the test meal may play a role in determining the reproducibility of the lipaemic response.

Therefore, the purpose of the present study was to determine the reproducibility of the lipaemic, insulinaemic and glycaemic responses to the high fat test meal used in this laboratory, under well-controlled conditions. Subjects were instructed to refrain from exercise for three days prior to each oral fat tolerance test, to weigh and record their dietary intake for two days prior to the first trial and to replicate this intake prior to the second trial, and to abstain from alcohol for 24 hours prior to each trial. During the oral fat tolerance tests subjects were only allowed to consume mineral water and this intake was replicated in the second trial. Subjects rested throughout the postprandial observation period.

Author/Date	Number of subjects	Meal Composition	Results
Patsch et al. (1983)	1 (3 pairs of tests)	130F/48C/10P per 2m ² surface area	Difference in lipaemia between trials: 151 %, 37 % and 17 % for the 1st, 2nd and 3rd pair of tests respectively
Kashyap et al. (1983)	6 (3 male, 3 female)	Saturated: 100F/63C/26P Unsaturated: 100F/63C/25P	Saturated fat meal (n = 5): intra-individual differences in lipaemia: 40 %, 46 %, 20 %, 8 %, 26 %. mean difference between trials: 35 %. Unsaturated fat meal (n = 6): intra-individual differences in lipaemia: 31 %, 12 %, 18 %, 28 %, 17 %, 172 %. mean difference between trials: 26 %.
Patsch (1987)	3	130F/48C/10P per 2m ² surface area	intra-individual differences in lipaemia: 32 %, 4 %, 5 %. mean difference between trials: 6 %.
Cohen et al. (1988)	8 males	80F	mean intra-subject variance in lipaemia: 19 %
Cohn et al. (1988)	5	1F/1C/1P per kg body mass	"an individual's response to the fat rich meal was sometimes, but not always, reproducible."
Brown et al. (1992)	10 (3 males, 7 females)	130F/50C/11P per 2m ² surface area	correlation and mean intra-subject difference between trials 1 and 2 for a) fasting TAG: 0.67, 8 % b) 3.5-hour TAG: 0.69, 2 % c) 9-hour TAG: 0.87, 9 %

Table 3.8 Data from published studies examining the reproducibility of lipaemic responses to oral fat loads. Meal composition values in grams, F = fat, C = carbohydrate, P = protein.

Author/Date	Number of subjects	Meal Composition	Results
Ryu et al. (1992)	24	65F per m ² body surface area	correlation for peak TAG concentrations between trials: 0.81
Aldred and Hardman, (1993)	6	A) 1.3F per kg body mass B) 1.2F/1.3C/0.2P per kg body mass	A) mean (\pm SEM) intra-subject difference between trials: 35 ± 11 %, correlation: 0.14. B) mean (\pm SEM) intra-subject difference between trials: 26 ± 7 %, correlation: 0.83.
Kaminsky et al. (1997)	10 males	70F	Subjects each performed 3 trials. Mean intra-individual variability and correlation coefficients between pairs of trials were 19 %, 22 % and 17 % and 0.79, 0.79 and 0.91 respectively.

Table 3.8 (cont.) Data from published studies examining the reproducibility of lipaemic responses to oral fat loads. Meal composition values in grams, F = fat, C = carbohydrate, P = protein, SEM = standard error of mean.

3.11.2 Subjects and Methods

3.11.2.1 Subjects

Eight male subjects volunteered to participate in this study which was conducted with the approval of the University Ethical Advisory Committee. Their physical characteristics are shown in Table 3.9. Subjects were informed of risks of participation and written informed consent was given prior to testing.

Subject	age (years)	height (m)	mass (kg)	BMI (kg.m ⁻²)	waist/hip ratio	% bodyfat	fat-free mass (kg)
1	32	1.83	85.9	25.6	0.94	17.3	71.0
2	54	1.73	80.1	26.7	0.92	29.3	56.6
3	43	1.72	84.9	28.7	0.95	34.7	55.4
4	31	1.77	73.7	23.5	0.80	20.2	58.8
5	43	1.85	101.4	29.6	0.89	33.9	67.0
6	45	1.73	78.9	26.3	0.85	29.5	55.6
7	56	1.72	71.4	24.1	0.94	21.8	55.8
8	59	1.80	82.1	25.3	1.00	36.1	52.4
mean	45.4	1.77	82.3	26.3	0.91	27.9	59.1
SD	10.5	0.05	9.2	2.1	0.06	7.2	6.5

Table 3.9 Physical characteristics of the subjects.

3.11.2.2 Study protocol

Oral fat tolerance tests were conducted on two occasions separated by a week, as described in section 3.7.1. Blood samples were obtained in the fasted state and for six hours postprandially as described in section 3.8, with plasma and serum frozen for subsequent analysis. The test meal was cereal-based and provided 1.70 g fat, 1.65 g carbohydrate and 0.25 g protein per kg body mass.

Subjects refrained from exercise for the three days prior to each trial, and consumed no alcohol on the day before each trial. In addition, subjects weighed and recorded

their food and drink intake for two days prior to the first trial and replicated this intake on the days preceding the second trial (see section 3.7.3).

3.11.2.3 Analysis of blood samples

Haemoglobin concentrations and haematocrit were determined at baseline and at the end of each trial, in order to estimate plasma volume changes between trials. Plasma concentrations of HDL and total cholesterol (in the fasted state) and TAG and glucose (in the fasted and postprandial states) were measured. Insulin concentrations were measured in serum. Details of the analyses are shown in section 3.9.

3.11.2.4 Data analysis

TAG and cholesterol (both total and HDL) concentrations in Trial 2 were corrected to account for changes in plasma volume relative to Trial 1 according to the method described by Dill and Costill (1974). The six-hour areas under the plasma or serum concentration vs. time curves, calculated using the trapezium rule, were defined as the respective total responses to the test meal, with these areas normalised to the baseline concentrations defined as the incremental responses. Data were compared using a ratio limits-of-agreement approach (Nevill and Atkinson, 1997), two tailed paired t-tests and Spearman rank order correlation. Statistical methods used in previous studies were also employed, despite not being the most appropriate, to enable direct comparisons between this and previous work. Data are presented as mean \pm standard error (SEM) unless otherwise stated.

3.11.3 Results

The test meal was well tolerated by the subjects, with all completing it and none reporting any nausea.

3.11.3.1 Estimation of plasma volume changes

Mean haemoglobin concentration was 14.9 ± 0.3 g.dl⁻¹ in Trial 1 and 14.5 ± 0.5 g.dl⁻¹ in Trial 2 ($p = 0.26$) and mean haematocrits were 43.8 ± 0.7 % and 43.0 ± 1.0 % in Trials 1 and 2 respectively ($p = 0.22$). These values were not significantly different between trials suggesting that the amount of haemoglobin circulating in Trial 2 was not systematically reduced by the blood sampled in Trial 1. Thus it was considered

possible to estimate differences in plasma volume between trials using the method described by Dill and Costill (1974) without abusing the assumption that the amount of circulating haemoglobin was constant between trials. It was therefore decided to correct lipid concentrations for differences in plasma volume between trials. Plasma volume was 4.9 ± 3.6 % greater in Trial 2 than Trial 1, but this difference was not statistically significant ($p = 0.22$).

3.11.3.2 Plasma and serum concentrations in the fasted state

Total cholesterol, HDL cholesterol and TAG concentrations in the fasted state, both uncorrected and corrected for differences in plasma volume between trials, are presented in Table 3.10. After correction for plasma volume changes, total cholesterol concentrations were higher in Trial 2 than Trial 1 ($p < 0.05$) and Spearman rank order correlations between Trial 1 and Trial 2 for fasting TAG, total cholesterol and HDL cholesterol concentrations were 0.95, 0.90 and 0.95 respectively (Pearson product moment correlations were 0.99, 0.99 and 0.99 respectively). The mean (\pm SEM) difference between trials was 4.8 ± 4.7 % for TAG, 2.5 ± 1.0 % for total cholesterol and 1.5 ± 2.0 % for HDL cholesterol, with 95 % confidence intervals for the differences between trials of -30.8 % to +21.2 % for TAG, -2.9 % to +7.9 % for total cholesterol and -10.1 % to +13.1 % for HDL cholesterol. Insulin and glucose concentrations were not corrected for differences in plasma volume (see discussion for justification). These concentrations in the fasted state are presented in Table 3.11. Spearman rank order correlations between Trial 1 and Trial 2 were 0.48 and 0.24 for glucose and insulin concentrations respectively (Pearson product moment correlations were 0.84 and 0.58 respectively). Mean (\pm SEM) differences between trials were 2.8 ± 2.0 % for glucose and 3.7 ± 10.9 % insulin with respective 95 % confidence intervals for the differences being -8.5 % to +14.1 % (glucose) and -49.4 % to +71.2 % (insulin).

Subject	Trial 1			Trial 2 (uncorrected)			Trial 2 (corrected)		
	TAG (mmol.l ⁻¹)	Tchol (mmol.l ⁻¹)	HDL (mmol.l ⁻¹)	TAG (mmol.l ⁻¹)	Tchol (mmol.l ⁻¹)	HDL (mmol.l ⁻¹)	TAG (mmol.l ⁻¹)	Tchol (mmol.l ⁻¹)	HDL (mmol.l ⁻¹)
1	0.90	3.47	1.28	0.92	3.79	1.31	0.90	3.70	1.27
2	1.07	5.07	0.78	0.91	5.33	0.91	0.87	5.10	0.87
3	1.18	4.24	1.33	1.20	4.08	1.25	1.22	4.16	1.27
4	0.80	4.05	1.05	0.84	4.09	1.00	0.87	4.21	1.03
5	0.76	5.84	2.00	0.59	5.59	1.82	0.65	6.19	2.01
6	0.63	4.09	1.48	0.71	4.02	1.42	0.74	4.18	1.47
7	3.24	6.12	1.03	2.31	4.99	0.91	2.93	6.34	1.16
8	1.44	6.80	1.72	1.29	6.97	1.73	1.26	6.83	1.69
Mean	1.25	4.96	1.33	1.10	4.86	1.29	1.18	5.09*	1.35
SEM	0.30	0.42	0.14	0.19	0.38	0.12	0.26	0.43	0.13

Table 3.10 Fasting TAG, total cholesterol (Tchol) and HDL cholesterol concentrations in Trial 1, Trial 2 (not corrected for changes in plasma volume) and in Trial 2 (corrected for changes in plasma volume). *different from Trial 1, $p < 0.05$.

Subject	Trial 1		Trial 2	
	Glucose (mmol.l ⁻¹)	Insulin (μ U.ml ⁻¹)	Glucose (mmol.l ⁻¹)	Insulin (μ U.ml ⁻¹)
1	4.66	8.7	4.45	7.3
2	4.65	10.5	4.85	11.7
3	5.06	10.1	5.00	11.3
4	4.80	11.6	4.55	5.8
5	6.07	17.8	5.93	15.6
6	4.71	7.3	4.80	7.0
7	5.10	7.7	4.35	12.2
8	5.44	10.4	5.41	10.1
Mean	5.06	10.5	4.92	10.1
SEM	0.2	1.2	0.2	1.2

Table 3.11 Glucose and insulin concentrations in the fasted state in Trial 1 and Trial 2.

3.11.3.3 Postprandial plasma and serum concentrations

The mean plasma TAG responses to the test meal in Trials 1 and 2, both uncorrected and corrected for differences in plasma volume between trials are shown in Figure 3.6. The total and incremental lipaemic responses to the test meal for individual subjects in Trials 1 and 2 (both uncorrected and corrected for differences in plasma volume between trials) are presented in Table 3.12. Neither response differed significantly between Trials 1 and 2 (total lipaemic response: $p = 0.17$ and $p = 0.58$ for uncorrected and corrected concentrations respectively; incremental lipaemic response: $p = 0.31$ and $p = 0.46$ for uncorrected and corrected concentrations respectively). Figure 3.7 shows scatter diagrams displaying the relationships between Trials 1 and 2 for the total and incremental lipaemic responses. These relationships were strong, with Spearman rank order correlation coefficients of 0.95 and 0.95 for the corrected total and incremental lipaemic responses respectively (Pearson product moment correlation coefficients: 0.99 and 0.95 for the total and incremental responses respectively). The mean (\pm SEM) difference in the total lipaemic response between

Trial 1 and Trial 2 was $2.0 \pm 4.7 \%$ ($6.6 \pm 5.1 \%$ for uncorrected concentrations) with the incremental lipaemic responses differing by $2.2 \pm 10.1 \%$ ($4.1 \pm 7.7 \%$ for uncorrected concentrations) between trials. The 95 % confidence interval for the differences in total lipaemic response between trials ranged from -28 % to +24 % (-20.9 % to +7.7 % for uncorrected concentrations). For the differences between trials in the incremental lipaemic responses, the 95 % confidence interval ranged from -53.5 % to +57.9 % (-46.8 % to +38.6 % for uncorrected concentrations).

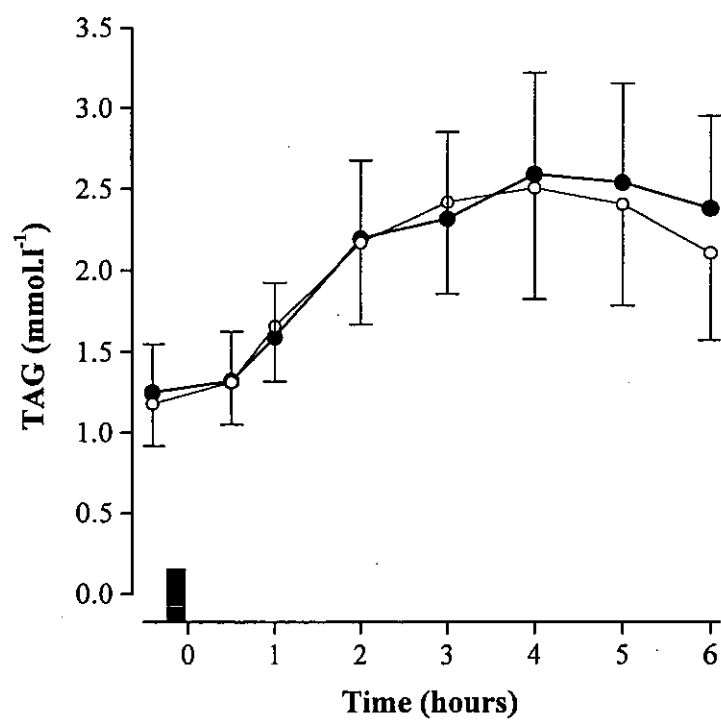
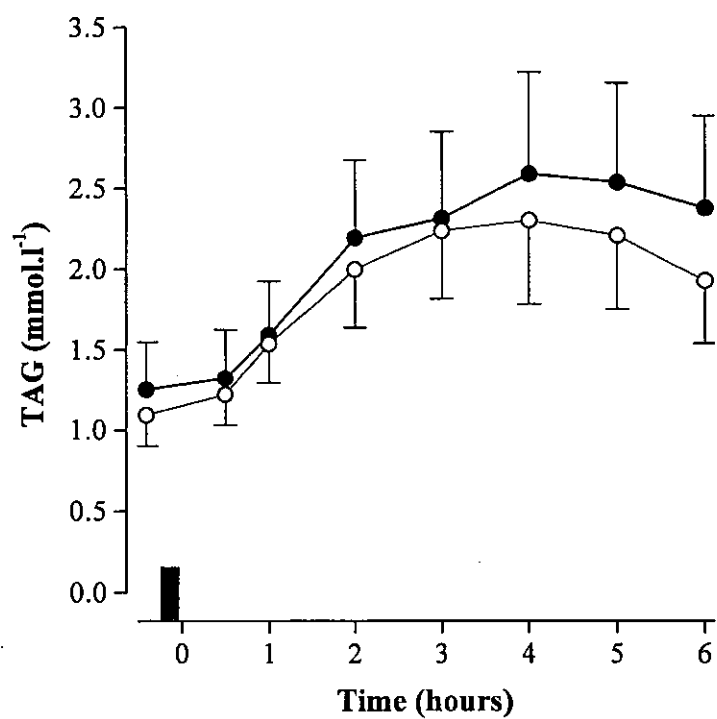


Figure 3.6 Plasma TAG response to the test meal in Trial 1 (filled) and Trial 2 (open). The top and bottom panels display TAG concentrations not corrected, and corrected, for differences in plasma volume between trials, respectively.

Subject	Trial 1		Trial 2 (uncorrected)		Trial 2 (corrected)	
	TLR	ILR	TLR	ILR	TLR	ILR
	(mmol.l ⁻¹ .h)	(mmol.l ⁻¹ .h)	(mmol.l ⁻¹ .h)	(mmol.l ⁻¹ .h)	(mmol.l ⁻¹ .h)	(mmol.l ⁻¹ .h)
1	9.24	3.87	8.44	2.95	8.24	2.75
2	10.45	4.06	9.36	3.9	8.94	3.49
3	13.55	6.47	12.13	4.93	12.4	5.2
4	7.11	2.34	8.17	3.12	8.43	3.39
5	9.98	5.42	8.32	4.81	9.22	5.71
6	5.58	1.8	6.66	2.43	6.93	2.69
7	32.51	13.11	26.24	12.41	33.4	19.57
8	15.52	6.88	14.69	6.95	14.44	6.7
Mean	12.99	5.49	11.75	5.19	12.75	6.19
SEM	3.0	1.3	2.3	1.1	3.1	2.0

Table 3.12 Individual total lipaemic responses (TLR) and incremental lipaemic responses (ILR) in Trial 1 and Trial 2 (with concentrations not corrected for differences in plasma volume between trials) and Trial 2 (with concentrations corrected for differences in plasma volume between trials).

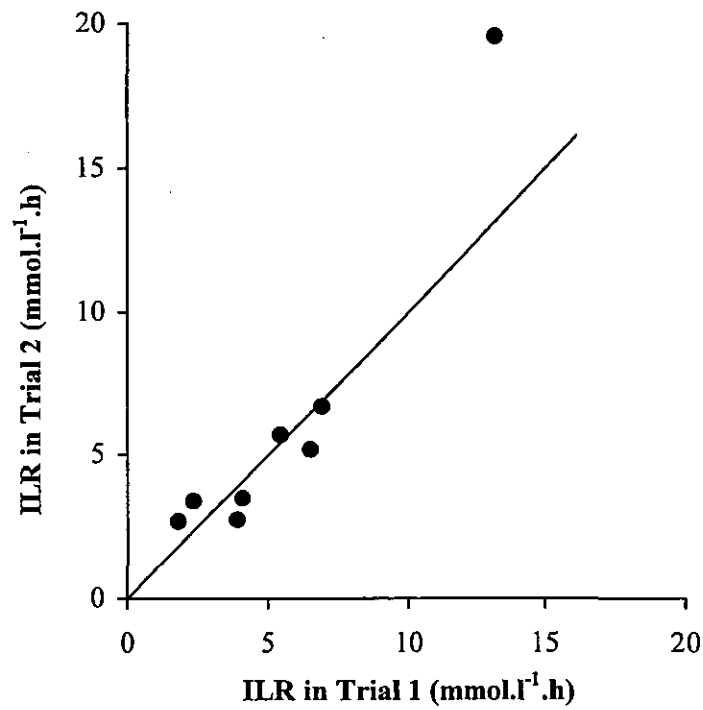
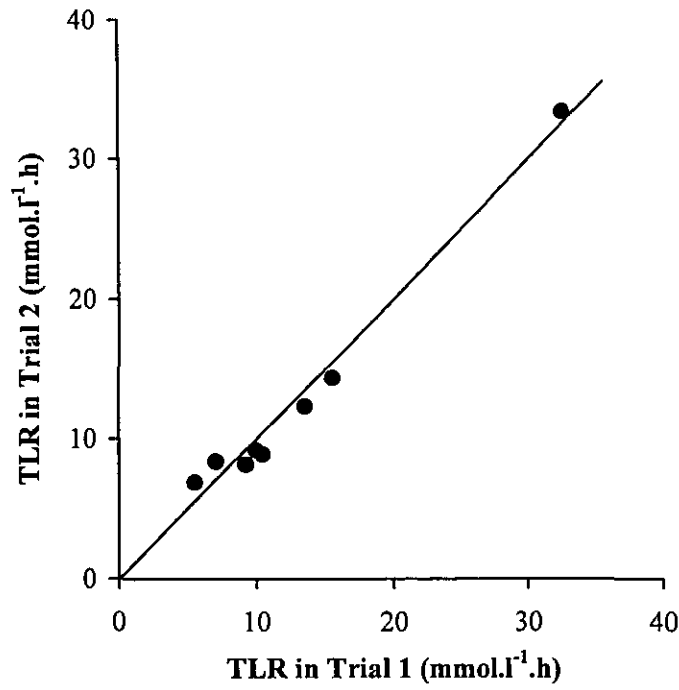


Figure 3.7 Scatter diagrams showing the relationship in total (TLR, top panel) and incremental (ILR, bottom panel) lipaemic responses and between Trial 1 and Trial 2, with the lines of equality shown. Spearman rank order correlation coefficient; TLR: 0.95, ILR: 0.95 (Pearson product moment correlation coefficient; TLR: 0.99, ILR: 0.95)

The plasma glucose and serum insulin responses to the test meal, which were not corrected for differences in plasma volume between trials, are presented in Figure 3.8. Neither response differed significantly between trials ($p = 0.26$ and $p = 0.64$ for the glycaemic and insulinaemic responses respectively). Table 3.13 shows individual insulinaemic and glycaemic responses to the test meal in Trials 1 and 2, and Figure 3.9 shows scatter diagrams displaying the relationships between Trials 1 and 2 for the glycaemic and insulinaemic responses. The mean (\pm SEM) difference between trials was 1.9 ± 1.6 % for the glycaemic response and 3.5 ± 10.5 % for the insulinaemic response. The 95 % confidence interval for the difference between trials was -6.8 % to +10.6 % for the glycaemic response and -54.8 to +61.8 % for the insulinaemic response.

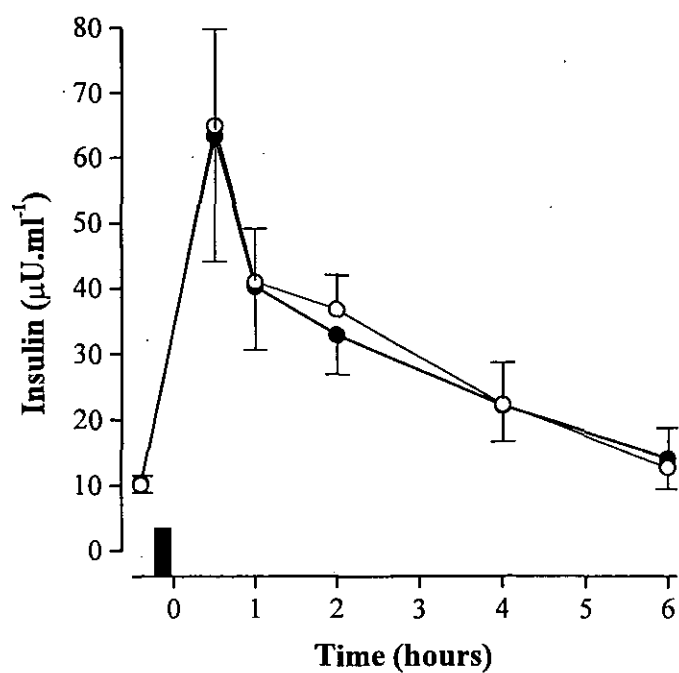
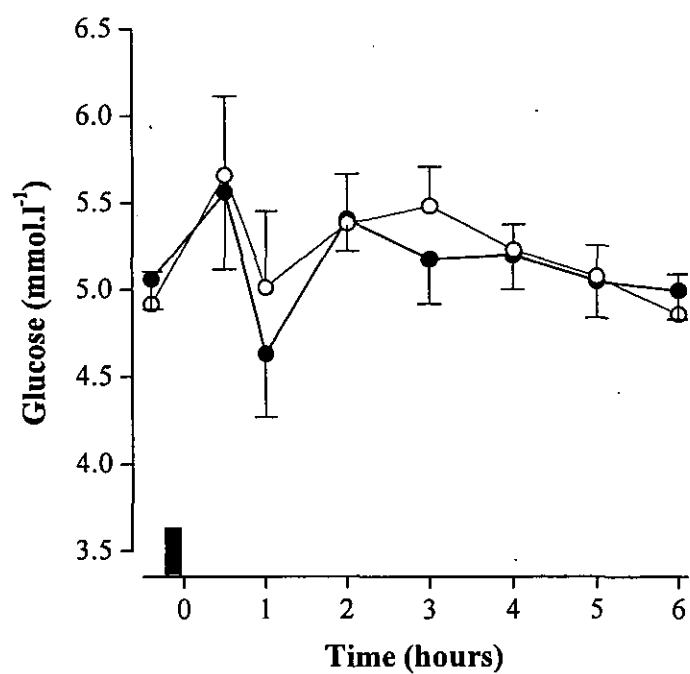


Figure 3.8 Plasma glucose (top panel) and serum insulin (bottom panel) responses to the test meal in Trial 1 (filled) and Trial 2 (open).

Subject	Trial 1		Trial 2	
	Glycaemic response (mmol.l ⁻¹ .h)	Insulinaemic response (μU.ml ⁻¹ .h)	Glycaemic response (mmol.l ⁻¹ .h)	Insulinaemic response (μU.ml ⁻¹ .h)
1	26.7	125.2	27.0	84.4
2	28.9	169.9	31.4	161.8
3	30.1	106.9	31.5	124.0
4	28.4	82.0	29.7	133.1
5	36.7	424.1	35.8	398.2
6	28.8	100.8	27.2	79.2
7	30.9	142.9	31.2	172.0
8	36.1	227.0	37.5	274.8
Mean	30.8	172.3	31.4	178.4
SEM	1.3	39.4	0.2	38.2

Table 3.13 Glycaemic and insulinaemic responses to the test meal in Trial 1 and Trial 2 (concentrations not corrected for differences in plasma volume).

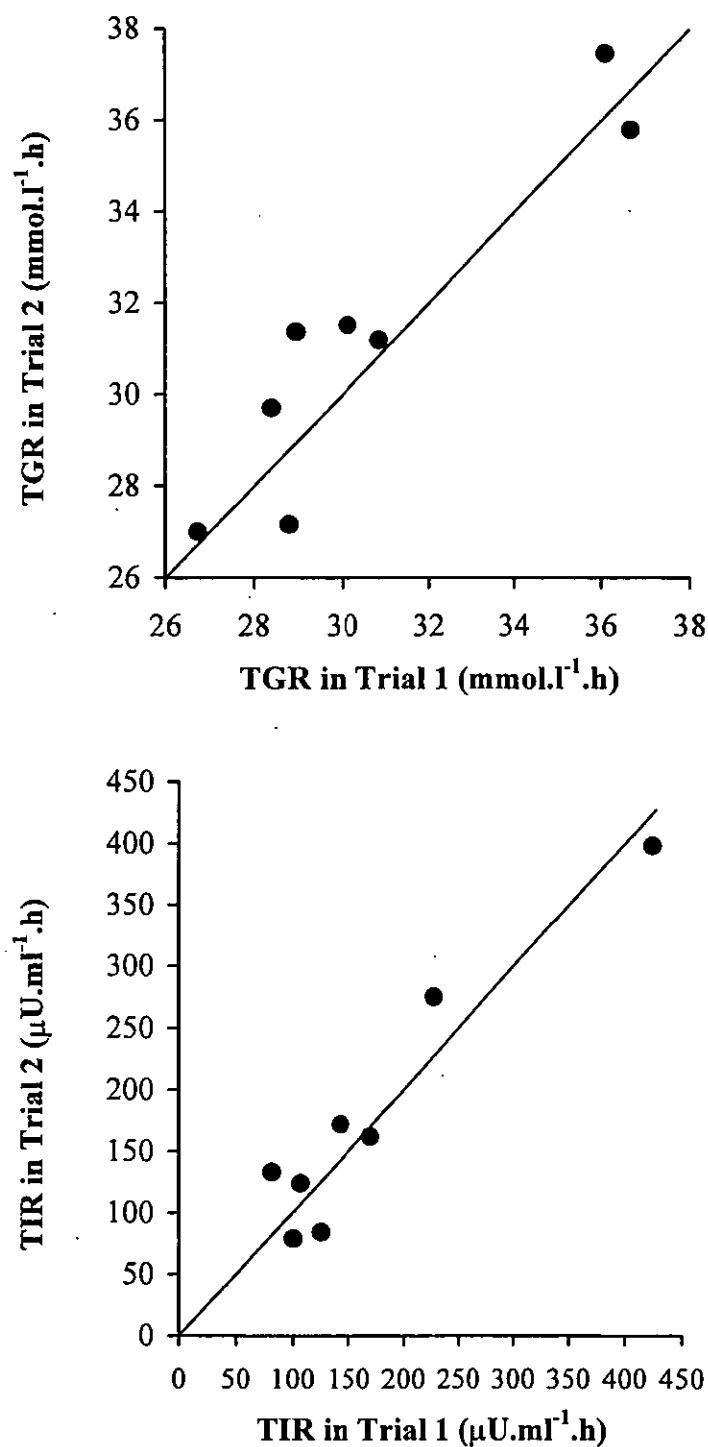


Figure 3.9 Scatter diagrams showing the relationship in glycaemic (TGR, top panel) and insulinaemic (TIR, bottom panel) responses between Trial 1 and Trial 2, with the lines of equality shown. Spearman rank order correlation coefficient; TGR: 0.88, TIR: 0.81 (Pearson product moment correlation coefficient; TGR: 0.94, TIR: 0.95)

3.11.4 Discussion

This study aimed to determine the reproducibility of the postprandial responses to a high-fat mixed test meal under well-controlled conditions both prior to, and during, the oral fat tolerance tests. Neither the total nor incremental lipaemic responses differed significantly between trials and the correlations between trials for both responses were high.

A number of studies have been published reporting the reproducibility of lipaemia following ingestion of a test meal (see Table 3.8 for results from previously reported studies). The highest correlation coefficient previously published (to the author's knowledge) was from Kaminsky and co-workers (1997), who reported a correlation 0.91 for the lipaemic response between two out of three repeated trials, with lipaemia differing by an average of 17 %. However, the correlation coefficients between the other pairs of trials were lower (0.79 and 0.79), with the mean differences being larger (19 % and 22 %). Other groups have reported correlation coefficients between indices of lipaemia between trials ranging from 0.14 (Aldred and Hardman, 1993) to 0.87 (Brown et al. 1992), although the latter considered TAG concentrations at just a single postprandial time point. Reported mean differences between trials in indices of lipaemia have ranged from 6 % (Patsch, 1987), for a study of just three subjects, to 35 % (Kashyap et al. 1983), with individual differences in lipaemia being as high as 151 % (Patsch et al. 1983). Indeed, it is possible that the published reproducibility data has been distorted by publication bias with laboratories with poor reproducibility data not publishing results. The vagueness of the statement by one group that "an individual's response to the fat meal was sometimes, but not always, reproducible" (Cohn et al. 1988), without any supporting data, suggests that the reproducibility of the lipaemic response in some laboratories may be poor. Thus, the results of the present study (Pearson product moment correlations of 0.99 and 0.95 for the total and incremental lipaemic responses respectively, with mean differences in lipaemia of 2.0 % and 2.2 %, for the two responses, when concentrations were corrected for differences in plasma volume between trials), indicate that reproducibility of the postprandial plasma TAG response in the current study was considerably better than previously reported data. This greater reproducibility is likely to be due to the tighter control of lifestyle (i.e. diet and exercise) before and during the oral fat tolerance tests in the current study, compared with many of the previous reports.

The reproducibility of the plasma TAG response to the test meal in the current study was shown to be better than previously published data when the same statistical techniques as those employed by the prior studies were used to compare the data. However, the traditional techniques used to assess reproducibility might not be the most revealing measures of test precision. Tests comparing means (e.g. *t*-tests) have been frequently used to assess reproducibility, but this approach gives no indication of the range of values. With a data set containing a high degree variance, it is difficult to detect a difference between groups. Thus, the response to two tests could differ considerably without being deemed significantly different.

Most commonly correlations are used to measure association between two tests – with a high correlation indicating a strong relationship between variables. However, caution is advised when utilising correlations as a high association does not necessarily imply good agreement – if, for example, all Trial 2 values were twice as high as values in Trial 1, then the correlation would be high but the agreement poor. Nevertheless, this problem can be overcome by seeing how close points on a plot of Trial 2 against Trial 1 lie to the line of equality. An additional criticism of using correlations is that data spread over a wide range would produce a higher correlation coefficient than data spanning a narrow range. This is only the case if parametric statistics are employed. Non-parametric statistics, which are based on ranks, are not affected by the spread of the data. Thus, if both parametric and non-parametric correlation coefficients are high, with the points following the line of equality – as is the case with the present data (both Pearson product moment and Spearman correlation coefficients ≥ 0.95 , with points lying close to the line of equality (see Figure 3.7)) – the agreement between trials can be considered to be good.

More recently, a limits of agreement approach, which gives a confidence interval for the differences between two trials (Bland and Altman, 1986; Nevill and Atkinson, 1997), has become increasingly popular. However, as the technique gives a 95 % confidence interval range for the differences between trials, it provides a ‘worst case scenario’ reflecting the reproducibility of the least reproducible individual. This information is invaluable if knowledge of the reproducibility of a test is required on

an individual level (e.g. when using a test to assess the efficacy of an intervention or treatment for an individual), but does not provide an accurate representation of the precision of the test measured at a group level.

Perhaps the best measure of the reproducibility of test on a group basis is to consider the mean and standard error of the differences between trials, as this reveals what the mean difference between trials might be if the reproducibility study were repeated. If the expected mean difference induced by an intervention fell outside the standard error range, then the precision of the instrument should be adequate to detect the difference. In a way, the mean difference between Trial 1 and Trial 2 is of secondary importance to the standard error of this difference, as use of a cross-over experimental design eliminates systematic order of testing effects. However, a large mean difference between trials would increase variance and therefore reduce statistical power. The mean (\pm SEM) difference between trials was 2.0 ± 4.7 % for the total lipaemic response and 2.2 ± 10.1 % for the incremental lipaemic response (when concentrations were corrected for differences in plasma volume between trials). This, indicates that the resolution of the oral fat tolerance test, under the conditions studied, is adequate to detect the effect of 90 minutes of brisk walking on postprandial lipaemia, which is typically of the order of 20 %, when eight subjects are studied. As the standard error is the standard deviation divided by the square root of the mean, increasing the number of subjects studied increases the precision of the measuring instrument. For example increasing the subject number from eight to twelve would increase the resolution of the measurement by 22 %, assuming the same spread of data.

An important issue to address is whether concentrations should be corrected for differences in plasma volume. When fat is consumed, it is digested, absorbed and enters the circulation in chylomicron particles. These chylomicrons are distributed throughout the plasma, and therefore one determinant of their concentration is the plasma volume. Thus, if plasma volume differences between oral fat tolerance test trials are not corrected for, invalid inferences regarding differences in TAG metabolism between trials might be made. Indeed, in the present study, correcting

TAG concentrations for differences in plasma volume between trials improved the reproducibility of the TAG responses.

Although it could be argued that, in principle, plasma TAG concentrations should be corrected for differences in plasma volume between trials, under certain circumstances (e.g. when a large volume of blood has been sampled, thus reducing the amount of circulating haemoglobin) a valid plasma volume correction cannot be made. In these situations, an erroneous plasma volume correction would add to, rather than reduce, experimental error and therefore perhaps the best option would be to leave concentrations uncorrected. If plasma TAG concentrations were not corrected for plasma volume differences in the present study, the mean (\pm SEM) difference between trials would have been 6.6 ± 5.1 % for the total lipaemic response and 4.1 ± 7.7 % for the incremental lipaemic response. Thus, although the mean difference between trials was slightly greater for the uncorrected data, the standard errors of the difference were not appreciably different. Therefore, whether or not TAG concentrations were corrected for differences in plasma volume between trials, the precision of the oral fat tolerance test protocol was adequate to detect the effect of a moderate exercise session on postprandial lipaemia.

While it is reasonable to suggest that TAG concentrations should be corrected for differences in plasma volume, it could also be argued that plasma volume differences should not be corrected for when considering glucose and insulin responses. This is due to fundamental differences between the control of plasma glucose and plasma TAG. Maintenance of glucose homeostasis is vital to man. It is important to keep plasma glucose concentrations within a narrow band in order that tissues dependent on glucose have an adequate substrate supply (DeFronzo et al. 1983) and to avoid the damaging effects of hyperglycaemia (Ganong, 1985; Frayn, 1996). As a consequence a fast-acting integrated control system is in place to ensure maintenance of blood glucose concentrations. When a glucose load is administered, insulin is secreted to ensure glucose concentrations stay within a narrow band (Vander et al. 1990; Frayn, 1996). As the body is working to oppose changes in glucose concentrations, (as tissues are exposed to glucose concentrations, and not the total amount of circulating glucose), these should be compared between trials without correction for differences

in plasma volume. Following on from this argument, insulin concentrations should also not be corrected for plasma volume differences, as insulin is secreted to counteract changes in glucose concentration and therefore occurs on a per unit volume (i.e. concentration) basis.

Data examining the reproducibility of the glucose and insulin responses to a mixed high fat test meal have not previously been published (to the author's knowledge). In the present study, the reproducibility of the glycaemic response to the test meal was good. Although the correlations in glycaemic response between Trials 1 and 2 were not as high as those observed for the lipaemic response (Spearman rank order correlation coefficient: 0.88, Pearson product moment correlation coefficient: 0.94), these were still high in absolute terms and the mean (\pm SEM) difference between trials was just 1.9 ± 1.6 %, suggesting that the resolution of the technique to detect differences in glycaemia was good. However, as the pancreas secretes insulin to maintain glucose homeostasis, the reproducibility of the glucose response would, almost by definition, be good and differences in insulin-glucose dynamics would be reflected more in the insulin concentrations. The reproducibility of the insulinaemic response was poorer than that observed for either the lipaemic or glycaemic responses in the present study. However, this reproducibility was still comparable to, or better than, the reproducibility of the lipaemic responses in previously published data (Spearman rank order correlation coefficient: 0.81, Pearson product moment correlation coefficient: 0.95, mean difference between trials: 3.5 %). The standard error of the difference in insulinaemia between trials was 10.5 % for the eight subjects studied. Thus, although the reproducibility of the insulinaemic response to the test meal was poorer than the reproducibility of the TAG response, it should be sufficient to allow detection of physiologically important differences in insulinaemia between trials in an intervention study, especially if larger subject numbers were studied.

The reproducibility of plasma and serum concentrations in the fasted state followed a similar pattern to the respective postprandial responses with standard errors of the difference between trials being less than 5 % for the fasting TAG, glucose, total cholesterol and HDL cholesterol concentrations but 10.9 % for the fasting insulin

concentration. Thus, as for the postprandial responses, the reproducibility of plasma and serum concentrations in the fasted state appears to be adequate to detect physiologically important intervention effects.

In conclusion, this study has shown the reproducibility of the oral fat tolerance test protocol in this laboratory to be good. The agreement between the lipaemic responses from two identical oral fat tolerance tests was better than observed in previously published data and the data on the reproducibility of the insulinaemic and glycaemic responses to a high-fat mixed test meal are the first available. Although the reproducibility of the insulinaemic response was poorer than that seen with lipaemia, this was sufficient to detect a physiologically important effect of exercise, particularly if more than eight subjects were studied.

CHAPTER 4

POSTPRANDIAL LIPAEMIA: EFFECTS OF EXERCISE AND ENERGY INTAKE RESTRICTION COMPARED

4.1 Introduction

Several studies have demonstrated that a single session of exercise, performed prior to the ingestion of a fat-rich meal, attenuates postprandial lipaemia (Aldred et al. 1994; Tsetsonis and Hardman, 1996a; Tsetsonis and Hardman, 1996b; Tsetsonis et al. 1997; Zhang et al. 1998; Gill et al. 1998; Malkova et al. 1999), and the magnitude of this reduction appears to be linked to the energy expended during the exercise session (Tsetsonis and Hardman, 1996a; Tsetsonis and Hardman, 1996b; Gill et al. 1998; Malkova et al. 1999). However, in all these investigations food intake was standardised during the day prior to the oral fat tolerance test so that exercise brought about a short-term energy deficit, relative to the control trials.

Intuitively, it is possible that in response to an energy deficit, the body would clear TAG more rapidly from the circulation in order to replace energy stores. Increased activity of LPL – the rate limiting enzyme for TAG hydrolysis (Taskinen and Kuusi, 1987a) – may play a role in this process. In humans it has been shown that after restriction of energy intake the postprandial rise in adipose tissue LPL activity was increased (Taskinen and Nikkilä, 1987b). Moreover, in the postprandial state, adipose tissue LPL activity was higher in previously energy-restricted rats than in control rats fed *ad libitum* (Fried et al. 1983; Cruz and Williamson, 1992). In addition, body mass loss – a consequence of prolonged energy deficit – has been shown to increase adipose tissue LPL activity (Schwartz and Brunzell, 1981). Consequently, it is possible that the attenuation of lipaemia by exercise is attributable to the associated energy deficit rather than exercise *per se*. The purpose of this study was therefore to compare the effects on postprandial lipaemia of prior exercise and of an equivalent energy deficit induced by restriction of food intake.

Postmenopausal women were studied because, despite the popular perception that coronary heart disease (CHD) is a male disease, it is responsible for 20 % of deaths in women, making it the leading cause of female mortality (Office for National Statistics, 1998). With the onset of menopause women lose much of the

cardioprotective effect of endogenous oestradiol and their CHD risk approaches that of males (Sotelo and Johnson, 1997); thus, any intervention to reduce CHD risk is particularly relevant for this group. In addition, the effects of cyclical hormonal concentrations on lipid and carbohydrate metabolism may confound the study of women prior to menopause.

4.2 Subjects and Methods

4.2.1 Subjects

Twelve moderately active but untrained postmenopausal (amenorrhoea for >2 years) female volunteers participated in the study, which was conducted with the approval of the Loughborough University Ethical Advisory Committee. All subjects were fully informed of the procedures and risks involved and gave their written consent to take part. One subject did not complete the study so data are presented for 11 women. All women were apparently healthy non-smokers and none was receiving hormone replacement therapy or any drugs thought to affect lipid or carbohydrate metabolism. Nine subjects exhibited the commonest E3/E3 apo E phenotype, one subject possessed the E3/E2 phenotype and one possessed the E4/E3 phenotype. Some physical characteristics of the subjects (n = 11) are presented in Table 4.1.

