The Regulation of Triglyceride Metabolism in the Liver and Adipose Tissue.

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Abstract.

The overall aim of this thesis is to understand better the control of triglyceride metabolism in both human and rat models.

Triglyceride metabolism is governed to a large part by the action of two enzymes, lipoprotein lipase (LPL) and hormone-sensitive lipase (HSL), whose activities are co-ordinately regulated by insulin. This thesis has two inter-related aims: firstly to investigate the effect of postprandial chylomicronaemia on the metabolism of VLDL subfractions in both normal and diabetic subjects and secondly to investigate the antilipolytic mechanism of action of several agents on HSL activity and adipocyte lipolysis in isolated primary rat adipocytes. Nicotinic acid was administered to Alderley Park (Wistar-derived) and Zucker rats to study its effects on plasma free fatty acid (FFA) levels *in vivo* with a view to using it as a model compound to influence the postprandial chylomicron-VLDL interaction in man.

Postprandial lipoprotein metabolism has been extensively studied in normals, hypertriglyceridaemics and diabetics using oral fat loads and measuring plasma triglyceride levels. It is only in recent years that immunoaffinity chromatography techniques have been developed and implemented by a few groups, with studies concentrating on normal and hypertriglyceridaemic subjects. Before the present human studies could begin an immunoaffinity chromatographic method using a monoclonal (LDL A4) antibody for the separation of triglyceride-rich lipoproteins (TRL) (chylomicrons and VLDL) had to be developed and tested to ensure it was operating under optimal conditions.

In normal subjects, mean plasma, apoB48, apoB100 and $VLDL_1$ triglyceride concentrations were significantly elevated *($p < 0.05$)* above the fasting concentration 1 to 8, 1 to 8, 2 to 5 and 2 to 4 hours respectively, postprandially. ApoB100 and $VLDL_1$ triglyceride concentrations decreased rapidly after 8 hours. $VLDL_2$ triglyceride concentrations did not change significantly during the course of the experiment. The increase in apoB48 TRL triglyceride was 5 times greater than the increase in apoB100 TRL triglyceride. Seventy nine percent of the postprandial increase in TRL ($d < 1.006$g/ml) triglyceride was due to apoB48 TRL, with apoB100 TRL accounting for 15%. The increase in $VLDL_1$ triglyceride was 11 times greater than the increase in $VLDL_2$ triglyceride. Seventy five percent of the postprandial increase in total apoB100 triglyceride was due to $VLDL_1$, with 10% accounted for by $VLDL_2$. Plasma FFA concentrations were significantly increased *($p < 0.05$)* above fasting levels 3 - 11 hours

postprandially with a consistent initial decrease in plasma FFA concentrations 1 - 2 hours after ingestion of the fat meal in virtually all subjects followed by the subsequent rise and a fall back towards baseline concentrations. Plasma insulin concentrations were significantly elevated above fasting levels 2 hours postprandially. This increase was significantly correlated with apoB100 and $VLDL_1$ areas under the curve and also with the changes in apoB100 and $VLDL_1$ triglyceride concentrations.

Twenty five diabetic subjects were screened and from those, 5 diabetic subjects agreed to take part in the fat feeding study. From the initial fasting blood sample obtained from the normal and diabetic patients during the screening procedure, plasma triglyceride, cholesterol and apoB concentrations were found to be significantly elevated *($p < 0.005$)* in the diabetic patient population.

In diabetic subjects, triglyceride response curves showed similar behaviour to those observed in normal subjects with the mean plasma, apoB48, apoB100, and $VLDL_1$ triglyceride concentrations significantly elevated *($p < 0.05$)* above fasting concentrations from 2 to 5 hours postprandially. $VLDL_2$ triglyceride concentrations remained unchanged and relatively constant. In the latter hours of lipaemia, differences were observed between diabetic and normal subjects, diabetic triglyceride response curves were elevated and prolonged. Diabetic mean apoB100 and $VLDL_1$ triglyceride response curves were significantly elevated above that of the normal subject group throughout the course of the experiment (0 - 11 hours postprandially). The increase in apoB48 TRL triglyceride was again almost 5 times greater than the increase in apoB100 TRL triglyceride. Eighty three percent of the postprandial increase in TRL ($d < 1.006$ g/ml) triglyceride was due to apoB48 TRL, apoB100 TRL accounted for 16%. The increase in $VLDL_1$ triglyceride was 12 times greater than the increase in $VLDL_2$ triglyceride. Seventy three percent of the postprandial increase in total apoB100 triglyceride was due to $VLDL_1$, with 13% accounted for by $VLDL_2$. Plasma insulin concentrations were significantly elevated above fasting concentrations 2 hours postprandially. Mean FFA concentrations were not increased significantly but there was a significant correlation between FFA area under the curve and apoB48 area under the curve; a relationship which failed to reach significance in the normal subjects.