Age (year)	60.2 ± 3.8
Height (m)	1.60 ± 0.08
Body mass (kg)	64.1 ± 7.3
Sum of four skinfolds (mm)	60.9 ± 11.5
Estimated fat-free mass (kg)	41.2 ± 5.4
BMI (kg.m ⁻²)	24.8 ± 1.3
Estimated $\dot{V}O_2$ max (ml.kg ⁻¹ .min ⁻¹)	30.7 ± 3.2
TAG, in the fasted state (mmol.l ⁻¹)	1.00 ± 0.31
Total cholesterol, in the fasted state (mmol.l ⁻¹)	5.78 ± 0.88
HDL cholesterol, in the fasted state (mmol.l ⁻¹)	1.43 ± 0.40

Table 4.1 Physical characteristics of subjects (n = 11), (mean ± standard deviation)

4.2.2 Preliminary testing

Anthropometric measurements were made as described in section 3.3. Subjects were familiarised with treadmill walking (section 3.6) and then, on a separate occasion, performed a submaximal incremental treadmill test to estimate $\dot{V}O_2$ max and determine the gradient necessary to elicit 60 % of this estimated maximum at their self-selected speed (section 3.6.1.).

On a separate visit, subjects performed a test designed to estimate the net energy expenditure of a 90-minute walk at approximately 60% of estimated $\dot{V}O_2$ max. When they arrived at the laboratory subjects sat quietly for 30 minutes to ensure that they were in a rested state. A six-minute expired air sample was then taken to estimate their resting metabolic rate by indirect calorimetry (section 3.4). Subjects then walked on the treadmill for 90 minutes at an intensity of approximately 60% estimated $\dot{V}O_2$ max (section 3.6.3). Upon completion of the walk, subjects were seated and expired air samples were collected continuously for 15 minutes in order to estimate the excess post-exercise oxygen consumption (a pilot study indicated that oxygen consumption had returned to baseline values within this time period). The net energy expenditure of exercise (i.e. the energy expenditure over and above the resting

metabolic rate) was calculated on an individual basis using indirect calorimetry (section 3.4).

Subjects weighed and recorded their diet for three days (two weekdays and one weekend day) prior to the study so that their normal dietary intake could be assessed. Food diaries were analysed using a computerised version (Comp-Eat 5, Nutrition Systems, London) of food composition tables (Holland et al. 1991). The mean (\pm SEM) reported energy intake for the subjects was 7.3 ± 0.4 MJ per day. Although this was slightly lower than the estimated energy requirements for women of this age and body mass (~ 8.2 MJ per day) (James and Schofield, 1990), the subjects informed the author that their food diaries provided an accurate representation of their habitual diets.

4.2.3 Study protocol

Subjects participated in three trials; a control trial, an exercise trial and an energy intake restriction trial, in a balanced design at intervals of approximately 7 days. Each trial was conducted over 2 days, with the intervention administered on day 1 and an oral fat tolerance test on day 2. The study protocol is shown in Figure 4.1.

On day 1 of the control trial, subjects refrained from exercise and carried out only the activities of daily living. They were requested to sit quietly for 90 minutes during the afternoon throughout the period during which they would be walking in the exercise trial, as the additional energy expenditure of the walk was calculated relative to sitting. They consumed only food provided by the experimenters, the energy value of which had been calculated to correspond with their normal dietary intake. For all subjects, 50 % of energy came from carbohydrate, 36 % from fat and 14 % from protein.

On day 1 of the exercise trial, subjects attended the laboratory during the afternoon (midway between lunch and dinner) and walked on the treadmill for 90 minutes at the same speed and gradient as they did in the earlier walk to estimate energy expenditure (section 3.6.3). Subjects consumed the same diet as in the control trial, which was provided by the experimenters.

On day 1 of the energy intake restriction trial, subjects performed only the activities of daily living and sat quietly for 90 minutes during the afternoon (as in the control trial). However, they consumed a diet (provided by experimenters), with the same macronutrient composition as the diets consumed in the other two trials, but reduced in energy by the net energy expenditure of exercise (i.e. energy expended during exercise and the post-exercise period over and above resting energy expenditure). Thus, the energy deficit elicited in this trial (relative to control) was similar to that elicited in the exercise trial. The energy intakes at lunch and dinner were each reduced by half the net expenditure of exercise, so that the deficit occurred at a similar time of day in both the walk and restriction trials.

On day 2 of each trial subjects underwent an oral fat tolerance test as described in section 3.7.1. Blood was sampled and stored as described in section 3.8. The test meal (section 3.7.1.1) provided 1.70 g fat, 1.65 g carbohydrate and 0.25 g protein per kg of fat-free body mass. It was anticipated that there would be large differences in fat mass between females in this age group. It was therefore decided to administer the meal according to fat-free body mass, rather than body mass, to avoid giving fatter individuals, who are often more sedentary and have lower energy intakes than their leaner peers, a disproportionately large fat load.

On the day prior to the intervention in the first trial, subjects weighed and recorded their dietary intake. This diet was replicated prior to subsequent trials. In addition, for the two days prior to the intervention in each trial, subjects refrained from exercise.

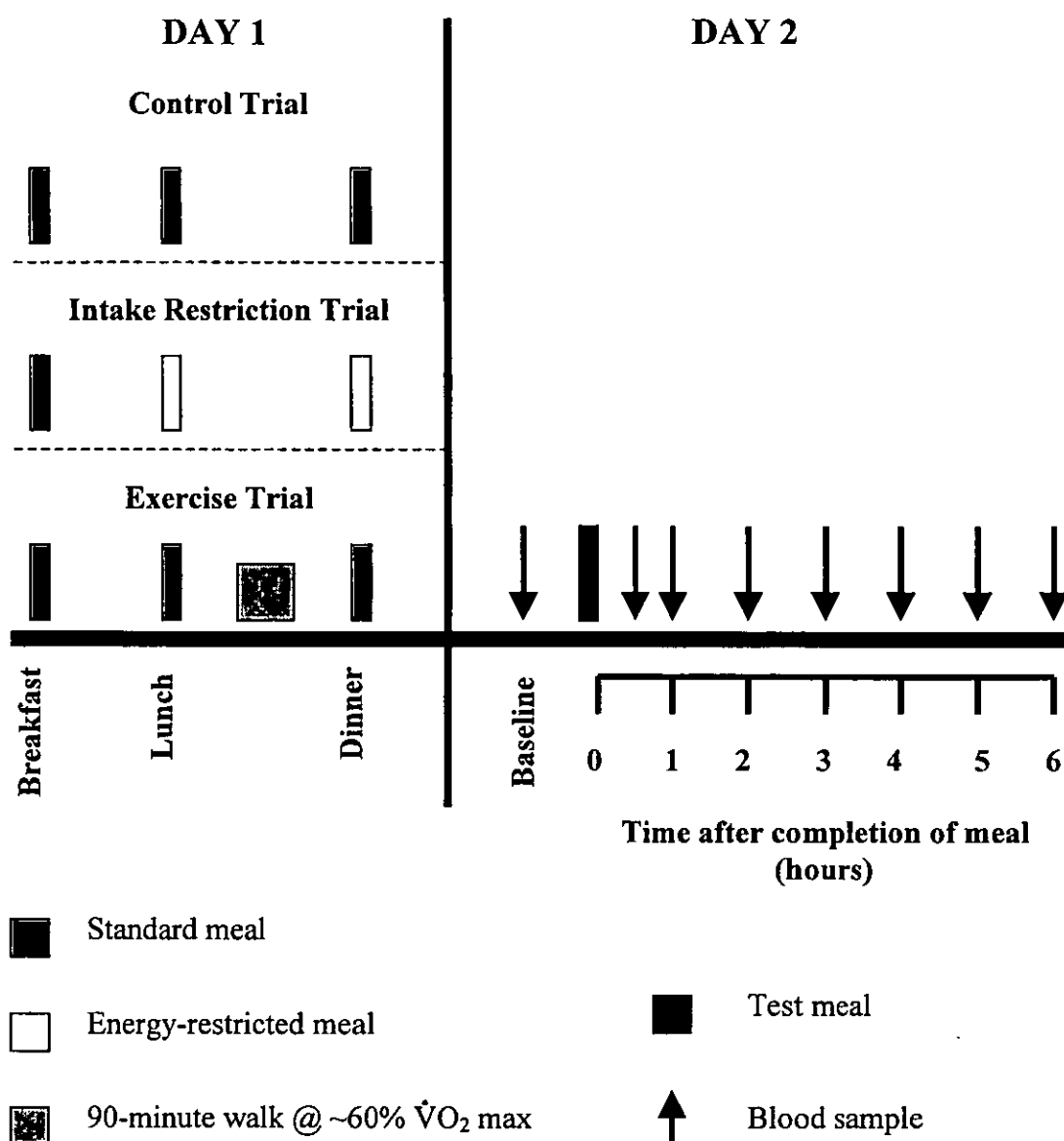


Figure 4.1 Schematic representation of the study protocol

4.2.4 Analysis of blood samples

Haemoglobin concentration and haematocrit were measured at baseline and at the end of each oral fat tolerance test, for estimation of plasma volume changes. Plasma samples were analysed for total cholesterol, HDL cholesterol and glucagon (in the fasted state only), and TAG, NEFA and glucose (in the fasted and postprandial states). Serum samples were analysed for insulin. Details of the analyses are shown in section 3.9.

4.2.5 Data analysis

The areas under the plasma or serum concentration vs. time curves, calculated using the trapezium rule, were defined as the respective total responses to the test meal, with these areas normalised to the baseline concentrations defined as the incremental responses. Means were compared using one-way ANOVA for correlated means, with post-hoc Tukey tests to identify where the differences lay. Statistical significance was accepted at the 5% level and data are presented as mean \pm SEM.

4.3 Results

4.3.1 Responses during treadmill walking.

Figure 4.2 shows oxygen uptake at rest prior to, during and in the recovery from the walk to estimate energy expenditure. Mean oxygen uptake during the walk was $19.0 \pm 0.6 \text{ ml.kg}^{-1}.\text{min}^{-1}$ which represented $62.2 \pm 1.6 \%$ of estimated $\dot{V}\text{O}_2$ max. From the graph it is clear that oxygen uptakes had returned to baseline values within 15 minutes of the cessation of exercise. As the Falconia tubing used in the collection of expired air samples was later discovered to be permeable, the data were corrected to provide true values for $\dot{V}\text{O}_2$ and $\dot{V}\text{CO}_2$ (section 3.4.1). Two different pieces of Falconia tubing were used in this data collection and it was not known when each tube was used. Thus, correction factors for both tubes were applied to each expired air value and the mean of the two corrected values was adopted. The additional error incurred by this uncertainty was small; mean $\dot{V}\text{O}_2$ was calculated as $19.4 \pm 0.6 \text{ ml.kg}^{-1}.\text{min}^{-1}$ using the correction factors for one tube and $18.7 \pm 0.5 \text{ ml.kg}^{-1}.\text{min}^{-1}$ using the factors for the other. After correction it was found that the net energy expenditure of this walk, including the post-exercise elevation of metabolic rate was $1.73 \pm 0.08 \text{ MJ}$, compared with the originally determined value of $1.44 \pm 0.08 \text{ MJ}$.

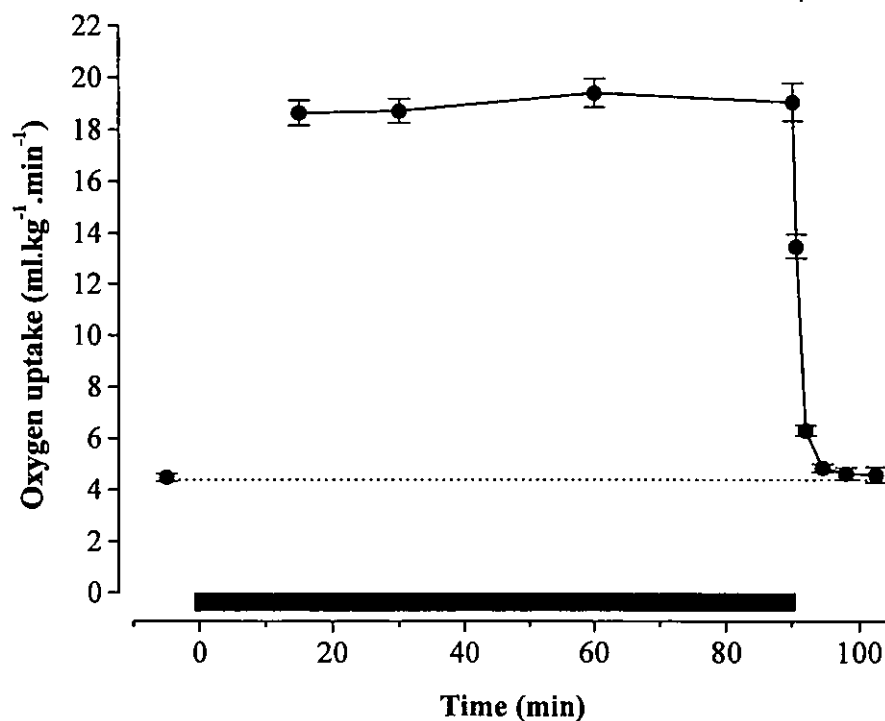


Figure 4.2 Oxygen uptake at rest prior to, during and in the recovery from the 90-minute walk to estimate energy expenditure of 90 minutes of brisk walking. The rectangle denotes the walk and the dotted line denotes the resting oxygen uptake.

The mean $\dot{V}O_2$ during the 90-minute walk on day one of the exercise trial (19.3 ± 0.6 ml.kg⁻¹.min⁻¹, which represented 62.9 ± 1.7 % estimated $\dot{V}O_2$ max), was similar to that in the preliminary walk to estimate energy expenditure. The mean heart rate was 130 ± 1.9 beat.min⁻¹. The subjects rated the walk as 12.1 ± 0.3 on the Borg scale, which corresponds to a perception of exertion between 'fairly light' and 'somewhat hard' (Borg, 1973). All subjects completed the walk without difficulty.

4.3.2 Exercise and dietary-induced energy deficits

The energy intakes on day one of the control, exercise and intake restriction trials were 7.48 ± 0.41 MJ, 7.47 ± 0.41 MJ and 6.04 ± 0.41 MJ respectively, resulting in an energy deficit of 1.44 ± 0.08 MJ in the intake restriction trial, relative to control.

Although the aim of the experiment was to produce a dietary energy deficit equivalent to the one induced by exercise, this was not quite achieved as the true energy expenditure of the exercise session was not known until after data collection was completed. The energy deficit incurred in the energy intake restriction trial was approximately 17 % smaller than the 1.73 ± 0.08 MJ energy deficit produced by exercise.

4.3.3 Estimation of plasma volume changes

Mean haemoglobin concentrations, haematocrits and plasma volumes (relative to plasma volume in control trial equalling 100) in the first, second and third trials completed are shown in Table 4.2. There were significant reductions in both haemoglobin concentration and haematocrit with successive trials. When these data were used to estimate changes in plasma volume using the method described by Dill and Costill (1974), plasma volumes were apparently increasing in consecutive trials. However, approximately 110 ml of blood (~2-3 % of total blood volume) were sampled in each trial, and the trials were separated by only one week. Thus, it is likely that the red cells removed were not completely replaced between trials and that the decreasing haemoglobin concentrations and haematocrits reflected reductions in the amount of circulating haemoglobin rather than increases in plasma volume. The fundamental assumption of the Dill and Costill method is that the amount of circulating haemoglobin is constant; thus, it would be inappropriate to use the method to correct concentrations for differences in plasma volumes when the validity of this assumption was in doubt. It was therefore decided not to correct for plasma volume differences.

	Trial 1	Trial 2	Trial 3
Haemoglobin (g.dL ⁻¹)	13.1 ± 0.2	12.6 ± 0.2	12.4 ± 0.3*
Haematocrit (%)	38.2 ± 0.7	36.9 ± 0.7*	36.7 ± 0.6*
Plasma volume (% of control trial value)	92.7 ± 2.6	99.7 ± 3.4*	101.3 ± 1.9*

Table 4.2 Mean haemoglobin concentration, haematocrit and plasma volume in Trials 1, 2 and 3. *Significantly different from Trial 1 ($p < 0.05$)

4.3.4 Plasma and serum concentrations in the fasted state.

Table 4.3 shows plasma and serum concentrations in the fasted state. Plasma TAG concentrations were significantly lower in the exercise trial than the control trial (by $18 \pm 2\%$) and the intake restriction trial (by $14 \pm 4\%$) (both $p < 0.05$), and plasma NEFA concentrations were $26 \pm 7\%$ higher in the exercise trial than the control trial ($p < 0.05$). Plasma NEFA concentrations were $18 \pm 9\%$ higher in the intake restriction trial than control, but this was not statistically significant ($p = 0.20$). Plasma total cholesterol, HDL cholesterol, glucose and glucagon concentrations and serum insulin concentrations did not differ between trials.

	Control	Intake restriction	Exercise
TAG (mmol.l ⁻¹)	1.00 ± 0.09	0.98 ± 0.12	0.81 ± 0.08 ^{a,b}
NEFA (mmol.l ⁻¹)	0.39 ± 0.04	0.45 ± 0.04	0.49 ± 0.05 ^a
Glucose (mmol.l ⁻¹)	4.46 ± 0.04	4.50 ± 0.03	4.54 ± 0.02
Insulin (μU.ml ⁻¹)	8.2 ± 0.3	8.2 ± 0.2	8.0 ± 0.2
Glucagon (ng.l ⁻¹)	93.4 ± 5.0	93.0 ± 3.6	89.4 ± 5.0
Total cholesterol (mmol.l ⁻¹)	5.78 ± 0.27	5.90 ± 0.24	5.72 ± 0.27
HDL cholesterol (mmol.l ⁻¹)	1.43 ± 0.12	1.45 ± 0.14	1.47 ± 0.14

Table 4.3 Plasma and serum concentrations measured in the fasted state in the control, intake restriction and exercise trials. ^aSignificantly different from control trial ($p < 0.05$), ^bSignificantly different from intake restriction trial ($p < 0.05$).

4.3.5 Postprandial plasma and serum concentrations.

Figure 4.3 shows the plasma TAG responses to the test meal in the three trials. The total and incremental lipaemic responses are shown in Figure 4.4. Both the total and incremental lipaemic responses were lower in the exercise trial than in the control trial ($p < 0.05$). Although the total lipaemic response was not significantly attenuated by intake restriction ($p = 0.17$), there was a tendency for the incremental lipaemic response to be lower than control ($p = 0.07$). Numerically, the reduction in the total lipaemic response by exercise was almost three times as great as the reduction in the intake restriction (2.14 mmol.l⁻¹.h vs. 0.79 mmol.l⁻¹.h), but the reductions in the incremental lipaemic response were more similar between the two interventions (Exercise: 1.03 mmol.l⁻¹.h, Intake restriction: 0.68 mmol.l⁻¹.h).

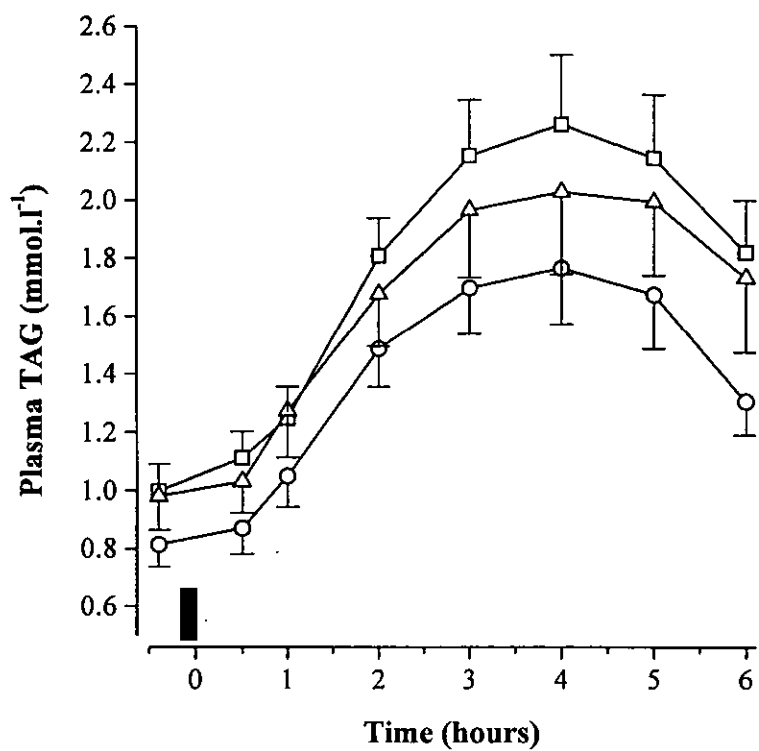


Figure 4.3 Plasma TAG concentrations in the fasted state and for 6 hours after consumption of test meal in the control (\square), intake restriction (Δ) and exercise (O) trials. Black rectangle denotes ingestion of test meal.

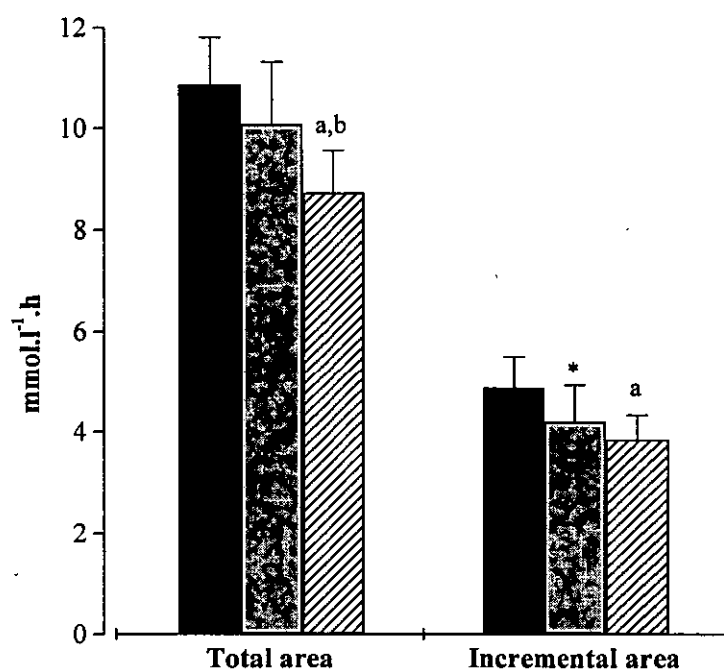


Figure 4.4. Total and incremental lipaemic responses in the control (black), intake restriction (grey) and exercise (hatched) trials. ^aSignificantly different from control trial ($p < 0.05$), ^bSignificantly different from intake restriction trial ($p < 0.05$), ^{*} $p = 0.07$ compared with control.

Figures 4.5, 4.6 and 4.7 show the concentrations in the fasted state and following the test meal for plasma glucose, serum insulin and plasma NEFA, respectively, with Table 4.4 showing the summary responses. The total glycaemic response tended to be higher than control in the exercise trial ($p = 0.07$), but the incremental glycaemic response did not differ between trials. The total and incremental insulinaemic responses were lower in the exercise trial than both the control and intake restriction trials ($p < 0.05$), and the total NEFA response was higher in the exercise trial than control ($p < 0.05$). In the exercise trial total NEFA response also tended to be higher than in the intake restriction trial ($p = 0.07$).

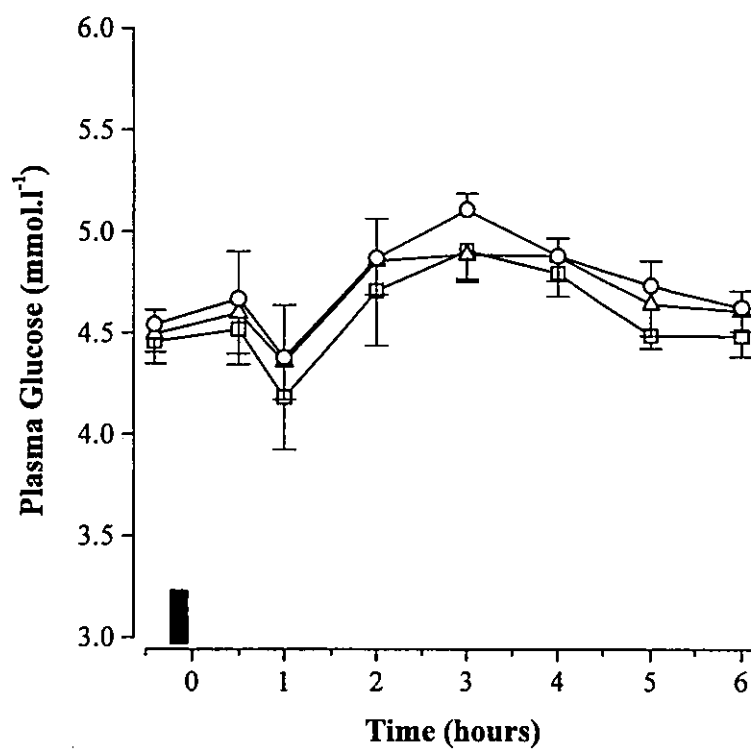


Figure 4.5 Plasma glucose concentrations in the fasted state and for 6 hours after consumption of test meal in the control (□), intake restriction (Δ) and exercise (O) trials. Black rectangle denotes ingestion of test meal.

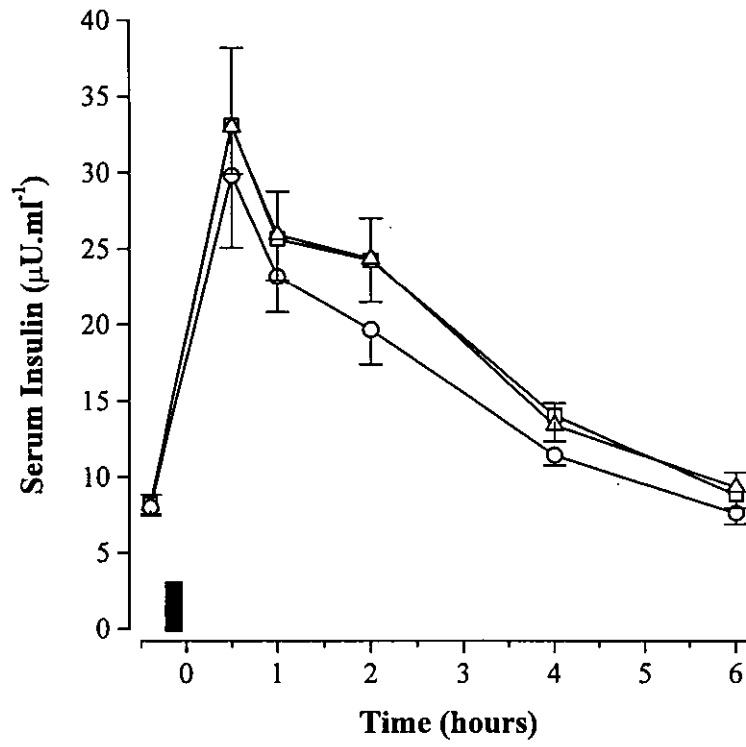


Figure 4.6 Serum insulin concentrations in the fasted state and for 6 hours after consumption of test meal in the control (\square), intake restriction (Δ) and exercise (\circ) trials. Black rectangle denotes ingestion of test meal.

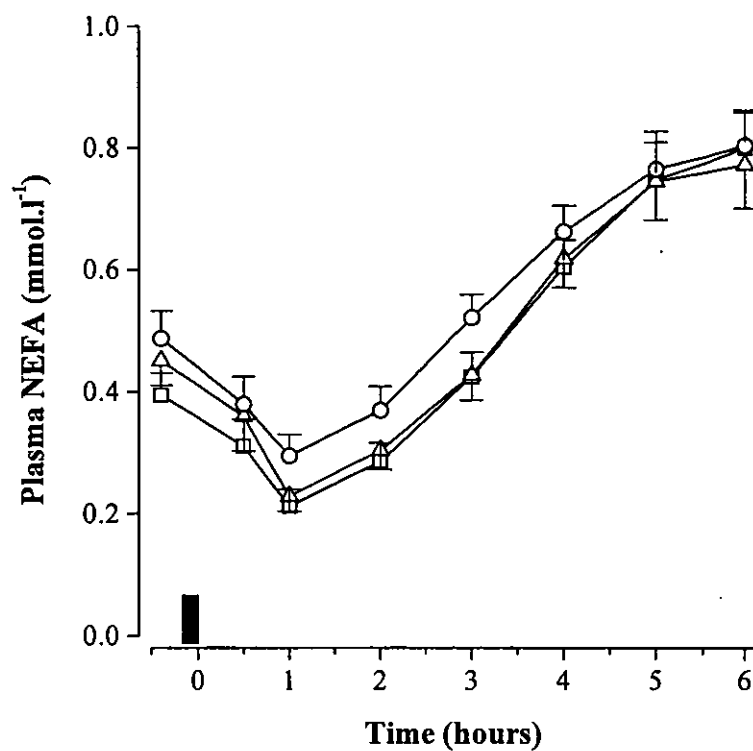


Figure 4.7 Plasma NEFA concentrations in the fasted state and for 6 hours after consumption of test meal in the control (□), intake restriction (Δ) and exercise (O) trials. Black rectangle denotes ingestion of test meal.

	Control	Intake restriction	Exercise
Total glycaemic response (mmol.l ⁻¹ .h)	27.3 ± 0.3	28.1 ± 0.2	28.4 ± 0.2 [#]
Incremental glycaemic response (mmol.l ⁻¹ .h)	1.1 ± 0.2	1.6 ± 0.2	1.6 ± 0.2
Total insulinaemic response (μU.ml ⁻¹ .h)	108.6 ± 3.6	107.9 ± 2.9	92.5 ± 2.3 ^{a,b}
Incremental insulinaemic response (μU.ml ⁻¹ .h)	59.2 ± 7.5	59.0 ± 7.4	44.4 ± 7.3 ^{a,b}
Total NEFA response (mmol.l ⁻¹ .h)	2.87 ± 0.17	2.95 ± 0.18	3.27 ± 0.20 ^{a,*}

Table 4.4 Glycaemic, insulinaemic and NEFA responses to the test meal in the control, intake restriction and exercise trials. ^aSignificantly different from control ($p < 0.05$), ^bSignificantly different from intake restriction ($p < 0.05$), [#] $p = 0.07$ with control, ^{*} $p = 0.07$ with intake restriction.

4.4 Discussion

There were two main findings in this study. The first was that a single moderate exercise session performed some hours prior to the consumption of a high-fat mixed meal attenuated postprandial lipaemia in this group of postmenopausal female subjects. This adds to the body of evidence which has demonstrated that moderate exercise can attenuate lipaemia in a number of different populations including, young male adults (Aldred et al. 1994; Tsetsonis and Hardman, 1996a; Tsetsonis and Hardman, 1996b; Gill et al. 1998; Malkova et al. 1999), young female adults (Aldred et al. 1994; Tsetsonis and Hardman, 1996a; Tsetsonis and Hardman, 1996b) and middle-aged trained and untrained females (Tsetsonis et al. 1997). Previous research suggests that, for an individual, the energy expended during an exercise session is a major determinant of the magnitude of the exercise effect on postprandial lipaemia (Tsetsonis and Hardman, 1996; Tsetsonis and Hardman, 1996; Gill et al. 1998; Malkova et al. 1999). Thus, it was encouraging to see that these untrained, older

women – with lower functional capacities for exercise than their younger, fitter counterparts – were able to reduce lipaemia by performing an amount of exercise which they could manage comfortably.

The second main finding was that the exercise-induced reduction in postprandial lipaemia was almost three times as great as the reduction due to a similar dietary-induced energy deficit. In addition, the effect of exercise on lipid and carbohydrate metabolism was qualitatively, as well as quantitatively, different from the effect of intake restriction. Only exercise reduced TAG concentrations in the fasted state and reduced postprandial insulin concentrations. In the fasted state, the plasma NEFA concentrations were only significantly elevated by exercise and only exercise resulted in elevated postprandial NEFA concentrations. Thus, it is clear that the action of exercise on postprandial lipid metabolism was different from the effect of an energy deficit.

Due to the problem with the Falconia tubing, the energy deficit incurred in the exercise trial was slightly greater than that induced by energy intake restriction. However, the effect of exercise was different in nature from the effect of intake restriction, and so much greater, that the small difference between the two energy deficits does not affect the interpretation of the results. Since the effect of exercise on postprandial lipaemia is closely related to the energy expended during exercise (Tsetsonis and Hardman, 1996a; Tsetsonis and Hardman, 1996b), the slightly differing deficits between the two interventions would result in only slightly different effects on lipaemia if the mechanisms via which both acted were the same.

Exercise, but not intake restriction, reduced TAG concentrations in the fasted state. This reduction could have been due to increased clearance of TAG from the circulation into peripheral tissues, or reduced hepatic VLDL secretion. Evidence exists in the literature supporting increased TAG clearance following prolonged exercise (Dufaux et al. 1981; Sady et al. 1986; Annuzzi et al. 1987) and, in rats, there are data suggesting that TAG secretion is reduced following exercise training (Simonelli and Eaton, 1978; Mondon et al. 1984). However, from the present data it is not possible to ascertain the relative importance of the possible TAG attenuating mechanisms, although it is clear that the effect was not mediated by energy deficit.

In the fasted state, NEFA concentrations were significantly elevated in the exercise trial relative to control and numerically, although not significantly, elevated in the intake restriction trial. In the exercise trial low muscle glycogen concentrations may have mediated the increased plasma NEFA concentrations (Weltan et al. 1998). Alternatively, elevated growth hormone concentrations during exercise (Hodgetts et al. 1991) and in response to intake restriction (Carlson et al. 1994), may have contributed to the elevated NEFA concentrations in the two interventions, as the lipolytic effect of this hormone is delayed and acts over several hours (Møller et al. 1990).

Exercise reduced both the total and incremental lipaemic responses relative to control, and the total response was also attenuated relative to intake restriction. As in the fasted state, this reduction could have been due to increased hydrolysis of circulating TAG mediated by increased activity of LPL, or reduced secretion of VLDL from the liver. Again, from the present data it is not possible to determine the relative importance of these two mechanisms. Theoretically, it is also possible that the appearance of chylomicrons into the circulation was delayed in the exercise trial. This seems unlikely, however, as peak TAG concentrations occurred at a similar time in the all three trials.

Intake restriction did not reduce the total lipaemic response to the test meal relative to control, but the incremental lipaemic response tended to be attenuated ($p = 0.07$). The finding that intake restriction only moderated the postprandial rise in plasma TAG and did not influence TAG concentrations in the fasted state is consistent with the literature which suggests that energy intake restriction promotes an increased postprandial rise in adipose tissue LPL activity and not an increase in activity in the fasted state (Fried et al. 1983; Taskinen and Nikkilä, 1987b; Cruz and Williamson, 1992).

The uptake of TAG from circulating chylomicrons and VLDL is a two-stage process. Lipoprotein-TAG is first hydrolysed by the action of LPL on the capillary endothelium, liberating NEFA. These NEFA then move into the adipocyte where they are entrapped and re-esterified. Retention of these fatty acids in adipose tissue is

not complete. Frayn and co-workers (1994) found that after consumption of a high-fat mixed meal, which was of similar size and composition to the meal used in the present study (80 g fat, 80 g carbohydrate, 18 g protein), about half of the fatty acids derived from lipoprotein-TAG hydrolysis were not entrapped into adipose tissue and 'spilt over' into the circulation (Frayn et al. 1994). Indeed, the majority of plasma NEFA in the postprandial state appear to arise from the action of LPL (Frayn et al. 1996a). In the exercise trial, postprandial plasma NEFA concentrations were higher than in the control trial ($p < 0.05$) and tended to be higher than in the intake restriction trial ($p = 0.07$). This might imply a greater 'spill-over' of NEFA into the circulation in the exercise trial – which could have been due to an increased action of adipose tissue LPL, without a corresponding increase in esterification within the adipocyte, or reduced re-esterification with similar LPL activity. Both possibilities are plausible and they are not mutually exclusive, but if the former mechanism were working exclusively we might expect elevated NEFA in the intake restriction trial compared with control as it is likely that adipose tissue LPL activity was increased in intake restriction trial, and this was not seen. The postprandial insulin response was lower in the exercise trial compared with control and intake restriction. As insulin is a potent stimulator of the re-esterification of NEFA in the adipocyte (Sniderman et al. 1998), it is not unreasonable to speculate that uptake of NEFA into the adipocyte was reduced in the exercise trial. Alternatively, the elevated NEFA concentrations in the exercise trial might have been due to increased lipolysis of adipose tissue TAG. This could also have been mediated by the lower postprandial insulin concentrations in the exercise trial, as insulin inhibits the action of hormone sensitive lipase (Coppack et al. 1992). Whatever the relative contributions of these two mechanisms to the elevated plasma NEFA concentrations in the exercise trial, the implications would be the same: namely, that in the exercise trial, TAG was being partitioned away from storage in the adipose tissue into the plasma NEFA pool where it could be directed to muscle and the liver for oxidation and/or to replace depleted intramuscular TAG (Kiens and Richter, 1998), or – speculatively – depleted hepatic cytoplasmic TAG stores. Clearly, this might have implications for obesity and the regulation of body fatness. Thus the elevated NEFA concentrations in the exercise trial, compared with the control and intake restriction trials, appear to be entirely appropriate, in contrast to the inappropriately elevated NEFA concentrations associated with the insulin resistance syndrome (Frayn et al. 1996b).

The total glucose response was somewhat higher in the exercise trial than control ($p = 0.07$), which might have been a consequence of the elevated NEFA concentrations reducing the uptake of glucose into muscle via the glucose-fatty acid cycle (Randle et al. 1963). Thus one might argue that the present data indicate a reduction in insulin sensitivity in the exercise trial. However, both the total and incremental insulinaemic responses to the test meal were lower in the exercise trial than in the intake restriction or control trials and the incremental glycaemic responses did not differ between trials. This suggests improved insulin sensitivity in the exercise trial because a lower insulin concentration was needed to control the rise in plasma glucose concentrations. The latter interpretation is probably more valid, as a slightly reduced uptake of glucose into muscle when insulin concentrations are markedly reduced is not inconsistent with improved insulin sensitivity.

It is clear that prior moderate exercise and a dietary energy deficit of similar magnitude did not have comparable effects on fasted and postprandial lipid metabolism. However, it is still possible that the effect of exercise was a consequence of energy deficit, but at a tissue rather than whole body level. A dietary-induced energy deficit shifts metabolism towards fasting. Thus, to compensate, fatty acids are released from adipose tissue, resulting in a reduction in adipose tissue stores, and liver glycogen is converted to glucose and released into the circulation, reducing liver glycogen stores (Cahill, 1970). However, the fuel requirements of exercise are met by muscle glycogen and intramuscular TAG (Romijn et al. 1993), in addition to circulating NEFA and liver glycogen. Indeed, exercise might even have reduced fuel depots not usually considered, such as hepatic cytoplasmic TAG stores. Thus, exercise is likely to have induced fuel deficits, which, although quantitatively similar to those induced by energy intake restriction, were qualitatively different in terms of fuel types and tissues affected. It is possible that the mitigating effect of exercise on postprandial lipaemia might be linked to the state of energy deficit in specific fuel depots. Thus, increasing the energy expenditure of exercise would increase its attenuation of postprandial lipaemia by placing the relevant fuel depots into a greater negative energy balance. Conversely, the energy deficit induced by energy intake restriction would not target the appropriate fuel depots and thus would not have an equivalent effect on fasting and postprandial lipid metabolism. While, speculatively,

fuel deficits in key depots might mediate the exercise-induced attenuation to lipaemia, the location and type of fuel depot involved are not immediately obvious. If the predominant effect of exercise were the upregulation of LPL activity in skeletal muscle, then fuel deficits in this tissue might be key. However, if exercise were primarily affecting hepatic secretion of VLDL, then fuel deficits in the liver might mediate.

Alternatively, exercise might be influencing TAG metabolic capacity by mechanisms other than energy deficit at a tissue level. Further exploration into the qualitative and quantitative effects of exercise on fasting and postprandial lipid metabolism might not only help elucidate the mechanisms via which exercise is acting, but also help explain why the effects of exercise and energy intake restriction are so different.

A high TAG metabolic capacity – revealed by low postprandial lipaemia – is an important determinant of elevated plasma HDL concentrations, particularly in the HDL₂ subfraction (Patsch et al. 1983). HDL concentrations can increase (Williams et al. 1994) or decrease (Thompson et al. 1979) with dietary-induced weight loss, but exercise can elevate HDL concentrations with (Wood et al. 1991) or without a loss of body fat (Thompson et al. 1997). This supports the data from the present study suggesting that, in the long term, although energy deficit may improve TAG metabolism, the mechanisms via which exercise enhances TAG metabolic capacity are independent of, or at least additive to, the effects of whole body energy deficit.

CHAPTER 5

EFFECTS OF MODERATE EXERCISE ON POSTPRANDIAL LIPOPROTEIN METABOLISM AND METABOLIC PARTITIONING OF [1,1,1-¹³C] TRIPALMITIN

5.1 Introduction

Although the attenuating effect of prior moderate exercise on postprandial plasma TAG concentrations has been well established (Aldred et al. 1994; Tsetsonis and Hardman, 1996a; Tsetsonis and Hardman, 1996b; Zhang et al. 1998; Gill et al. 1998; Malkova et al. 1999), a detailed description of the qualitative changes to postprandial TAG metabolism elicited by a single session of moderate exercise is lacking.

In the fasted state, where VLDL are the only circulating TAG-rich lipoproteins, both exercise training (Després et al. 1990) and a vigorous exercise session (Dufaux et al. 1981; Baumstark et al. 1993) have been shown to reduce VLDL-TAG concentrations. However, in the postprandial state, where chylomicrons and VLDL compete for clearance by a common saturable pathway (Brunzell et al. 1973; Björkegren et al. 1996), the data are limited. Studies have shown that exercise training, with (Drexel et al. 1992) or without (Weintraub et al. 1989) weight loss, can produce large reductions to the postprandial chylomicron response. In addition, recent work from this laboratory has shown that a single moderate exercise session reduced TAG concentrations by 53 % in a 'chylomicron-rich' fraction and by 17 % in a 'non-chylomicron-rich' TAG fraction (Herd et al. 1997). Although the absolute TAG reduction in the two fractions was similar ($\sim 1 \text{ mmol.l}^{-1}.\text{h}$), the decrease was only statistically significant for the 'chylomicron-rich' fraction. However, in Herd and co-workers' (1997) study, the lipoprotein fractions were separated using a high-speed refrigerated centrifuge rather than an ultracentrifuge, so definitive quantification of TAG in the chylomicron and VLDL fractions was not possible. Thus, further data investigating the effects of moderate exercise on chylomicron and VLDL are necessary to help elucidate the mechanisms via which this type of exercise reduces lipaemia.