In vitro rat adipocyte studies to elucidate the antilipolytic mechanism of action of a series of compounds on HSL showed insulin to be the most potent antilipolytic agent, nicotinic acid (and analogues) were found to be G_i receptor agonists (depending on adenosine removal to exert their antilipolytic effect), and compound 304205 was observed to be a direct inhibitor of HSL. Thiazolidinediones (TZD), fibrates and a $ß_3$

agonist had no inhibitory effects on HSL activity in the isolated rat adipocyte. Studies administering nicotinic acid to Zucker rats were conducted and allowed the *in vivo* antilipolytic effects of nicotinic acid, primarily on plasma FFA concentrations, to be observed.

This thesis extends the current knowledge of the regulation of triglyceride metabolism, namely the effects of postprandial chylomicronaemia on VLDL subfraction metabolism in normal and diabetic subjects, and on the antilipolytic mechanism of action of a series of compounds on adipocyte lipolysis. It demonstrates that the lipid and lipoprotein responses to a fat meal in normal and NIDDM subjects depends on the fasting triglyceride concentration and not necessarily the underlying diabetic state. *In vitro* adipocyte work targeting HSL showed that not even the best antilipolytic agent was as potent as insulin.

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iv *Author's Declaration.*

The work presented in this thesis was performed solely by the author, except where the assistance of others is acknowledged.

Lynne Mary Crawford,
(April 1998).

v *Dedication.*

This thesis is dedicated to my family for all their encouraging words, love and support over the past few years. To Morag for her sisterly support, my Dad for being the first oral fat load test subject and in particular to my mum, who without all her encouragement, constant reassurance and faith in my abilities, this thesis would not have been possible. Thankyou.

Chapter I Introduction.

1 The regulation of triglyceride metabolism.

1.1 An overview.

The regulation of triglyceride metabolism is complex. Transport of this lipid through the circulation involves the plasma lipoprotein system, while the adipose tissue, liver and muscle are the most important tissue sites of triglyceride storage and utilisation *(Coppack, Jensen and Miles 1994)*. The plasma lipoprotein system comprises 5 major groups: chylomicrons, VLDL, IDL, LDL, and HDL, which can be differentiated by size, density, and composition *(Alaupovic 1971)*. Two enzymes have a particular involvement in lipolysis, lipoprotein lipase (LPL) and hormone-sensitive lipase (HSL).

Lipolysis refers to processes in which triglyceride is hydrolysed, via di- and monoglyceride intermediates, to fatty acids and glycerol. Fatty acids (derived from the action of two different lipases; LPL and HSL) circulate in plasma bound to albumin *(Fredrickson, Levy and Lees 1967, Spector 1975 and Coppack et al 1994)*. Lipolysis occurs both intra- and extracellularly, and is an essential component of both lipid storage and mobilisation.

LPL *(Goldberg 1996)* is an extracellular enzyme associated with the luminal side of capillaries and arteries where it hydrolyses triglyceride in triglyceride-rich lipoproteins (TRL) to produce glycerol and free fatty acids (FFA). Fatty acids, from either dietary or endogenous lipoproteins (chylomicrons and VLDL respectively), can be released and delivered to the adipose tissue for storage or taken up by the muscle or liver for their metabolic requirements. LPL *(Eckel 1989)* is therefore of prime importance for the regulation of fat deposition in adipose tissue.

HSL is responsible for the mobilisation of fatty acids in adipose tissue which are then transported to the liver. It *(Yeaman 1990, Frayn et al 1993)* is an intracellular enzyme, which hydrolyses the adipose tissue stores of triglyceride and is the major source of FFAs used for fuel in the fasting state. The fatty acids liberated by lipolysis may also be re-esterified into newly synthesised triacylglycerol *(Arner 1990, Edens, Liebel and Hirsch 1990)*. Adipose tissue lipolysis is the major regulator of the body's supply of lipid energy because it controls the release of FFA into the plasma, (the rate limiting step of adipose tissue triglyceride lipolysis being HSL), which are transported back to the liver bound to albumin *(Spector 1975)*. Both LPL and HSL are under hormonal control, and are co-ordinately regulated by insulin. The hormone activates LPL activity and inhibits HSL activity *(Eckel 1989)*.