There is a body of evidence suggesting that prolonged vigorous exercise can increase skeletal muscle LPL activity (at least in the fasted state) (Lithell et al. 1979; Taskinen

et al. 1980; Lithell et al. 1984; Lithell et al. 1981), thus, increased hydrolysis and clearance of lipoprotein-TAG into this tissue would be expected following a vigorous exercise session. If moderate exercise was reducing plasma TAG concentrations by the same mechanism, it would be anticipated that reductions in chylomicron-TAG would account for a large proportion of the postprandial plasma TAG attenuation, as chylomicrons are the preferred substrate for LPL in vivo (Potts et al. 1991; Björkegren et al. 1996).

Of course, TAG concentrations in the circulation are determined by the rate of appearance of lipoprotein-TAG into, as well as the rate of clearance of TAG from, the bloodstream. Thus, the lower TAG concentrations seen after moderate exercise might reflect, to some degree, a reduced rate of chylomicron appearance into the circulation. While it is unlikely that the digestion and absorption of the fat would be affected by an exercise session performed over 15 hours prior to ingestion of the meal (as is the case in the present experimental model), there is at present no direct evidence to support this claim. Similarly, it is also possible that a reduced rate of hepatic VLDL secretion might have contributed to the reduced plasma TAG concentrations seen after exercise, but to the author's knowledge there have been no human studies in this area.

There is evidence to suggest that postprandial fat oxidation can be elevated by prior moderate exercise (Tsetsonis and Hardman, 1996b; Tsetsonis et al. 1997), but the nature of this increase is not known. Lipoprotein-TAG cleared into skeletal muscle is likely to be oxidised to meet the tissue's energy demands, whereas TAG cleared into adipose tissue is more commonly stored. Thus, if chylomicron-TAG clearance into skeletal muscle was increased following moderate exercise, a postprandial increase in exogenous fat oxidation would be anticipated. However, to the author's knowledge, there are currently no direct data to suggest whether or not this is the case.

Therefore, the purpose of the present study was to examine the effects of prior moderate exercise on chylomicron-TAG and VLDL-TAG concentrations in the fasted and postprandial states and to determine its effects on exogenous and endogenous fat oxidation. The latter aim was addressed by incorporating a [1,1,1-¹³C] tripalmitin label into the test meal. In addition, the postprandial observation period was extended

from six to eight hours so that the effects of exercise on TAG concentrations in the late postprandial phase could be observed. To provide some insight as to whether gut function might have been affected in the exercise trial, subjects ingested 1.5 g paracetamol with the test meal as a marker of gastric emptying (Heading et al. 1973; Clements et al. 1978).

5.2 Subjects and Methods

5.2.1 Subjects

Twelve male volunteers participated in the study, which was conducted with the approval of the Loughborough University Ethical Advisory Committee. All subjects were fully informed of the procedures and risks involved and gave their written consent to take part. All subjects were apparently healthy non-smokers and none was receiving any drugs thought to affect lipid or carbohydrate metabolism. One subject was hypertriglyceridaemic (fasting TAG 3.88 mmol.l^{-1}) and was excluded from the main analyses; his data are presented separately as a case study in section 5.3.6. Seven subjects (including the hypertriglyceridaemic subject) exhibited the commonest E3/E3 apo E phenotype, three subjects possessed the E4/E3 phenotype and two possessed the E3/E2 phenotype. Some physical characteristics of the subjects ($n = 11$) are presented in Table 5.1.

Age (year)	51.7 ± 6.1
Height (m)	1.75 ± 0.06
Body mass (kg)	74.7 ± 13.5
Sum of 4 skinfolds (mm)	58.0 ± 19.2
BMI (kg.m ⁻²)	24.2 ± 3.7
$\dot{V}O_2$ max (ml.kg ⁻¹ .min ⁻¹)	38.9 ± 5.6
TAG, in the fasted state (mmol.l ⁻¹)	1.03 ± 0.11
Total cholesterol, in the fasted state (mmol.l ⁻¹)	4.84 ± 0.25
HDL cholesterol, in the fasted state (mmol.l ⁻¹)	1.02 ± 0.10

Table 5.1 Physical characteristics of subjects (n = 11), (mean ± standard deviation)

5.2.2 Preliminary testing

Subjects all underwent a clinical exercise stress test (section 3.2) prior to inclusion in the study to ensure they were free from existing cardiovascular disease and had no cardiovascular contraindications to prolonged exercise. Subjects were familiarised with treadmill walking (section 3.6) then, on separate occasions, performed submaximal (section 3.6.1) and maximal (section 3.6.2) incremental exercise tests to find the gradient required to elicit 60 % $\dot{V}O_2$ max.

5.2.3 Study protocol

Subjects participated in two trials, an exercise trial and a control trial, at an interval of 7 days, in a balanced crossover design. Each trial was conducted over 2 days with the intervention administered on day 1 and an oral fat tolerance test on day 2. The protocol is shown in Figure 5.1.

On day 1 of the control trial, subjects did no exercise and performed only the activities of daily living. On day 1 of the exercise trial, subjects performed 90 minutes of treadmill walking in the afternoon, at an intensity of approximately 60 % $\dot{V}O_2$ max (section 3.6.3).

On day 2 subjects performed an oral fat tolerance test as described in section 3.7.1., with blood samples obtained in the fasted state and for 8 hours postprandially (see section 3.8). Plasma and serum were refrigerated or frozen as described in section 3.8. The test meal comprised two parts; a lipid-casein-glucose-sucrose emulsion containing [1, 1, 1-¹³C] tri-palmitin to assess the metabolism of exogenous lipid, and a cereal based meal (section 3.7.1.1) which together provided 1.32 g fat, 1.36 g carbohydrate and 0.30 g protein per kg body mass. Subjects also ingested 1.5 g paracetamol with the meal as a marker of gastric emptying (Heading et al. 1973; Clements et al. 1978). Six-minute expired air samples were collected into Douglas bags in the fasted state and immediately prior to each hourly postprandial blood sample, in order to estimate substrate utilisation and energy expenditure by indirect calorimetry. To assess excretion of ¹³C on the breath, end-tidal breath samples were collected into a 750 ml alveolar breath collection bag (Quintron, Milwaukee, USA) through a one-way valve. Triplicate 10 ml aliquots of expired air were removed from the bag and transferred into evacuated 11 ml gas sample containers (Exetainers; Labco, High Wycombe, UK), using a system of two-way taps, a 10 ml syringe and a needle. These samples were obtained in the fasted state, immediately prior to the hourly blood samples, and 15 and 24 hours after ingestion of the test meal. They were subsequently analysed for ¹³C enrichment by continuous flow isotope ratio mass spectrometry (section 3.10).

After the 8-hour postprandial period, subjects were given a standardised meal, with low natural enrichment of ¹³C and were allowed to leave the laboratory. Subjects were instructed to consume an evening meal with low ¹³C enrichment and not consume breakfast until after the 24-hour end-tidal volume breath sample was taken, to ensure that ¹³C enrichment of the samples was affected by nothing other than the [1,1,1-¹³C] tripalmitin in the test meal.

Subjects weighed and recorded their dietary intake for the two days prior to the first oral fat tolerance test and replicated this prior to the second fat tolerance test. The evening meal consumed after the oral fat tolerance test in the first trial was also repeated in the second trial. In addition, subjects were asked to avoid foods naturally enriched in ¹³C and performed no exercise, other than the treadmill walk in the

exercise trial, for the three days preceding each oral fat tolerance test (see section 3.7.3).

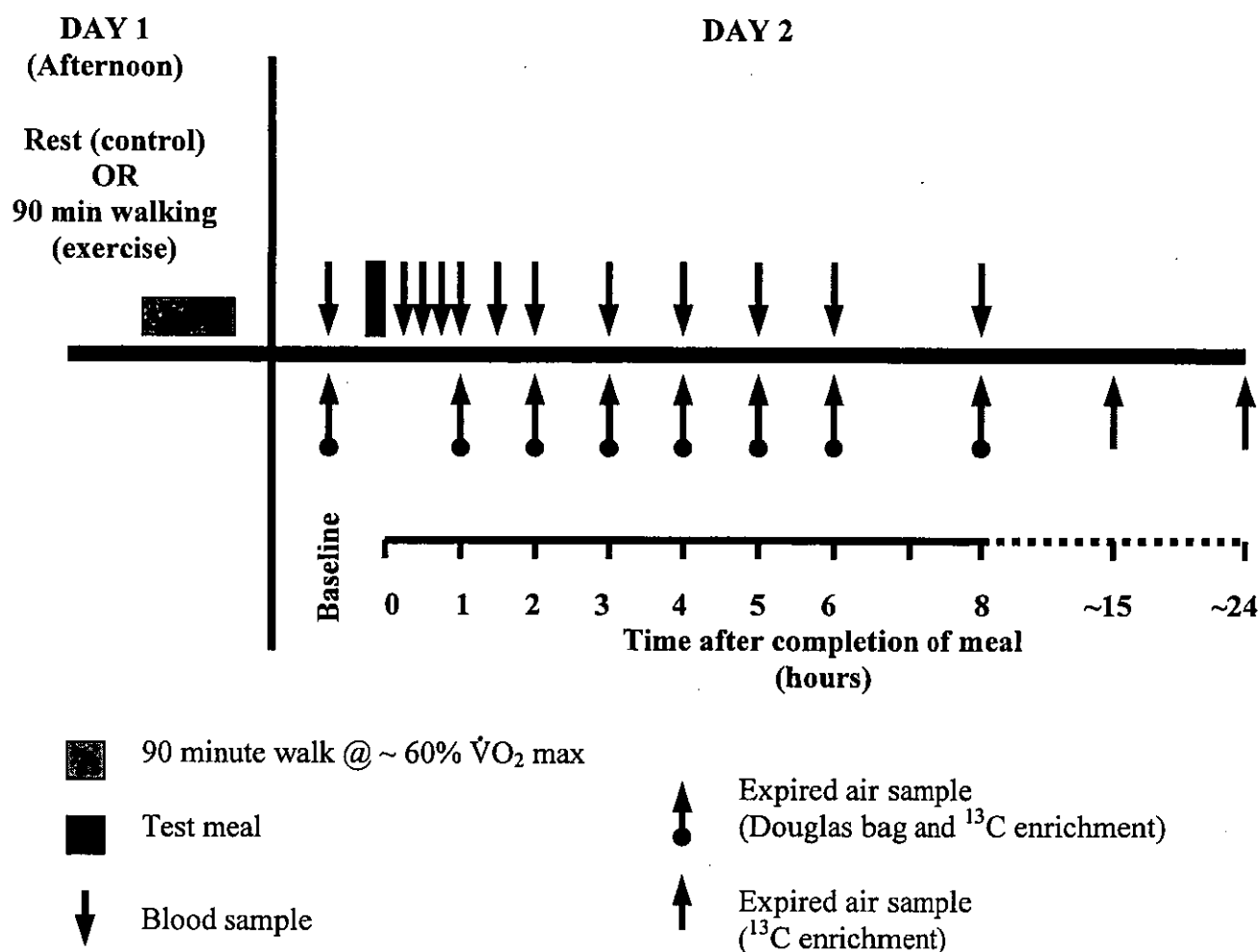


Figure 5.1 Schematic representation of the study protocol

5.2.4 Analysis of blood samples

Haemoglobin concentration and haematocrit were measured at baseline and at the end of each oral fat tolerance test, for estimation of plasma volume changes. Lipoprotein fractionation, with subsequent analysis for TAG, was conducted on fresh plasma samples on the day after sample collection (section 3.9.5). Frozen plasma samples were analysed for total cholesterol and HDL cholesterol (in the fasted state and eight hours postprandially) and for NEFA, glucose and paracetamol (in the fasted and postprandial states). Serum samples were analysed for insulin and 3-hydroxybutyrate. The concentration of plasma ^{13}C palmitic acid was also determined at the 4-hour postprandial time point. Details of the analyses are shown in section 3.9 and 3.10.

5.2.5 Correction of expired air data

As the expired air collections were made using old pieces of Falconia tubing which were permeable to oxygen and carbon dioxide, it was necessary to correct the expired air data to achieve true values for $\dot{V}O_2$ and $\dot{V}CO_2$ (section 3.4.1). This process was complicated by the fact that two different pieces of Falconia tubing were used in the data collection and it was not known exactly when each tube was used. When correction factors for both tubes were applied to each expired air value it was discovered that the two correction factors produced oxygen uptake values differing by approximately 10 %, for resting values. There was an almost identical discrepancy in carbon dioxide production between the two corrections, resulting in near identical respiratory exchange ratios with the two correction factors. Thus, without knowing exactly when each tube was used, it would be possible to determine the ratios of fat to carbohydrate oxidation for each expired air value, but comparisons of rates of fat and carbohydrate oxidation between trials could not be securely made. However, this problem was resolved by making the assumption that the resting oxygen uptakes would be similar in the control and exercise trials. It is unlikely that subjects' metabolic rates would have been lower in the exercise trial compared with the control trial (Tsetsonis and Hardman, 1996; Herd, 1997), and if anything metabolism may have been slightly higher following exercise as prior exercise may enhance the thermic effect of feeding (Young, 1995) – thus the assumption was conservative and likely to diminish, rather than enhance, the chances of observing any differences in substrate utilisation occurring as a result of prior exercise. For some subjects, where different pieces of tubing were used in the two trials, only one combination (i.e. tube 1 for control, tube 2 for exercise or vice versa) satisfied the assumption of similar oxygen uptakes, however, for other subjects where the same tube was used for both trials, there were two possibilities which satisfied the assumption (i.e. tube 1 for control and exercise, and tube 2 for control and exercise). In the latter situation, the choice of correction factor was not critical because in both instances the data in the two trials would be correct relative to each other, so any effect of exercise on substrate utilisation would be apparent. In these instances, the correction factor which resulted in the baseline oxygen uptake being closest to $3.5 \text{ ml.kg}^{-1}.\text{min}^{-1}$ (i.e. 1 metabolic equivalent (MET) – the approximate oxygen requirement at rest) was adopted.

5.2.6 Data analysis

The eight-hour areas under the plasma or serum concentration vs. time curves calculated using the trapezium rule were defined as the respective total responses to the test meal, with these areas normalised to the baseline concentrations defined as the incremental responses. The peak paracetamol concentration and time to peak concentration were used to assess gastric emptying in the two trials. These indices have been shown to correlate well with rate of gastric emptying (Heading et al. 1973; Clements et al. 1978). Mean values in the fasted state and areas under curves were compared using t-tests for correlated means with a 5 % level of significance being adopted. Two-way ANOVA with repeated measures, with post-hoc Tukey tests, were used to compare data within and between trials. Data are presented as mean \pm SEM.

5.3 Results

5.3.1 Responses during treadmill walking

The mean (corrected) $\dot{V}O_2$ during the 90-minute walk on day one of the exercise trial was $25.1 \pm 0.8 \text{ ml.kg}^{-1}.\text{min}^{-1}$, which represented $64.9 \pm 1.2 \% \dot{V}O_2 \text{ max}$. As described in section 4.3.1, the mean of the two possible corrected oxygen uptake values was adopted. The mean heart rate was $140 \pm 4 \text{ beat.min}^{-1}$ and the perceived rate of exertion (Borg, 1973) was 12.6 ± 0.5 (between 'fairly light' and 'somewhat hard'). All subjects completed the walk without undue difficulty.

5.3.2 Estimation of plasma volume changes

Mean haemoglobin concentration was $14.4 \pm 0.2 \text{ g.dl}^{-1}$ in Trial 1 and $13.8 \pm 0.2 \text{ g.dl}^{-1}$ in Trial 2 and mean haematocrits were $41.5 \pm 0.6 \%$ and $39.9 \pm 0.5 \%$ in trials 1 and 2 respectively. Both these values were significantly lower in Trial 2 than in trial 1 ($p < 0.05$). As approximately 200 ml of blood was sampled in each trial and the interval between trials was relatively short (1 week), it is likely that the lower haemoglobin concentrations and haematocrits in trial 2 reflected lower amounts of circulating haemoglobin rather than increases in plasma volume. Therefore, advancing the same argument as described in section 4.3.2, it was decided not to correct concentrations for differences in plasma volume.

5.3.3 Plasma and serum concentrations in the fasted state

Table 5.2 shows plasma and serum concentrations in the fasted state. Plasma TAG concentration was 0.21 mmol.l^{-1} ($22 \pm 4 \%$) lower in the exercise trial than control ($p < 0.05$) and this reduction could largely be accounted for by the 0.17 mmol.l^{-1} reduction in the VLDL fraction ($p < 0.05$). HDL cholesterol concentrations were higher in the exercise trial than control, as were NEFA and 3-hydroxybutyrate concentrations ($p < 0.05$). Insulin concentrations were lower in the exercise trial than control ($p < 0.05$) and glucose concentrations were somewhat lower in the exercise trial ($p = 0.08$).

	Control	Exercise
Plasma TAG (mmol.l ⁻¹)	1.03 ± 0.11	0.82 ± 0.12*
Chylomicron TAG (mmol.l ⁻¹)	0.02 ± 0.004	0.01 ± 0.002
VLDL TAG (mmol.l ⁻¹)	0.54 ± 0.09	0.37 ± 0.09*
Total cholesterol (mmol.l ⁻¹)	4.84 ± 0.25	4.80 ± 0.25
HDL cholesterol (mmol.l ⁻¹)	1.02 ± 0.10	1.06 ± 0.10*
NEFA (mmol.l ⁻¹)	0.40 ± 0.08	0.56 ± 0.09*
Glucose (mmol.l ⁻¹)	5.66 ± 0.16	5.47 ± 0.12 [#]
Insulin (μU.ml ⁻¹)	13.8 ± 1.4	11.9 ± 1.1*
3-hydroxybutyrate (mmol.l ⁻¹)	0.06 ± 0.02	0.23 ± 0.07*

Table 5.2. Plasma and serum concentrations in the fasted state in the control and exercise trials. *Significantly different from control ($p < 0.05$), [#] $p = 0.08$ with control.

5.3.4 Postprandial plasma and serum concentrations

Figure 5.2 shows the TAG responses to the test meal in plasma and Figure 5.3 the TAG responses in the chylomicron ($S_f > \sim 400$) and VLDL ($S_f \sim 20-400$) fractions. Table 5.3 shows the total and incremental responses. The plasma total and incremental lipaemic responses were 3.31 mmol.l⁻¹.h ($25 \pm 3\%$) and 1.66 mmol.l⁻¹.h ($25 \pm 5\%$) lower respectively in the exercise trial than the control trial (both $p < 0.05$). Almost four-fifths ($79 \pm 10\%$) of the reduction in the total plasma lipaemic response could be accounted for by the $36 \pm 5\%$ reduction in the total VLDL response, whereas less than a fifth ($19 \pm 4\%$) of the total plasma TAG reduction was attributable to the $25 \pm 4\%$ reduction in the chylomicron response, although both the total chylomicron and VLDL responses were significantly reduced by exercise. The incremental VLDL response was $31 \pm 5\%$ lower in the exercise trial than the control trial ($p < 0.05$) and this accounted for almost two-thirds ($61 \pm 14\%$) of the reduction in plasma incremental lipaemic response, with the reduced chylomicron response accounting for just over a third ($35 \pm 5\%$) of the attenuation.

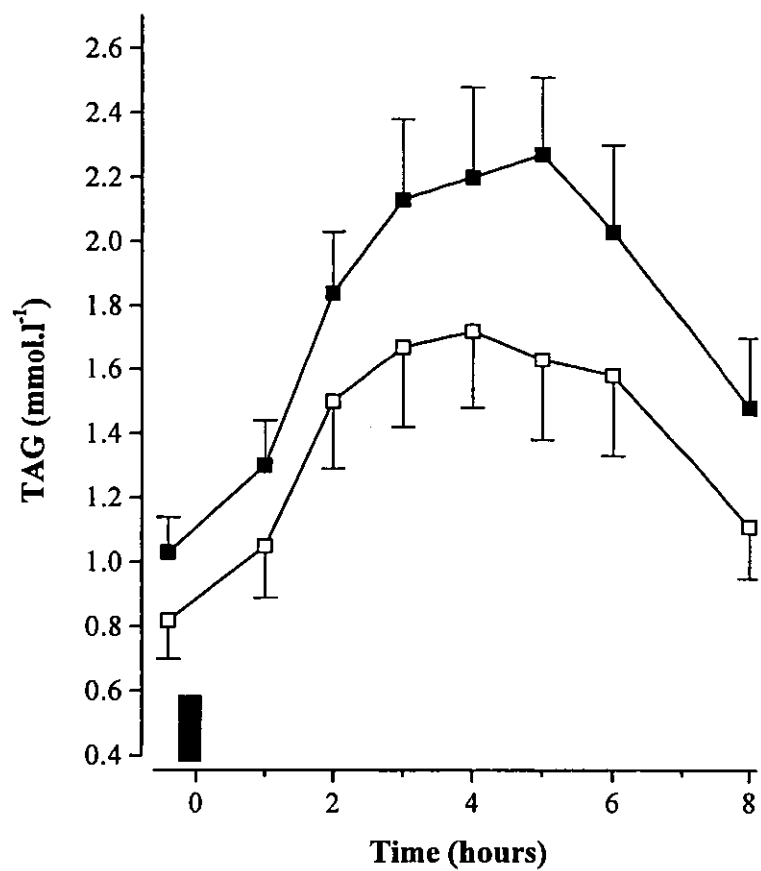


Figure 5.2 Plasma TAG concentrations in the fasted state and for 8 hours after consumption of test meal in the control (filled) and exercise (open) trials. Black rectangle denotes ingestion of test meal.

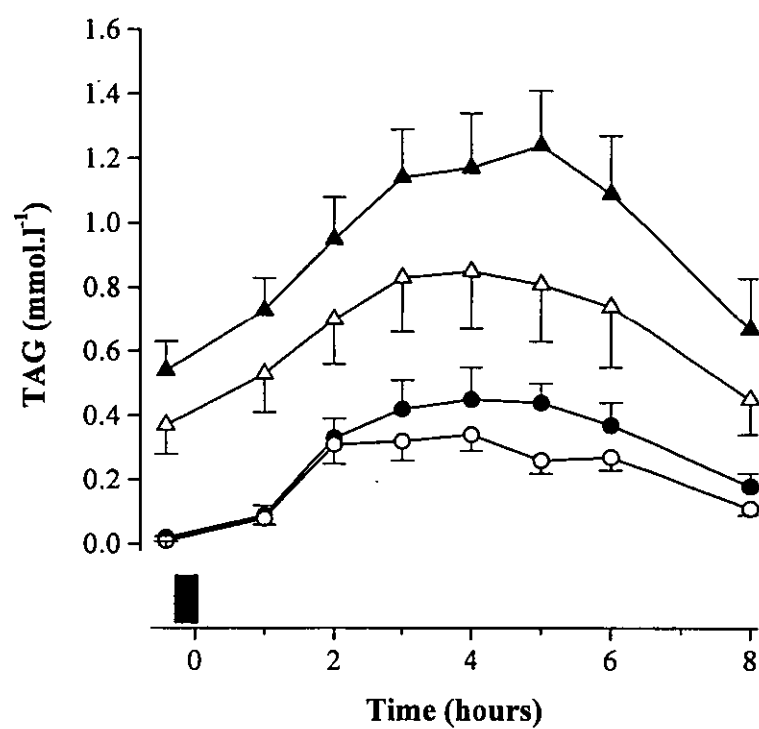


Figure 5.3 Chylomicron (circles) and VLDL (triangles) TAG concentrations in the fasted state and for 8 hours after consumption of test meal in the control (filled) and exercise (open) trials. Black rectangle denotes ingestion of test meal.

The total NEFA response (Figure 5.4, Table 5.3) was higher in the exercise trial than the control trial ($p < 0.05$) and 3-hydroxybutyrate concentrations (Figure 5.5, Table 5.3) were 67 ± 14 % higher in the exercise trial than control ($p < 0.05$). The total glycaemic response did not differ between trials but the incremental response was higher in the exercise trial than control ($p < 0.05$) (Figure 5.6, Table 5.3). Total and incremental insulinaemic responses were lower in the exercise trial than the control trial ($p < 0.05$ and $p = 0.05$ respectively) (Figure 5.7, Table 5.3).

For five subjects, plasma concentrations of ^{13}C palmitic acid were measured 4 hours postprandially. This time point was chosen as NEFA concentrations were high enough to allow detection of ^{13}C enrichment of palmitic acid by GC-IRMS and the difference in NEFA concentrations between trials was clear. The concentration of ^{13}C palmitic acid at this time was $82.3 \pm 12.0 \mu\text{g.ml}^{-1}$ in the control trial and $78.9 \pm 7.7 \mu\text{g.ml}^{-1}$ in the exercise trial. At the same time point plasma NEFA concentrations were $0.41 \pm 0.03 \text{ mmol.l}^{-1}$ and $0.49 \pm 0.03 \text{ mmol.l}^{-1}$ in the control and exercise trials for these five subjects. Thus, these limited data imply that the elevated NEFA concentrations seen at this time point were not attributable to an increased quantity of exogenous fatty acids in the circulation.

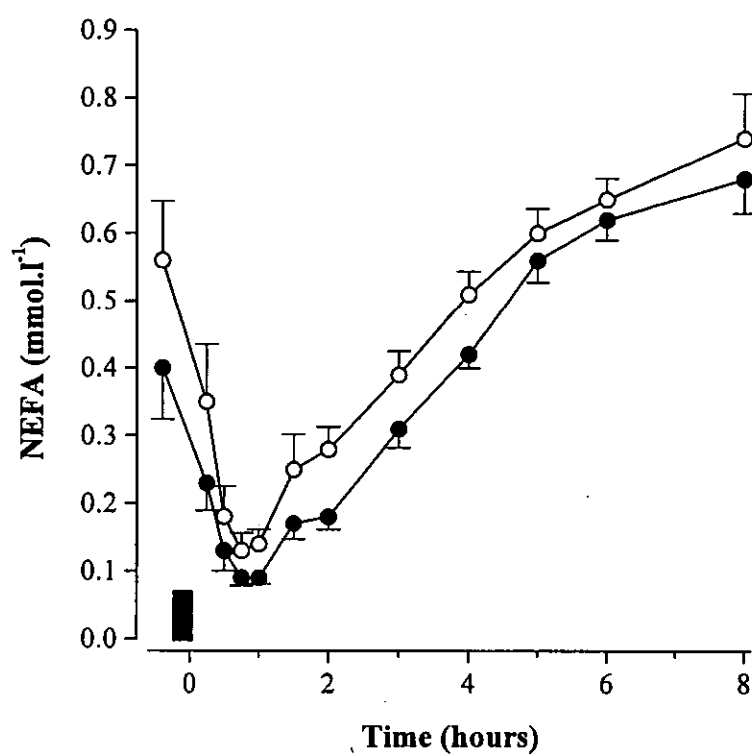


Figure 5.4 Plasma NEFA concentrations in the fasted state and for 8 hours after consumption of test meal in the control (filled) and exercise (open) trials. Black rectangle denotes ingestion of test meal.

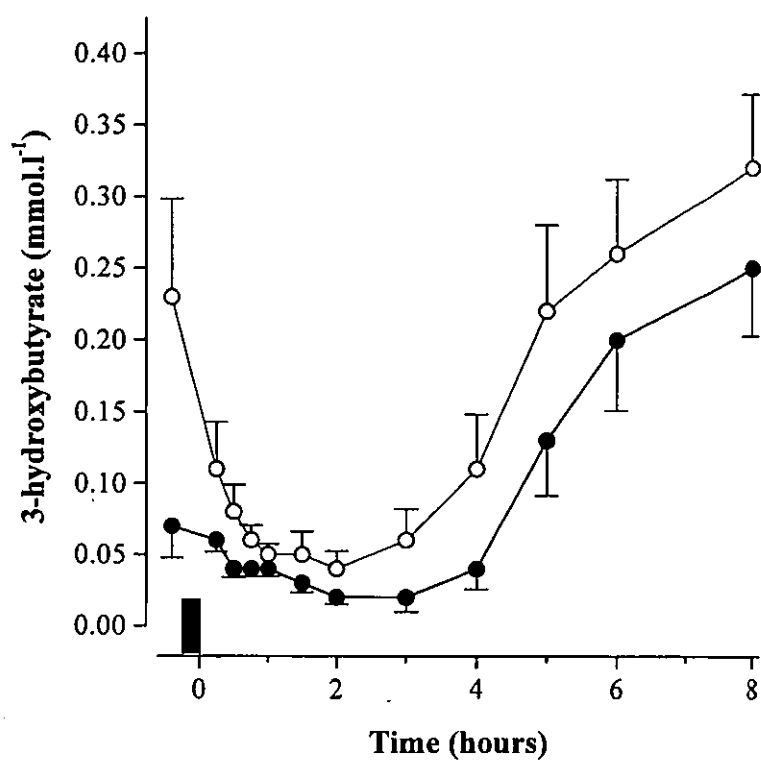


Figure 5.5 Serum 3-hydroxybutyrate concentrations in the fasted state and for 8 hours after consumption of test meal in the control (filled) and exercise (open) trials. Black rectangle denotes ingestion of test meal.

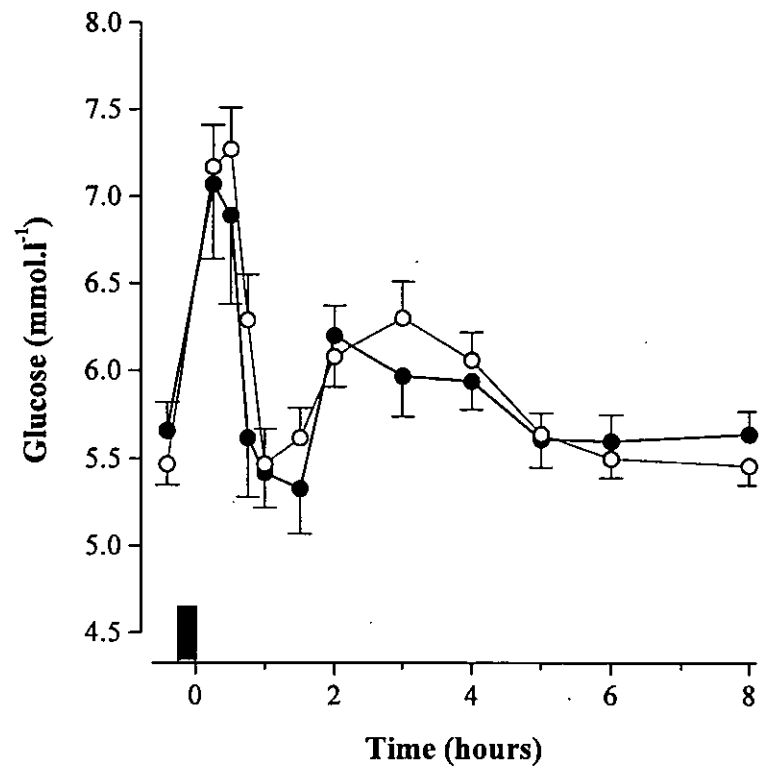


Figure 5.6 Plasma glucose concentrations in the fasted state and for 8 hours after consumption of test meal in the control (filled) and exercise (open) trials. Black rectangle denotes ingestion of test meal.

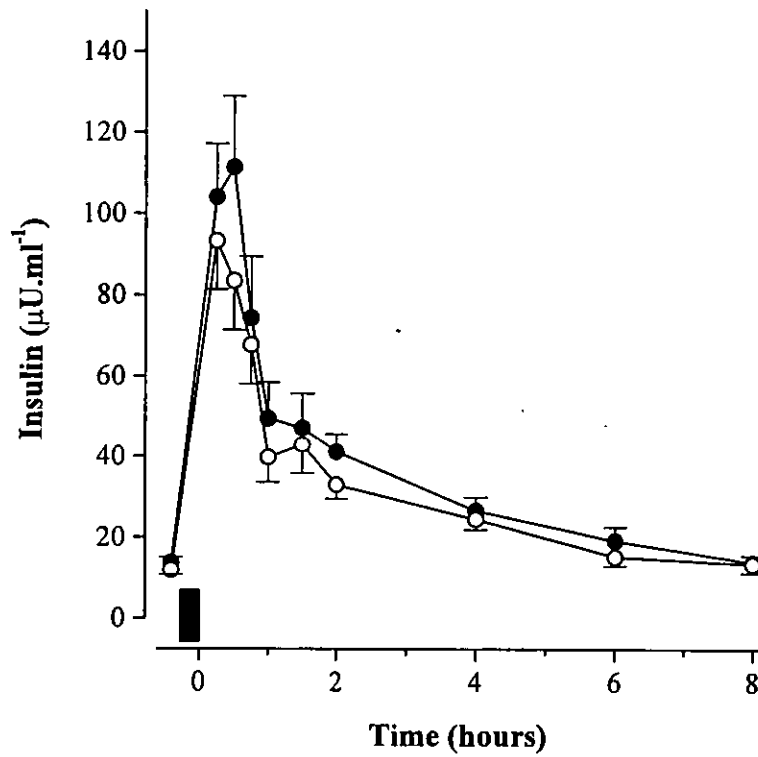


Figure 5.7 Serum insulin concentrations in the fasted state and for 8 hours after consumption of test meal in the control (filled) and exercise (open) trials. Black rectangle denotes ingestion of test meal.

	Control	Exercise
Plasma total lipaemic response (mmol.l ⁻¹ .h)	14.37 ± 1.61	11.06 ± 1.52*
Plasma incremental lipaemic response (mmol.l ⁻¹ .h)	6.12 ± 1.10	4.46 ± 0.79*
Total chylomicron response (mmol.l ⁻¹ .h)	2.42 ± 0.42	1.77 ± 0.26*
Total VLDL response (mmol.l ⁻¹ .h)	7.64 ± 1.08	5.26 ± 1.12*
Incremental VLDL response (mmol.l ⁻¹ .h)	3.31 ± 0.62	2.27 ± 1.04*
Total NEFA response (mmol.l ⁻¹ .h)	3.25 ± 0.14	3.85 ± 0.25*
Total insulinaemic response (μU.ml ⁻¹ .h)	268 ± 31	228 ± 21*
Incremental insulinaemic response (μU.ml ⁻¹ .h)	158 ± 21	132 ± 13 [#]
Total glycaemic response (mmol.l ⁻¹ .h)	46.5 ± 1.2	47.0 ± 0.8
Incremental glycaemic response (mmol.l ⁻¹ .h)	1.2 ± 0.9	3.2 ± 0.6*
Total 3-hydroxybutyrate response (mmol.l ⁻¹ .h)	0.81 ± 0.15	1.24 ± 0.26*

Table 5.3 Total and incremental plasma and serum responses to the test meal in the control and exercise trials. *Significantly different from control ($p < 0.05$), [#] $p = 0.05$ with control

Figure 5.8 shows the plasma paracetamol responses to the test meal in the control and exercise trials. The peak plasma paracetamol concentration observed was $0.134 \pm 0.022 \text{ mmol.l}^{-1}$ in the control trial and $0.125 \pm 0.014 \text{ mmol.l}^{-1}$ in the exercise trial ($p = 0.64$) with the median, and mode, time to peak concentration being 60 minutes in both the exercise and control trials.

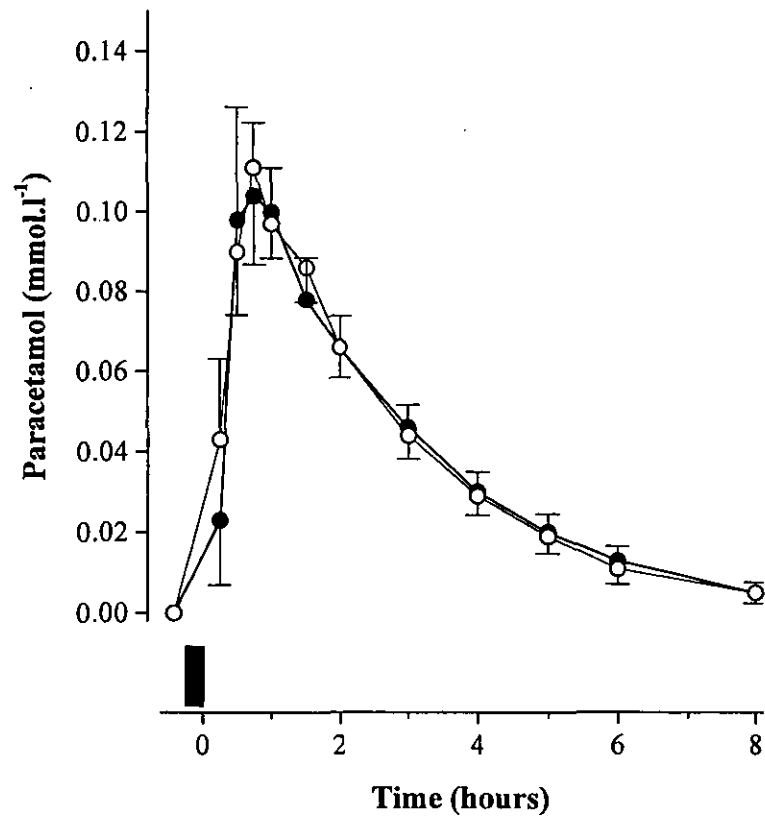


Figure 5.8 Plasma paracetamol concentrations for 8 hours after consumption of the test meal which contained 1.5 g of paracetamol, in the control (filled) and exercise (open) trials. Black rectangle denotes ingestion of test meal.

HDL cholesterol concentrations in the fasted state and eight hours postprandially are shown in Figure 5.9. HDL concentrations were higher in the exercise trial than the control trial both in the fasted state and eight hours postprandially ($p < 0.05$). In both trials, the eight hour concentrations were lower than the respective fasting values ($p < 0.05$), but there was a near significant trial by time interaction ($p = 0.10$) suggesting that, in the exercise trial, the postprandial reduction to HDL cholesterol concentration was somewhat attenuated.

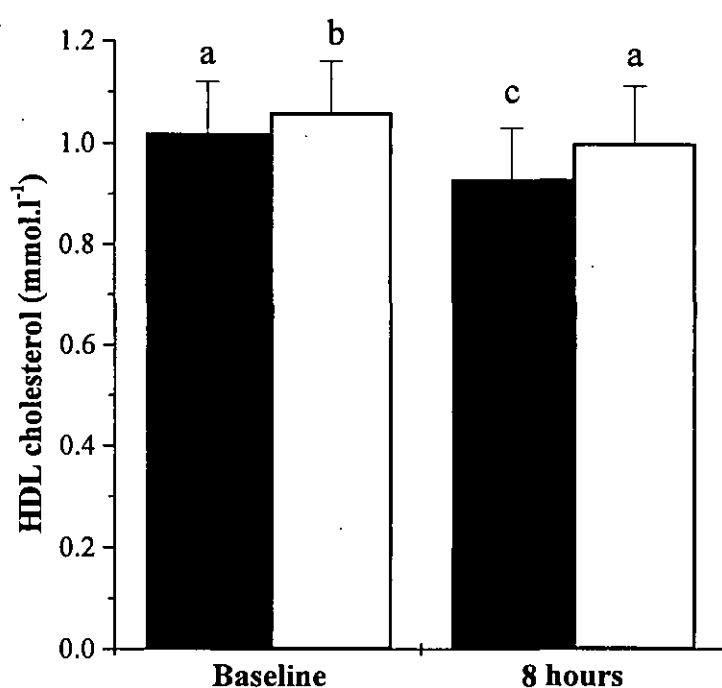


Figure 5.9 HDL cholesterol concentrations at baseline and eight hours postprandially in the control (black) and exercise (white) trials. Unlike letters significantly different from each other ($p < 0.05$). Trial by time interaction, $p = 0.10$.

Total cholesterol concentrations in the fasted state and eight hours postprandially are shown in figure 5.10. Concentrations did not differ between trials either in the fasted state or eight hours postprandially, but in both trials concentrations were higher than baseline eight hours after ingestion of the test meal ($p < 0.05$). The trial by time interaction was not significant.

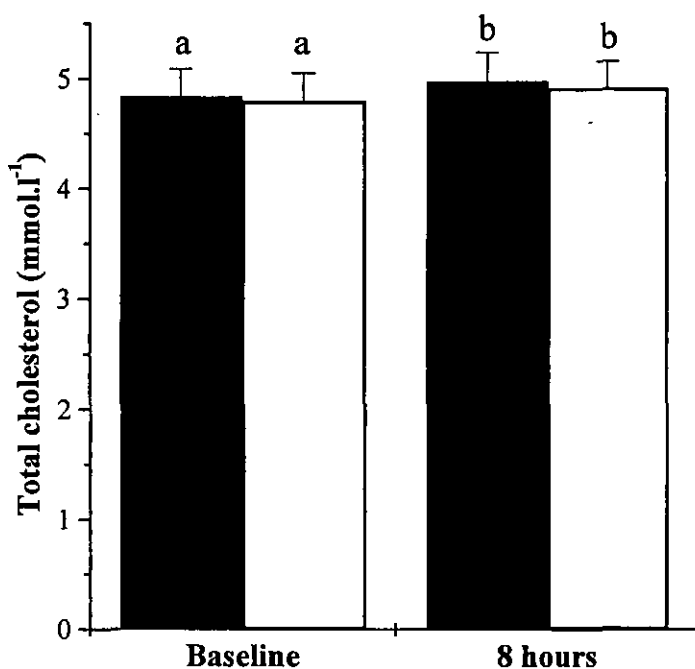


Figure 5.10 Total cholesterol concentrations at baseline and eight hours postprandially in the control (black) and exercise (white) trials. Unlike letters significantly different from each other ($p < 0.05$). Trial by time interaction, $p = 0.60$.

5.3.5 Substrate utilisation and metabolic rate

5.3.5.1 Substrate utilisation and metabolic rate in the fasted state

In the fasted state, respiratory exchange ratios were numerically lower in the exercise trial (0.75 ± 0.01) than the control trial (0.78 ± 0.02), but these differences were not statistically significant ($p = 0.11$). Calculated rates of fat oxidation were somewhat higher in the exercise trial ($6.4 \pm 0.3 \text{ g.h}^{-1}$) than the control trial ($5.5 \pm 0.6 \text{ g.h}^{-1}$), but this difference was not statistically significant ($p = 0.11$). In a reciprocal manner, carbohydrate oxidation was somewhat lower in the exercise trial ($3.2 \pm 0.6 \text{ g.h}^{-1}$) than the control trial ($5.4 \pm 1.6 \text{ g.h}^{-1}$), but again this was not statistically significant ($p = 0.14$). Neither oxygen uptake (Control: $3.44 \pm 0.14 \text{ ml.kg}^{-1}.\text{min}^{-1}$, Exercise: $3.46 \pm 0.12 \text{ ml.kg}^{-1}.\text{min}^{-1}$), nor metabolic rate (Control: $302 \pm 12 \text{ kJ.h}^{-1}$, Exercise $303 \pm 13 \text{ kJ.h}^{-1}$) differed significantly between trials.

5.3.5.2 Substrate utilisation and metabolic rate in the postprandial state

Mean oxygen uptake values (Control: $3.85 \pm 0.09 \text{ ml.kg}^{-1}.\text{min}^{-1}$, Exercise: $3.90 \pm 0.10 \text{ ml.kg}^{-1}.\text{min}^{-1}$) over the 8-hour postprandial period were not different between trials. Similarly, calculated eight-hour values for energy expenditure were not different between trials (Control: $2734 \pm 140 \text{ kJ}$, Exercise: $2741 \pm 122 \text{ kJ}$). Figure 5.11 shows the respiratory exchange ratio values over the 8-hour postprandial period. Mean respiratory exchange ratio values were significantly lower in the exercise trial (0.75 ± 0.01) than the control trial (0.79 ± 0.01) ($p < 0.05$).

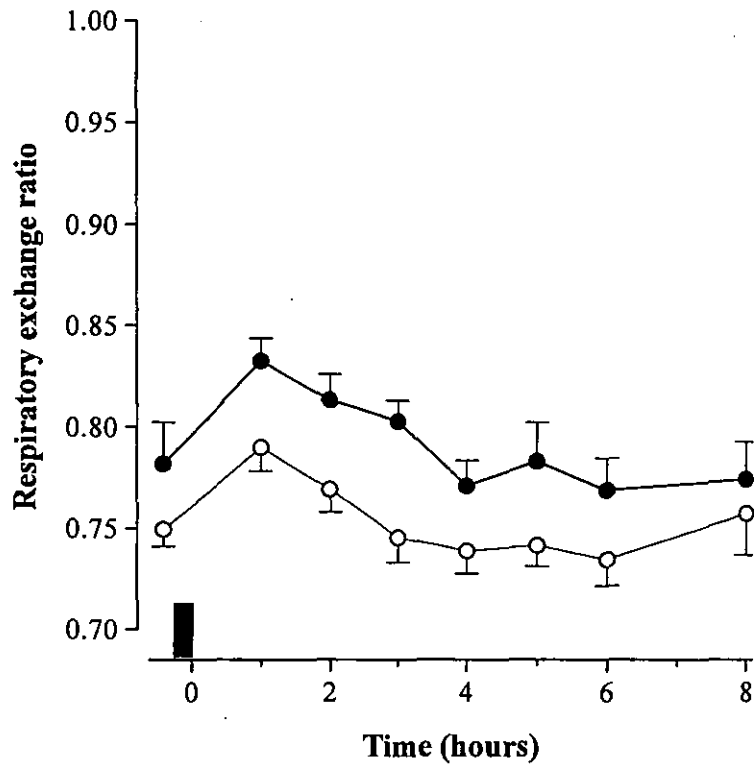


Figure 5.11 Respiratory exchange ratios in the fasted state and for 8 hours after consumption of the test meal in the control (filled) and exercise (open) trials. Black rectangle denotes ingestion of test meal.

Figure 5.12 shows total whole body fat oxidation and Figure 5.13 shows total whole body carbohydrate oxidation over the 8-hour postprandial period in the control and exercise trials. Total fat oxidation over this period was $22 \pm 6\%$ higher in the exercise trial (56.9 ± 2.8 g) than in the control trial (47.3 ± 2.7 g) ($p < 0.05$), and there was a concomitant $47 \pm 9\%$ decrease in carbohydrate oxidation in the exercise trial (30.6 ± 6.4 g) compared with control (54.4 ± 8.0 g) ($p < 0.05$).

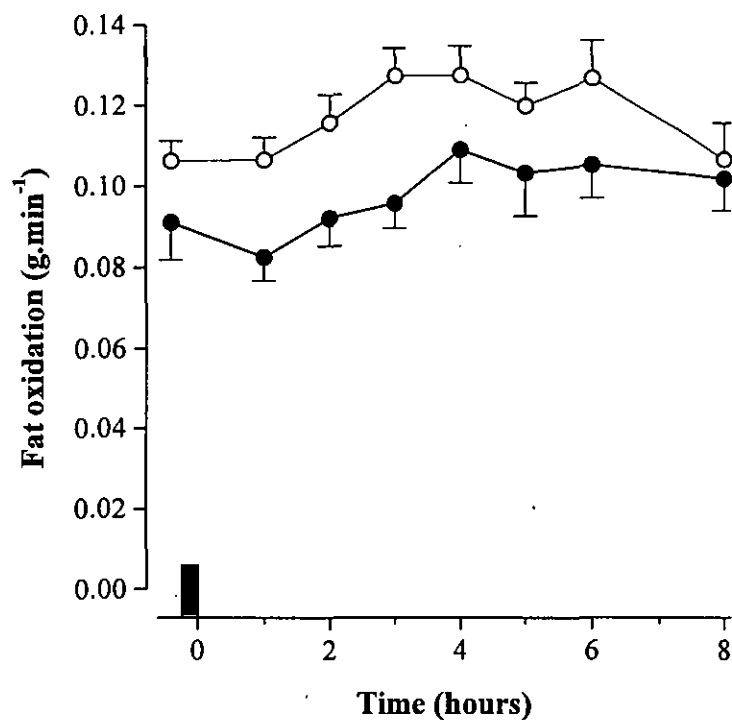


Figure 5.12 Rates of fat oxidation in the fasted state and for 8 hours after consumption of the test meal in the control (filled) and exercise (open) trials. Black rectangle denotes ingestion of test meal.

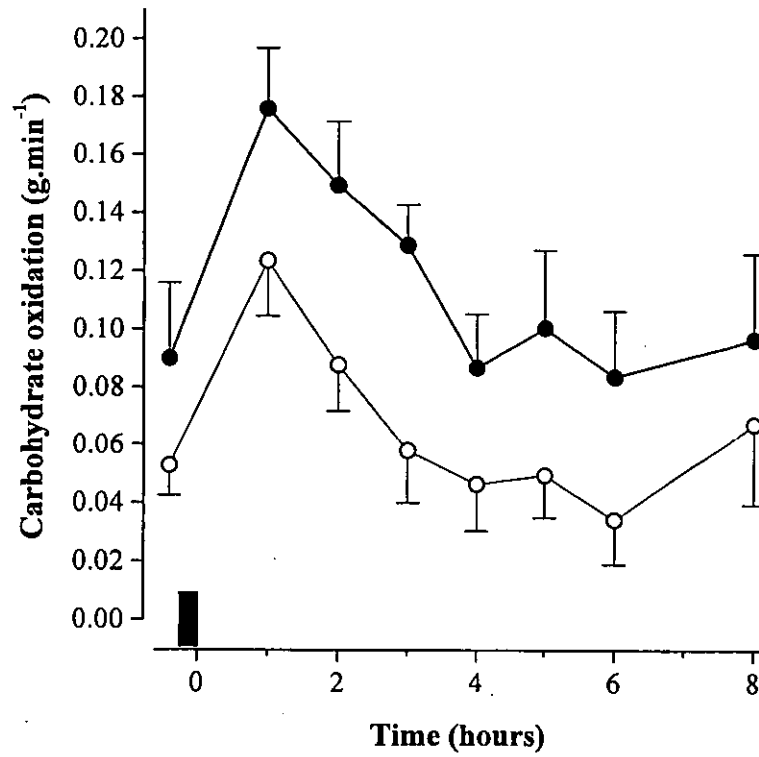


Figure 5.13 Rates of carbohydrate oxidation in the fasted state and for 8 hours after consumption of the test meal in the control (filled) and exercise (open) trials. Black rectangle denotes ingestion of test meal.

Figure 5.14 shows the cumulative excretion of ^{13}C on the breath as a percentage of the ingested dose. Cumulative excretion of ^{13}C on the breath was $11.9 \pm 0.8\%$ and $15.1 \pm 0.9\%$ in the control and exercise trials respectively for the 8 hour period during which directly measured $\dot{V}\text{CO}_2$ measurements were obtained. Over the full 24-hour observation period, excretion of ^{13}C on the breath was $22.0 \pm 1.6\%$ and $27.6 \pm 1.5\%$ of the ingested dose in the control and exercise trials respectively. Over both time scales, ^{13}C excretion on the breath was significantly higher in the exercise trial than the control trial ($p < 0.05$). After 24 hours, ^{13}C breath enrichments were within 2 % of baseline values indicating that, for the short term, oxidation of the ingested [1,1,1- ^{13}C] tripalmitin was almost completed in this time period.

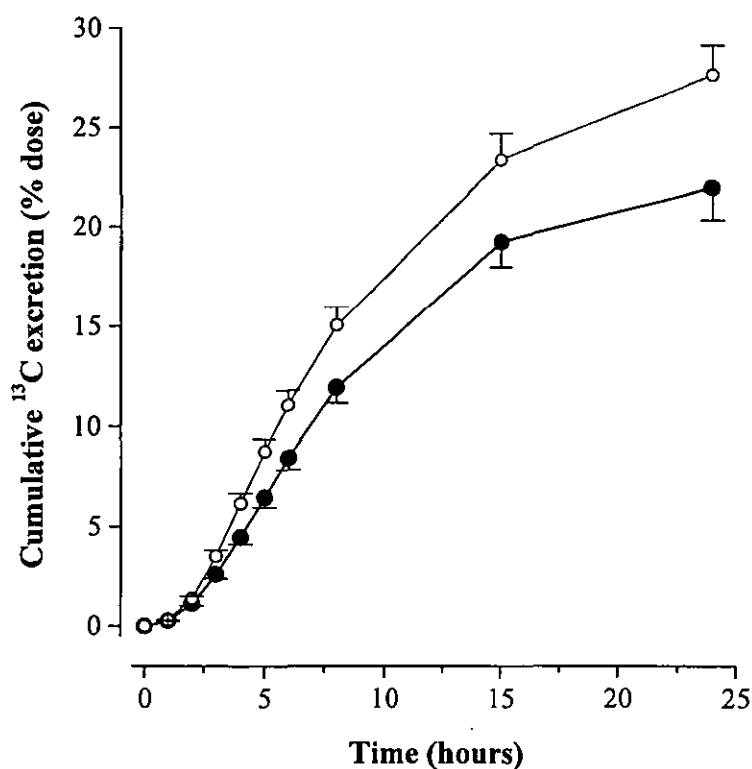


Figure 5.14 Cumulative excretion of ^{13}C label on the breath over 24 hours in the control (filled) and exercise (open) trials.

In order to determine exogenous fat oxidation from ^{13}C excretion on the breath, it is necessary to apply a correction factor for $^{13}\text{CO}_2$ generated from the oxidation of [1,1,1- ^{13}C] tripalmitin, but not excreted on the breath. The literature is equivocal on the extent of this phenomenon, but it is clear the magnitude of this correction factor

influences estimation of exogenous fat oxidation. The application of a correction factor, and the determination of exogenous fat oxidation using ^{13}C excretion on the breath with an appropriate correction factor, has been addressed in the discussion of this chapter (section 5.4).

5.3.6 Subject 6: A case study

One of the 12 subjects in this study (subject 6) was hypertriglyceridaemic and badly skewed the data for fasting and postprandial TAG concentrations, both in whole plasma and the lipoprotein fractions. He was 49 years old with a BMI of 28.7 kg.m^{-2} and his sum of 4 skinfolds was 56.6 mm, his $\dot{V}\text{O}_2$ max was $38.4 \text{ ml.kg}^{-1}.\text{min}^{-1}$ and he possessed the commonest E3/E3 apo E phenotype.

The fasted state - Data for subject 6 in the fasted state are presented in Table 5.4. Plasma TAG concentrations were about four times the mean concentrations for the group in both trials, with high VLDL TAG concentrations accounting for most of this elevation. Exercise reduced the plasma TAG concentration by 7 %. Total cholesterol concentration was moderately elevated, and HDL cholesterol concentration was low. Fasting insulin concentration was twice the group mean in control trial, but this concentration was almost halved in the exercise trial. NEFA concentration was not elevated in the exercise trial, and plasma 3-hydroxybutyrate concentrations were negligible in both trials. Substrate utilisation rates were comparable to the rest of the group, but fat oxidation rate was not elevated in the exercise trial.

	Control	Exercise
Fasting plasma TAG (mmol.l ⁻¹)	3.88	3.60
Fasting VLDL TAG (mmol.l ⁻¹)	3.23	2.88
Fasting chylomicron TAG (mmol.l ⁻¹)	0.09	0.11
Fasting total cholesterol (mmol.l ⁻¹)	6.54	6.06
Fasting HDL cholesterol (mmol.l ⁻¹)	0.82	0.82
Fasting insulin (μU.ml ⁻¹)	24.9	13.8
Fasting NEFA (mmol.l ⁻¹)	0.38	0.39
Fasting 3-hydroxybutyrate (mmol.l ⁻¹)	0.010	0.025
Fasting respiratory exchange ratio	0.80	0.78
Fasting fat oxidation rate (g.h ⁻¹)	5.5	5.6
Fasting carbohydrate oxidation rate (g.h ⁻¹)	7.2	4.7

Table 5.4 Data for subject 6, in the fasted state, in the control and exercise trials

The postprandial state - It was in the postprandial state that the differences between subject 6 and the rest of the group became more apparent. Postprandial data are presented in Table 5.5. Clearly, exercise did not attenuate the whole plasma or lipoprotein fraction TAG concentrations and the total lipaemic response was over four times the mean value for the group and over twice the value of the next highest subject's. This high response was due to elevations in both the chylomicron and VLDL fractions. The NEFA response for this subject was very similar in the control and exercise trials, but these concentrations were not markedly different from the values observed in other subjects in the group. The insulin response to the test meal was high, with the total response being attenuated by exercise. This reduction was attributable to the lower fasting insulin concentration in the exercise trial, and the incremental insulin response was in fact higher in the exercise trial than control. In both trials, 3-hydroxybutyrate concentrations were very low. The pattern of postprandial substrate utilisation was similar to the rest of the group with an increase in fat oxidation and concomitant decrease in carbohydrate oxidation, however, excretion of ¹³C on the breath was not increased in the exercise trial.

	Control	Exercise
Plasma total lipaemic response (mmol.l ⁻¹ .h)	53.53	56.23
Total VLDL response (mmol.l ⁻¹ .h)	34.73	34.54
Total chylomicron response (mmol.l ⁻¹ .h)	13.35	16.21
Total NEFA response (mmol.l ⁻¹ .h)	4.36	4.32
Total insulinaemic response (μU.ml ⁻¹ .h)	390.3	347.1
Incremental insulinaemic response (μU.ml ⁻¹ .h)	191.1	236.4
Total glycaemic response (mmol.l ⁻¹ .h)	46.3	45.4
Total 3-hydroxybutyrate response (mmol.l ⁻¹ .h)	0.04	0.07
Mean postprandial respiratory exchange ratio	0.81	0.76
8 hour fat oxidation (g)	46.5	55.6
8 hour carbohydrate oxidation (g)	73.5	41.6
8 hour ¹³ C excretion (% of ingested dose)	11.54	10.57
24 hour ¹³ C excretion (% of ingested dose)	26.28	25.23

Table 5.5 Data for subject 6, in the postprandial state, in the control and exercise trials

5.4 Discussion

The present study has shown that prior moderate exercise significantly attenuated plasma TAG concentrations in the postabsorptive and postprandial states in this group of normotriglyceridaemic and predominantly normocholesterolaemic middle-aged, untrained men. With the exception of subject 6, who has been considered separately, exercise reduced both postabsorptive and postprandial TAG concentrations in every subject. Thus, together with the results in the previous chapter, these data indicate that moderate exercise can improve TAG metabolic capacity in groups of subjects who, by nature of their age (Dawber, 1980; Keys, 1980; Wood et al. 1998) and/or gender (Dawber, 1980; Wood et al. 1998) are at increased risk of CHD compared with the young adults (Aldred et al. 1994; Tsetsonis and Hardman, 1996a; Tsetsonis and Hardman, 1996b; Herd et al. 1997; Herd, 1997; Gill et al. 1998; Malkova et al. 1999), and middle-aged women (Tsetsonis et al. 1997) previously studied. Importantly, the amount of moderate exercise necessary to achieve these results was

attainable for these men, who were not well trained. For the first time, it has been possible to quantify the effect of moderate exercise on TAG concentrations in the chylomicron (Sf > 400) and VLDL (Sf 20-400) fractions. Exercise reduced TAG concentrations in both the Sf > 400, and Sf 20-400 fractions, but unexpectedly, the reduction in the Sf 20-400 fraction accounted for almost four-fifths of the reduction in plasma TAG. Plasma TAG concentrations were lower in the fasted state in the exercise trial and predictably this reduction was primarily due to reduced VLDL concentrations. This shift in baseline VLDL-TAG concentration clearly contributed to the lower area under the VLDL-TAG vs. time curve in the exercise trial, but even when the incremental lipaemic response was considered, the reduction in VLDL-TAG was a larger contributor to the exercise-induced TAG attenuation than the reduction in chylomicron-TAG. In this study, lipoprotein classes were separated on the basis of Svedberg flotation rates. As lipoproteins do not form discrete classes on the basis of flotation rates, it is theoretically possible that the reduction in the Sf 20-400 fraction seen after exercise was attributable, at least in part, to lower concentrations of small chylomicrons and/or chylomicron remnants. However, as the concentration of apo B48 in the Sf 20-400 fraction is at least an order of magnitude lower than the concentration of apo B100 in this fraction (Karpe et al. 1993; Karpe et al. 1997), it is unlikely that the differences in the Sf 20-400 fraction seen in the present study were attributable to reduced concentrations of lipoproteins of intestinal origin.

These findings appear to differ from the limited data available in the literature, which suggests that exercise largely influences the chylomicron fraction, but there are a number of possible explanations for these differences. In the training studies by Drexel et al. (1992) and Weintraub et al. (1989), data on the postprandial VLDL-TAG responses to the test meal were not reported and it is possible that these were substantially attenuated by training. If this was the case, the data would be in agreement with the present data as in the present study exercise did attenuate the chylomicron-TAG as well as the VLDL-TAG response to the test meal. In addition, subjects in Drexel et al.'s (1992) study lost 4.3 kg of body mass; this may have influenced the attenuation to the chylomicron response as weight loss in itself can stimulate LPL activity (Schwartz and Brunzell, 1981). Both the above studies considered training and not a single exercise session. Although skeletal muscle LPL activity might be upregulated in response to a prolonged exercise session (Lithell et

al. 1979; Taskinen et al. 1980; Lithell et al. 1984; Lithell et al. 1981), with this increase being transient (Simsolo et al. 1993), it is possible that there is an interaction between exercise training and an acute session due to the increased capillarisation of trained muscle, which augments the exercise-mediated increase in LPL activity. If this were the case, training would reduce chylomicronaemia to a greater extent than a single exercise session does.

In the study by Herd et al. (1997), which used the same 2-day model as the present study, a 'chylomicron-rich' fraction was separated using a long spin in a bench-top centrifuge rather than by ultracentrifugation. This was not a validated method and it is unlikely that the supernatant fraction contained solely chylomicrons, so perhaps this fraction could have been better described as a 'TAG-rich lipoprotein' fraction. Thus, the apparent discrepancy between the present study and Herd et al's (1997) study, might be a consequence of differing analytical methodologies.

For the first time using this experimental model, there is evidence, albeit indirect, to suggest that the lower TAG concentrations seen in the exercise trial were not due to a reduced rate of chylomicron appearance into the circulation. As chylomicron-TAG concentrations peaked at the same time point (4 hours) in both trials, and the shape of the chylomicron-TAG responses were similar in the two trials, it appears that chylomicron appearance was not delayed in the exercise trial. In addition, time to peak and peak paracetamol concentrations did not differ between trials, suggesting that the rate of gastric emptying was not slower in the exercise trial (Heading et al. 1973; Clements et al. 1978). Thus, it appears that the lower chylomicron-TAG concentrations in the exercise trial were not due to a reduced chylomicron appearance rate.

The lower TAG concentrations in the fasted state and for the first two hours postprandially of the exercise trial were almost entirely due to reduced concentrations of TAG in the VLDL fraction. These lower concentrations could be due to reduced hepatic VLDL secretion, or increased VLDL hydrolysis and clearance from the circulation (mediated by increased LPL activity and/or increased blood flow to the peripheral tissues). Both are theoretically possible but the data suggest that reduced VLDL secretion is the more likely alternative. At the two hour time point – the first

where chylomicron-TAG concentrations were appreciable – chylomicron-TAG concentrations were $0.33 \pm 0.06 \text{ mmol.l}^{-1}$ and $0.31 \pm 0.06 \text{ mmol.l}^{-1}$ in the control and exercise trials respectively. At the same time point VLDL-TAG concentrations were $0.95 \pm 0.13 \text{ mmol.l}^{-1}$ in the control trial and $0.70 \pm 0.14 \text{ mmol.l}^{-1}$ in the exercise trial. Thus, at this time point, exercise had reduced VLDL-TAG concentrations by over a quarter but had very little effect on chylomicron-TAG concentrations. As chylomicrons and VLDL compete for hydrolysis by LPL (Brunzell et al. 1973; Björkegren et al. 1996) with chylomicrons being the preferred substrate for LPL *in vivo* (Potts et al. 1991; Björkegren et al. 1996), these data suggest that, at least in the fasted and early postprandial states, exercise was not acting to increase lipoprotein-TAG hydrolysis.

Further support for this interpretation comes from two studies using the same experimental model as the one employed in the present study. These found that, in the fasted state, muscle LPL activity was not significantly higher in the exercise trial than control (Herd et al. 1997) and that postheparin LPL activity was not significantly elevated 18 hours after a 2 hour moderate intensity exercise session (Herd, 1997). Indeed, the assertion that exercise might be reducing VLDL secretion is not unprecedented. There are data showing that, in rats, hepatic VLDL secretion rates are reduced by training (Simonelli and Eaton, 1978; Mondon et al. 1984; Fukuda et al. 1991), but data on a single exercise session, or in humans, are (to the author's knowledge) lacking.

While in the fasted state and early in the postprandial period it seems likely that the reduced plasma TAG concentrations are attributable to reduced hepatic VLDL secretion, later in the postprandial state the evidence is not as clear cut. At later time points, lower VLDL-TAG concentrations still accounted for the majority of the reduction in plasma TAG concentration, but chylomicron-TAG concentrations were also significantly reduced by exercise. As the data suggest that the lower chylomicron-TAG concentrations were unlikely to have been due to a reduced rate of chylomicron appearance, increased chylomicron-TAG clearance is the probable mechanism for the lower values. This is likely to have been mediated by reduced competition for LPL binding sites, due to the lower VLDL-TAG concentrations in the

exercise trial. However, it is also possible that the lower chylomicron TAG concentrations may have been a consequence of increased TAG clearance mediated by upregulation of LPL activity and/or increased blood flow, although the magnitude of this increase would have been small.

As in the study described in the previous chapter, plasma NEFA concentrations were elevated in both the fasted and postprandial states. Although the mechanisms behind the elevated NEFA concentrations in the fasted state are still unclear, the present study provides some preliminary data that shed light on the postprandial NEFA elevation in the exercise trial. In the five subjects for whom data was obtained, plasma ^{13}C palmitic acid concentrations measured 4 hours postprandially were not higher in the exercise trial than control, despite the exercise trial plasma NEFA concentrations being 20 % higher (i.e. ^{13}C enrichment of plasma palmitic acid was lower in the exercise trial). These data suggest that the exercise-induced elevation in plasma NEFA concentrations was not due to increased spill-over of LPL derived fatty acids. Therefore, increased lipolysis of adipose tissue TAG appears to be the likely mechanism. However, as these data were only obtained for five subjects, at one time point, this finding should be interpreted with caution and further analysis is necessary to confirm these results. In any case, whether the increase in plasma NEFA was due to increased LPL or HSL derived fatty acids the implications are the same – lipid fuels are being directed away from adipose tissue for storage into the circulation for oxidation or storage in skeletal muscle and the liver.

As the present data suggest that reduced hepatic VLDL secretion is the major contributory mechanism for the reduced plasma TAG concentrations seen in the exercise trial, the elevated NEFA concentrations in the exercise trial are of particular interest. It has been suggested that substrate delivery to the liver is the major determinant of VLDL apoB-100 secretion (Sniderman and Cianflone, 1993), thus, an elevated plasma NEFA concentration would normally result in a higher, not lower, rate of VLDL secretion. However, the situation is not clear cut, as in the liver fatty acids are either re-esterified into TAG, or enter the mitochondrial matrix for β -oxidation and ketone body formation, depending on the concentration of malonyl CoA (Stryer, 1988). As the rate of hepatic oxidation is a major determinant of ketone

body formation (Frayn, 1996) and the liver lacks the enzymes necessary to oxidise ketone bodies (Stryer, 1988), plasma ketone body concentrations provide a surrogate measure of hepatic β -oxidation. In the exercise trial, 3-hydroxybutyrate (a ketone body) concentrations were over 50 % higher than in the control trial, suggesting a shift in partitioning of the fatty acid flux to the liver towards ketogenesis and oxidation rather than re-esterification and VLDL synthesis. At rest, the liver is a very metabolically active organ – its contribution to the whole body metabolic rate is equivalent to that of skeletal muscle, accounting for over a quarter of the body's oxygen uptake (Frayn, 1992). Thus, it is not unreasonable to suggest that the substantial shift in hepatic fatty acid metabolism towards oxidation that occurred in the exercise trial was a large mediator of reduced VLDL secretion. Support for this hypothesis comes from Fukuda and co-workers (1991) who found that, in rats, the exercise training-induced reduction in TAG was matched by a similar decrease in the hepatic TAG secretion rate, with a concomitant increase in hepatic ketone body production. On the basis of these data the authors concluded that the altered partitioning of NEFA between esterification and oxidation was one of the causative factors for the TAG-lowering effect of exercise.

While an increase in hepatic fat oxidation and ketogenesis is likely to be at least a contributory mechanism for reduced VLDL synthesis in the exercise trial, how and why exercise might elicit this shift is not immediately obvious. Indeed, exercise affects almost every aspect of fat metabolism in the liver (Gorski et al. 1990), so other mechanisms and pathways might be acting synergistically with increased oxidation and ketogenesis to reduce VLDL secretion. As there is a dearth of available data on exercise and liver metabolism, exactly how exercise might be acting on the liver to reduce VLDL secretion is largely a matter of speculation and will be discussed further in the general discussion section of this thesis.

Exercise also had a profound effect on substrate utilisation. This, in itself, is not a novel finding as data are available to suggest that in both the fasted (Calles-Escandón et al. 1996; Weststrate et al. 1990) and postprandial (Tsetsonis and Hardman, 1996; Tsetsonis et al. 1997) states a prior exercise session increases whole body fat oxidation. However, what was not known was the extent that the postprandial

elevation in fat oxidation was attributable to an increase in the oxidation of exogenous lipid. To the author's knowledge, the current study provides the first data to address this issue. However, before any conclusions can be made, it is necessary to address the limitations to the data. As discussed earlier, it was necessary to correct the expired air data, to take into account the fact that the Falconia tubing was permeable to oxygen and carbon dioxide. Although the correction was cross-validated, it is possible that there were systematic errors in the correction. After the data were corrected it became apparent that the respiratory exchange ratios were at the lower end of the physiological range (i.e. 0.7 to 0.85) with a couple of values falling below 0.7 – the RER for 100 % fat oxidation. This is probably due to the correction factors applied 'overcorrecting' the data to some extent and was probably due to some systematic error introduced in the correction process (see section 3.4.1.4). This error is likely to have been small as the oxygen uptakes fell within the expected range, but in any case, as the same error would have been present in both the exercise and control data sets, the data sets would have been correct relative to each other. Thus at the very least, the changes in substrate utilisation due to prior exercise could be ascertained from the present data.

To determine exogenous fat oxidation from ^{13}C excretion on the breath it is necessary to apply a correction factor for $[1,1,1-^{13}\text{C}]$ oxidised but not excreted on the breath. The majority of this loss is due to $^{13}\text{CO}_2$ being incorporated and trapped in metabolic intermediates (Elia, 1990) and it is possible to measure the recovery of $^{13/14}\text{CO}_2$ on the breath after administering $\text{NaH}^{13/14}\text{CO}_3$ either orally or intravenously and calculate a 'bicarbonate correction' to account for these losses. The magnitude of such a correction is matter of debate with different authors reporting recoveries of label on the breath ranging from 51 % (Irving et al. 1983) to 95 % (Elia et al. 1992). Recent data suggest that for an orally administered bolus of $\text{NaH}^{13}\text{CO}_3$, recovery of ^{13}C on the breath was 71.3 % of the ingested dose in males (Jones et al. 1999). As the route of administration and the subject group were similar to those in the present study, this seems like the most appropriate correction to use. In the present study, ^{13}C excretion on the breath over the 8 hour postprandial period was $11.9 \pm 0.8 \%$ and $15.1 \pm 0.9 \%$ of the ingested dose in the control and exercise trials respectively. If a bicarbonate correction assuming that 100 % oxidation of the label would result in 71.3 % recovery

on the breath was applied, this label excretion would imply that $16.7 \pm 1.1 \%$ and $21.2 \pm 1.3 \%$ of the ingested dose was oxidised in the control and exercise trials respectively. This corresponds to $16.4 \pm 1.2 \text{ g}$ and $20.5 \pm 1.2 \text{ g}$ of exogenous fat oxidation in the control and exercise trials respectively – i.e. 4.1 ± 0.4 extra grams of exogenous fat were oxidised in the exercise trial. In the exercise trial, whole body fat oxidation was $9.6 \pm 2.2 \text{ g}$ greater than in the control trial over the 8 hour postprandial period. Thus, the increase in fat oxidation seen postprandially following exercise cannot be accounted for solely by an increase in exogenous fat oxidation. If extreme values for bicarbonate recovery are applied (i.e. 50 % and 100 %), the calculated extra exogenous fat oxidation in the exercise trial would range from $2.9 \pm 0.3 \text{ g}$ (100 % bicarbonate recovery) to $5.9 \pm 0.6 \text{ g}$ (50 % recovery). Thus, even when the limitations of the data are fully considered, it is clear that both exogenous and endogenous fat oxidation were increased in the exercise trial.

This knowledge can help our understanding of the tissues mobilising and oxidising additional fat in the exercise trial. It is clear that hepatic fat oxidation was enhanced in the exercise trial. As the fatty acid flux to the liver would have included chylomicron and VLDL fatty acids derived from the hydrolysis of TAG-rich lipoproteins ‘spilt-over’ into the circulation and fatty acids derived from the lipolysis of adipose tissue-TAG, the increases in both exogenous and endogenous fat oxidation are likely to have contributed to the increase in hepatic fat oxidation. Muscle is the other tissue in which fat oxidation is likely to have been elevated in the exercise trial. Like the liver, increases in both endogenous and exogenous fat oxidation probably occurred. Recent data from Kiens and Richter (1998) have shown that intramuscular TAG concentrations were still decreasing 18 hours after completion of an exercise session indicating that intramuscular TAG oxidation was still elevated at this time. The authors reasoned that this increase in intramuscular fat oxidation occurred to cover the muscle’s energy needs while muscle glycogen was being resynthesised. Thus, in the present study an increase in intramuscular TAG oxidation is likely to have contributed to the increase in endogenous fat oxidation. For an increase in exogenous fat oxidation to have occurred in skeletal muscle in the exercise trial, one or more of the following must have taken place:

- 1) Chylomicron derived fatty acids sourced either from the circulation or from the action of skeletal muscle LPL were oxidised at a greater rate once entrapped in the muscle.
- 2) Greater entrapment of chylomicron derived fatty acids derived from the action of skeletal muscle LPL, increasing the flux of exogenous substrate for oxidation.
- 3) Activity of muscle LPL activity in skeletal muscle was increased leading to increased hydrolysis of chylomicron-TAG thereby increasing the flux of exogenous substrate for oxidation.
- 4) The flux of chylomicron derived fatty acids 'spilt-over' into the circulation after poor entrapment in adipose tissue was increased and these fatty acids were entrapped and oxidised in skeletal muscle.

The first option is likely to have contributed because after exercise muscle is primed to oxidise fat while carbohydrate resynthesis is occurring (Kiens and Richter, 1998). Therefore it is not unreasonable to suppose that oxidation of fatty acids derived from TAG-rich lipoproteins, as well as from the muscle's endogenous stores, was increased. The second option might also have contributed as it is not inconceivable that a muscle with reduced TAG stores would entrap fatty acids more efficiently to facilitate intramuscular TAG replenishment. However, the limited data available suggest that fatty acid entrapment in muscle is good even in the unexercised state (Coppack et al. 1990), diminishing the likelihood that this mechanism is a major contributor to increased exogenous fat oxidation. For the third mechanism to contribute, muscle LPL activity must be increased in the exercise trial. While the data suggest that increased muscle LPL activity was not the predominant mechanism via which exercise was reducing plasma TAG concentrations, it is likely to have been a contributory mechanism and thus might have contributed to the exogenous fat oxidation. The final mechanism requires increased 'spill-over' of lipoprotein derived fatty acids from adipose tissue. Preliminary data from this study suggests that this did not occur, thus this mechanism's contribution to increased exogenous fat oxidation in skeletal muscle is likely to have been small.

A similar oxygen uptake rate was assumed in both trials to enable corrections to the expired air data to be made but this assumption is unlikely to be erroneous considering the findings from previous studies (Tsetsonis and Hardman, 1996; Herd,

1997). Thus, with the increase in fat oxidation, there was a concomitant decrease in carbohydrate oxidation in the exercise trial, resulting in a similar rate of energy expenditure in the two trials. However, although exercise did not appear to influence resting metabolic rate, the shift in energy metabolism towards fat oxidation may have important implications for the regulation of body fat mass and the prevention of obesity. These issues have been addressed in the general discussion section of this thesis.

In one subject, exercise did not reduce postprandial TAG concentrations. He was markedly hypertriglyceridaemic, so it is likely that he possessed some defects to lipoprotein metabolism. However, the nature of his postprandial responses to the test meal are of particular interest given the proposed mechanism for the exercise-induced reduction in plasma TAG concentrations – i.e. reduced VLDL secretion. This subject had very low fasting and postprandial 3-hydroxybutyrate concentrations in the control trial and, importantly, these concentrations did not rise in the exercise trial. Thus, he appeared to be unable to partition the NEFA flux to the liver towards oxidation and ketogenesis and therefore overproduced VLDL. As he could not upregulate hepatic fatty acid oxidation in the exercise trial, his VLDL-TAG concentration was not attenuated. This also could, at least in part, explain his excessive chylomicronaemia – the elevated VLDL concentrations would reduce chylomicron clearance as they would profoundly increase competition for hydrolysis by lipoprotein lipase. Interestingly he upregulated fat oxidation in the exercise trial, although this increase was completely accounted for by an increase in endogenous fat oxidation. As his NEFA concentrations were not elevated in the exercise trial, suggesting no increase in adipose tissue TAG mobilisation, it is likely this increase was due increased oxidation of intra-muscular TAG stores, by the same mechanism as that proposed for the other subjects.

In conclusion, although moderate exercise attenuated both fasting and postprandial TAG concentrations as expected, the qualitative nature of these changes was unexpected. The present findings suggest that moderate exercise attenuated plasma TAG concentrations by reducing the rate of VLDL appearance into the circulation rather than increasing the rate of TAG clearance and shifts the focus of attention from skeletal muscle to the liver. Further research is required both to confirm these

findings and to investigate the effects of moderate exercise on the hepatic regulation of lipid metabolism.

CHAPTER 6

THE EFFECTS OF PRIOR MODERATE EXERCISE ON POSTPRANDIAL LIPAEMIA AND ON CLEARANCE OF A LIPID EMULSION

6.1 Introduction

In chapter 5, it was shown that moderate exercise attenuated postprandial lipaemia primarily by reducing VLDL-TAG concentrations, suggesting that mechanisms other than increased TAG clearance were mediating the exercise-induced plasma TAG reduction. Although this finding was unexpected, there is currently little direct evidence to suggest whether a 90-minute moderate intensity exercise session can in fact increase TAG clearance.

It has been demonstrated that athletes clear an intravenous fat emulsion from the circulation at an accelerated rate compared to matched controls (Ericsson et al. 1982; Sady et al. 1988; Podl et al. 1994) and that a prolonged period (32 to 52 weeks) of endurance exercise training can improve TAG clearance (Thompson et al. 1988; Zmuda et al. 1998). In addition, marathon running (Sady et al. 1986) and three-hour vigorous (Dufaux et al. 1981) or moderate intensity (Annuzzi et al. 1987) exercise sessions have been shown to increase TAG clearance measured on the morning after exercise. However, Annuzzi et al (1987) found that after 90-minutes of moderate exercise, TAG clearance was not increased. Thus, although exercise training and prolonged single exercise sessions can increase TAG clearance, it is not clear whether a 90-minute moderate intensity exercise session provides a sufficient stimulus for this effect, despite the wealth of data suggesting that this amount of exercise is sufficient to reduce postprandial lipaemia (Aldred et al. 1994; Tsetsonis and Hardman, 1996a; Tsetsonis and Hardman, 1996b; Tsetsonis et al. 1997; Zhang et al. 1998; Gill et al. 1998; Malkova et al. 1999).

To the author's knowledge, no study has compared the effect of a moderate intensity exercise session on TAG clearance and postprandial lipaemia in the same group of subjects. Such a study would be necessary to determine whether the attenuating effect of moderate exercise on postprandial lipaemia was in fact attributable to increased TAG clearance or whether there are other mechanisms mediating the reduced TAG concentrations observed post-exercise. Therefore, the purpose of the present study

was to compare the effect of a 90-minute moderate intensity exercise session on both postprandial lipaemia, and clearance of a bolus injection of Intralipid (a chylomicron-like emulsion), in a group of middle-aged men.

In the previous chapter, the fat load in the oral fat tolerance tests was 1.32 g.kg^{-1} body mass, which corresponded to $99 \pm 18 \text{ g}$ (mean \pm SEM) of fat being ingested for this subject group. This large fat load was employed to provide a large degree of stress to TAG metabolism and therefore maximise potential differences between the control and exercise conditions. As the previous chapter established a clear attenuation of postprandial lipaemia by exercise in middle-aged men, it was decided to reduce the oral fat load to 1 g.kg^{-1} body mass to make the fat challenge more representative of daily fat intake.

6.2 Pilot study

6.2.1 Introduction

The intravenous fat tolerance test protocol is shown in section 3.7.2. but, briefly, involves a bolus injection of an Intralipid emulsion through an indwelling cannula placed in an antecubital vein with subsequent blood sampling to assess TAG clearance. As blood samples were to be taken fairly rapidly following the Intralipid injection (starting 2.5 minutes post injection), it is possible that early blood samples taken via the same cannula might have been contaminated with Intralipid which had not completely dispersed from the injection site. To minimise subject discomfort it was desirable to avoid placing a cannula in the other arm for blood sampling, unless this was absolutely necessary. Therefore, a pilot study was performed in which blood samples were obtained from the Intralipid injection site as well as from a cannula placed in the contralateral arm, to assess whether sampling from the injection site distorted the data. The pilot study also provided an opportunity to determine the Intralipid dose to administer in the main trials.

6.2.2 Methods

Two female subjects (subject A: age 40 years, body mass 68 kg, subject B: age 47, body mass 80 kg) were cannulated in the antecubital fossa of both arms. After baseline blood samples were obtained from both arms, a bolus dose of 20 % Intralipid

(0.75 ml (0.15g fat) and 0.5 ml (0.10g fat) per kg body mass for subjects A and B respectively) was injected through one cannula over a two-minute period. The cannula extension through which the Intralipid was injected was changed after the injection for subject B, as it was observed that for subject A, the extension remained coated with Intralipid after the injection possibly contaminating the subsequent blood samples. Blood samples were simultaneously obtained from both arms 2.5, 5, 7.5, 10, 15, 20, 25, 30, 40 and 60 minutes after the Intralipid injection and plasma was subsequently analysed for TAG.

6.2.3 Results

Fasting TAG concentrations were 0.57 mmol.l^{-1} and 0.44 mmol.l^{-1} for subjects A and B respectively. Figure 6.1 shows post Intralipid plasma TAG concentrations from both arms for subject A. Peak TAG concentration was 4.15 mmol.l^{-1} at the injection site and 3.88 mmol.l^{-1} in the contralateral arm, and TAG concentrations remained higher in blood sampled from the injection site until 20 minutes after the injection. First order (exponential) TAG clearance kinetics (i.e. the TAG clearance rate was directly proportional to the plasma TAG concentration) were observed with goodness-of-fit R^2 values (where $R^2 = \text{total sum of squares} - \text{residual sum of squares} / \text{total sum of squares}$) for exponential regression lines of 0.9955 and 0.9941 for the injection site and contralateral arms respectively.

Figure 6.2 shows the same data for subject B. Peak TAG concentration was 2.76 mmol.l^{-1} and 2.72 mmol.l^{-1} for the the injection site and contralateral arms respectively and observation of the Figure shows that plasma TAG concentrations were virtually identical from the two arms. When the entire TAG clearance curves were considered, the goodness-of-fit for exponential regression lines was poor with R^2 values of 0.8997 at the injection site and 0.8743 for the contralateral arm. However, when the first 20 minutes of the clearance curves were considered, the R^2 values for the exponential regression lines rose to 0.9939 and 0.9800 for the injection site and contralateral arms respectively, suggesting that first order clearance kinetics were being observed during the early part of the TAG clearance curves.

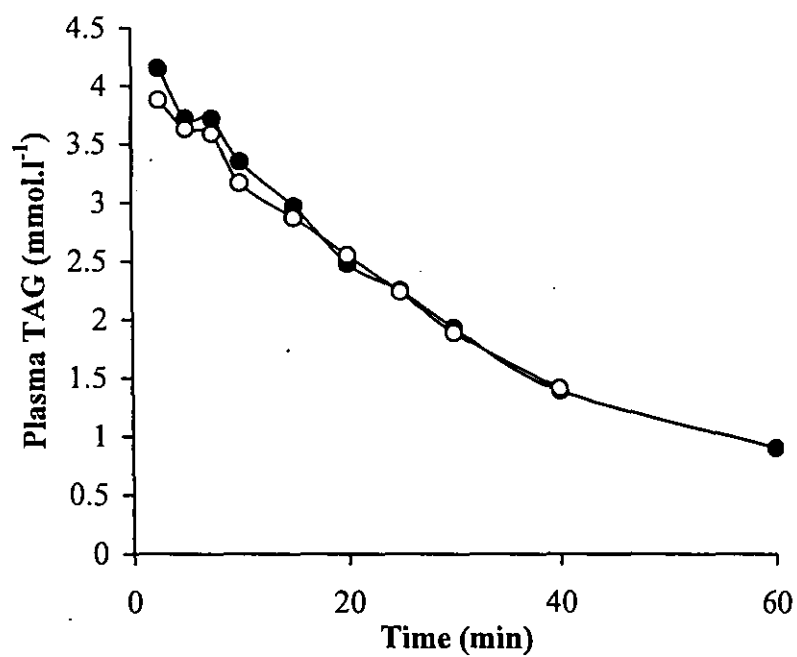


Figure 6.1 Plasma TAG concentrations, after a 0.75 ml.kg^{-1} injection of Intralipid, at the injection site (filled) and in the contralateral arm (open), for subject A.