Triglyceride-rich lipoproteins (TRL) are a heterogeneous population of lipoprotein particles comprising chylomicrons and VLDL, which compete for a common lipolytic pathway *(Brunzell et al 1973)*, involving LPL. Chylomicrons are formed in the intestinal cells *(Green and Glickman 1981)* during fat absorption, and are released into the intestinal lymphatic system entering the bloodstream via the thoracic duct. They appear as a wave of particles following a fatty meal and are cleared rapidly from the bloodstream. Triglyceride-rich VLDL are secreted by the liver to provide tissues with a source of lipoprotein triglyceride in the absence of chylomicrons *i.e.* in fasting conditions. The regulation of hepatic VLDL production is primarily substrate driven, the most important regulatory substrate being fatty acids *(Lewis 1997)*. Hepatic fatty acids are derived from 4 sources (figure 1.1):

- *de novo* lipogenesis,
- cytoplasmic triglyceride stores,
- fatty acids derived from lipoproteins taken up directly by the liver or
- plasma free fatty acids.

In the postprandial state the plasma is flooded with chylomicrons. They are delipidated by the action of LPL (mediated by apolipoprotein CII as cofactor) and the remnants produced are cleared rapidly by the liver. Chylomicrons and VLDL compete for a common lipolytic pathway. Chylomicrons are the preferred substrate for LPL, therefore postprandially VLDL, mainly large $VLDL_1$, accumulates in the plasma as a result of increased synthesis and/ or decreased catabolism *(Karpe et al 1993)*, but the majority of the triglyceride increase is due to the presence of chylomicrons in the plasma. HDL exchanges neutral lipid with chylomicrons by the action of CETP. The longer chylomicrons reside in the plasma the more cholesterol enriched they become.

These cholesterol rich chylomicrons are believed to be highly atherogenic *(Packard and Shepherd 1990)*. Conversely, HDL becomes triglyceride rich. VLDL are secreted into the plasma by the liver in response to hormonal control of hepatic triglyceride synthesis, entering at the top of the delipidation cascade. They are sequentially delipidated first to IDL by LPL and then to LDL by hepatic lipase (HL). LDL are then cleared from the plasma by the LDL receptor, with binding mediated by apolipoprotein (apo) B on the particles surface.

The homoeostasis of triglyceride metabolism and the metabolism of lipoproteins between fasted and fed states is largely controlled by local factors such as lipid concentration, but also responds to more distant hormonal influences such as sex steroids and more importantly insulin.

Insulin is the major antilipolytic hormone *(Coppack et al 1994)* functioning, primarily in the postprandial state, to produce the following *(Frayn 1993)*:

- inhibition of HSL, thereby suppressing fatty acid release from adipocytes,
- decreasing hepatic VLDL production and
- increasing LPL activity in adipose tissue, so clearing triglyceride from the circulation.

Primary hyperlipidaemias are genetically determined while secondary hyperlipidaemias such as those seen in diabetes and obesity, account for approximately 40% of all hyperlipidaemias, with the lipid abnormalities being reversible on correction of the underlying condition. In insulin resistant states where the antilipolytic action of insulin is lost *(Reaven 1988, Garg 1996, and Lewis and Steiner 1996) e.g.* diabetes, obesity and coronary heart disease (CHD), increased hepatic triglyceride production and secretion of VLDL is observed, as a result of increased lipolysis in adipose tissue, and increased delivery of FFAs to the liver. Decreased clearance of TRL and low HDL levels result also from reduced LPL activity in these insulin resistant states *(Howard 1987, Bianchi and Erkelens 1994, Boden 1996)*. Thus the development of antilipolytic drugs, directed at selective inhibition of HSL and hereby improving insulin resistance and lowering fasting plasma triglyceride levels, are attractive targets.