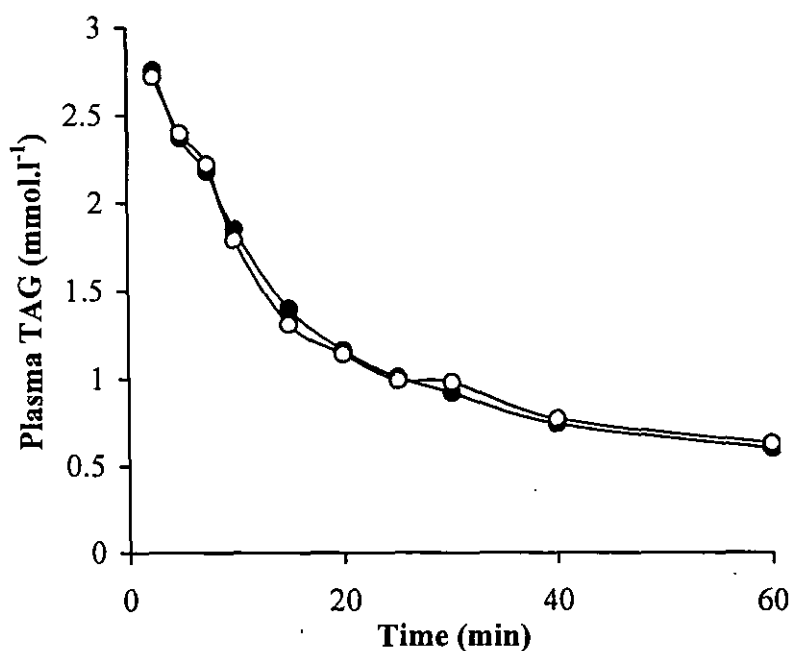


Figure 6.2 Plasma TAG concentrations, after a 0.5 ml.kg^{-1} injection of Intralipid, at the injection site (filled) and in the contralateral arm (open), for subject B.

6.2.4 Discussion

For subject A, who received an Intralipid dose of 0.75 ml.kg^{-1} , TAG concentrations were higher at the injection site than the contralateral arm. This discrepancy between sampling sites may have been a consequence of the Intralipid not fully dispersing from the injection site prior to the first sample or of Intralipid remaining in the cannula extension and contaminating blood samples. However for subject B, who received a lower Intralipid dose of 0.5 ml.kg^{-1} and whose cannula extension was changed, post-injection TAG concentrations were similar in both arms. This suggested that if this Intralipid dose was used and the cannula extension was changed after the injection, the same cannula could be used for the Intralipid injection and blood sampling, reducing subject discomfort.

Although Intralipid clearance from the circulation is usually reported to observe first order kinetics (Rössner, 1974; Rössner, 1976; Redheendran et al. 1979; Rössner and Kirstein, 1984; Harris and Muzio, 1993), in the present data set exponential regression lines only provided a good fit for the 60-minute TAG clearance curve for subject A

(who received the higher Intralipid dose). However, the poor exponential fit for subject B might have been a consequence of Intralipid TAG being recirculated into the endogenous TAG pool at the later time points, as this has a proportionally greater effect on plasma TAG concentrations when the Intralipid dose is lower (Hallberg, 1965). By eliminating this subject's later data points from the analysis, a good exponential fit was obtained, supporting the idea that recirculating Intralipid influenced the later TAG concentrations. Thus, for subject B, it was possible to use the first 20 minutes of the TAG clearance curve – which followed first order clearance kinetics – to obtain a TAG clearance constant.

On balance, it was decided to use an Intralipid dose of 0.5 ml.kg^{-1} body mass in the experimental trials, as with this dose it was clear that blood could be sampled from the same site as the Intralipid injection without contamination, eliminating the need for a second cannulation. Although first order clearance kinetics were only observed for the first portion of the TAG clearance curve with this Intralipid dose, this was adequate for determination of a TAG clearance constant (k_2), thus, a higher Intralipid dose was not needed. Indeed, 0.1 g fat per kg body mass is an Intralipid dose commonly utilised in intravenous fat tolerance tests (Rössner, 1974; Rössner, 1976; Redheendran et al. 1979; Rössner, 1982; Rössner and Kirstein, 1984). It was decided to retain the 60-minute post injection observation period so that the portion of the curve which obeyed first order kinetics could be assessed on an individual basis.

6.3 Subjects and Methods

6.3.1 Subjects

Eight male volunteers participated in the study, which was conducted with the approval of the Loughborough University Ethical Advisory Committee. All subjects were fully informed of the procedures and risks involved and gave their written consent to take part. All subjects were apparently healthy non-smokers and none was receiving any drugs thought to affect lipid or carbohydrate metabolism. Five subjects exhibited the E3/E3 apo E phenotype, two subjects possessed the E4/E3 phenotype and one possessed the E4/E2 phenotype. Some physical characteristics of the subjects are presented in Table 6.1.

Age (year)	48.3 ± 7.3
Height (m)	1.76 ± 0.07
Body mass (kg)	79.1 ± 7.0
Sum of 4 skinfolds (mm)	73.9 ± 22.1
BMI (kg.m ⁻²)	25.5 ± 2.8
$\dot{V}O_2$ max (ml.kg ⁻¹ .min ⁻¹)	39.0 ± 6.1
TAG, in the fasted state (mmol.l ⁻¹)	1.37 ± 0.62
Total cholesterol, in the fasted state (mmol.l ⁻¹)	4.99 ± 1.11
HDL cholesterol, in the fasted state (mmol.l ⁻¹)	1.01 ± 0.30

Table 6.1 Physical characteristics of subjects (n = 8), (mean ± standard deviation)

6.3.2 Preliminary testing

Subjects all underwent a clinical exercise stress test (section 3.2) prior to inclusion in the study to ensure they were free from existing cardiovascular disease and had no cardiovascular contraindications to prolonged exercise. Subjects were familiarised with treadmill walking (section 3.6) then, on separate occasions, performed submaximal (section 3.6.1) and maximal (section 3.6.2) incremental exercise tests to find the gradient required to elicit 60 % $\dot{V}O_2$ max.

6.3.3 Study protocol

In order to assess the effect of moderate exercise on both postprandial lipaemia and TAG clearance, subjects participated in four fat tolerance tests – two oral fat tolerance tests and two intravenous fat tolerance tests. One oral and one intravenous fat tolerance test was preceded by a moderate intensity exercise session on the prior afternoon, with the other oral and intravenous fat tolerance tests being no-exercise control trials. To balance the study design each subject performed the four fat tolerance tests in a different order as shown in Table 6.2. The protocols for the oral and intravenous fat tolerance tests are shown in Figures 6.3 and 6.4 respectively.

Subject	Trial 1	Trial 2	Trial 3	Trial 4
1	Ex OFTT	Con OFTT	Ex IVFTT	Con IVFTT
2	Ex OFTT	Con OFTT	Con IVFTT	Ex IVFTT
3	Con OFTT	Ex OFTT	Ex IVFTT	Con IVFTT
4	Con OFTT	Ex OFTT	Con IVFTT	Ex IVFTT
5	Ex IVFTT	Con IVFTT	Ex OFTT	Con OFTT
6	Ex IVFTT	Con IVFTT	Con OFTT	Ex OFTT
7	Con IVFTT	Ex IVFTT	Ex OFTT	Con OFTT
8	Con IVFTT	Ex IVFTT	Con OFTT	Ex OFTT

Table 6.2 Order of testing for subjects 1 to 8 ensure a balanced experimental design. (Ex: exercise trial, Con: control trial, OFTT: oral fat tolerance test, IVFTT: intravenous fat tolerance test)

On day 1 of each of the control trials (i.e. the control oral fat tolerance test and the control intravenous fat tolerance test), subjects did no exercise and performed only the activities of daily living. On day 1 of each exercise trial subjects performed identical 90-minute treadmill walking sessions in the afternoon, at intensities of approximately 60 % $\dot{V}O_2$ max (section 3.6.3).

On day 2 subjects either underwent an oral fat tolerance test as described in section 3.7.1., or an intravenous fat tolerance test as described in section 3.7.2. In the oral fat tolerance tests, blood samples were obtained prior to the test meal (in the fasted state), for six hours postprandially and 24 hours after the test meal (in the fasted state). In the intravenous fat tolerance tests blood was obtained in the fasted state and for 60 minutes after the Intralipid injection (0.5 ml 20 % Intralipid (0.1 g fat) per kg body mass). All blood was sampled, and plasma or serum stored, as described in section 3.8., but for blood samples obtained after the Intralipid injection, particular care was taken when aspirating the plasma layer from the red cells after centrifugation to ensure that the lipid was homogenous throughout the plasma and that no lipid

remained on the walls of the Monovette. The test meal ingested in the oral fat tolerance tests was cereal based and provided 1.00 g fat, 0.97 g carbohydrate and 0.15 g protein per kg body mass (section 3.7.1.1). Subjects also ingested 1.5 g paracetamol with the meal as a marker of gastric emptying (Heading et al. 1973; Clements et al. 1978).

Subjects weighed and recorded their dietary intake for the two days prior to the first fat tolerance test and replicated this prior to all subsequent fat tolerance tests. After the six-hour observation period of their first oral fat tolerance test, subjects were given a light meal and this was replicated at the end of their second oral fat tolerance test. In addition, subjects weighed and recorded their evening meal after the first oral fat tolerance test and repeated this after their second oral fat tolerance test.

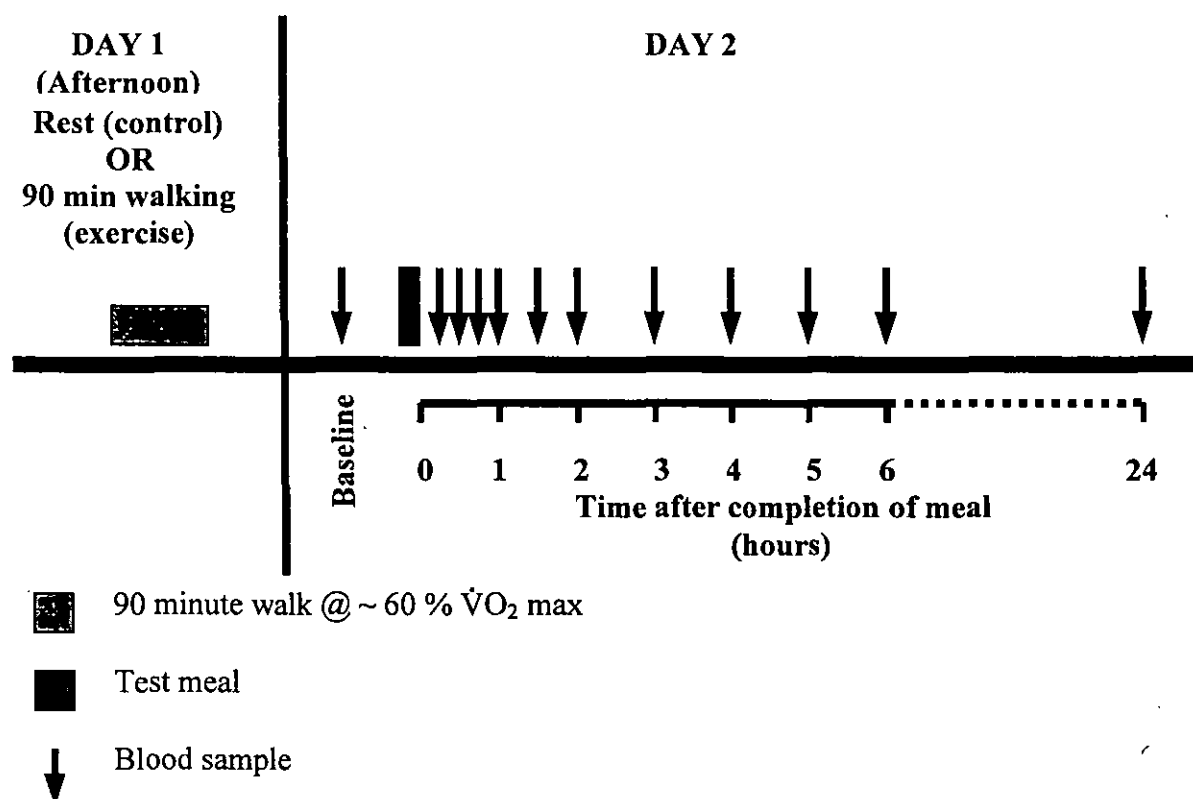


Figure 6.3 Schematic representation of the oral fat tolerance test protocol

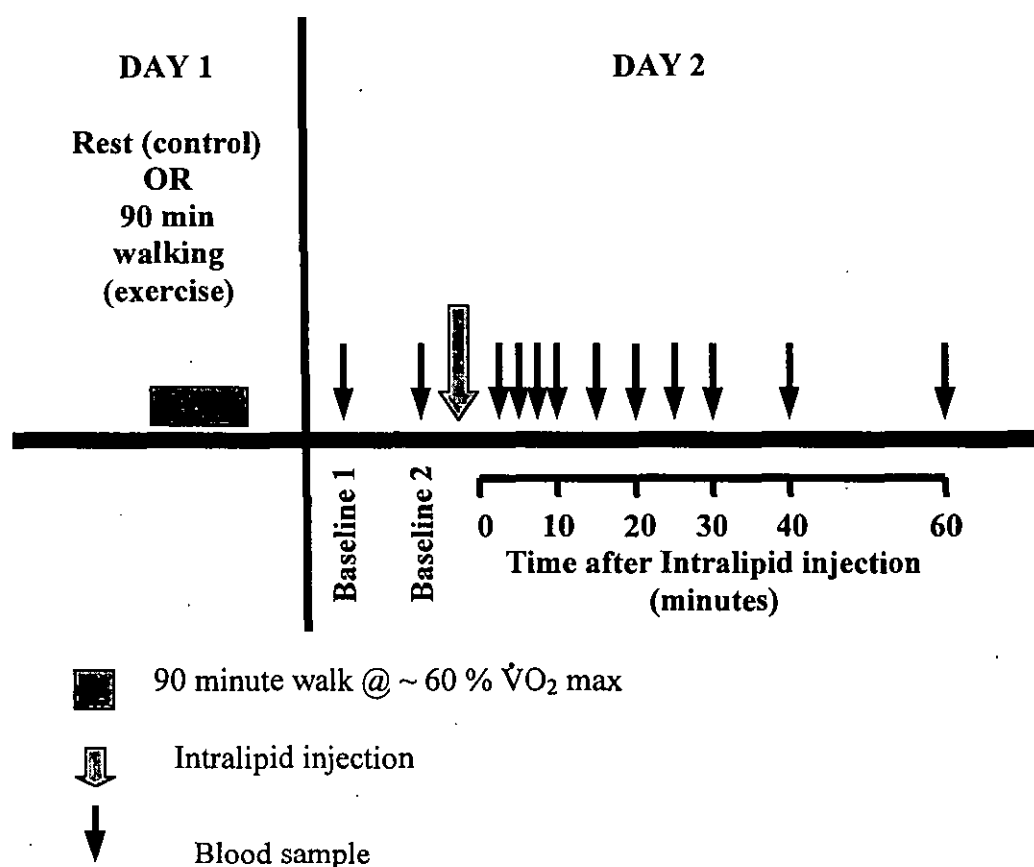


Figure 6.4 Schematic representation of the intravenous fat tolerance test protocol

6.3.4 Analysis of blood samples

Haemoglobin concentration and haematocrit were measured at baseline and the end of each oral fat tolerance test and at baseline in each intravenous fat tolerance test.

However, for reasons described in section 4.3.2 and 5.3.2, it was decided not to correct concentrations for differences in plasma volume between trials. In the oral fat tolerance tests, plasma samples were analysed for HDL and total cholesterol concentrations at baseline (in the fasted state), six hours after ingestion of the test meal and again 24 hours after the test meal (in the fasted state). In the intravenous fat tolerance tests plasma was analysed for HDL and total cholesterol concentrations at baseline. In the oral fat tolerance tests, plasma was analysed for TAG at all time points and for glucose, paracetamol and NEFA at all time points except 24 hours. Serum samples were analysed for insulin at all time points except 24 hours. Plasma

was analysed for TAG and NEFA at all time points in the intravenous fat tolerance tests. Details of the analyses are shown in section 3.9.

6.3.5 Data analysis

In the oral fat tolerance tests the six-hour areas under the plasma or serum concentration vs. time curves calculated using the trapezium rule were defined as the respective total responses to the test meal. These areas normalised to the baseline concentrations were defined as the incremental responses. The peak paracetamol concentration and time to peak concentration were used to assess gastric emptying in the two trials (Heading et al. 1973; Clements et al. 1978).

To calculate TAG clearance rates in the intravenous fat tolerance tests, the mean of the two baseline TAG concentrations was subtracted from each post Intralipid TAG concentration to give values for TAG rise and all values were expressed as a percentage of the peak value. Exponential regression lines were fitted to the TAG clearance curves to determine the first-order TAG clearance constant (k_2) (Rössner, 1974). It was decided to evaluate TAG clearance using plasma TAG quantification rather than nephelometry, as the latter technique has been reported to produce less reproducible results (Sady et al. 1986). Sixty-minute areas under the NEFA concentration vs. time curves were calculated using the trapezium rule.

Mean values in the fasted state, areas under curves and TAG clearance rates were compared using t-tests for correlated means adopting a 5 % level of significance. Two-way ANOVA with repeated measures, with post-hoc Tukey tests, were used to compare data within and between trials. Data are presented as mean \pm SEM.

6.4 Results

6.4.1 Responses during treadmill walking

The mean $\dot{V}O_2$ during the 90-minute walk on day one of the exercise oral fat tolerance test trial was $23.2 \pm 1.5 \text{ ml.kg}^{-1}.\text{min}^{-1}$, which represented $59.4 \pm 1.0 \% \dot{V}O_2 \text{ max}$. In the corresponding walk on day 1 of the intravenous fat tolerance test trial, mean oxygen uptake was $23.4 \pm 0.9 \text{ ml.kg}^{-1}.\text{min}^{-1}$, which corresponded to $59.8 \pm 0.9 \% \dot{V}O_2 \text{ max}$. Mean heart rate was $134 \pm 3 \text{ beat.min}^{-1}$ in the oral fat tolerance test walk and $135 \pm 3 \text{ beat.min}^{-1}$ in the intravenous fat tolerance test walk. The perception of effort was between 'fairly light' and 'somewhat hard' for both the walks, corresponding to values of 12.6 ± 0.6 and 12.2 ± 0.6 on the Borg scale (Borg, 1973) for the oral and intravenous test walks, respectively.

6.4.2 Plasma and serum concentrations in the fasted state

Table 6.3 shows plasma and serum concentrations in the fasted state. Plasma TAG concentrations in the fasted state were reduced by $0.25 \pm 0.10 \text{ mmol.l}^{-1}$ ($16 \pm 7 \%$) in the exercise oral fat tolerance test compared with the control oral fat tolerance test ($p < 0.05$). In the exercise intravenous fat tolerance test there was a similar reduction in plasma TAG of $0.26 \pm 0.12 \text{ mmol.l}^{-1}$ ($18 \pm 7 \%$) compared with the intravenous control trial ($p = 0.06$). Fasting total cholesterol and HDL cholesterol concentrations were not altered by exercise prior to either the oral or intravenous fat tolerance tests. Plasma NEFA concentrations were $15 \pm 7 \%$ and $10 \pm 8 \%$ higher than control in the exercise oral and intravenous fat tolerance tests respectively, but these differences were not statistically significant ($p = 0.06$ and $p = 0.26$). Neither fasting insulin nor fasting glucose concentrations differed between the two oral fat tolerance test trials.

	Control OFTT	Exercise OFTT	Control IVFTT	Exercise IVFTT
Plasma TAG (mmol.l ⁻¹)	1.37 ± 0.22	1.12 ± 0.18*	1.33 ± 0.24	1.07 ± 0.18 [#]
Total cholesterol (mmol.l ⁻¹)	4.99 ± 0.39	4.82 ± 0.29	4.95 ± 0.33	4.89 ± 0.30
HDL cholesterol (mmol.l ⁻¹)	1.00 ± 0.11	1.00 ± 0.10	1.01 ± 0.10	1.04 ± 0.11
NEFA (mmol.l ⁻¹)	0.47 ± 0.03	0.53 ± 0.03 [#]	0.48 ± 0.01	0.52 ± 0.04
Glucose (mmol.l ⁻¹)	5.52 ± 0.16	5.56 ± 0.16	-	-
Insulin (μU.ml ⁻¹)	12.1 ± 1.6	11.3 ± 1.5	-	-

Table 6.3 Plasma and serum concentrations in the fasted state in the control and exercise oral fat tolerance tests (OFTT) and intravenous fat tolerance tests (IVFTT).

*Significantly different from corresponding control trial. [#] p = 0.06 with corresponding control trial.

6.4.3 Plasma TAG and NEFA responses to a bolus injection of Intralipid

Figure 6.5 shows the plasma TAG clearance after the Intralipid injection. Peak plasma TAG concentrations after the Intralipid injection were 3.47 ± 0.26 mmol.l⁻¹ and 3.13 ± 0.27 mmol.l⁻¹ in the control and exercise trials respectively (p = 0.16). On all but two occasions, when the peak TAG concentration was observed 5 and 7.5 minutes after the Intralipid injection, peak TAG concentration was observed 2.5 minutes after the Intralipid administration. The maximum rise in plasma TAG concentration after the Intralipid injection was 2.13 ± 0.10 mmol.l⁻¹ in the control trial and 2.15 ± 0.17 mmol.l⁻¹ in the exercise trial. Fitting exponential regression lines to the entire 60 minute TAG clearance curves resulted in goodness-of-fit R² values (where R² = total sum of squares – residual sum of squares/total sum of squares) of only 0.924 ± 0.024 and 0.924 ± 0.020 in the control and exercise trials respectively and observation of the curves (see appendix C1 for individual TAG clearance curves) showed that they did not adequately fit the complete data set. These poor fits were probably a consequence of Intralipid recirculating in the endogenous pool at later time points (Hallberg, 1965). By eliminating the later data points and considering only the first 20 or 25-minute portion of the TAG clearance curves and removing obvious outlying data points by visual inspection of the data (see appendix C1), the R² values

rose to 0.988 ± 0.003 for the control trial and 0.964 ± 0.011 for the exercise trial. This indicated that first order kinetics were being observed during the early part of the TAG clearance curve. TAG clearance rates (k_2) derived from these truncated exponential plots were $4.69 \pm 0.49 \text{ \%} \cdot \text{min}^{-1}$ in the control trial and $4.85 \pm 0.40 \text{ \%} \cdot \text{min}^{-1}$ in the exercise trial. The TAG clearance rate was $8 \pm 12 \text{ \%}$ faster in the exercise trial, but this was not statistically significant ($p = 0.71$).

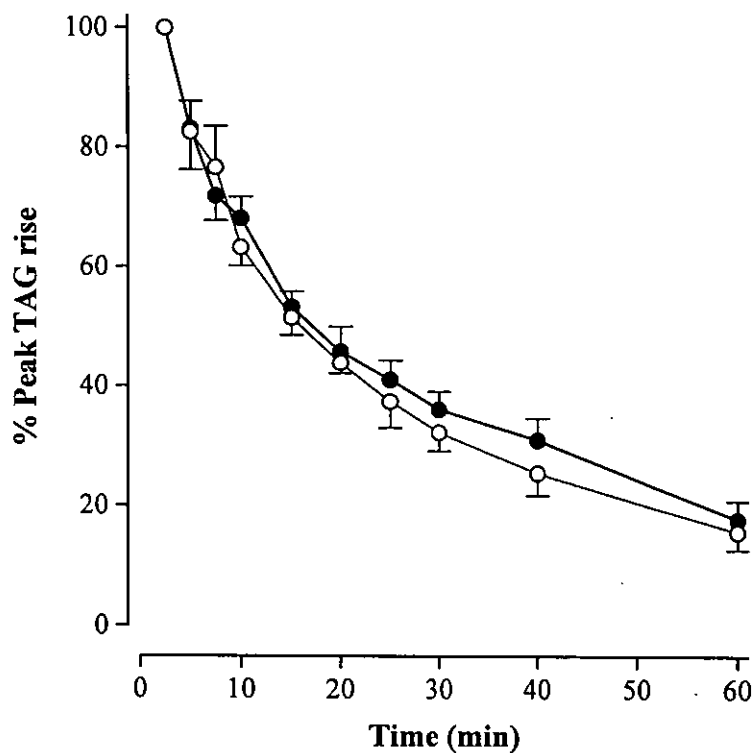


Figure 6.5 TAG clearance after the Intralipid injection in the control (filled) and exercise (open) trials.

Figure 6.6 shows plasma NEFA concentrations in the fasted state and in response to the Intralipid injection. The 60-minute area under the NEFA concentration vs. time curve was $29.27 \pm 0.98 \text{ mmol.l}^{-1}.\text{min}$ in the control trial and $32.60 \pm 1.56 \text{ mmol.l}^{-1}.\text{min}$ in the exercise trial. This response was $11 \pm 7 \%$ higher in the exercise trial, but the difference was not significant ($p = 0.12$). In the control trial, NEFA concentration was significantly higher than the 2.5-minute value, 30 minutes after the Intralipid injection. In the exercise trial, NEFA concentrations at 20, 25, 30 and 40 minutes were significantly higher than the 2.5-minute value.

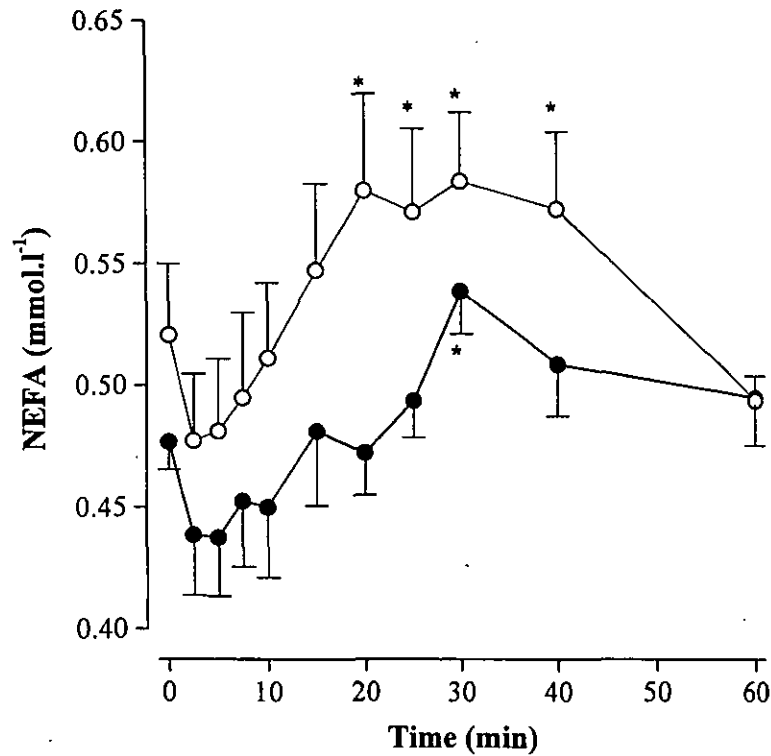


Figure 6.6 Plasma NEFA concentrations in the fasted state and for 60 minutes after the Intralipid injection in the control (filled) and exercise (open) trials.
*Significantly different from 2.5-minute NEFA concentration.

6.4.4 Postprandial plasma and serum concentrations in the oral fat tolerance tests

Figure 6.7 shows the six-hour plasma TAG response to the test meal. The total and incremental lipaemic responses are shown in Table 6.4. These responses were both significantly lower in the exercise trial than control by $18 \pm 6 \%$ and $18 \pm 6 \%$ for the total and incremental responses respectively (both $p < 0.05$). Plasma TAG concentrations in the fasted state 24 hours after ingestion of the test meal were $1.49 \pm 0.26 \text{ mmol.l}^{-1}$ and $1.36 \pm 0.21 \text{ mmol.l}^{-1}$ in the control and exercise trials respectively, these values did not differ significantly.

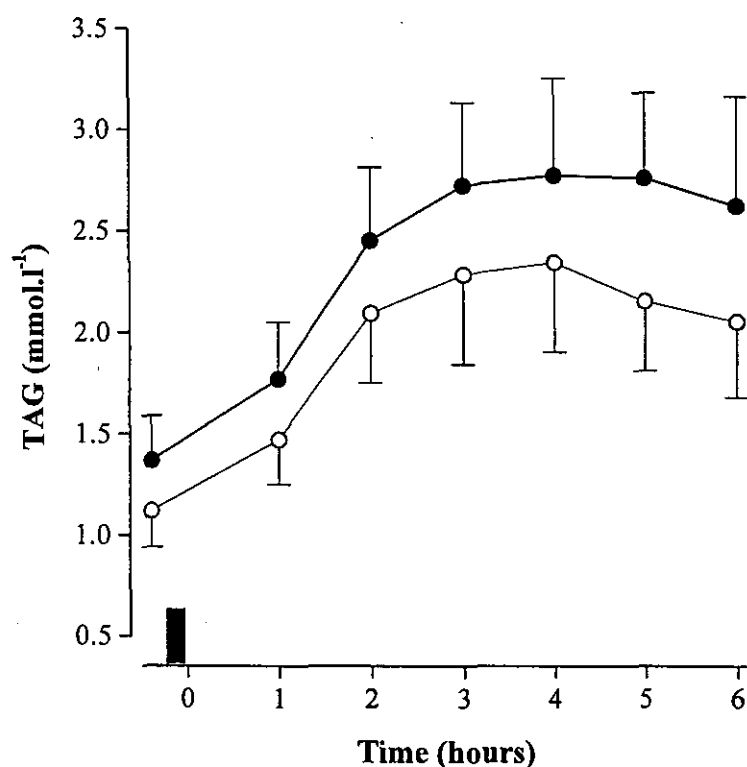


Figure 6.7 Plasma TAG concentrations in the fasted state and for six hours after consumption of the test meal in the control (filled) and exercise (open) trials. Black rectangle denotes ingestion of the test meal.

Plasma NEFA concentrations in the fasted state and after the test meal are shown in Figure 6.8, with the summary response displayed in Table 6.4. Although the area under the NEFA concentration vs time curve was $14 \pm 7\%$ higher in the exercise trial than control, this was not significantly different between trials ($p = 0.09$).

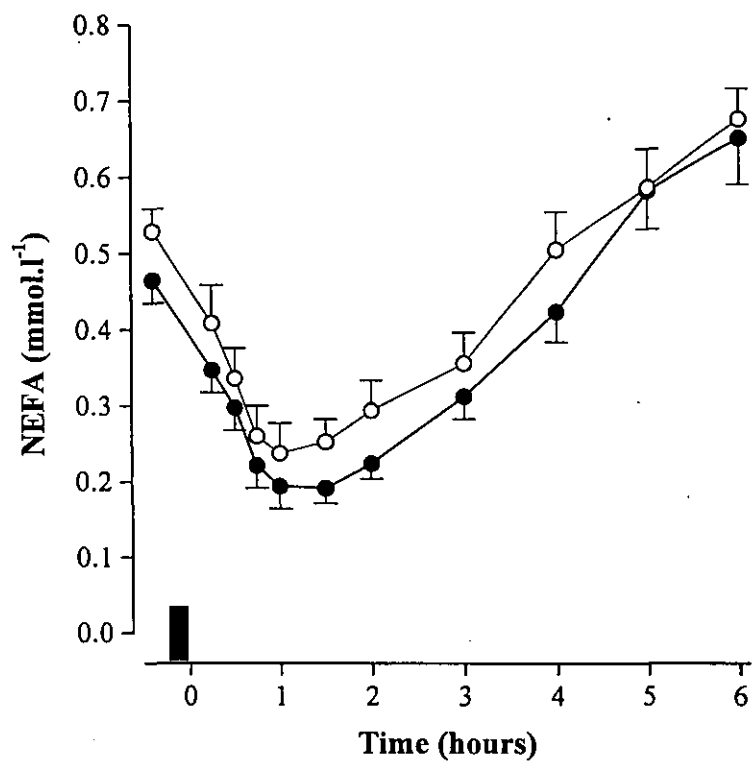


Figure 6.8 Plasma NEFA concentrations in the fasted state and for six hours after consumption of the test meal in the control (filled) and exercise (open) trials. Black rectangle denotes ingestion of the test meal.

Figure 6.9 shows serum insulin concentrations in the fasted and postprandial states with Table 6.4 showing the total and incremental responses. Although the total and incremental insulinaemic responses were $9 \pm 5 \%$ and $8 \pm 6 \%$ lower respectively in the exercise trial than the control trial, neither response differed significantly between trials ($p = 0.10$ and $p = 0.18$ for the total and incremental responses respectively).

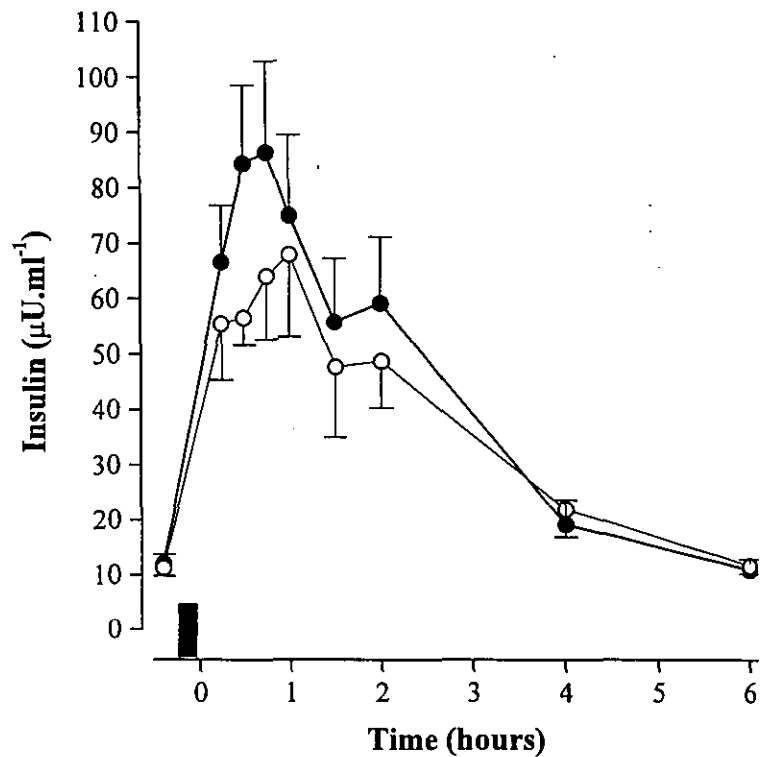


Figure 6.9 Serum insulin concentrations in the fasted state and for six hours after consumption of the test meal in the control (filled) and exercise (open) trials. Black rectangle denotes ingestion of the test meal.

Figure 6.10 shows plasma glucose concentrations in the fasted state, with the summary total and incremental responses shown in Table 6.4. Neither the total or incremental glycaemic responses differed between trials.

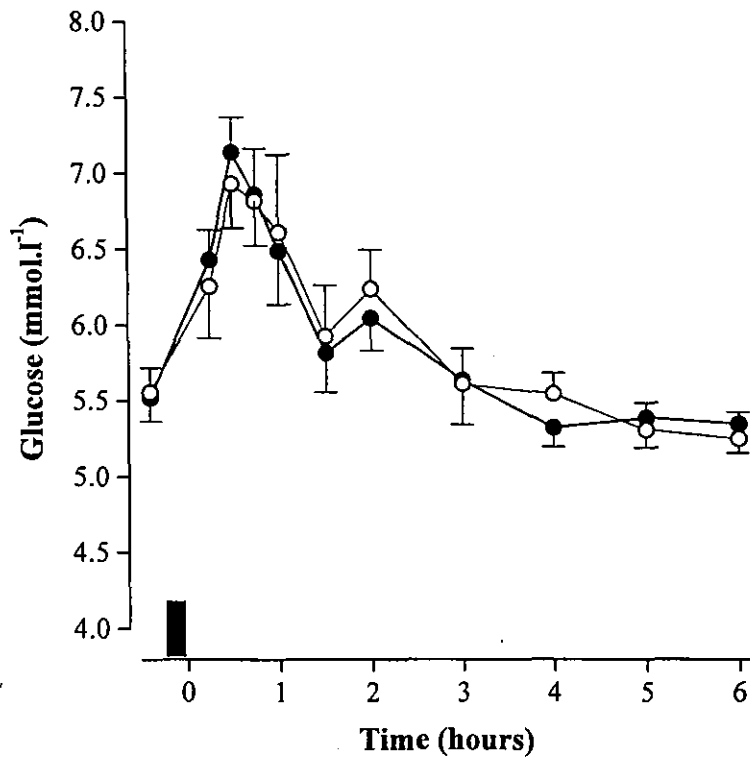


Figure 6.10 Plasma glucose concentrations in the fasted state and for six hours after consumption of the test meal in the control (filled) and exercise (open) trials. Black rectangle denotes ingestion of the test meal.

	Control	Exercise
Plasma total lipaemic response (mmol.l ⁻¹ .h)	14.48 ± 2.26	11.92 ± 1.95*
Plasma incremental lipaemic response (mmol.l ⁻¹ .h)	6.27 ± 1.02	5.20 ± 0.99*
Total NEFA response (mmol.l ⁻¹ .h)	2.26 ± 0.13	2.55 ± 0.17
Total insulinaemic response (μU.ml ⁻¹ .h)	241 ± 42	211 ± 30
Incremental insulinaemic response (μU.ml ⁻¹ .h)	168 ± 34	143 ± 23
Total glycaemic response (mmol.l ⁻¹ .h)	34.7 ± 0.7	35.0 ± 0.8
Incremental glycaemic response (mmol.l ⁻¹ .h)	1.6 ± 0.9	1.6 ± 1.0

Table 6.4 Total and incremental plasma and serum responses to the test meal in the control and exercise trials. *Significantly different from control ($p < 0.05$).

Figure 6.11 shows the plasma paracetamol responses to the test meal in the control and exercise trials. The peak plasma paracetamol concentration observed was $0.145 \pm 0.02 \text{ mmol.l}^{-1}$ in the control trial and $0.177 \pm 0.02 \text{ mmol.l}^{-1}$ in the exercise trial ($p = 0.06$), with the mode time to peak concentration being 15 minutes, and the median time to peak 22.5 (range 15 to 60 minutes) minutes, in both the exercise and control trials.

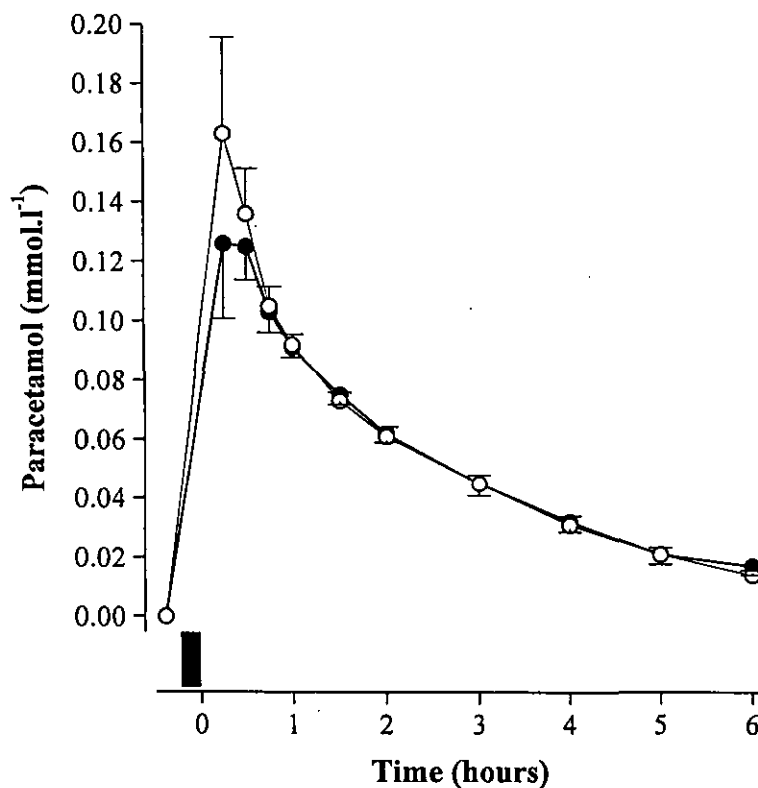


Figure 6.11 Plasma paracetamol concentrations for six hours after consumption of the test meal which contained 1.5 g of paracetamol, in the control (filled) and exercise (open) trials. Black rectangle denotes ingestion of test meal.

HDL cholesterol concentrations at baseline (in the fasted state), six hours after ingestion of the test meal and 24 hours after ingestion of the test meal (in the fasted state) are shown in Figure 6.12. HDL cholesterol concentrations did not differ between trials at baseline or 24 hours but were significantly higher in the exercise trial than the control trial six hours after ingestion of the test meal ($p < 0.05$). HDL cholesterol was lower than baseline six hours postprandially in the control ($p < 0.05$), but not the exercise trial ($p = 0.11$) and 24 hour concentrations were higher than baseline in both trials ($p < 0.05$). There was a significant trial by time interaction ($p < 0.05$), indicating that the pattern of change for HDL cholesterol differed between trials.

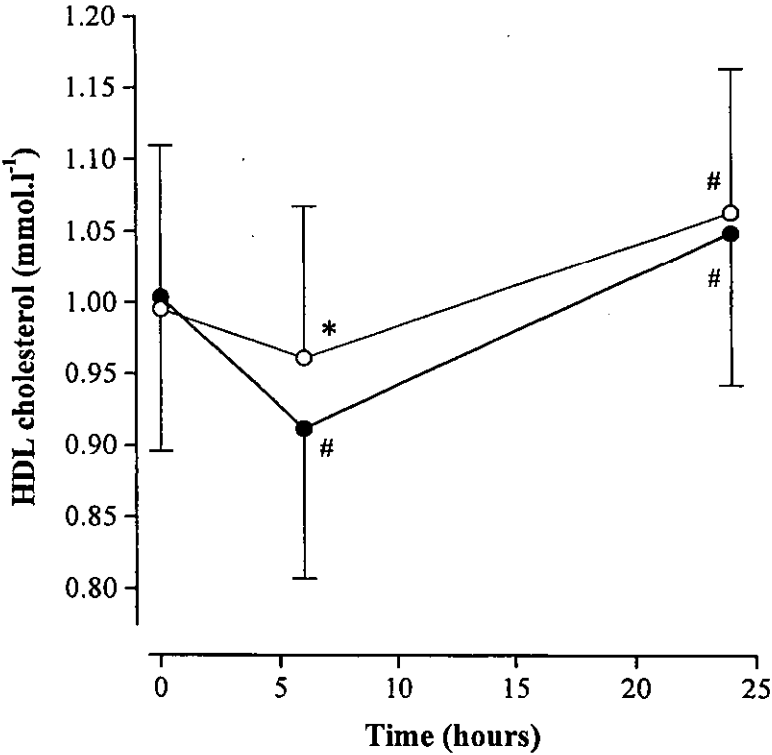


Figure 6.12 HDL cholesterol concentrations in the fasted state prior to ingestion of the test meal, six hours postprandially and in the fasted state 24 hours after the test meal in the control (filled) and exercise (open) trials.

*Different from control trial ($p < 0.05$), [#]Different from zero hour value ($p < 0.05$)

Figure 6.13 shows total cholesterol concentrations at baseline (in the fasted state), six hours after ingestion of the test meal and 24 hours after ingestion of the test meal (in the fasted state). Total cholesterol concentrations measured 24 hours after the test meal were significantly higher than the baseline values in both trials ($p < 0.05$) and, at this time point, total cholesterol concentrations were significantly lower in exercise than the control trial ($p < 0.05$). In these analyses, where total cholesterol concentrations were compared by 2-way ANOVA, the difference between trials for baseline total cholesterol concentration approached statistical significance ($p = 0.05$). This is in contrast to the results reported in section 6.4.2 where baseline total cholesterol concentrations were not significantly different between trials, when compared with a t -test ($p = 0.27$). The trial by time interaction was not significant ($p = 0.74$).

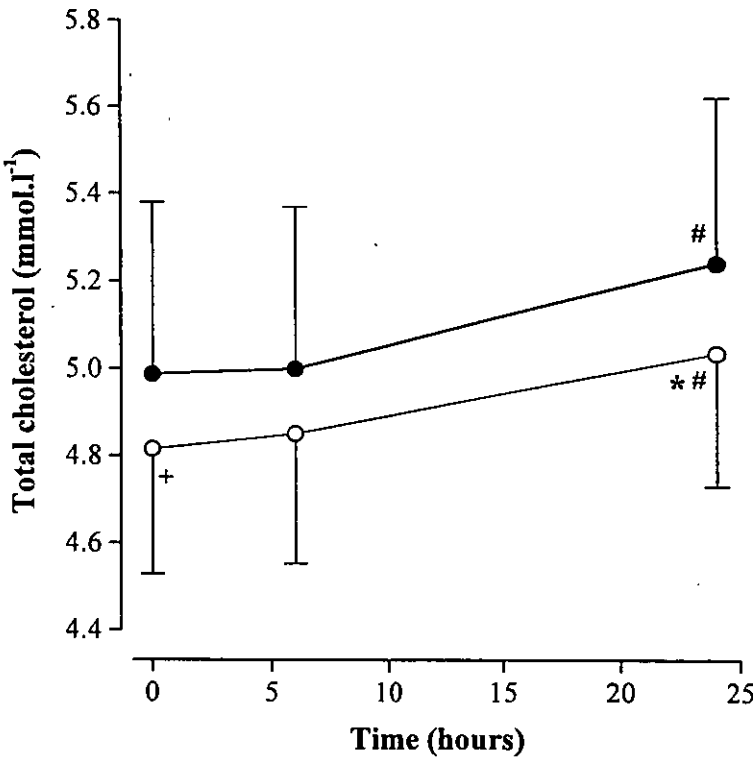


Figure 6.13 Total cholesterol concentrations in the fasted state prior to ingestion of the test meal, six hours postprandially and in the fasted state 24 hours after the test meal in the control (filled) and exercise (open) trials.

*Different from control trial ($p < 0.05$), #Different from zero hour value ($p < 0.05$),
⁺ $p = 0.05$ with control trial.

6.5 Discussion

The main finding of this study was that a moderate-intensity exercise session reduced fasting and postprandial TAG concentrations in this group of normolipidaemic middle aged-men but did not increase the clearance rate of an intravenously-administered Intralipid emulsion.

Intralipid is an artificial lipid emulsion with chylomicron-like properties. The particles are about the same size of chylomicrons (Lutz et al. 1989) but they do not contain apolipoproteins prior to injection. However, they rapidly acquire these, including apo C-II, from circulating lipoproteins once in the circulation (Carlson, 1980; Iriyama et al. 1988; Tonouchi et al. 1990), and are therefore able to be hydrolysed by LPL, in a similar manner to TAG-rich lipoproteins. The few studies directly comparing the metabolism of lipid-emulsion particles with chylomicrons have shown that both emulsion-TAG and chylomicron-TAG are cleared from the circulation with first order kinetics with emulsion-TAG being cleared almost as quickly as chylomicron-TAG (Carlson and Hallberg, 1963; Hultin et al. 1995). However, despite similar TAG clearance kinetics, lipid emulsion catabolism is not entirely analogous to chylomicron catabolism, as Hultin et al. (1995) found that core emulsion droplets were removed from the circulation with less TAG hydrolysis than was seen for chylomicrons. In addition, there are some data which suggest that the metabolic responses to intravenous infusion of lipid are not entirely similar to those seen after oral administration of lipids (Samra et al. 1998; Evans et al. 1999). However, despite the limitations of the intravenous fat tolerance test as a model for the clearance of chylomicrons from a meal, it is clear that a large proportion of lipid emulsion-TAG is removed in a similar manner to chylomicron-TAG and that there is a high correlation between chylomicron-TAG clearance and intravenous fat tolerance (Cohen, 1989). Thus, the finding that Intralipid-TAG clearance was not accelerated by exercise in the present study is strongly suggestive that lipoprotein-TAG clearance was not enhanced by exercise under the conditions studied.

It is unlikely that blood flow to peripheral tissues in the fasted state was increased in the exercise trials, as Intralipid-TAG clearance was similar in the exercise and control trials and increased peripheral blood flow mediates accelerated TAG clearance (for a given LPL activity). However, it is not immediately obvious whether exercise might

have influenced postprandial blood flow. An increase in adipose tissue (Coppack et al. 1990) and skeletal muscle (Baron et al. 1990) blood flow occurs in response to a mixed meal, although increased forearm (which is mainly muscle) blood flow has not always been observed (Coppack et al. 1990). This is likely to be mediated by insulin action, rather than gut hormone stimulation by the presence of food, as increased blood flow is not observed after a meal containing negligible carbohydrate (Evans et al. 1999). Thus, if exercise altered insulin action, postprandial blood flow might have been higher in the exercise trial than control. Therefore an insulin-mediated increase in postprandial TAG clearance, which was not evident in the intravenous fat tolerance test, was theoretically possible in the exercise oral fat tolerance test. This is unlikely to have occurred, because exercise did not significantly alter postprandial insulin concentrations in the current study and because previous research from this laboratory has demonstrated that an exercise-induced attenuation in postprandial lipaemia can occur without co-existing changes in serum insulin concentrations (Tsetsonis et al. 1997). Thus, it is unlikely that the reduced postprandial TAG concentrations were attributable to an insulin-mediated increase in blood flow. In any case, the results from chapter 5 of this thesis which showed that the exercise-induced TAG reduction was predominantly due to lower TAG concentrations in the VLDL, and not chylomicron fraction, argue against elevated postprandial blood flow in the exercise trial.

The Intralipid emulsion contains negligible carbohydrate and consequently an insulin response is not seen in response to an Intralipid infusion (Evans et al. 1999). Thus, the physiological responses to a mixed meal and an intravenous Intralipid injection differ markedly, as in the former marked elevations in serum insulin concentrations are evident. As insulin is an important co-ordinator in the postprandial period, increasing LPL activity in adipose tissue (Sadur and Eckel, 1982) while suppressing skeletal muscle LPL activity (Kiens et al. 1989), the absence of an insulin response could potentially influence interpretation of the intravenous fat tolerance test data. However, in the present study, postprandial insulin concentrations were not significantly affected by exercise in the oral fat tolerance tests, so it seems unlikely that an insulin-mediated increase in TAG clearance occurred following exercise in the oral fat tolerance test which was not evident in the intravenous fat tolerance test.

It must be noted that the intravenous fat tolerance test was conducted with subjects in the fasted state and, under these conditions, clearance of the Intralipid-TAG from the circulation models the occurrences of the early postprandial state. In the postprandial state LPL activity in adipose tissue can almost treble, peaking about five hours after a meal (Frayn et al. 1995), whereas LPL activity in skeletal muscle falls after consumption of a meal (Lithell et al. 1978). Thus, the body is in a very different physiological state late in the postprandial period compared with the first hours after consumption of a meal. Therefore, it is not possible from the present results to ascertain whether prior exercise might have increased TAG clearance late in the postprandial state, although the data do suggest that when fasted and early in the postprandial period, increased TAG clearance was not the predominant mediator of the reduced TAG concentrations.

The exercise sessions performed prior to the exercise oral and intravenous fat tolerance tests were performed at identical treadmill speeds and gradients and the physiological responses to exercise were remarkably similar in the two exercise trials (see section 6.4.1). In addition, the exercise-induced reduction to fasting TAG concentrations was very similar in the oral and intravenous fat tolerance tests ($16.2 \pm 7.1\%$ ($p = 0.046$) in the oral fat tolerance tests and $18.3 \pm 7.2\%$ ($p = 0.059$) in the intravenous fat tolerance tests). Thus, it cannot be argued that the absence of an exercise effect on Intralipid clearance was due to a smaller exercise stimulus in the intravenous fat tolerance test trials compared with the oral fat tolerance tests.

Intuitively, one might have expected exercise to accelerate Intralipid clearance given the body of evidence indicating that exercise can increase LPL activity. However, there is little evidence in the literature to suggest that a single exercise session of the intensity and duration used in the current study can increase TAG clearance. Although there is evidence to suggest that intense (Thompson et al. 1988) or moderate (Zmuda et al. 1998) exercise training can increase TAG clearance, the studies showing an increase in TAG clearance following a single exercise session have considered larger volumes of exercise than that used in the present study. Sady et al. (1986) found an increase in TAG clearance following a marathon race, and Annuzzi et al. (1987) and Dufaux et al. (1981) found that TAG clearance was increased the morning after three

hours of moderate or vigorous exercise, respectively. Indeed, Annuzzi et al. (1987) found that TAG clearance was not increased on the morning following 90 minutes of moderate intensity exercise (50 % peak power output, 70 to 85 % maximum heart rate). In addition, previous data from this laboratory suggested that neither post-heparin (Herd, 1997) nor skeletal muscle (Herd et al. 1997) LPL activity were significantly elevated on the morning following 90 minutes of moderate intensity exercise. Thus, the weight of available evidence appears to be in accord with the present finding that 90 minutes of moderate exercise did not increase clearance of an intravenous TAG emulsion.

A recent study by Zmuda et al. (1998) has suggested that exercise training had little effect on TAG clearance in men with initially low HDL cholesterol concentrations ($<1.03 \text{ mmol.l}^{-1}$) in contrast to its facilitatory effect on TAG clearance in men with normal HDL cholesterol concentrations ($>1.13 \text{ mmol.l}^{-1}$). At first glance these data support the present findings, as the mean HDL cholesterol concentration of the current subject group was low ($1.01 \pm 0.10 \text{ mmol.l}^{-1}$ in the control intravenous fat tolerance test). However, in Zmuda and colleagues' (1998) study exercise training had no significant effect on any aspect of TAG metabolic capacity, including fasting TAG concentrations, in the low HDL cholesterol subjects, whereas in the current study a moderate exercise session reduced fasting and postprandial TAG concentrations. Thus, unlike the subjects in the study by Zmuda and co-workers, the present subjects were not 'non-responders' to exercise, rather exercise was influencing TAG metabolic capacity by mechanisms other than increasing TAG clearance.

In one subject, Intralipid-TAG clearance was 90 % faster in the exercise trial than the control trial (2.98 \%.min^{-1} in the control trial, 5.66 \%.min^{-1} in the exercise trial). In fact, if his data were excluded from the analyses the mean TAG clearance rate was $4 \pm 4 \text{ \%}$ slower in the exercise trial than the control trial. Interestingly, he was the only subject who did not have lower postprandial TAG concentrations in the exercise trial than the control trial (total lipaemic response: $10.95 \text{ mmol.l}^{-1}.\text{h}$ in control trial vs $12.95 \text{ mmol.l}^{-1}.\text{h}$ in the exercise trial). The fact that exercise increased TAG clearance, but did not attenuate lipaemia in this subject, adds to the evidence

suggesting that increased TAG clearance was not the predominant mediator of the reduced postprandial lipaemia seen after exercise.

To calculate the TAG clearance rates, only the first 20 or 25 minute portions of the TAG clearance curves were considered, as at later time points first order kinetics were not observed, probably due to Intralipid-TAG recirculating in the endogenous pool (Hallberg, 1965). The argument that increased hepatic VLDL secretion at later time points in the intravenous fat tolerance test confounded calculations of TAG clearance rates is supported by the NEFA data. Plasma NEFA concentrations rose after the Intralipid injection and were significantly higher than the 2.5-minute value at later time points; this rise probably reflecting 'spill-over' into the circulation of fatty acids derived from Intralipid-TAG hydrolysis. Thus the hepatic NEFA flux was probably increased late in the intravenous fat tolerance test. As substrate delivery to the liver is a major determinant of VLDL secretion (Sniderman and Cianflone, 1993), it is not unreasonable to suppose that the endogenous TAG pool was larger at the end of the 60-minute observation period than at the start. Observation of Figure 6.5 suggests, that from about the 25-minute time point, plasma TAG concentrations tended to be lower in the exercise trial than control, although the curves were not significantly different. (Logarithmic regression lines were fit to the entire TAG curves with R^2 values of 0.978 ± 0.004 and 0.979 ± 0.004 in the control and exercise trials respectively ($p = 0.22$ between trials for the regression lines)). As the TAG clearance rates were similar in the control and exercise trials and TAG concentrations at the late time points were likely to be influenced by increased VLDL secretion, these data might suggest a tendency for lower VLDL secretion in the exercise trial late in the observation period. As plasma NEFA concentrations were numerically (although not significantly) higher in the exercise trial, the reduced VLDL secretion would be occurring despite a hepatic NEFA flux which was probably higher. This is supportive of a shift in hepatic fatty acid partitioning away from re-esterification and VLDL production in the exercise trial (and towards oxidation) and is identical to the proposed shift in hepatic metabolism occurring after exercise in the oral fat tolerance tests. However, it is also possible that the tendency for lower TAG concentrations late in the intravenous fat tolerance test reflect less competition for LPL binding sites

in the exercise trial because TAG concentrations were lower in the fasted state in this trial.

In this study exercise did not significantly attenuate postprandial insulin concentrations and similarly did not significantly elevate postprandial NEFA concentrations. This is in contrast to the study described in chapter 5, where exercise significantly changed both these indices. It is important to note that, despite no significant changes to insulin and NEFA, exercise significantly attenuated postprandial lipaemia. This suggests that the exercise-induced changes in TAG metabolism occur independently of changes to insulin and NEFA responses. However, it is interesting that the exercise induced changes to insulin and NEFA appear to co-exist, supporting the argument advanced in chapter 4 that the exercise-induced elevations in NEFA concentration were mediated by lower insulin concentrations.

The similar time to peak plasma paracetamol concentration in the control and exercise trials, together with the higher peak paracetamol concentration in the exercise trial, is indicative that gastric emptying was not slowed, and if anything was slightly faster, in the exercise trial. Thus, as in the study described in chapter 5, it is unlikely that a reduced rate of chylomicron appearance into the circulation contributed to the reduced postprandial TAG concentrations seen in the exercise trial.

The results of this study suggest that mechanisms other than increased TAG clearance are largely responsible for the lower plasma TAG concentrations seen on the day following a moderate intensity exercise session, at least in the fasted state and early in the postprandial period. This supports the data from chapter 5. In light of the paracetamol data, it is unlikely that the rate of chylomicron appearance was slower in the exercise than the control trial, thus the most likely mediator of the lower TAG concentrations is reduced hepatic VLDL secretion. However, it should be noted that it is not clear from the results of this study whether exercise might have influenced TAG clearance late in the postprandial period and further research is necessary to address this issue. The relative importance of all the possible mechanisms for exercise-induced reduction to fasting and postprandial plasma TAG concentrations

will be addressed in the general discussion, together with the implications of the findings of this and the other experimental chapters of this thesis.

CHAPTER 7

GENERAL DISCUSSION

There is a growing body of evidence implicating elevated plasma TAG concentrations, especially in the postprandial state, as an important marker of CHD risk. Case control studies (Simons et al. 1987; Simpson et al. 1990; Groot et al. 1991; Patsch et al. 1992; Meyer et al. 1996), limited epidemiological evidence (Stensvold et al. 1993; Stampfer et al. 1996) and a growing body of scientific research suggest that exaggerated postprandial lipemia is either directly (Zilversmit, 1979; Mamo et al. 1998) or indirectly (Miesenböck and Patsch, 1992) linked to the progression of atherosclerosis. Thus, interventions which can attenuate TAG concentrations in the postprandial state are likely to be beneficial in delaying atherogenic progression. It has become increasingly evident that the postprandial state is the most critical phase of TAG transport (Miesenböck and Patsch, 1992) and that subtle deficiencies in lipoprotein metabolism, not necessarily apparent in the fasted state, may be evident postprandially (Tall, 1986). In addition, measurements made in the fasted state are not representative of 24-hour lipoprotein metabolism as the majority of the day is spent in the postprandial state. Thus, in order to obtain accurate and revealing information about an individual's 'TAG metabolic capacity', it is not sufficient to consider only the fasted state and postprandial studies are necessary. This thesis sought to build on work carried out in this laboratory over the last decade, elucidating the nature of the changes that moderate exercise elicits on this critical and revealing phase of lipoprotein transport.

The data from this thesis have shown that moderate exercise can reduce postprandial lipaemia in middle-aged men and postmenopausal women. This adds to the body of evidence which has suggested that this type of exercise can attenuate lipaemia in young adults (Aldred et al. 1994; Tsetsonis and Hardman, 1996a; Tsetsonis and Hardman, 1996b; Herd et al. 1997; Herd, 1997; Gill et al. 1998; Malkova et al. 1999), and middle-aged women (Tsetsonis et al. 1997), and shows that an attainable amount of exercise can improve TAG metabolic capacity in groups of individuals who by nature of their age (Dawber, 1980; Keys, 1980; Wood et al. 1998) and, for the female subjects, postmenopausal status (Sotelo and Johnson, 1997), were at an

increased risk of developing CHD compared with the subject groups previously studied.

The effects of exercise were not trivial. A single moderate intensity exercise session reduced postprandial TAG concentrations by about a fifth in these untrained individuals, with no obvious gender differences. Importantly, the exercise was prescribed at a 'relative exercise intensity' of 60 % $\dot{V}O_2$ max, thus each individual walked at a speed and gradient which they could manage comfortably. As the exercise was prescribed according to individual ability and fitness, the least fit subjects performed less exercise in absolute terms than their fitter counterparts, but even these subjects experienced substantial reductions in plasma TAG concentrations. In fact exercise attenuated postprandial lipaemia in 29 of the 31 subjects studied. This suggests that, in practice, exercise could be a viable tool for the enhancement of 'TAG metabolic capacity'.

Recent studies have suggested that the potential for exercise to influence an individual's HDL cholesterol concentrations might be dependent on their apo E phenotype. Individuals possessing the E2 isoform appear to be more responsive to exercise than carriers of the E3 isoform and exercise appears to be least effective in altering HDL cholesterol concentrations of those possessing of the E4 isoform (St.-Amand et al. 1999; Hagberg et al. 1999). In the studies described in this thesis, 20 (nine female) normolipidaemic subjects possessed the E3/3 phenotype and these experienced a mean reduction in postprandial lipaemia of 20.4 % after exercise. Subjects possessing the E4/3 phenotype (n = 6, 1 female) experienced a 14.3 % reduction in postprandial lipaemia, whereas exercise induced a 26.8 % reduction in lipaemia in subjects possessing the E3/2 phenotype (n = 3, 1 female). Thus, the present data suggest that apo E polymorphism similarly affects the potential for exercise to influence postprandial TAG and fasting HDL cholesterol concentrations. This is not surprising given the relationship between TAG metabolism and HDL cholesterol (Miesenböck and Patsch, 1992), but it should be noted that for the present data the number of subjects in each group was very small, particularly for the E3/2 phenotype, and thus the results should be interpreted with caution.

Prior to the studies described in this thesis, the mechanisms behind the moderate exercise-induced attenuation in postprandial lipaemia were unclear but, based on studies of vigorous and/or prolonged exercise, increased lipoprotein-TAG clearance, mediated by an upregulation of LPL activity in skeletal muscle, seemed probable. However, the present data suggest that this is not the case. The moderate exercise-induced attenuation in plasma TAG concentrations was largely attributable to lower VLDL-TAG concentrations and that, especially early in the postprandial phase, the exercise-induced reductions in chylomicron-TAG concentrations were small. Plasma ketone body concentrations were markedly higher in both the fasted and postprandial states after exercise, implying that ketogenesis and hepatic fat oxidation were markedly enhanced by exercise and, at least in the fasted state, moderate exercise did not augment the clearance of a chylomicron-like lipid emulsion. Taken together, these data suggest that changes to the liver, and not skeletal muscle, were largely responsible for the shifts in TAG metabolism seen after moderate exercise, with the attenuation in plasma TAG concentration being predominantly attributable to reduced hepatic VLDL secretion rather than increased TAG clearance. This interpretation is supported by preliminary unpublished data from this laboratory, in collaboration with the Oxford Lipid Metabolism Group, which suggests that the reduction in postprandial lipaemia observed after a moderate exercise session cannot be accounted for by increased TAG uptake into skeletal muscle (D. Malkova, personal communication).

It is perhaps surprising that changes in the liver, which only accounts for two or three percent of body mass, might be responsible for the changes in TAG metabolism seen after moderate exercise. However, the liver is a very metabolically active organ and accounts for over a quarter of whole body oxygen uptake at rest, which is equivalent to the oxygen uptake of skeletal muscle (Frayn, 1992). In addition blood flow to the splanchnic bed is greater than blood flow to skeletal muscle at rest (Åstrand and Rodahl, 1986). Thus, although changes in the liver have been largely overlooked as a mediator of the exercise-induced changes to TAG metabolism, it is not unreasonable to suggest that changes in this tissue, rather than muscle, were predominantly responsible for the reductions in plasma TAG concentration evident after a moderate exercise session.

The fact that moderate exercise did not markedly influence TAG clearance in the present studies might have been a characteristic of the subjects studied – who were middle-aged and predominantly untrained. It is possible that TAG clearance would be enhanced by moderate exercise in other subject groups. The effect of exercise training on skeletal muscle LPL activity appears to be transient, with activity rapidly diminishing on cessation of training (Simsolo et al. 1993). In addition, muscle LPL activity can increase after a short training period (Seip et al. 1995) or prolonged intense exercise (Lithell et al. 1979; Taskinen et al. 1980; Lithell et al. 1981; Lithell et al. 1984), suggesting that increases in muscle LPL activity might be due to the acute effect of recent exercise. At first glance, these data appear to be at odds with research linking increased muscle capillarisation – a long-term effect of training – with elevated LPL activity (Kiens and Lithell, 1989). However, it is not unreasonable to suggest that in trained individuals the acute effects of exercise on muscle LPL activity might be enhanced, compared to their untrained counterparts, due to their high degree of muscle capillarisation providing more potential binding sites for the enzyme. Thus, in well trained subjects, a moderate exercise session may increase TAG clearance as well as reducing VLDL secretion. This proposed interaction between training status and an acute exercise session implies that, although the attenuating effect of exercise on postprandial lipaemia is short lived (Hardman et al. 1998), the beneficial effects of exercise training on TAG metabolism might extend beyond the ‘last bout’ effect, with background training enhancing effectiveness of a single exercise session at reducing plasma TAG concentrations. Previous work from this laboratory which found that moderate exercise reduced postprandial lipaemia more markedly in endurance trained middle-aged women than their untrained peers (Tsetsonis et al. 1997) supports the concept of a synergistic interaction between training and acute exercise. However, in that study the mechanism(s) behind the different exercise effects in the trained and untrained women were unclear, and were likely to include factors other than differences in LPL activity, as the trained women expended more energy in exercise than the untrained subjects (due to their higher functional capacities) and also experienced an exercise-induced attenuation in the postprandial insulin response which was not evident in the untrained women. Thus, further research is required to determine whether in well trained subjects moderate exercise can, in fact, increase TAG clearance, thereby augmenting the TAG lowering potential of a single exercise session.

The study described in chapter 4 discovered that the effect of exercise on fasting and postprandial lipid metabolism was qualitatively different and quantitatively far greater than the effect of an equivalent dietary induced energy deficit. These data implied that the influence of exercise was not a consequence of the energy deficit incurred, at least at a whole body level. This has practical implications, suggesting that additional metabolic benefits can be gained by regulating body mass through increasing physical activity rather than by restricting energy intake. This finding is supported by recent epidemiological data, which found that leanness (a function of prolonged energy balance) only conferred a low risk of cardiovascular disease mortality when accompanied by cardiorespiratory fitness (a characteristic of regular exercisers), and that obese, but fit, men were at lower risk of CVD than their lean, but unfit, counterparts (Lee et al. 1999). Thus, the lower cardiovascular disease risk experienced by regular exercisers cannot be accounted for by improved regulation of energy balance and this might reflect, at least in part, the different effects of exercise and dietary restriction on postprandial TAG metabolism.

Although the present data imply that whole body energy deficit was not mediating the exercise changes, it is possible that the exercise-induced reduction in plasma TAG concentrations was a consequence of substrate deficit in a specific tissue. Substrate mobilisation during and after exercise differs qualitatively and quantitatively from that elicited in response to energy intake restriction, and this may explain the differing effects of the two interventions on TAG metabolism (see the discussion in chapter 4). It is unlikely that a substrate deficit in skeletal muscle mediated the exercise-induced TAG reductions as the data suggest that predominant mechanism for the plasma TAG attenuation was reduced VLDL secretion, and not increased lipoprotein-TAG clearance. However, a reduction in hepatic TAG stores could mediate reduced VLDL production by partitioning fatty acids entering the liver towards replacing depleted TAG stores, rather than towards VLDL assembly. To the author's knowledge, there are no data in humans showing the effect of exercise on hepatic TAG stores. However, data in rats have shown that, after a rise during exercise and immediately post-exercise, hepatic TAG concentration falls to below pre-exercise concentrations in the hours post-exercise – despite free access to food – during which time liver glycogen concentration steadily rises (Gorski and Kiryluk, 1980). This is analogous

to the post-exercise recovery of substrates in skeletal muscle in humans (Kiens and Richter, 1998) and might reflect reliance on hepatic TAG as a fuel post-exercise to facilitate the rapid recovery of muscle glycogen stores. Clearly, studies are needed to ascertain whether, in humans, hepatic TAG concentration falls post-exercise in the same way that it does in rats and indeed, whether this contributes to the reduced plasma TAG concentrations seen after exercise. If this mechanism were acting, reductions in plasma TAG, particularly in the VLDL fraction, would not be observed immediately post-exercise but would occur after a delay. Thus, a recent study from Børsheim and co-workers (1999) reporting that the attenuating effect of a 90-minute moderate exercise session on plasma VLDL-TAG concentration was delayed, being evident 4.5 hours but not immediately post-exercise, provides indirect evidence that hepatic TAG deficit is at least a contributing mechanism for the exercise-induced reduction in plasma TAG concentrations.

As it seems likely that the attenuating effect of exercise on plasma TAG was, at least in part, a consequence of a substrate deficit in the liver, it would be interesting to consider whether exercise would still attenuate lipaemia if the energy expended during the exercise session was replaced. Clearly a permanent exercise-induced energy deficit cannot be sustained, as this would require individuals to constantly be in negative energy balance. Thus the issue of energy replacement is of great practical importance as it influences the efficacy of exercise as a long-term moderator of plasma TAG concentrations. However, it is not unreasonable to speculate that exercise would still attenuate plasma TAG concentrations if the energy expended during exercise were replaced. At least in rats, hepatic TAG concentration falls for a number of hours after exercise while hepatic glycogen stores are replaced, despite free access to food (Gorski and Kiryluk, 1980). As the repletion of hepatic TAG following exercise is delayed, it would be a number of hours before additional ingested energy would go towards replacing these stores. Thus, even if the energy expended during exercise was replaced, the time lag in the replenishment in hepatic TAG stores would mean that plasma TAG concentrations would still be attenuated. Indeed, the time-course for repletion of hepatic TAG might explain why the effect of exercise on postprandial lipaemia is transient, with much of the effect of exercise training on postprandial lipaemia being lost within two to three days of the last exercise session (Hardman et al. 1998). This hypothesis warrants further research but

is supported, at least in the fasted state, by the data of Gynzelberg et al. (1977) which found that the effect of individual exercise sessions on plasma TAG concentrations was the same whether or not energy intake was increased to compensate for the energy expenditure of exercise.

The higher fasting and postprandial 3-hydroxybutyrate concentrations in the exercise trial, compared with the control trial, in chapter 5 implied increased ketogenesis and hepatic fat oxidation following exercise, which probably reflects a shift in partitioning of fatty acids toward ketogenesis and oxidation and away from re-esterification and VLDL synthesis. This shift in hepatic fatty acid metabolism towards oxidation might reflect the liver's reliance on fat as a substrate while hepatic glycogen stores were replenished – an interpretation supported by data in rats which has shown that post-exercise ketosis is inversely related to liver glycogen concentrations (Beattie and Winder, 1984; Adams and Koeslag, 1988). However, if exercise affected the VLDL assembly process in some manner, thereby preventing hepatic TAG from being 'packaged' into VLDL, an increase in hepatic ketogenesis and oxidation in the exercise trial might have occurred to prevent an accumulation of TAG in the liver (i.e. a fatty liver). How moderate exercise might have affected VLDL assembly is unclear, but Mondon et al. (1984) proposed that the apolipoprotein synthesis might be decreased in response to low serum insulin concentrations. Although insulin concentrations were not necessarily lower in the exercise trials than the control trials on the day after exercise, subjects were exposed to low insulin concentrations during exercise and this, in itself, might have affected subsequent apolipoprotein synthesis.

Unfortunately 3-hydroxybutyrate was not measured in the study described in chapter 4 as its importance was not appreciated until the study described in chapter 5 was completed. Thus it is not known whether serum ketone body concentrations were higher, relative to the control trial, in the intake-restriction trial as well as the exercise trial. However, as the subjects ingested almost 200 g of carbohydrate on day one of the intake restriction trial (seven subjects ingested > 194 g, three subjects ingested 168–177 g and one subject ingested 112 g of carbohydrate) and a daily carbohydrate consumption of 65 to 180 g of carbohydrate is considered necessary to prevent adaptive responses to fasting (Klein and Wolfe, 1992), it seems unlikely that ketogenesis would have been markedly higher in the intake-restriction trial than the

control trial – in contrast to the ketogenic response to exercise. Knowledge of the ketogenic response to intake-restriction of this magnitude might help further elucidate why the effects of exercise and intake-restriction on fasting and postprandial TAG metabolism differed so markedly.

As one reported effect of endurance exercise is to increase insulin sensitivity (for review see Henriksson (1995)) and insulin is an important co-ordinator of the postprandial state, it would not be unreasonable to hypothesise that the mitigating effect of exercise on postprandial lipaemia was in some way attributable to differences in insulin concentrations or sensitivity between the control and exercise trials. An exercise effect on insulin could potentially influence VLDL secretion in a number of ways. Insulin suppresses plasma NEFA concentrations both by inhibiting mobilisation of adipose tissue-TAG (Frayn, 1996) and by increasing entrapment of LPL derived fatty acids in to the adipocyte (Sniderman et al. 1998). Thus, if exercise increased the sensitivity of the adipocyte to insulin, this could potentially have played a role in reducing VLDL secretion in the exercise trial by reducing the NEFA flux to the liver. However, in the exercise trials plasma NEFA concentrations were either significantly higher than the control trial (chapters 4 and 5) or tended to be higher (chapter 6), suggesting that the proposed reduction in VLDL secretion in the exercise trials was not a consequence of insulin-mediated suppressions of plasma NEFA and, indeed, occurred in spite of increased substrate delivery to the liver. Insulin acts to increase the concentration of hepatic malonyl-CoA, thus reduced insulin concentrations in the exercise trials would promote a shift in hepatic fatty acid metabolism towards oxidation and ketogenesis and away from re-esterification, thereby reducing VLDL production. Insulin may also have a direct suppressive effect on VLDL secretion, possibly by promoting the degradation of apolipoprotein B (Mason, 1998). Thus, if exercise enhanced insulin sensitivity, the effectiveness of insulin at suppressing VLDL secretion might have been increased in the exercise trial. While all these mechanisms are theoretically possible, the data from chapter 6 of this thesis and other research from this laboratory (Aldred et al. 1995; Tsetsonis et al. 1997; Hardman et al. 1998; Malkova et al. 1999) indicate that exercise-induced changes to lipaemic and insulinaemic responses to a meal can occur independently, suggesting that that differences in postprandial insulin concentrations were not the primary mediator of the exercise-induced attenuation to postprandial lipaemia.

Insulin sensitivity was not directly measured in the studies described in this thesis, so it is theoretically possible that exercise enhanced tissue-sensitivity to insulin without necessarily affecting serum insulin concentrations, although this seems unlikely given that in chapter 6 – where postprandial insulin concentrations were not significantly affected by exercise – postprandial glucose concentrations also did not differ between trials. However, although moderate exercise-induced reductions in postprandial lipaemia can occur independent of changes in insulin concentration, it is possible that, for individuals who experienced reduced insulin concentrations and/or increased insulin sensitivity after exercise, the exercise-induced effect on plasma TAG concentrations was enhanced. In addition, it seems likely that the elevated plasma NEFA concentrations seen in the exercise trials in chapters 4 and 5 were due, at least in part, to the reduced serum insulin concentrations seen in these studies. These increased NEFA concentrations imply increased partitioning of lipids away from storage in adipose tissue towards skeletal muscle and the liver – where their ultimate fate is likely to be oxidation – and are perhaps a key factor mediating the enhanced ability of regular exercisers to regulate their body mass, compared with their sedentary counterparts (Williamson et al. 1993). Thus, although insulin may not be an important mediator of the exercise-induced reduction in plasma TAG concentrations, by regulating NEFA concentrations, it might play a key role in the overall effect of exercise on lipid metabolism.

There is a growing body of evidence that implies that whole-body fat balance is poorly regulated, with an increase in fat intake leading directly to increased fat storage (Flatt et al. 1985; Schutz et al. 1989; Bennett et al. 1992; Griffiths et al. 1994), in contrast to the increase in carbohydrate oxidation which accompanies carbohydrate ingestion (Acheson et al. 1982). There also appears to be a close relationship between fat balance and energy balance which does not exist between carbohydrate balance and energy balance (Schutz et al. 1989). As fat oxidation is not regulated by fat intake, the importance of interventions which promote fat oxidation and therefore promote negative fat balance, and consequentially negative energy balance is clear. The data from chapter 5 have indicated that prior moderate exercise can increase both endogenous and exogenous postprandial fat oxidation. While the nature of this

increase in fat oxidation has mechanistic implications, discussed in chapter 5, the practical implications of the increased fat oxidation – i.e. a reduction in net fat storage – are dependent only on the magnitude of the increase. At the end of the postprandial observation period, 24 hours after the cessation of exercise, fat oxidation was higher in the exercise trial than the control trial – i.e. in response to a single moderate exercise session fat oxidation was increased for over 24 hours. During the eight-hour postprandial observation period, total fat oxidation was almost 10 g (9.6 ± 2.2 g) greater in the exercise trial than the control trial, thus – if the assumption that fat oxidation was increased by the same amount in the post-exercise hours preceding the oral fat tolerance test was made (a conservative assumption because the influence of an exercise session on fat oxidation is likely to decrease, and not increase, with time) – an estimated 30 g more fat would have been oxidised in the 24 hour period following exercise in the exercise trial than the control trial, during which time macro-nutrient and energy intake in the two trials were matched. The magnitude of the post-exercise increase in fat oxidation was comparable to the amount of fat oxidised during the 90-minute exercise session (26.9 ± 3.6 g) and thus including the post-exercise period doubled the overall effect of the exercise session on fat oxidation. Thus, net fat storage was probably over 50 g lower in the exercise trial than control trial, an important amount in comparison to the ~95 g recommended daily fat intake for a male adult (Ministry of Agriculture, Fisheries and Food, 1995). The increased postprandial fat oxidation was accompanied by a concomitant decrease in carbohydrate oxidation, implying increased carbohydrate storage. However, as this probably reflects replenishment of depleted muscle glycogen stores (which were not depleted in the control trial) the long term energy balance consequences of this decreased carbohydrate oxidation would be minimal and the magnitude of the exercise effect on energy balance is likely to reflect the increase in fat oxidation. Thus, although the metabolic rate may only be elevated for a few minutes after a moderate exercise session (see Figure 4.2), changes in substrate utilisation occurring during the hours after moderate exercise make a large contribution to the overall effect of exercise on fat, and consequentially energy, balance. In the present study, subjects were in a negative energy balance in the exercise trial compared with the control trial and theoretically this might have contributed to the increased fat oxidation seen post-exercise. However, there are data from Calles-Escandón and co-workers (1996)

which have shown that the increase in fat oxidation post-exercise is the same whether or not the energy expended during exercise was replaced, thus a similar increase in fat oxidation might have been seen in chapter 5 even if the energy expended during exercise was replaced post-exercise – a dissociation between exercise and energy balance analogous to the one observed for plasma TAG.

HDL cholesterol concentrations were not consistently increased following the moderate exercise sessions, in the fasted state. However, at the end of the postprandial observation period in chapters 5 and 6, HDL cholesterol concentrations were higher in the exercise trial than the control trial. In the control trials, HDL cholesterol concentrations fell by about 9 % during the postprandial period, a finding consistent with data from Patsch et al. (1984) which suggested that a single postprandial event can reduce HDL cholesterol concentrations by upto 10 %. This postprandial reduction in HDL cholesterol was attenuated in the exercise trial, which was probably a consequence of reduced neutral lipid exchange between TAG-rich lipoproteins and HDL particles due to the lower postprandial TAG concentrations in this trial. Interestingly, the difference between trials in HDL cholesterol was abolished 24 hours after ingestion of the test meal, suggesting that a single moderate intensity exercise session did not provide sufficient stimulus to substantially alter HDL cholesterol concentrations. This is perhaps not surprising, given that the half life of HDL particles in the circulation is approximately five days (Durstine and Haskell, 1994), and perhaps exercise sessions on a number of days are necessary to elicit prolonged changes in HDL cholesterol.

The subjects who participated in the studies described in this thesis were predominantly normolipidaemic, and with the exception of one subject in the study described in chapter 5, all normotriglyceridaemic. Thus, the results from this thesis indicate that moderate exercise attenuates postprandial lipaemia in normotriglyceridaemic middle-aged men and women, but its influence on TAG metabolism in dyslipidaemic subjects is not known. Intuitively, it might be hypothesised that those with higher initial TAG concentrations might have a larger scope for TAG lowering after exercise. However, this might be a naive argument given that exercise did not reduce postprandial lipaemia in the only hypertriglyceridaemic subject studied. The underlying cause of this subject's

elevated plasma TAG concentrations was not known, although the data imply that his rates of ketogenesis and hepatic fat oxidation were low and not upregulated by exercise. As this subject appeared to have a deficiency in the proposed pathway via which exercise reduced plasma TAG concentrations, it was clear that exercise would not attenuate his postprandial lipaemia. However, the fact that moderate exercise did not attenuate postprandial lipaemia in this one subject does not necessarily mean that moderate exercise would not attenuate postprandial lipaemia in all individuals with elevated fasting TAG concentrations. Indeed, in the study described in chapter 6, two subjects (both of South Asian origin) had fasting TAG concentrations at the upper end of the normal range (2.19 and 2.25 mmol.l⁻¹) and for these individuals exercise attenuated six-hour area under the plasma TAG vs. time curve by 5.00 mmol.l⁻¹.h (20 %) and 2.68 mmol.l⁻¹.h (12 %) respectively – greater reductions, in absolute terms, than the mean plasma TAG attenuation for the group (2.56 mmol.l⁻¹.h, 18 %). It is clear that fasting hypertriglyceridaemia can have a number of underlying causes and moderate exercise might have a substantial effect on fasting and postprandial plasma TAG concentrations in some hypertriglyceridaemic groups, but a minimal effect in others. Thus, further research is required to determine in which dyslipidaemias moderate exercise might be effective in attenuating postprandial lipaemia and whether exercise might be used as an adjuvant therapy, together with drug treatment, for TAG lowering in certain groups of hypertriglyceridaemic patients. It should be noted that, even in individuals for whom moderate exercise does not attenuate postprandial lipaemia, physical activity can confer a number of benefits potentially acting to reduce the CHD risk; including reducing blood pressure (American College of Sports Medicine Position Stand, 1993), improving insulin/glucose dynamics (Henriksson, 1995), regulating body mass and improving haemostatic function (Meade, 1995). Thus, unless there are specific contraindications, regular moderate exercise is likely to be beneficial to a majority of people, irrespective of its influence on TAG metabolism.

The data from this thesis suggest that the liver, rather than skeletal muscle, is the dominant mediator of the moderate exercise effect on TAG metabolism. This is novel reasoning and as a consequence there are few data available to confirm or deny this interpretation of the present data. In the next few months data will be published by this group in collaboration with the Oxford Lipid Metabolism Group suggesting that

increased clearance into skeletal muscle cannot explain the lower postprandial TAG concentrations seen after moderate exercise, but data focussing on the liver, in humans, are lacking. Kinetic tracer studies are required to determine whether, indeed, reduced hepatic VLDL secretion was the predominant mediator of the attenuated postprandial lipaemia seen following moderate exercise. Magnetic resonance spectroscopy studies to assess the pattern of post-exercise hepatic substrate recovery would help determine whether the proposed reduced VLDL secretion and increased hepatic fat oxidation seen after moderate exercise were a consequence of a reduction in hepatic TAG stores. Indeed, mechanisms not considered in this thesis may have played a role in the moderate exercise-induced attenuation to VLDL secretion and/or postprandial lipaemia these might be elucidated by further research. It is clear that further research into the mechanisms via which moderate exercise reduces plasma TAG concentrations would be expensive and require considerable specialist expertise. These factors may constrain further research into this area but, even without detailed knowledge about the mechanisms via which moderate exercise affects TAG metabolism, it is clear that this intensity of exercise attenuates postprandial lipaemia in a broad spectrum of subject groups and is therefore potentially a valuable tool in preventing the progression of atherosclerosis.