1.2 Epidemiology of triglyceride and CHD.

Large intra-individual variation in triglyceride, relatively imprecise measurements, individual genetic susceptibility and the inverse correlation with HDL-cholesterol all play a role in masking the relationship between plasma triglyceride levels and CHD *(Lippel et al 1981, Austin 1991)*. In univariate analysis, fasting plasma triglyceride levels are associated significantly with the risk of coronary artery disease (CAD). However in multivariate analyses including other lipid parameters, this association is often not maintained, primarily because HDL cholesterol, by virtue of its strong inverse association with triglycerides eliminates triglyceride as a risk factor for CAD *(Hulley et al 1980, Austin 1991)*. The large variability of triglyceride measurements and the correlation of triglyceride values with other lipid measurements appears to result in the underestimation of the association between triglyceride and disease in multivariate analyses.

A relationship between postprandial lipaemia and coronary heart disease (CHD) was originally suggested in the 1950's. A recent study *(Patsch et al 1992)* reaffirmed this idea. It provided evidence that the postprandial triglyceride concentration is an independent predictor of CAD, finding that postprandial but not fasting triglyceride levels exhibited an association with CAD that was statistically independent and stronger than that of HDL cholesterol.

As humans spend most of their lives in a postprandial state, in order to to look at the effects of postprandial lipaemia on the progression of CAD, research should include postprandial studies of TRL metabolism in addition to the study of traditional fasting state levels of plasma risk factors *(Patsch et al 1992)*.

2 Lipids, lipoproteins and their metabolism.

2.1 Lipids *(Feher and Richmond 1991)*.

Three unique lipid families are found in the human body: cholesterol and its esters, triglycerides and phospholipids *(Fredrickson et al 1967)*. As a group they are water insoluble organic molecules characterised by the hydrocarbon nature of a major portion of their structure. Fatty acids (FA) are simple lipids classified into three groups according to the presence of double bonds in their hydrocarbon chain: saturated (no double bonds), monounsaturated (1 double bond) and polyunsaturated (2 or more double bonds). Fatty acids are an important energy source and an integral component of

complex lipids. FFA are the form in which fatty acids are transported from their storage site in adipose tissue to their sites of utilisation in the liver and muscle. Fatty acids are stored in the adipose tissue as triglyceride. The rate limiting step in the mobilisation of this triglyceride is HSL. Lipolysis (via the action of HSL) releases FFA and glycerol into the plasma, where the FFA circulate bound to albumin *(Spector 1975)*. Once in the plasma the FFA are taken up by liver and muscle and oxidised or re-esterified to triglyceride (figure 1.2).

Complex lipids are produced when fatty acids combine with molecules containing alcohol (-OH) groups to form esters. The major complex lipids in the plasma are cholesteryl esters and glyceryl esters (triglycerides and phospholipids).

2.1.1 Cholesteryl esters.

Cholesterol with its free alcohol group is relatively polar but becomes non-polar when esterified with fatty acids. The majority of plasma cholesterol (75%) is esterified with cholesteryl linoleate (43%) and cholesteryl oleate (24%) being the most abundant forms.

2.1.2 Glyceryl esters.

Triglycerides.

When all 3 hydroxyl groups of glycerol are esterified with fatty acids the molecule becomes non-polar and is called triacylglycerol (or triglyceride). Triglycerides (TG) are stored in adipose tissue and represent an energy source from which fatty acids can be released during periods of starvation.

Dietary triglycerides (exogenous) are lipolysed, absorped, and packaged into chylomicrons which transverse the intestinal lymphatics and enter the systemic circulation via the thoracic duct. Triglycerides derived from endogenous fatty acids also originate in the small intestine but their chief source is the liver where they are secreted in the form of VLDL.

Phospholipids.

They differ from triglycerides in that the terminal hydroxyl group of glycerol is esterified to phosphate containing molecules rather than fatty acids. Possession of a polar phosphate ester head group together with a non-polar hydrocarbon tail, enables phospholipids to play an important structural role in cell membranes and lipoprotein complexes.

2.1.3 Cholesterol. *(Grundy 1991)*

Cholesterol is an insoluble lipid containing a steroid ring nucleus. Cholesterol is structurally different from the other major lipids of the body, triglycerides and phospholipids, both of which have fatty acids as their major constituent. Cholesterol has several vital functions in the body: it is an essential component of cell membranes, important for stability and transmembrane transport functions. It also plays an important role in the transport of triglyceride in the plasma by being an essential component of plasma lipoproteins. It is the precursor of bile acids (required for fat absorption), adrenal steroids and sex hormones (oestrogens and androgens). Cholesterol entering the intestine originates from either bile or the diet, with about 50% being reabsorbed and returning to the liver while the remainder is excreted in stool.