Perhaps of equal importance to the elucidation of the mechanisms via which moderate exercise reduces postprandial lipaemia is research aimed at making exercise more accessible to the population at large. In the studies described in this thesis, the subjects performed 90 minutes of exercise, which even at a moderate intensity is a large amount of exercise, and while all the subjects managed to complete their walks without difficulty, this is not an amount of exercise that many people would perform on a regular basis. Seven out of ten men and eight out of ten women do not do enough exercise to benefit their health (Sports Council and Health Education Authority, 1992) and thus the majority of the population would be concerned about the minimum amount of exercise they could get away with to gain some health benefit. Therefore, studies are needed to explore the effects of smaller quantities of exercise than that used in this thesis, perhaps accumulated over several days rather than in one session, on TAG metabolism to assess whether exercise programmes which are attainable for the majority of the (sedentary) population, and sustainable in terms of time commitment, can markedly improve 'TAG metabolic capacity'. Such

research is vital to give the maximum number of people the potential to benefit from the TAG-lowering qualities of moderate exercise.

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APPENDICES

Appendix A: Questionnaires, forms and information for subjects

Appendix B: Blood, plasma and serum assays, and determination of breath enrichment of $^{13}\text{CO}_2$

Appendix C: Individual TAG clearance curves from chapter 6

APPENDIX A1 – INFORMATION FOR SUBJECTS

LOUGHBOROUGH UNIVERSITY, DEPARTMENT OF PHYSICAL EDUCATION, SPORTS SCIENCE AND RECREATION MANAGEMENT

BRITISH HEART FOUNDATION STUDY

The influence of one prolonged session of treadmill walking on postprandial lipaemia and clearance of an intravenous fat emulsion.

BACKGROUND

Work in the Muscle Metabolism Research Group in this department is looking at the effects of exercise on a person's ability to cope with fat contained in food. This capacity has important implications in determining one's risk of developing coronary heart disease.

After eating a meal, blood vessel walls are exposed to particles carrying fat from the food which may facilitate the build-up of fatty deposits on the artery surface. We have shown that if a high fat meal is consumed the day after a prolonged session of brisk walking, concentrations of fat in the bloodstream are lower than if no exercise was undertaken. To help us to understand whether these reduced fat concentrations are influenced by slower digestion of fat following exercise, we will compare the effects of exercise on blood fat responses to oral fat tolerance test with its effects on an intravenous fat tolerance test. As the latter approach is not dependant on the digestion of fat, a comparison of the two responses will enable us to determine whether the exercise induced reduction to blood fat concentrations is partly due to an effect on the digestive system. This will help to improve our understanding of the manner by which exercise influences fat metabolism, and has implications for prevention of heart disease.

STUDY DESIGN

All volunteers will undertake a series of tests during the study.
These tests involve the evaluation of:

- fitness, by exercise tests.
- body composition.
- blood fat responses to a test meal after three days without planned exercise.
- blood fat responses to a test meal the morning after 90 minutes of treadmill walking.
- blood fat responses to an intravenous infusion of a fat emulsion after three days without planned exercise.
- blood fat responses to an intravenous infusion of a fat emulsion the morning after 90 minutes of treadmill walking.

PRELIMINARY PROCEDURES

Before enrolling in the study, you will be asked to attend the laboratory for a preparatory session during which we will:

- (a) measure your blood pressure
- (b) discuss and complete with you confidential questionnaires regarding your health, physical activity and diet
- (c) provide an opportunity for you to ask questions

We will also ask you to come to the laboratory before breakfast one morning so we can take a small blood sample to check the triglyceride (fat) and cholesterol levels in your blood.

EXPERIMENTAL PROCEDURES

A. Preliminary Exercise Tests

At the beginning of the study, an exercise stress test will be undertaken at Glenfield Hospital. This will involve walking on a motorised treadmill with electrodes attached to your chest to monitor how hard your heart is working. This test will us whether it is safe for you to take part in this study. In the unlikely event of there being a problem, we would request your permission for the cardiologist to contact your GP and inform them of the finding.

In addition a submaximal and a maximal exercise test will be undertaken on the treadmill at Loughborough University. Heart rate will be monitored and recorded throughout using a heart rate monitor and expired air will be collected at intervals using a mouthpiece and respiratory valve.

- (i) The first test will be sub-maximal and last for 16 mins. The gradient will be increased at the end of each 4 min period. This test is designed to familiarise you with walking on a treadmill
- (ii) The second test will be maximal. The gradient will increase every three minutes and the test will end when you decide that you are able to walk for just one further minute. This test is designed to determine your body's ability to use oxygen and enables us to find the correct speed and gradient for you to walk at during the exercise trial.

B. Body Composition

The amount and distribution of your body fat will be determined by measuring body girths and by using callipers to measure skin fold thickness at four different sites (a sophisticated version of "pinch an inch"). Your height and weight will also be recorded.

You will need to wear only underclothing for these measurements which will be made in private.

C. Recording diet

You will be asked to weigh and record every thing that you eat and drink for two days before your first fat tolerance test. We will ask you to replicate this diet prior to subsequent fat tolerance tests. We will provide you with a set of scales to do this easily.

D. Main trials

There will be four main trial and these will be conducted in random order.

- (i) Two control trials - You will be asked to refrain from physical activity (other than 'everyday activities') for three days before attending the lab for an oral fat tolerance test or and intravenous fat tolerance test(described below).
- (ii) Two exercise trials - You will be asked to refrain from physical activity for two days prior to each trial. On the first day of each trial, you will come to the laboratory to walk for 90 minutes on the treadmill at a moderate intensity. On the second day you will attend the laboratory for an oral or an intravenous fat tolerance test.

E. Blood Fat Responses to a Test Meal (Oral fat tolerance test)

You will be asked to come to the laboratory after an overnight fast (i.e. having eaten nothing for 12 hours) and consume a test meal consisting consisting of cereal, bananas, apples, sultanas, chocolate, nuts and cream. In order to determine fat concentrations in the blood, we will take small blood samples before the test meal and and hourly afterwards for six hours. To minimise any discomfort the samples will be obtained via a cannula (tiny plastic tube) placed (under local anaesthetic) in a forearm vein. For six hours following the meal, you will not be able to eat or drink anything, except water. You will just be resting, watching TV, reading or working. After this time we will provide you with a meal and then you can go home.

F. Intravenous Fat Tolerance Test

You will be asked to come to the laboratory the morning after an over-night fast and a cannula will be placed (under local anaesthetic) into a forearm vein. A small blood sample will be taken. A doctor will then introduce ~10g of fat (~two teaspoons) into the circulation via the cannula. The fat will in a preparation identical to that used by hospitals to feed patients who are on a "drip". Small blood samples will be taken for 40 minutes after admistration of the fat. After this time we will provide you with breakfast.

These tests will be done at your convenience.

HOW MUCH TIME WILL THE TESTS TAKE?

Fitness tests: For the exercise stress test you will have to be at Glenfield Hospital for about an hour. For the exercise tests at the University you will be in the laboratory for no more than 40 minutes on each occasion.

Body composition: The procedure requires only a few minutes and will take place on the same visit as one of the exercise tests.

Exercise trial: The prolonged treadmill walks will take 90 minutes.

Oral fat tolerance tests: These tests will take place over a period of about 7 hours.

Intravenous fat tolerance tests: These tests will take about two hours.

The organisation of these tests is shown in the diagram at the end of this set of notes.

POSSIBLE RISKS AND DISCOMFORT

- Exercise testing will be at sub-maximal and maximal levels and the possibility exists that, very occasionally, certain changes may occur during or shortly after the tests. They include abnormal blood pressure, fainting or a change in the normal rhythm of the heart beat.
- Blood sampling via the cannula may cause minor bruising, an inflammation of the vein or haematoma (a small accumulation of blood under the skin). There is also an exceedingly small risk of a tiny piece of plastic or an air bubble entering the bloodstream if the cannula is incorrectly placed. Good practice, however, minimises this risk.
- The fat emulsion used in this study is the same as those used to feed patients on a “drip” in hospitals and will be administered by a doctor. The risks involved are no greater than those present with cannulation

The preliminary procedures before the start of the study are designed to minimise possible risks. All tests will be conducted and closely monitored by trained and experienced staff and in addition, only those at little risk from the procedures will be accepted into the study.

BENEFITS OF THE STUDY

This study is funded by the British Heart Foundation whose objective it is to support research into the causes, prevention, diagnosis and treatment of cardiovascular disease. The findings will be published in the scientific and medical literature so that understanding of the way in which exercise decreases the risk of heart disease and obesity can be increased. The BHF specifically requested that subjects for this study are men of ‘middle-age’ so that findings would be applicable to the group which is most at risk of developing coronary heart disease.

We will provide you with feedback about the group findings and also about your own results and would be delighted to explain results and discuss the implications with you.

CONFIDENTIALITY

Although information will be stored on computer, each subject will be entered as a number rather than by name and will not be identifiable. This is in accordance with the Data Protection Act.

Any questions about the procedures used in this study are encouraged. If you have any doubts or questions, please ask for further explanations by contacting Jason Gill on 01509 228183 (Office), 01509 236490 (Home), J.M.R.Gill@lboro.ac.uk (e-mail) or Dr. Adrienne Hardman on 01509 223265.

APPENDIX A2 – STATEMENT OF INFORMED CONSENT

**LOUGHBOROUGH UNIVERSITY, DEPARTMENT OF PHYSICAL
EDUCATION, SPORTS SCIENCE AND RECREATION MANAGEMENT**

BRITISH HEART FOUNDATION STUDY

**The influence of one prolonged session of treadmill walking on postprandial
lipaemia and clearance of an intravenous fat emulsion.**

Statement of Informed Consent:

Your permission to take part in this study is voluntary. You are free to deny consent or to withdraw from the study at any point and without explanation, if you so desire.

I have read the information regarding this study and had the opportunity to ask questions of the investigators. I understand the procedures involved and consent to participate in this study.

Signature of subject:

Date:

Signature of witness:

APPENDIX A3 – HEALTH SCREEN FOR STUDY VOLUNTEERS

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:

1. **At present**, do you have any health problem for which you are:
- | | | |
|--|---------|--------|
| (a) on medication, prescribed or otherwise | yes [] | no [] |
| (b) attending your general practitioner | yes [] | no [] |
| (c) on a hospital waiting list | yes [] | no [] |
2. **In the past two years**, have you had any illness which required you to:
- | | | |
|---|---------|--------|
| (a) consult your GP | yes [] | no [] |
| (b) attend a hospital outpatient department | yes [] | no [] |
| (c) be admitted to hospital | yes [] | no [] |
3. **Have you ever** had any of the following
- | | | |
|---|---------|--------|
| (a) Convulsions/epilepsy | yes [] | no [] |
| (b) Asthma | yes [] | no [] |
| (c) Eczema | yes [] | no [] |
| (d) Diabetes | yes [] | no [] |
| (e) A blood disorder | yes [] | no [] |
| (f) Head injury | yes [] | no [] |
| (g) Digestive problems | yes [] | no [] |
| (h) Hearing problems | yes [] | no [] |
| (i) Problems with bones or joints | yes [] | no [] |
| (j) Disturbance of balance/coordination | yes [] | no [] |
| (k) Numbness in hands or feet | yes [] | no [] |
| (l) Disturbance of vision | yes [] | no [] |
| (m) Thyroid problems | yes [] | no [] |
| (n) Kidney or liver problems | yes [] | no [] |
4. Do you currently smoke
- | | |
|---------|--------|
| yes [] | no [] |
|---------|--------|
- Have you ever smoked
- | | |
|---------|--------|
| yes [] | no [] |
|---------|--------|

If so, for how long did you smoke and when did you stop?

5. How many units of alcohol do you typically drink in a week?

If YES to any question, please describe briefly if you wish (e.g. to confirm whether problem was short-lived, insignificant or well controlled.)

.....
.....

Name and address of GP

Blood pressure measured at screening.....mm Hg

APPENDIX A4 – PHYSICAL ACTIVITY QUESTIONNAIRE

Name:

Date:

During one week, how many times on average do you do the following kinds of exercise for more than 15 minutes?

(a) Strenuous Exercise (heart beats rapidly)

For example; running, jogging, squash, hockey, football, basketball, vigorous swimming, vigorous long distance cycling.

_____ times per week.

(b) Moderate Exercise (not exhausting)

For example; fast walking, tennis, easy cycling, badminton, easy swimming, dancing.

_____ times per week.

(c) Mild Exercise (minimal effort)

For example; yoga, archery, fishing, bowling, golf, easy walking.

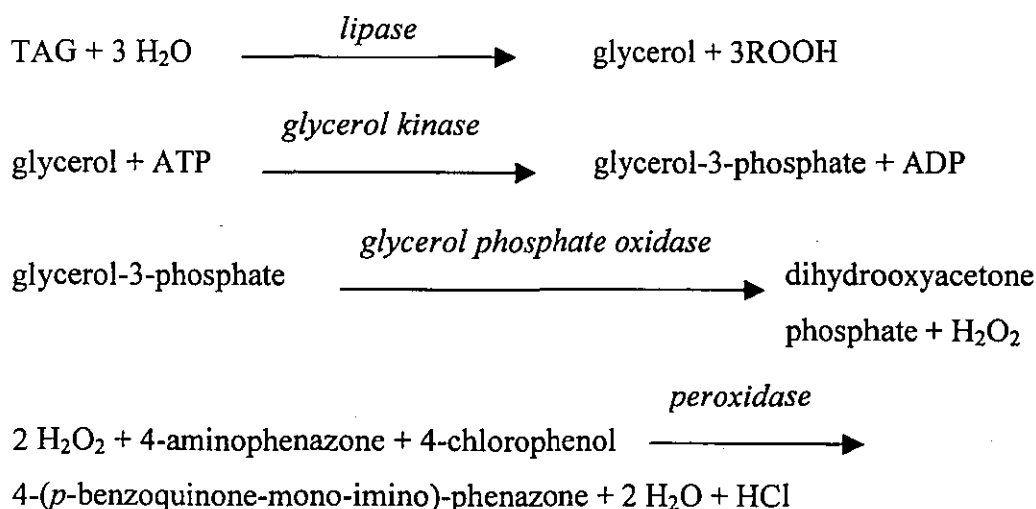
_____ times per week.

APPENDIX B1: DETERMINATION OF TAG CONCENTRATION

TAG concentrations were determined in plasma and lipoprotein fractions by an enzymatic colorimetric method using a commercially available kit (Peridochrom Triglylycerides GPO-PAP, Boehringer Mannheim, UK, Ltd., Lewes, UK). Samples were analysed in duplicate on an automated centrifugal analyser (Cobas Bio, Version 8326 (reproducibility study, chapter 4 and chapter 5) or Cobas Mira Plus (chapter 6); Roche Diagnostic Systems, Hertfordshire, UK).

Principle

This assay is based on lipase hydrolysis of TAG to liberate glycerol, which is subsequently determined by colorimetrically. Specifically, hydrogen peroxide is generated from the substrate by glycerol phosphate oxidase and this is coupled through peroxidase to produce a chromogen, which is detected by its absorbance at 500 nm (Mackness and Durrington, 1992). The following sequence of equations outlines the assay principle:



Reagents

The kit provided a buffer solution and reagent strips. A reagent strip was immersed in each bottle of buffer solution, stirred for 10 seconds and left for five minutes. The buffer was then stirred with the reagent strip for a further 10 seconds and discarded. The compositions of the buffer solution and reagent strips are shown below:

Buffer solution:

Tris buffer	0.15 mol.l ⁻¹ , pH 7.6
Magnesium sulphate	17.5 mmol.l ⁻¹
EDTA, di sodium salt	10 mmol.l ⁻¹
Pottassium hexacynoferrate	6 µmol.l ⁻¹
Sodium cholate	0.15 %
4-chlorophenol	3.5 mmol.l ⁻¹
Hydroxypolyethoxy n-alcanes	0.12 %

Reagent strips:

ATP	≥ 0.5 mmol.l ⁻¹
4-aminophenazone	0.35 mmol.l ⁻¹
Lipase	≥ 3 U.ml ⁻¹
Glycerol phosphate oxidase	≥ 2.5 U.ml ⁻¹
Glycerol kinase	≥ 0.15 U.ml ⁻¹
Peroxidase	≥ 0.15 U.ml ⁻¹

Standards

For analyses on the Cobas Bio, three working standards were prepared from a commercially available 2.29mmol.l⁻¹ glycerol solution (Boehringer Mannheim, UK, Ltd., Lewes, UK). This standard was serially diluted with distilled water to produce standards appropriate for the assay range. For analyses on the Cobas Mira, a single commercially available serum based standard was used (Calibrator for automated systems (CFAS), Boehringer Mannheim, UK, Ltd., Lewes, UK).

Quality control

Precinorm U and Precipath U, Boehringer Mannheim UK Ltd, Lewes, UK
Control Serum N (Human), Roche Diagnostic Systems, Welwyn Garden City, UK
Seronorm Lipid, Sero AS, Billingstad, Norway

Procedure

1. Samples were defrosted for 45 minutes (or removed from the refrigerator) and vortexed, before ~100 μ l was dispensed into plastic sample cups and loaded onto the Cobas Bio or Cobas Mira analyser.
2. For plasma TAG and VLDL-TAG analysis, 5 μ l of sample, standard or quality control was pipetted into each cuvette. For analysis of chylomicron- and Sf < 20-TAG, 10 μ l of sample was pipetted.
3. 350 μ l (plasma TAG and VLDL-TAG) or 300 μ l (chylomicron- and Sf < 20-TAG) of reagent was added to each cuvette.
4. Samples were mixed, incubated for five minutes at 37°C and the absorbances were read at a wavelength of 500 nm.
5. TAG concentrations of the samples and quality controls were determined by the analyser using a regression equation derived from analysis of the standards.

APPENDIX B2 – PRECIPITATION OF HDL FROM PLASMA

Principle

Apo B containing lipoproteins (i.e chylomicrons, VLDL, IDL and LDL) are precipitated from plasma after formation an insoluble complex with the polyanion-divalent cation molecules of magnesium chloride/phosphotungstic acid, leaving a supernatent containing only non-apo B containing lipoprotein (i.e. HDL) particles.

Reagent

The precipitant solution was supplied, ready to use, in a commercially available kit (HDL-C, Boehringer Mannheim, UK, Ltd., Lewes, UK), containing 0.55 mmol.l⁻¹ phosphotungstic acid and 25 mmol.l⁻¹ magnesium chloride.

Procedure

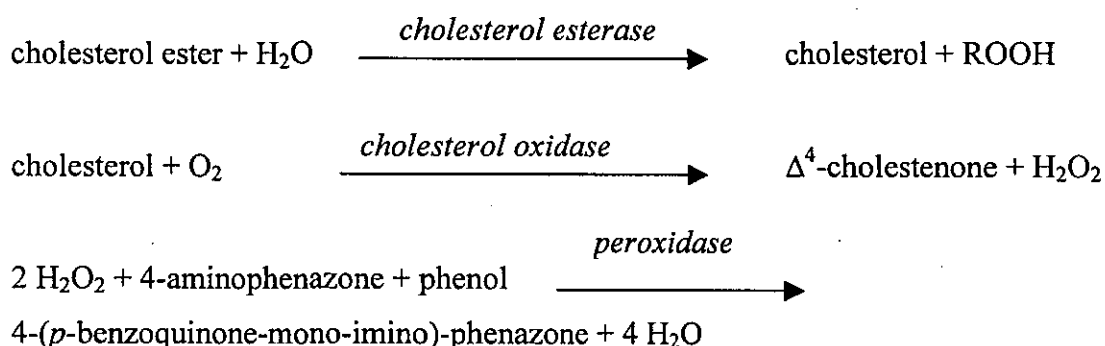
1. Plasma samples were defrosted for 45 minutes at room temperature and vortexed.
2. 500 µl of precipitant was added to 200 µl of sample or quality control and vortexed.
3. These were allowed to stand at room temperature for 10 minutes before centrifugation, in a microcentrifuge, for two minutes at 12 000 revs.min⁻¹.
4. The clear supernatent was removed for cholesterol analysis (Appendix B3).

APPENDIX B3: DETERMINATION OF CHOLESTEROL CONCENTRATION

Total plasma cholesterol and HDL cholesterol concentrations were determined in by an enzymatic colorimetric method using a commercially available kit (Cholesterol C system CHOD-PAP, Boehringer Mannheim, UK, Ltd., Lewes, UK). Samples were analysed in duplicate on an automated centrifugal analyser (Cobas Bio, Version 8326 (reproducibility study and chapter 4) or Cobas Mira Plus (chapters 5 and 6); Roche Diagnostic Systems, Hertfordshire, UK).

Principle

The test principle is analogous to the previously described TAG assay and is based n the generation of hydrogen peroxide from the substrate by the action of cholesterol oxidase (after the hydrolysis of esterified cholesterol), which is coupled through peroxidase to produce a chromogen, detected by its absorbance at 500 nm (Mackness and Durrington, 1992). The principle is displayed in the sequence of reactions below:



Standards

For analyses on the Cobas Bio, three standards from a commercially available set of calibrators (Preciset Cholesterol, Boehringer Mannheim, UK, Ltd., Lewes, UK) were used. These standards were in the appropriate range for total cholesterol determination, but needed to be serially diluted with distilled water to produce standards appropriate range for HDL cholesterol. For analyses on the Cobas Mira, a single commercially available serum based standard was used (Calibrator for automated systems (CFAS), Boehringer Mannheim, UK, Ltd., Lewes, UK).

Reagents

The kit supplied the reagents in powder form. This was reconstituted with distilled water and inverted gently mixed. The reagent was ready for use after 10 minutes. The composition is shown below:

Tris buffer	100 mmol.l ⁻¹ , pH 7.7
Magnesium aspartate	50 mmol.l ⁻¹
4-aminophenazone	1 mmol.l ⁻¹
Sodium cholate	10 mmol.l ⁻¹
Phenol	6 mmol.l ⁻¹
3,4 dichlorophenol	4 mmol.l ⁻¹
Hydroxypolyethoxy n alcanes	0.3 %
Cholesterol esterase	≥ 0.4 U.ml ⁻¹
Cholesterol oxidase	≥ 0.25 U.ml ⁻¹
Peroxidase	≥ 0.2 U.ml ⁻¹

Quality control

Precinorm U and Precipath U, Boehringer Mannheim UK Ltd, Lewes, UK

Control Serum N (Human), Roche Diagnostic Systems, Welwyn Garden City, UK

Procedure

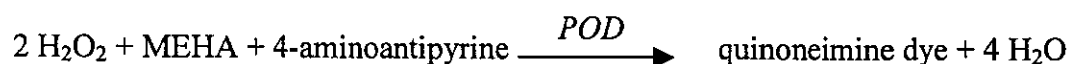
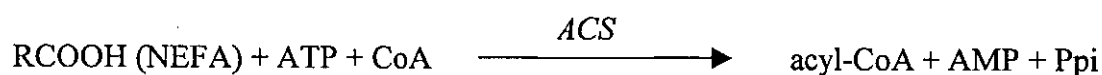
1. HDL supernatant (see appendix B2) or thawed plasma samples were vortexed, before ~100 µl was dispensed into plastic sample cups and loaded onto the Cobas Bio or Cobas Mira analyser.
2. 5 µl of sample, standard or quality control was pipetted into each cuvette.
3. 350 µl of reagent was added to each cuvette.
4. Samples were mixed, incubated for five minutes at 37°C and the absorbances were read at a wavelength of 500 nm.
5. Cholesterol concentrations of the samples and quality controls were determined by the analyser using a regression equation derived from analysis of the standards.

APPENDIX B4: DETERMINATION OF PLASMA NEFA CONCENTRATION

Plasma NEFA concentrations were determined in by an enzymatic colorimetric method using a commercially available kit (NEFA-C ACS-ACOD Method, Wako Chemicals GmbH, UK, Ltd). Samples were analysed on an automated centrifugal analyser (Cobas Bio, Version 8326 (chapter 4) or Cobas Mira Plus (chapters 5 and 6); Roche Diagnostic Systems, Hertfordshire, UK).

Principle

This method relies on the acylation of coenzyme A (CoA) by the fatty acids in the presence of added acyl-CoA synthetase (ACS). The acyl-CoA produced is oxidised by added acyl-CoA oxidase (ACOD), producing hydrogen peroxide. The hydrogen peroxide, in the presence of peroxidase (POD), enables the oxidative condensation of 3-metyhl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple adduct which can be measured at 550 nm. The series of reactions is shown below:



Standards

A single 1.0 mmol.l⁻¹ oleic acid standard was supplied with the kit.

Reagents

Two reagents (colour reagents A and B) were supplied in powder form with the kit. For analyses performed on the Cobas Bio, colour reagent A was reconstituted in 10 ml of 'diluent for colour reagent A' and colour reagent B was dissolved in 7.5 ml of 'diluent for colour reagent B'. For analyses performed on the Cobas Mira the reconstitution volumes were 10 ml and 7 ml for colour reagents A and B respectively.

Colour reagent A:

ACS	0.3 U.ml ⁻¹
Ascorbate oxidase	3 U.ml ⁻¹
CoA	0.7 mg.ml ⁻¹
ATP	3 mg.ml ⁻¹
4-aminoantipyrine	0.3 mg.ml ⁻¹

Diluent for colour reagent A:

Phosphate buffer	0.05 mol.l ⁻¹ , pH 6.9
Magnesium chloride	3 mmol.l ⁻¹
Surfactant	
Stabilisers	

Colour reagent B:

ACOD	17.6 U.ml ⁻¹ (Cobas Bio), 18.9 U.ml ⁻¹ (Cobas Mira)
POD	20 U.ml ⁻¹ (Cobas Bio), 21.4 U.ml ⁻¹ (Cobas Mira)

Diluent for colour reagent B:

MEHA	1.2 mmol.l ⁻¹
Surfactant	

Quality controls

Seronorm Lipid, Sero AS, Billingstad, Norway

Procedure

1. Plasma samples were defrosted for 45 minutes at room temperature and vortexed. Approximately 100 µl of sample was dispensed into plastic sample cups and loaded onto the Cobas Bio or Cobas Mira analyser. Samples were kept on ice until they were analysed.
2. 10 µl (Cobas Bio) or 6 µl of sample, standard or quality control was pipetted into each cuvette.
3. 100 µl (Cobas Bio) or 120 µl (Cobas Mira) of colour reagent A was added to each cuvette.

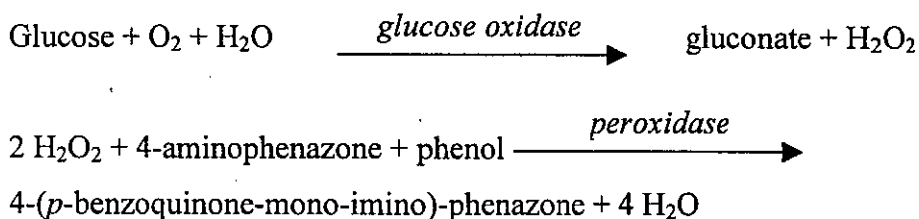
4. Samples were mixed and incubated for ten (Cobas Bio) or six (Cobas Mira) at 37°C.
5. 75 µl (Cobas Bio) or 85 µl (Cobas Mira) of colour reagent B was then added to each cuvette and mixed. Samples were incubated for a further five (Cobas Bio) or six (Cobas Mira) minutes and absorbances were read at 550 nm.
6. NEFA concentrations of the samples and quality controls were determined by the analyser using a regression equation derived from analysis of the standards.

APPENDIX B5 – DETERMINATION OF PLASMA GLUCOSE CONCENTRATION

Plasma glucose concentrations were determined by an enzymatic colorimetric method using a commercially available kit (Glucose GOD-PAP, Boehringer Mannheim, UK, Ltd., Lewes, UK). Samples were analysed in duplicate on an automated centrifugal analyser (Cobas Bio, Version 8326 (reproducibility study and chapter 4) or Cobas Mira Plus (chapters 5 and 6); Roche Diagnostic Systems, Hertfordshire, UK).

Principle

This assay is based on the generation of hydrogen peroxide from glucose by the action of glucose oxidase and the coupling of this through peroxidase to produce a chromogen which is analysed spectrophotometrically at 500 nm. The reaction sequence is shown in the equations below:



Standards

For analyses on the Cobas Bio, commercially available standards, with concentrations of 2.78 mmol.l⁻¹, 5.55 mmol.l⁻¹ and 8.53 mmol.l⁻¹ (Preciset Glucose, Boehringer Mannheim, UK, Ltd., Lewes, UK) were used. For analyses on the Cobas Mira, a single commercially available serum based standard was used (Calibrator for automated systems (CFAS), Boehringer Mannheim, UK, Ltd., Lewes, UK).

Reagents

The kit provided a reagent mixture of buffer, enzymes and chromogen in a powder form and a bottle of phenol. The reagent mixture was reconstituted in 200 ml of distilled water and phenol was added. This was mixed thoroughly and stored in an amber glass bottle. Composition of the reagent is shown below:

Phosphate buffer	100 mol.l ⁻¹ , pH 7.0
Glucose oxidase	17.5 U.ml ⁻¹
Peroxidase	≥ 1.1 U.ml ⁻¹
4-aminophenazone	0.77 mmol.l ⁻¹
Phenol	11 mmol.l ⁻¹

Quality control

Precinorm U and Precipath U, Boehringer Mannheim UK Ltd, Lewes, UK

Control Serum N (Human), Roche Diagnostic Systems, Welwyn Garden City, UK

Procedure

1. Plasma samples were defrosted for 45 minutes at room temperature and vortexed. Approximately 100 µl of sample was dispensed into plastic sample cups and loaded onto the Cobas Bio or Cobas Mira analyser.
2. 4 µl of sample, standard or quality control was pipetted into each cuvette.
3. 300 µl of reagent was added to each cuvette.
4. Samples were mixed, incubated for one minute at 30°C (Cobas Bio) or 37°C (Cobas Mira) and the absorbances were read at a wavelength of 500 nm.
5. Glucose concentrations of the samples and quality controls were determined by the analyser using a regression equation derived from analysis of the standards.

APPENDIX B6 – DETERMINATION OF PLASMA PARACETAMOL CONCENTRATION

Plasma paracetamol concentrations were determined in by an enzymatic colorimetric method using a commercially available kit (Cambridge Life Sciences plc, Cambridge, UK). Samples were analysed on an automated centrifugal analyser (Cobas Mira Plus, Roche Diagnostic Systems, Hertfordshire, UK).

Principle

This method relies on the use of a enzyme specific for the amide bond of acylated aromatic amines. This cleaves the paracetamol molecule, producing p-aminophenol, which reacts specifically with o-cresol in ammoniacal copper solution which results in a blue colour, detected spectrophotometrically.

Standard

A 2 mmol.l⁻¹ paracetamol standard was provided with the kit. His was diluted with distilled water to 0.2 mmol.l⁻¹ before use.

Reagents

The kit contains lyophilised enzyme (> 10 U aryl acylamidase), which is reconstituted with 10 ml of enzyme diluent, and colour reagents A (ortho creosol solution, 1.0 % w/v aqueous) and B (ammomiactal copper sulphate solution), which are ready for use.

Procedure

1. Plasma samples were defrosted for 45 minutes and vortexed, before ~100 µl was dispensed into plastic sample cups and loaded onto the Cobas Mira analyser.
2. 20 µl of sample, standard or quality control was pippetted into each cuvette.
3. 100 µl of enzyme was added to each cuvette.
4. Samples were mixed and incubated for one minute at 37°C.
5. 90 µl colour reagent A and 90 µl colour reagent B were added and samples were mixed, incubated at 37°C for 150 seconds and absorbance was measured at 600 nm..

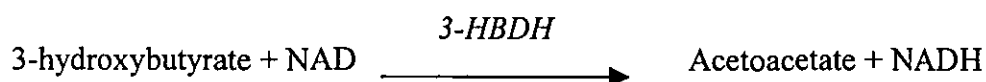
6. Paracetamol concentrations were determined by the analyser using a regression equation derived from analysis of the standards.

APPENDIX B7 – DETERMINATION OF SERUM 3-HYDROXYBUTYRATE CONCENTRATION

Serum 3-hydroxybutyrate concentrations were determined in by an enzymatic method using a commercially available kit (Sigma Diagnostics, Poole, UK). Samples were analysed on an automated centrifugal analyser (Cobas Mira Plus, Roche Diagnostic Systems, Hertfordshire, UK).

Principle

3-hydroxybutyrate dehydrogenase (3-HBDH) catalyses the oxidation of 3-hydroxybutyrate to acetoacetate. During this reaction, an equimolar amount of nicotinamide adenine dinucleotide (NAD) is reduced to NADH, which is detected spectrophotometrically at an absorbance of 340 nm. The equation for the reaction is shown below:



Standards

A 4.8 mmol.l⁻¹ 3-hydroxybutyrate calibrator solution was provided with the kit. This was diluted with distilled water to produce two working standards of 0.48 mmol.l⁻¹ and 0.24 mmol.l⁻¹.

Reagents

The 3-hydroxybutyrate reagent was supplied in lyophilised form and reconstituted with 10 ml of distilled water. 3-hydroxybutyrate dehydrogenase was supplied in a ready-to-use solution. Concentrations of the reagents are given below:

3-hydroxybutyrate reagent:

NAD	4.6 mmol.l ⁻¹
Buffer	pH 7.6 ± 0.2
Oxamic acid	

3-hydroxybutyrate dehydrogenase solution:

3-hydroxybutyrate dehydrogenase	50 U.ml ⁻¹
Buffer	pH 7.6 ± 0.2
Stabilisers and fillers	

Quality controls :

β-HBA Control-N (Human), Sigma Diagnostics, Poole, UK

Procedure

1. Plasma samples were defrosted for 45 minutes at room temperature and vortexed. Approximately 100 µl of sample was dispensed into plastic sample cups and loaded onto the Cobas Mira analyser.
2. 10 µl of sample, standard or quality control was pipetted into each cuvette.
3. 200 µl of 3-hydroxybutyrate reagent, then 11 µl of 3-hydroxybutyrate dehydrogenase solution was added to each cuvette.
4. Samples were mixed and incubated for ten minutes at 37°C. Absorbance was measured at 340 nm.
5. 3-hydroxybutyrate concentrations were determined by the analyser using a regression equation derived from analysis of the standards.

APPENDIX B8 – DETERMINATION OF SERUM INSULIN CONCENTRATION

Serum insulin concentrations were determined using a solid phase ^{125}I radioimmunoassay using a commercially available kit (COAT-A-COUNT Insulin, EURO/DPC Ltd., Caernarfon, UK). Radioactivity was measured using an automated gamma counting system (Cobra II, Packard Instrument Company Inc, USA).

Principle

In this procedure, ^{125}I labelled insulin competes with insulin in the serum sample for sites on insulin-specific antibodies which are immobilised on the wall of a polypropylene tube. After an incubation period, the antibody bound fraction is achieved by decanting the supernatant. The radioactivity of the tube is then measured, with the counts being inversely related to the amount of insulin present in the serum sample. Serum insulin concentration is then determined by comparing the counts to a standard curve.

Materials and reagents

- Plain (uncoated) polypropylene tubes for the measurement of total and non-specific binding counts.
- Green polypropylene tubes coated with antibodies to insulin.
- A concentrated solution of ^{125}I labelled insulin, which was diluted with 100 ml distilled water.

Standards

Lyophilised processed human standards were supplied with the kit, at insulin concentrations of 0, 5, 15, 50, 100, 200 and 400 $\mu\text{U}.\text{ml}^{-1}$. These were reconstituted with distilled at least 30 minutes before use.

Quality Controls

A three-level, human-serum based, quality control was used (CON6, EURO/DPC Ltd., Caernarfon, UK).

Procedure

1. Samples were defrosted and vortexed.
2. 200 μl of the 0 $\mu\text{U}.\text{ml}^{-1}$ calibrator was dispensed into the plain non-specific binding tubes.
3. 200 μl of each calibrator, serum sample or quality control was dispensed directly to the bottom of each antibody-coated tube.
4. 1.0 ml of ^{125}I labelled insulin was added to the plain total count tubes and each sample-containing tube, within 40 minutes of dispensing the sample.
5. These tubes were vortexed and allowed to incubate for 18 to 24 hours at room temperature.
6. After this incubation period, the contents of each tube (except the total count tubes) were decanted using a foam decanting rack. The tubes were allowed to drain for about 5 minutes and were then struck sharply on absorbant paper to remove residual droplets remaining in the tubes.
7. The radioactivity of each tube was then counted for 1 minute in the gamma counter.
8. Insulin concentrations was from a spline fit calibration curve calculated from the standard values.

APPENDIX B9 – DETERMINATION OF HAEMOGLOBIN CONCENTRATION

Haemoglobin concentrations were determined in duplicate using a commercially available kit (Test-Combination Haemoglobin, Boehringer Mannheim UK Ltd, Lewes, UK) and a spectrophotometer (Digital Grating Spectrophotometer Series 2, Model CE393; Cecil Instruments Ltd, Cambridge, UK).

Principle

Haemoglobin is oxidised to methaemoglobin by ferricyanide, which is converted to cyanmethaemoglobin by potassium cyanide. The absorbance of cyanmethaemoglobin is measured spectrophotometrically at 546 nm. The principle is outlined in the equation below.



Reagent

Drabkin's reagent was prepared using reagents supplied in the kit. 25 ml of potassium hexacyanoferrate (III) (0.6 mol.l^{-1}) and 25 ml of potassium cyanide (0.75 mol.l^{-1}) were diluted to one litre with distilled water.

Procedure

1. 20 μl of whole blood was dispensed into 5 ml of Drabkin's reagent and mixed.
2. The spectrophotometer was zeroed against a Drabkin's reagent blank and sample absorbances were read at 546 nm.
3. Haemoglobin concentration in g.dl^{-1} was determined by multiplying the absorbance value by 36.77.

APPENDIX B10 – DETERMINATION OF ^{13}C ENRICHMENT OF BREATH

The ^{13}C enrichment of breath samples was determined by researchers at the Institute of Human Nutrition using continuous flow isotope ratio mass spectrometry (CF-IRMS) (Europa Scientific Ltd, Crewe, UK).

The breath samples were loaded into a 220-hole auto-sampler rack, interspersed with samples of a reference gas (10 ml of a 5 % CO_2 , 95 % N_2 gas mix (BOC Gases, Manchester, UK)). These were injected into the system and were carried in a flow of helium (60 ml.s^{-1}) through a reduction tube to reduce nitrogen oxides (NO_x) to N_2 . The sample then passed through a water scrubber containing magnesium perchlorate to remove any water present in the sample before passing through a gas chromatography (GC) column kept at 125°C to separate the gases. A small proportion (about 1 %) of the GC effluent was passed through the mass spectrometer, which bombarded the samples to generate positive CO_2^+ ions. These passed through a magnetic field and were deflected according to their mass to charge ratio, with the mass spectrometer detecting three different molecular masses – 44, 45 and 46 – corresponding to $\text{C}^{12}\text{O}^{16}\text{O}^{16}$, $\text{C}^{13}\text{O}^{16}\text{O}^{16}$ and $\text{C}^{12}\text{O}^{18}\text{O}^{16}$, respectively. The system is fully automated and is operated via a personal computer (Visual Basic version 3.5, Europa Scientific Ltd., Crewe, UK).

The ^{13}C enrichment was expressed by isotopic ratio delta (δ) values in units per mil (mil = 1000), written ‰. These values were expressed relative to the international reference standard, Pee Dee Belemite (PDB), a carbonate from South Carolina with an isotopic ratio of 0.0112372 (1.1112328 atom % ^{13}C) (Murphy, 1992). Delta values can be positive or negative compared with this standard and, in fact, most biological materials contain less ^{13}C than the standard; the $\delta^{13}\text{C}$ for cane sugar is -11.65 ‰ (atom % 1.0964) and for beet sugar is -25.96 ‰ (atom % ^{13}C 1.0827) (Murphy, 1992). Delta values were calculated according to the following equation:

$$\text{Delta } ^{13}\text{C} (\text{‰}) = \frac{^{13}\text{C}:^{12}\text{C}_{\text{sample}} - ^{13}\text{C}:^{12}\text{C}_{\text{standard}}}{^{13}\text{C}:^{12}\text{C}_{\text{standard}}} * 1000$$

Excretion of $^{13}\text{CO}_2$ on breath was expressed as a percentage of the total dose administered using the equations described by Watkins et al (1982) (Watkins et al. 1982):

$$\% \text{ administered dose excreted per hour} = \frac{\text{mmol excess } ^{13}\text{C}/\text{mmol CO}_2 * \text{VCO}_2 * 100}{\text{mmol } ^{13}\text{C administered}}$$

where:

$$\text{mmol excess } ^{13}\text{C}/\text{mmol CO}_2 = (\delta ^{13}\text{C}_t - \delta ^{13}\text{C}_{t=0}) * R_{\text{PDB}} * 10^{-3}$$

$$\text{mmol } ^{13}\text{C administered} = \frac{\text{mg substrate} * P * n}{M * 100}$$

VO_2 is in mmol.h^{-1} (1 mmol \equiv 0.0224 l)

$R_{\text{PDB}} = 0.0112372$

P = isotopic purity of the labelled substrate (99 %)

n = number of carbon atoms labelled with ^{13}C per molecule of substrate (3)

M = molecular weight of labelled substrate (810.3)

Cumulative $^{13}\text{CO}_2$ excretion over the study period was determined as the cumulative area under the percentage excretion *versus* time curve, calculated using the trapezium rule.

APPENDIX B11 – DETERMINATION OF PLASMA ¹³C PALMITIC ACID CONCENTRATION

These procedures were performed by the author under the supervision of researchers at the Institute of Human Nutrition and comprised four stages: total lipid extraction, separation of lipid classes by solid phase extraction (Bond Elut), methylation of the NEFA extract to form fatty-acid-methyl-esters and measurement of total and ¹³C palmitic acid by GC-IRMS.

Lipids were extracted from plasma by mixing 0.8 ml of plasma with 5 ml of chloroform:methanol (2:1) for 15 minutes after the addition of 30 µl of a NEFA surrogate standard (containing 30 µg NEFA). 1 ml 1M NaCl was then added and the mixture was centrifuged for 10 minutes. The solvent layer was then aspirated and dried under nitrogen.

Lipid classes were then separated by using aminopropyl bonded phase (Bond Elut) columns based on a method described by Kalunzy et al (1985) (Kaluzny et al. 1995). The dried lipid extract was dissolved in 1 ml of chloroform. The sample was then pipetted onto the Bond Elut columns and allowed to drip through under gravity. The columns were then washed with 1 ml of chloroform twice, 1 ml of chloroform:methanol 60:40 and 1 ml of methanol under vacuum to remove CE, TAG and phosphatidylcholine from the lipid extract. The wash extract was discarded and NEFA were eluted by passing 1 ml of chloroform:methanol:acetic acid 100:2:2 through the Bond Elut columns under vacuum. The NEFA extract was then dried under nitrogen.

The NEFA extracts were then methylated to form fatty acid methyl esters (FAME) by adding 2 ml of 2 % sulphuric acid in methanol to the dried lipid and heating overnight at 50°C. The samples were allowed to cool and 2 ml of 'neutralising agent' (a potassium carbonate and potassium hydrogen carbonate solution) and 2 ml dry hexane was added. After mixing for 15 minutes and centrifugation for 10 minutes, the solvent was aspirated and dried under nitrogen. A further 2 ml of dry hexane was added and the mixing, centrifugation and drying process was repeated. The lipid was then dissolved in 200 µl of dry hexane, transferred to a mini-vial and dried under

nitrogen. The original sample tube was then washed with 200 μ l of dry hexane. This was transferred to the mini-vial and dried under nitrogen and the process was repeated twice. 30 μ l of a FAME recovery standard was then added before again drying the extract under nitrogen.

The dried lipid extracts were reconstituted in 30 μ l of dry hexane before analysis of the ^{13}C and total fatty acid composition by gas chromatography-IRMS (GC-IRMS) using the method described by Stolinski et al (1997) (Stolinski et al. 1997).

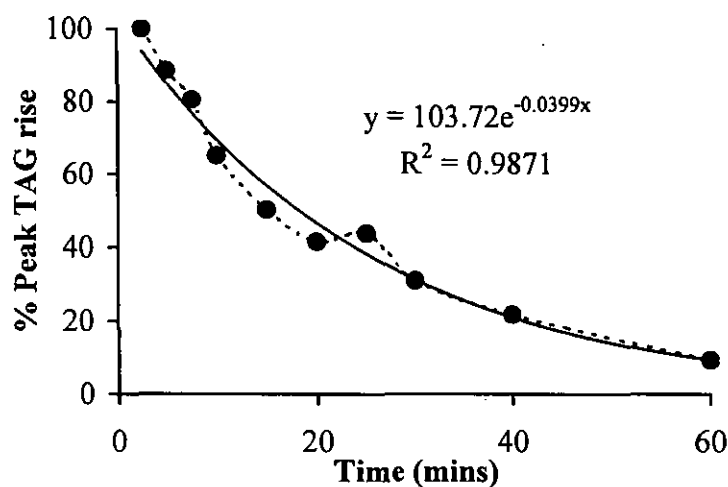
The ^{13}C enrichment of palmitic acid was calculated from the following equation derived from a calibration regression line:

$$^{13}\text{C enrichment} = \frac{\delta^{13}\text{C}_{\text{palmitic acid}} + 31.57}{53.80}$$

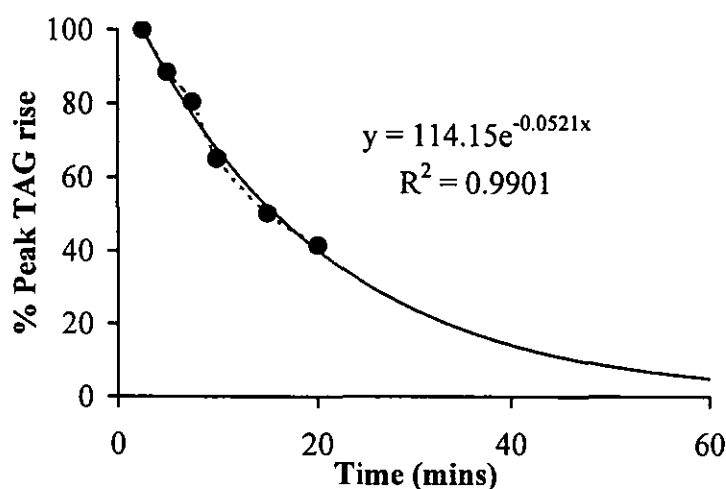
The concentration of palmitic acid in the sample was calculated from the ratio of the areas under the peaks for palmitic acid and the NEFA surrogate standard, taking into account the amount of surrogate used and the volume of the plasma sample. This was multiplied by the fractional enrichment of ^{13}C to determine the plasma ^{13}C palmitic acid composition.

APPENDIX C1 - INDIVIDUAL TAG CLEARANCE CURVES FROM CHAPTER 6

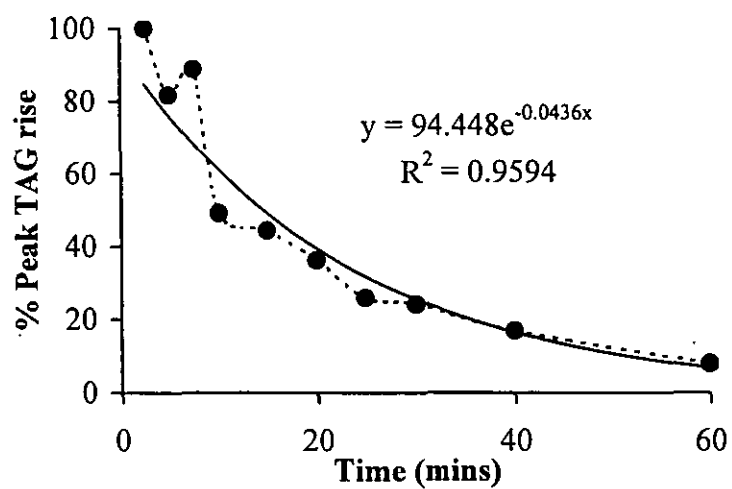
The figures below show exponential regression lines fitted to the entire 60 minute TAG clearance curves, and to the first 20 or 25 minutes of the TAG clearance curves (with obvious outlying data points removed), for the eight subjects in the control and exercise trials. Equations of the regression lines and goodness-of-fit R^2 values shown on the figures.



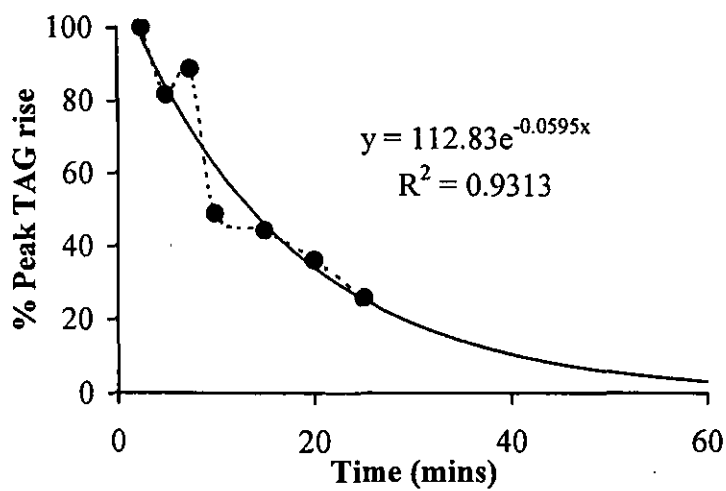
Subject 1, control trial: 60-minute TAG clearance curve



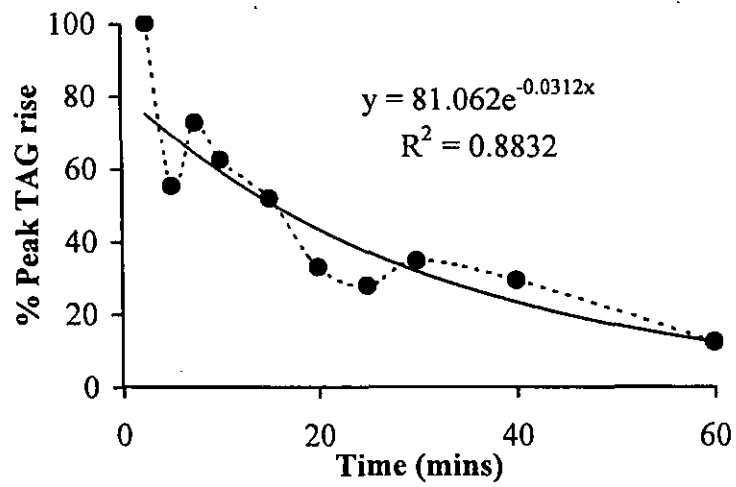
Subject 1, control trial: 20-minute TAG clearance curve (outlying points removed)



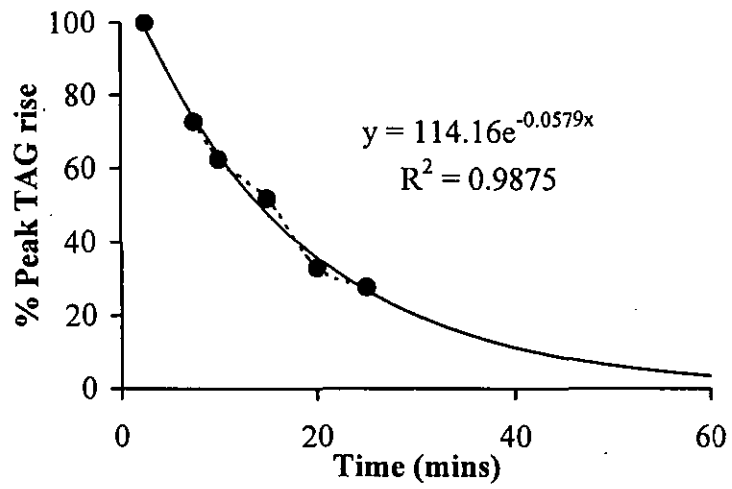
Subject 1, exercise trial: 60-minute TAG clearance curve



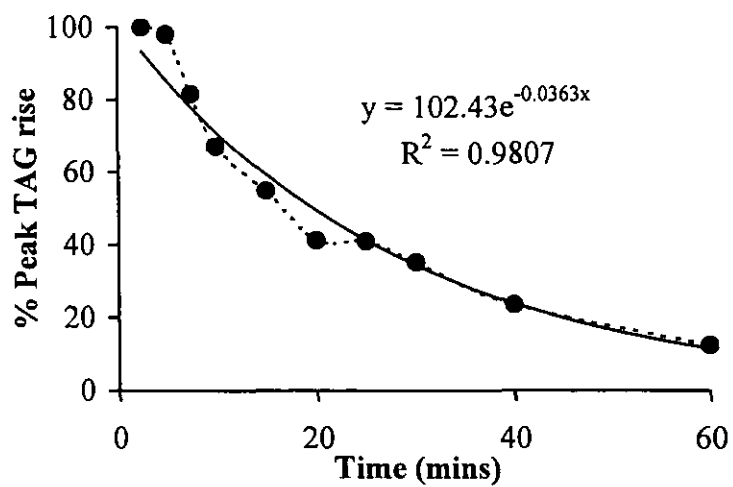
Subject 1, exercise trial: 25-minute TAG clearance curve (outlying points removed)



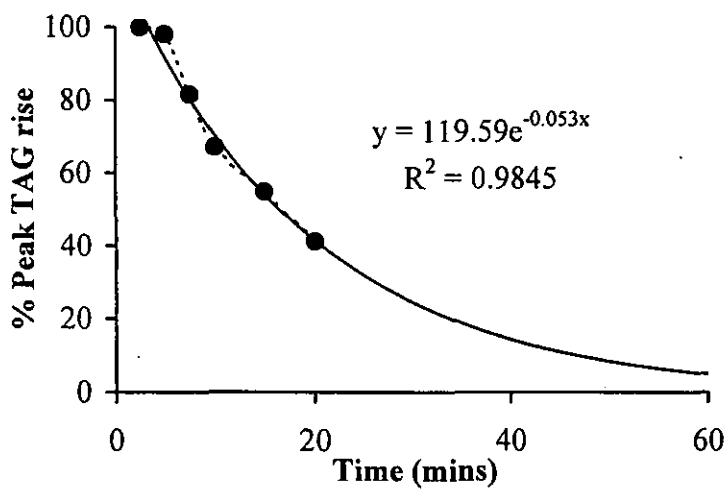
Subject 2, control trial: 60-minute TAG clearance curve



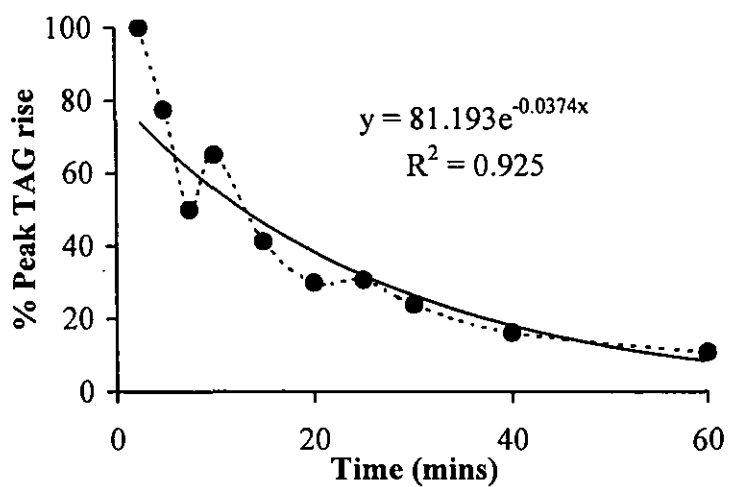
Subject 2, control trial: 25-minute TAG clearance curve (outlying points removed)



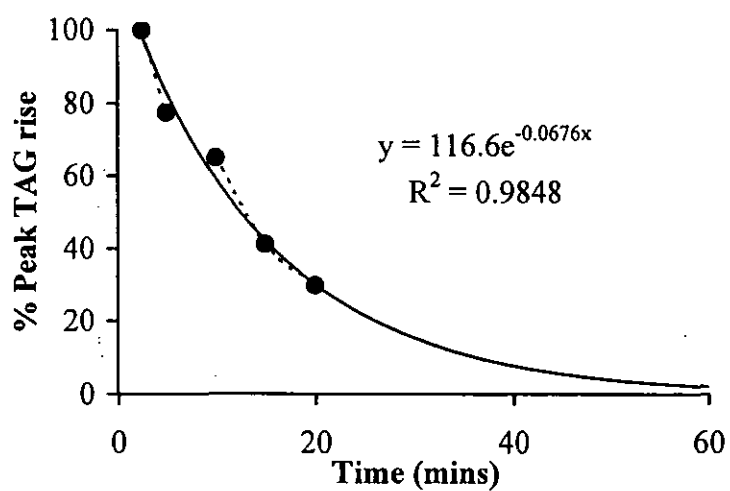
Subject 2, exercise trial: 60-minute TAG clearance curve



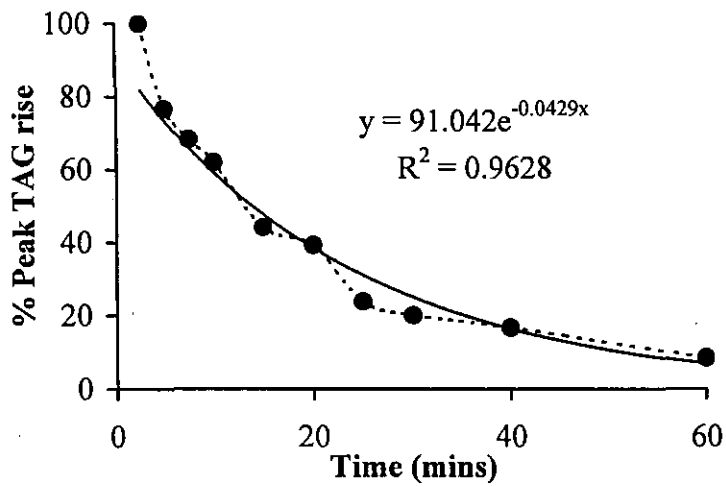
Subject 2, exercise trial: 20-minute TAG clearance curve (outlying points removed)



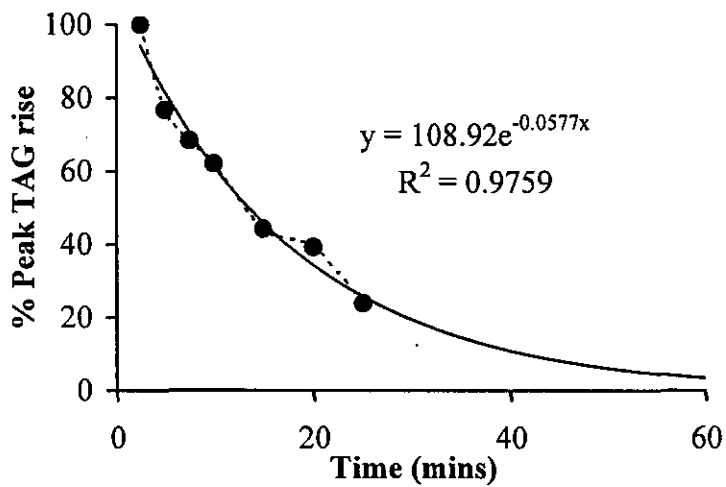
Subject 3, control trial: 60-minute TAG clearance curve



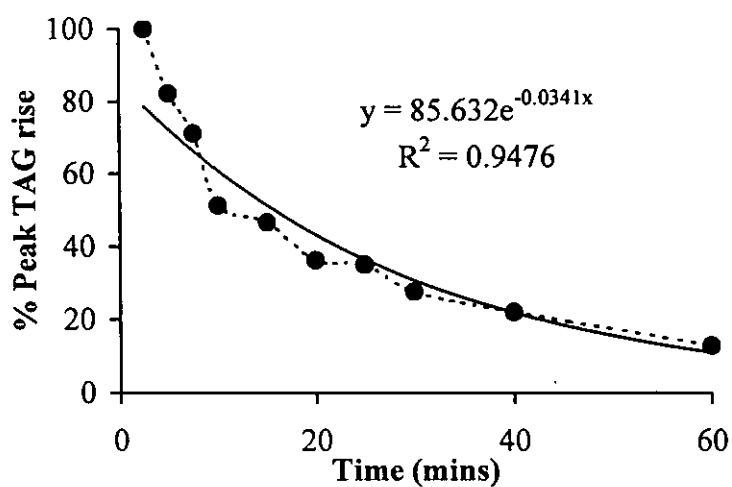
Subject 3, control trial: 20-minute TAG clearance curve (outlying points removed)



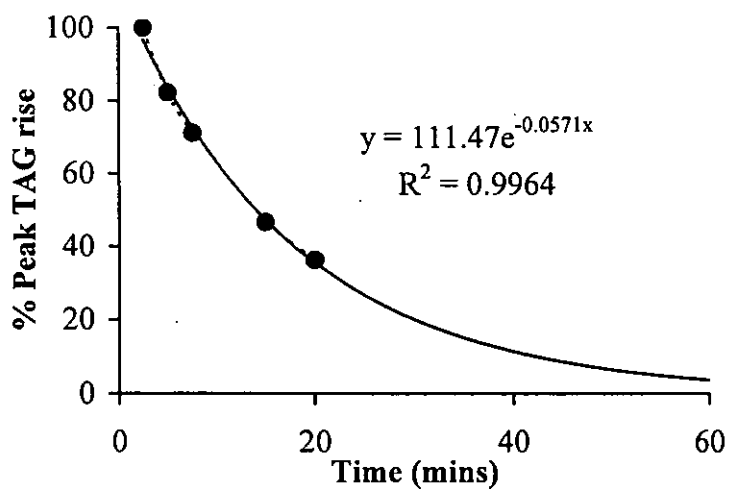
Subject 3, exercise trial: 60-minute TAG clearance curve



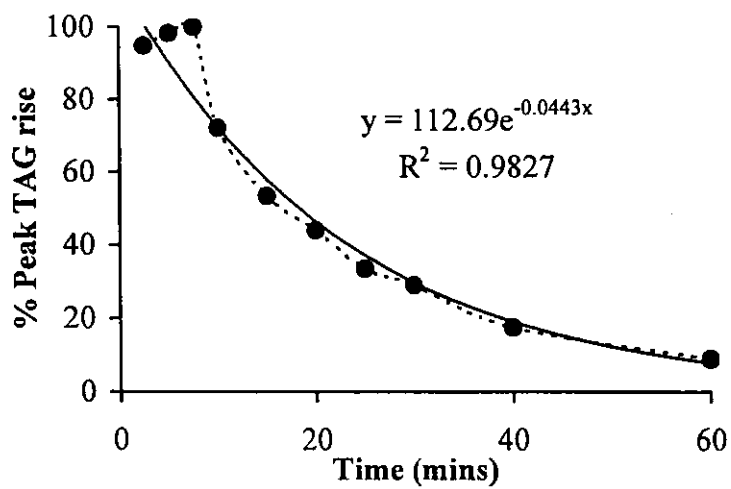
Subject 3, exercise trial: 25-minute TAG clearance curve (outlying points removed)



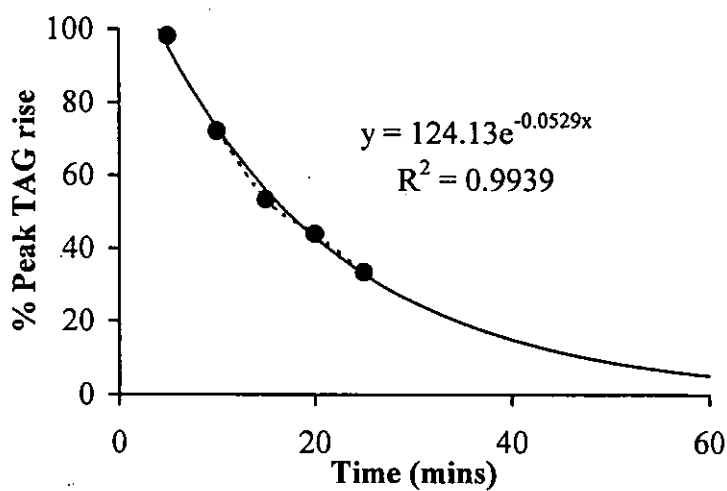
Subject 4, control trial: 60-minute TAG clearance curve



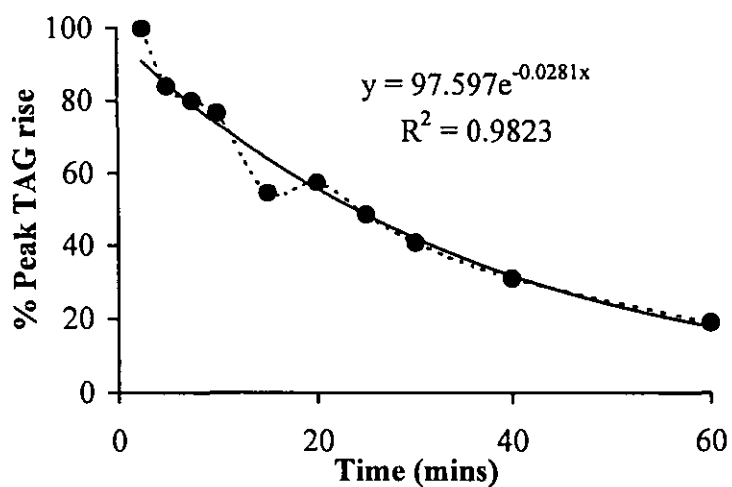
Subject 4, control trial: 20-minute TAG clearance curve (outlying points removed)



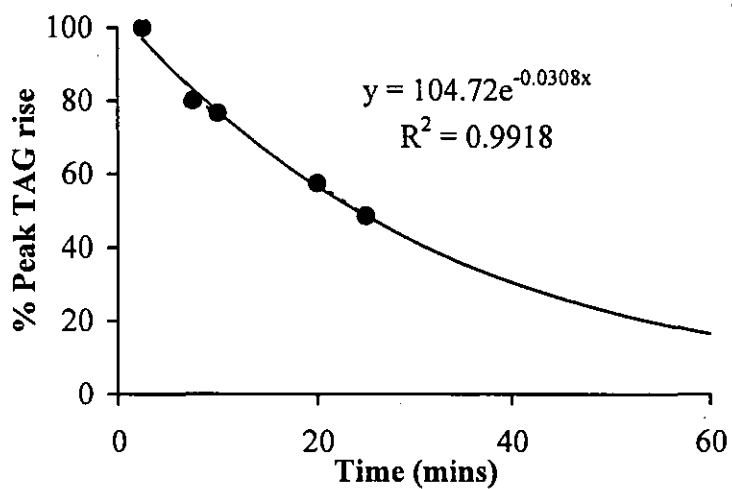
Subject 4, exercise trial: 60-minute TAG clearance curve



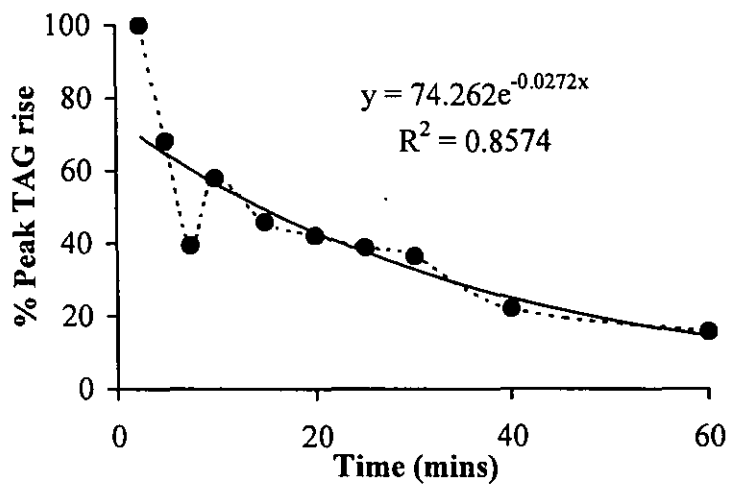
Subject 4, exercise trial: 25-minute TAG clearance curve (outlying points removed)



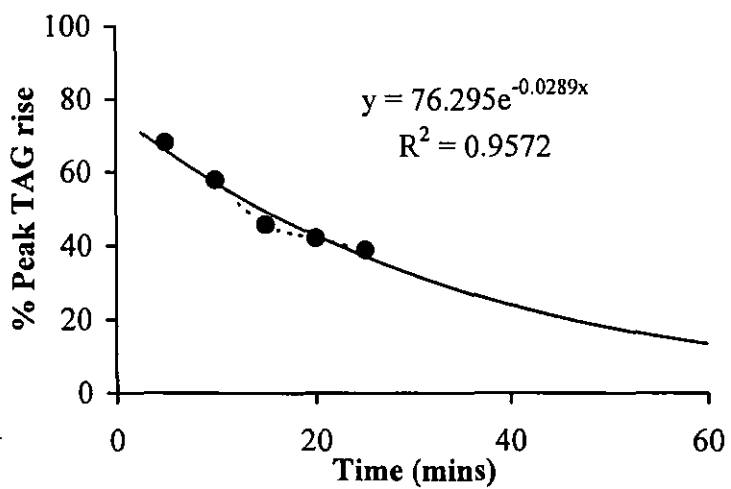
Subject 5, control trial: 60-minute TAG clearance curve



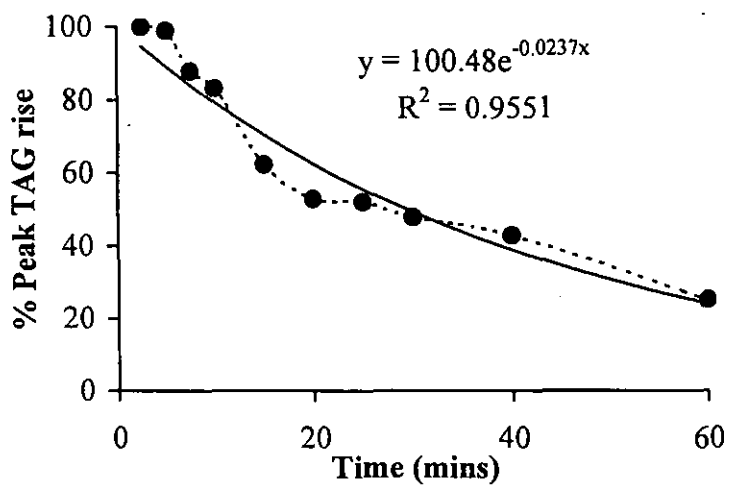
Subject 5, control trial: 25-minute TAG clearance curve (outlying points removed)



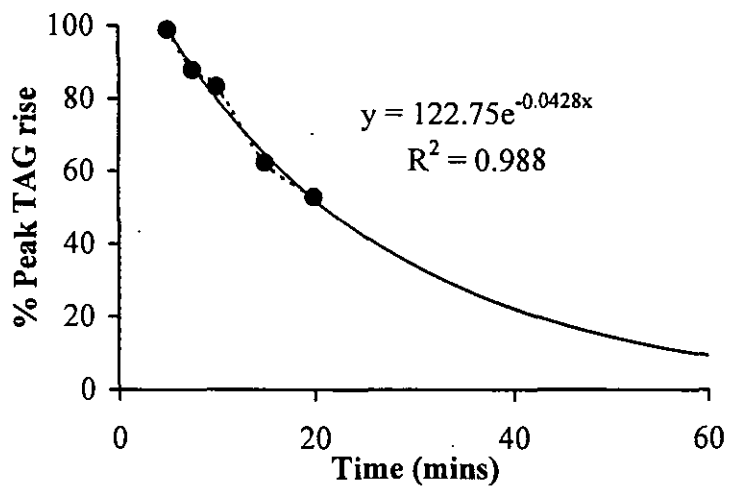
Subject 5, exercise trial: 60-minute TAG clearance curve



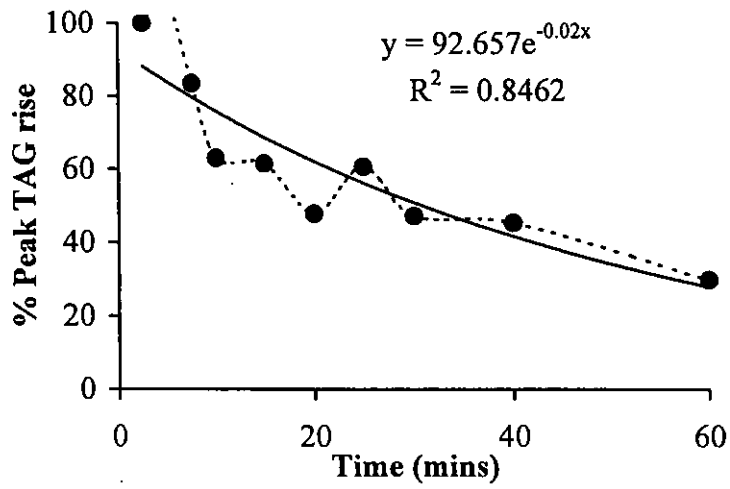
Subject 5, exercise trial: 25-minute TAG clearance curve (outlying points removed)



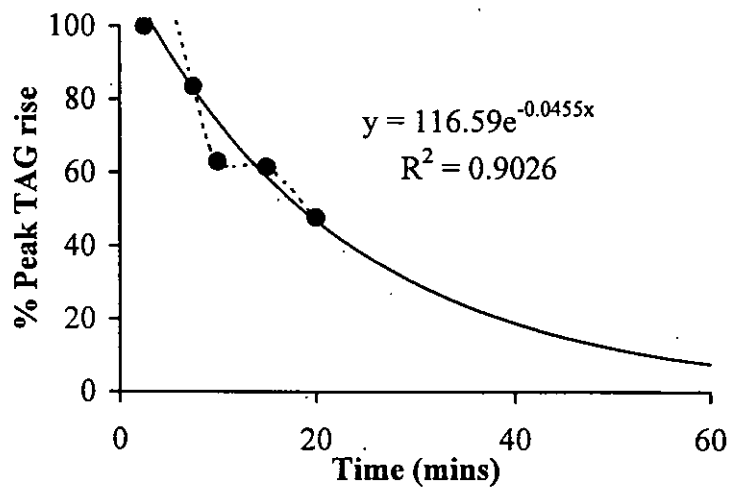
Subject 6, control trial: 60-minute TAG clearance curve



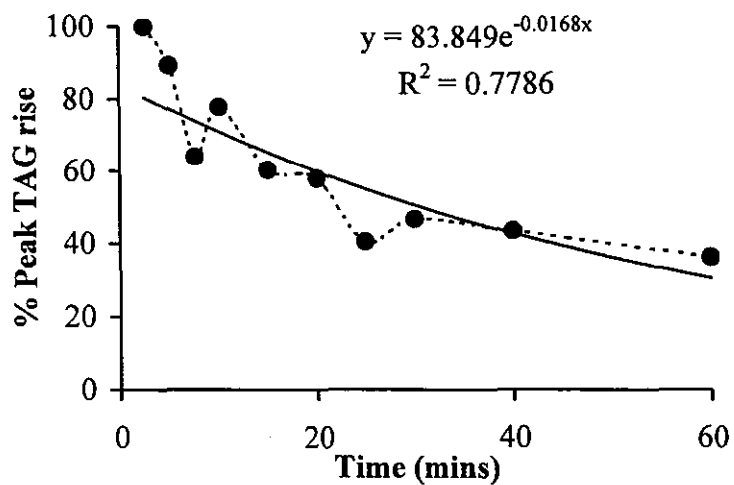
Subject 6, control trial: 20-minute TAG clearance curve (outlying points removed)



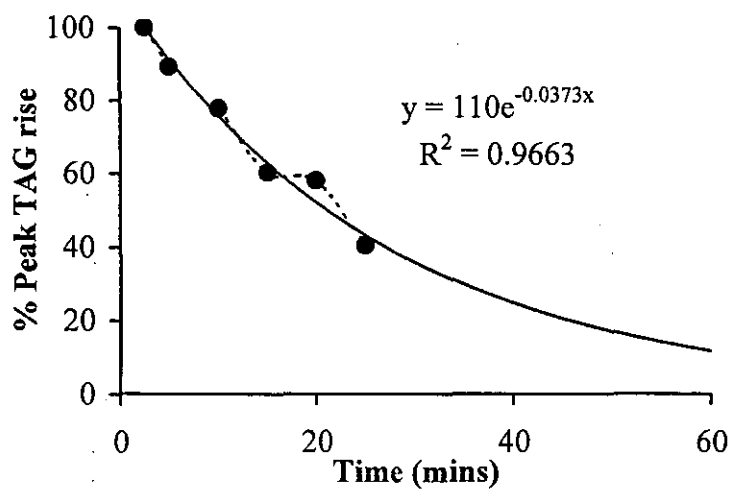
Subject 6, exercise trial: 60-minute TAG clearance curve



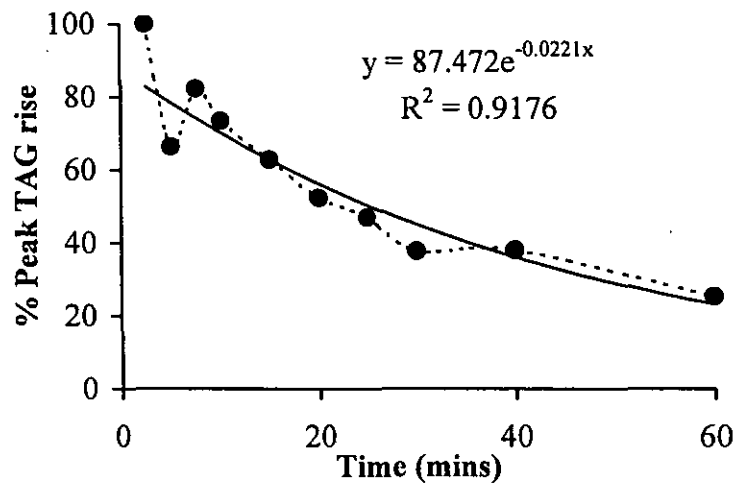
Subject 6, exercise trial: 20-minute TAG clearance curve (outlying points removed)



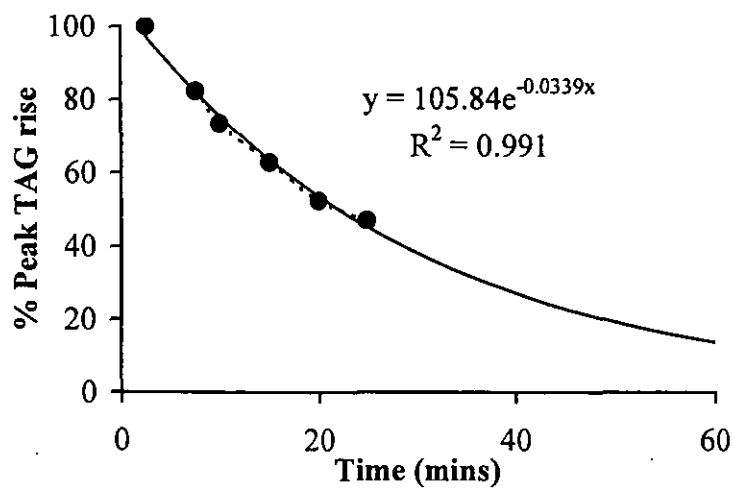
Subject 7, control trial: 60-minute TAG clearance curve



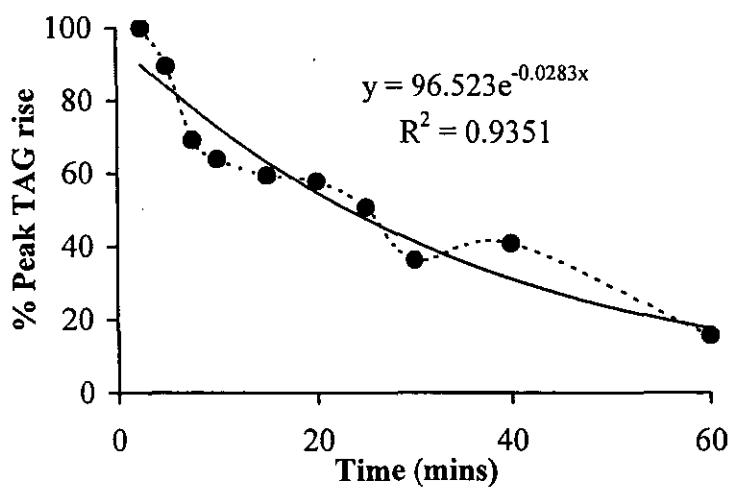
Subject 7, control trial: 25-minute TAG clearance curve (outlying points removed)



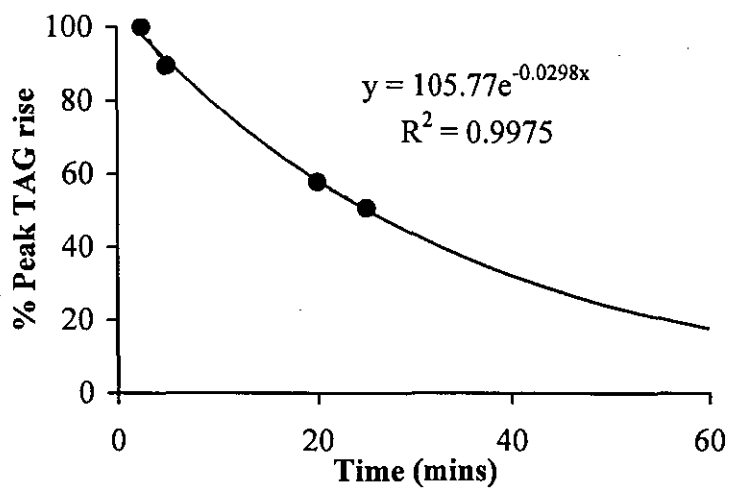
Subject 7, exercise trial: 60-minute TAG clearance curve



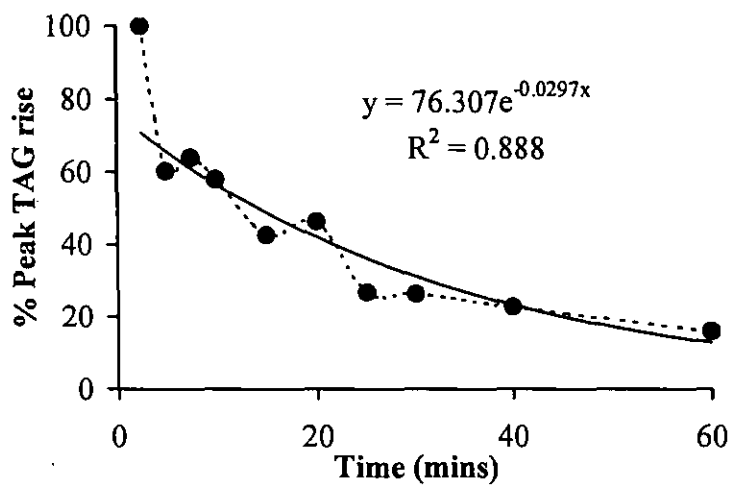
Subject 7, exercise trial: 25-minute TAG clearance curve (outlying points removed)



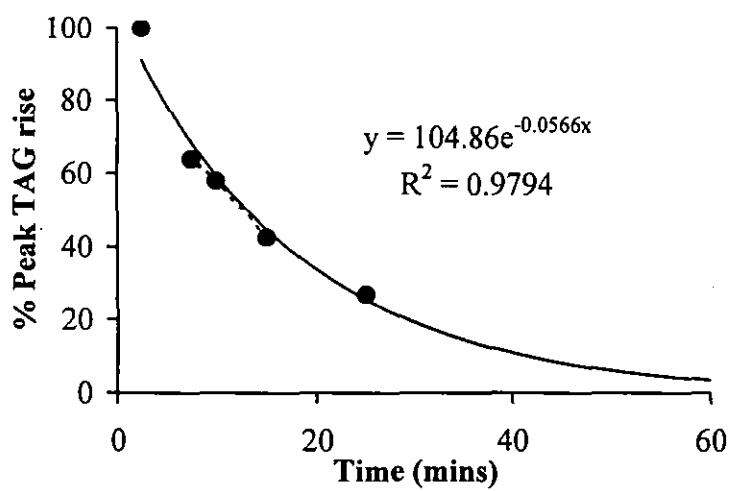
Subject 8, control trial: 60-minute TAG clearance curve



Subject 8, control trial: 25-minute TAG clearance curve (outlying points removed)



Subject 8, exercise trial: 60-minute TAG clearance curve



Subject 8, exercise trial: 25-minute TAG clearance curve (outlying points removed)