Lipids (triglyceride, cholesterol, cholesterol ester and phospholipids) being insoluble in aqueous solution are transported in plasma in association with specialised proteins, apolipoproteins, in spherical lipoprotein complexes.

2.2 Lipoproteins: structure and function.

The basic structure of lipoproteins consists of a core of neutral lipids, consisting of cholesterol ester and triglycerides and a surface coat of more polar lipids (unesterified cholesterol and phospholipids) and apolipoproteins *(Grundy 1991)* (figure 1.3).

Plasma lipoproteins are routinely fractionated by ultracentrifugation into 5 major groups: chylomicrons, VLDL, IDL, LDL and HDL *(Alaupovic 1971)* which can be differentiated according to size, density (table 1.1) and composition (table 1.2). The main cholesterol carrying lipoproteins are LDL and HDL. In a normal individual LDL and HDL contain approximately 70% and 20% respectively, *(Thompson 1989)* of the total plasma cholesterol. The main triglyceride carrying lipoproteins are chylomicrons

and VLDL. Chylomicrons are not normally present in the blood after a 12 hour fast; in the fasting state VLDL accounts for 60% of the total plasma triglyceride.

Table 1.1: The density classes of plasma lipoproteins (Thompson 1989).

Lipoprotein class	*Sf* values or F values*	Density (g/ml)	Sources	Mean diameter (nm)
Chylomicron	>400	<0.95	Intestine	500
VLDL	20 - 400	<1.006	Liver	43
IDL	12 - 20	1.006 - 1.019	VLDL delipidation	27
LDL	0 - 12	1.019 - 1.063	VLDL delipidation	22
HDL	0 - 9*	1.063 - 1.21	Chylomicron and VLDL catabolism; intestine and liver	8

**Sf values are measured at a background solute density of 1.063g/ml: F values at a density of 1.21g/ml.*

Table 1.2: Composition of human plasma lipoproteins by weight (% of total).

Constituent	Chylomicron	VLDL	IDL	LDL	HDL
Protein	2	10	18	25	55
Triglyceride	85	50	26	10	4
Cholesterol	1	7	12	8	2
Cholesterol ester	3	13	22	37	15
Phospholipid	9	20	22	20	24

Table 1.3: Apoprotein distribution in human lipoprotein classes.

Chylomicrons	VLDL	IDL	LDL	HDL
B48	B100	B100	B100	AI
CI	CIII	B48*		AII
CII	E	E		D
CIII	CI			CI
E	CII			CII
AI	AI			E
AII	AII			

**Chylomicron remnants only.*

2.2.1 Chylomicrons.

Chylomicrons (*Sf* > *400*) are triglyceride-rich lipoprotein (TRL) particles formed in the intestine during lipid absorption *(Fredrickson et al 1967, Green & Glickman 1981)*. They are heterogeneous with a size range from 100 - 1000 nm *(Fraser 1970)*. Size is largely determined by the flux of triglyceride through the intestinal cell. Chylomicrons are small at the beginning and larger during the peak of lipid absorption. The major lipid is triglyceride, reflecting the role of chylomicrons in lipid absorption *(Zilversmit 1965, Fredrickson et al 1967)* (table 1.2). Several apolipoproteins are found in the surface coat (figure 1.4, table 1.3). The major structural apolipoprotein of chylomicrons is apoB48 (chylomicrons are referred to as apoB48 containing particles). Apolipoproteins of the A series (AI, AII, AIV) are also secreted on chylomicrons. As chylomicrons leave the thoracic duct and enter into the plasma they undergo rapid structural changes essential for subsequent metabolism, acquiring apoC and E primarily by transfer from HDL *(Patsch et al 1987)*. Chylomicrons are the form in which most of the dietary triglyceride is transported from its intestinal site of absorption into the systemic circulation. Peak chylomicronaemia normally occurs between 3 - 6 hours after ingestion of a fat rich meal and then generally declines with a return to near fasting levels at 8 hours post ingestion.

ApoCII, as a cofactor, *(Eckel 1989, Goldberg et al 1990)* activates LPL. The major sites of LPL activity in human subjects are adipose tissue and skeletal muscle, which are also the most important determinants for the removal rate of triglyceride-rich particles. LPL does not circulate freely in plasma but is attached to the surface of cells

where its action occurs *(Taskinen and Kuusi 1987)*. LPL hydrolyses the triglyceride core of the chylomicron and liberates FFA and glycerol for utilisation by these tissues. As chylomicron delipidation proceeds much of the surface coat becomes redundant, with loss of part of the surface coat to HDL. The particle shrinks to a so-called 'remnant' while selectively retaining all of its apoB and the majority of the apoE. The LDL receptor binding site is absent from apoB48 but apoE is able to recognise and bind specific receptors on hepatocyte membranes, triggering remnant uptake and delivery of dietary cholesterol to the liver *(Mahley et al 1981, Sherrill 1980)* (figure 1.5).

2.2.2 Very low density lipoprotein (VLDL).

In the fasting state, VLDL is the main carrier of triglyceride *(Sata, Havel and Jones 1972)*. In contrast to the pattern of intermittent chylomicron production by the intestine, VLDL secretion by the liver is continuous. Both particle types share many metabolic characteristics. VLDL *(Sf 60 - 400)* has a size range from 25 - 100 nm *(Thompson 1989)*. The major structural apolipoprotein is apoB100 (hence they are referred to as apoB100 containing lipoproteins) (figure 1.6, table 1.3), but apoC & E are also present, acquired as for chylomicrons, primarily by transfer from HDL. VLDL are similar to chylomicrons, in that they are TRL but they have two important differences. Firstly, the triglyceride transported by VLDL is of endogenous origin *(Havel 1961)*, and secondly, the major apolipoprotein is apoB100 rather than apoB48. The major lipid component of the non-polar core of VLDL is triglyceride but cholesterol ester is also present *(Sata et al 1972)*. The surface coat contains unesterified cholesterol and phospholipids. Hepatic VLDL production is primarily substrate driven, the most important regulatory substrate being fatty acids *(Lewis 1997)*. Fatty acids can be derived from either *de novo* synthesis in the liver, cytoplasmic triglyceride stores, fatty acids derived from lipoproteins taken up directly by the liver, or from exogenous fatty acids (plasma free fatty acids) (figure 1.1).

VLDL is composed of a heterogeneous population of particles *(Streja, Kallai and Steiner 1977)* which can be usefully divided into 2 major subfractions with distinct metabolic properties *i.e.* larger, triglyceride-rich $VLDL_1$ *(Sf 60 - 400)* and a smaller relatively cholesterol-enriched $VLDL_2$ *(Sf 20 - 60) (Shepherd & Packard 1987)*. Both $VLDL_1$ and $VLDL_2$ can be synthesised directly by the liver and, in addition, $VLDL_2$ are formed by the delipidation of $VLDL_1$. It appears that smaller $VLDL_2$ particles continue through the lipolytic cascade to IDL, while large $VLDL_1$ remnants are more likely to be cleared directly from the circulation *(Packard et al 1984)*. Following hepatic synthesis the VLDL are released into the systemic circulation where they acquire

apoC and E before undergoing a stepwise delipidation by LPL (in a similar manner to chylomicrons). As the triglyceride core is hydrolysed, the particle becomes smaller forming a remnant particle or IDL. This is then delipidated further to LDL by hepatic lipase or cleared by the LDL receptor (figure 1.7).

2.2.3 Intermediate density lipoprotein (IDL).

Intermediate density lipoproteins (IDL) are formed in an intermediate step in the conversion of VLDL to LDL. The plasma concentration of IDL is usually low (one tenth that of LDL) due to the rapid turnover rate of these particles. IDL are smaller, denser (1.006 - 1.019g/ml) and more cholesteryl ester enriched than VLDL with the main protein component being apoB100 (table 1.3). As with VLDL, IDL can be further divided into several subfractions *(Musliner, Giotas and Krauss 1986)*.

2.2.4 Low density lipoprotein (LDL).

Low density lipoprotein (LDL) is the major cholesterol carrying lipoprotein of normal plasma. LDL consists of a lipid core composed almost entirely of cholesteryl esters, and is formed by the delipidation of IDL. The only protein is apoB100 (table 1.3). LDL is cleared from the plasma by receptor uptake and by non-receptor pathways. LDL can be fractionated into several subfractions varying in size, density and composition *(Shen et al 1981, Krauss & Burke 1982)* with small, dense LDL being particularly atherogenic.

2.2.5 High density lipoprotein (HDL) *(Thompson 1989)*.

High density lipoproteins (HDL) are the most abundant lipoproteins in particle number, in the plasma, isolated at a density of 1.063 - 1.21g/ml. HDL is subdivided into HDL_2 (1.063 - 1.125g/ml) and HDL_3 (1.125 - 1.21g/ml). They are synthesised in both the liver and small intestine. Over 90% of the protein on HDL is apoAI, and apoAII (table 1.3). The surface coat of HDL also contains apoC's and E which can be readily transferred to TRLs (chylomicrons and VLDL). HDL acts as a reservoir for apo C derived from chylomicrons and VLDL during lipolysis *(Patsch et al 1987)*, the apoC being picked up by chylomicrons entering the blood via the thoracic duct during alimentary lipaemia.

2.2.6 Lipoprotein (a).

Lipoprotein (a), (Lp(a)), is a relatively recently discovered lipoprotein whose physiological function is unclear. It is linked not only to coronary artery disease but also to stenosis of the carotid and cerebral arteries. Lp(a) has both atherogenic and thrombogenic potential (interference in several reactions involving plasminogen due to its lysine binding capability). Lp(a) is structurally related to LDL; both have 1 molecule of apoB100 per particle and similar lipid compositions. The distinctive feature of Lp(a) is that it contains a second polypeptide chain, disulphide linked to apoB100 and referred to as apolipoprotein (a). Lp(a) particles are spherical in shape, with an average diameter of 21 nm and a density pattern consistent with a low density core surrounded by a high density shell. Apo(a) is synthesised by the liver and has a high degree of sequence homology with plasminogen. It is a highly glycosylated, hydrophilic protein, composed of a kringle-containing domain and a serine protease domain, that has very little affinity for lipids. Lp(a) is recognised by the LDL receptor, although its affinity is considerably lower than that of LDL *(Marcovina & Morrisett 1995)*. As a consequence, little, if any, is metabolised by the LDL receptor *in vivo*.

2.3 Apolipoproteins.

Alaupovic *(1971)* was responsible for the classification and introduction of the alphabetical nomenclature that is now widely used for apolipoproteins. Lipoproteins cannot be synthesised and secreted from the liver or intestine without the corresponding structural apolipoprotein. In addition to their structural role in lipoprotein metabolism, apolipoproteins play a dynamic role as enzyme activators and receptor ligands. The apolipoproteins serve several important functions *(Thompson 1989, Grundy 1991, Mahley et al 1984)*. They are required for secretion of lipoproteins, stabilisation of the surface coat and hence maintain the structure of the whole lipoprotein transport system (table 1.4). They also serve as cofactors for activation of enzymes that modify lipoproteins (table 1.5) and finally they interact with specific cell-surface receptors that remove lipoproteins from the circulation thus determining the sites of uptake and rates of degradation (apoB100 and E mediate interactions of lipoproteins with receptors). There are 4 major classes of apolipoproteins A, B, C and E (table 1.6).

Table 1.4: The structural apolipoproteins (Grundy 1991).

Apolipoprotein	Lipoprotein	Source
AI	HDL	Liver, Intestine
B100	VLDL, IDL, LDL	Liver
B48	Chylomicrons	Intestine

Table 1.5: Apolipoproteins involved in enzyme activation.

Apolipoprotein	Lipoprotein	Enzyme
AI	HDL_3, nascent HDL	Lecithin cholesterol acyl-transferase (LCAT)
CII	Chylomicrons, VLDL	Lipoprotein lipase

Table 1.6: Human apolipoprotein classification.

Apolipoprotein	Molecular weight (kDa)	Lipoprotein association	Source	Plasma concentration (mg/dl)
B48	264	Chylomicrons	Intestine	-
B100	550	VLDL, IDL, LDL	Liver	80 - 100
AI	28	HDL, Chylomicrons	Intestine, Liver	100 - 150
AII	17	HDL, Chylomicrons	Intestine, Liver	30 - 40
AIV	46	HDL, Chylomicrons	Intestine	15
CI	5.8	Chylomicrons, VLDL, IDL, HDL	Liver	6
CII	9.1	Chylomicrons, VLDL, IDL, HDL	Liver	4
CIII	8.7	Chylomicrons, VLDL, IDL, HDL	Liver	12
E's	35	Chylomicrons, VLDL, IDL, HDL	Liver, Peripheral tissues	3 - 7

2.3.1 Apolipoprotein A.

The A apolipoproteins consist of 3 main proteins; apoAI, AII and AIV (table 1.6). ApoAI circulates in the plasma primarily as a component of HDL but it is also present on chylomicrons (being rapidly transferred to HDL during lipase-mediated hydrolysis of chylomicrons). The protein has two major sites of synthesis; the intestine and the liver. Since apoAI binds lipid and is the major protein constituent of HDL it is clear that it must be an important structural component of lipoproteins. Its other major function is to serve as a cofactor for lecithin cholesterol acyl-transferase (LCAT). ApoAII occurs primarily as the second most abundant protein component of HDL. It is a dimer with its major site of synthesis being the liver. It activates LCAT and appears to enhance the lipid binding properties of apoAI. ApoAIV is present on newly secreted chylomicrons, although the majority of apoAIV is found free in the plasma.

2.3.2 Apolipoprotein B.

Apolipoprotein B is the primary apolipoprotein of chylomicrons, VLDL, IDL, and LDL. It is heterogeneous and exists in 2 forms; apoB48 and apoB100 (table 1.6). ApoB100 is synthesised by the liver and is found on VLDL, IDL and LDL. ApoB100 is the protein determinant on LDL that recognises the LDL (apoB/ E) receptor. ApoB48 is synthesised by the intestine and secreted on chylomicrons. It is identical to the amino terminal 48% of apoB100 (hence the nomenclature), being formed by post-translational modification of apoB100 mRNA *(Scott 1990)*. The metabolism of apoB48 and apoB100 is different. In man apoB100 is produced by the liver *(Edge et al 1985)*, secreted on a nascent particle of VLDL and transferred with the particle to IDL and finally LDL. ApoB48 is produced by the intestine and secreted on a chylomicron particle. The triglyceride in chylomicrons is partially hydrolysed and the resulting remnants are taken up by the liver. In the rat, apoB48 and apoB100 are both synthesised in the liver with apoB48 found on both chylomicrons and VLDL.

2.3.3 Apolipoprotein C.

The C apolipoproteins are 3 low molecular weight apolipoproteins; CI, CII and CIII (table 1.6) that are surface components of chylomicrons, VLDL and HDL. The C apolipoproteins appear to be diverse in their metabolic functions but they share a common property of redistributing among lipoprotein classes (like apoE and apoAI, II and IV). The liver appears to be the major site of apoC protein synthesis. In the fasting state C apolipoproteins are mainly associated with HDL. During absorption of dietary

fat by the intestine and chylomicron production or during active VLDL synthesis by the liver, the C apolipoproteins redistribute to the surface of the triglyceride-rich chylomicrons and VLDL. In reverse, as the triglyceride core of chylomicrons and VLDL is hydrolysed and depleted by the action of LPL, C apolipoproteins are transferred back to HDL *(Patsch et al 1987)*. ApoCI is the smallest of the three and acts as an activator of LCAT. ApoCII is a cofactor of LPL *(Goldberg et al 1990, Eckel 1989)*, while apoCIII has an inhibitory effect on the hydrolysis and clearance of chylomicrons and VLDL *(Thompson 1989)*. CIII exists in 3 forms $CIII_0$, $CIII_1$ and $CIII_2$ depending on the number of sialic acid residues that are present.

2.3.4 Apolipoprotein E.

Apolipoprotein E is a constituent of chylomicrons, chylomicron remnants, VLDL and HDL (table 1.6). Its major site of synthesis is the liver. ApoE functions as a ligand for the hepatic uptake of chylomicron remnants and VLDL. Isoelectric focussing reveals three common apoE isoforms; E2, E3, and E4 *(Davignon, Gregg and Sing 1988)*. The 3 isoforms differ by an amino acid residue at one or both sites (residues 112 and 158) on the 299 amino acid chain of the mature apoE molecule.

The six common phenotypes revealed by isoelectric focussing, are homozygotes for E4/4, E3/3 and E2/2, the most common phenotype in the human population being E3/3 (present in approximately 60% of the population) and heterozygotes for E4/3, E4/2, E3/2. The different isoforms have varying effects on plasma lipoprotein metabolism, in particular on the metabolism of LDL precursors.

2.4 Enzymes involved in lipoprotein metabolism.

Lipoprotein metabolism is regulated by various enzymes.

2.4.1 Lipoprotein lipase.

Lipoprotein lipase (LPL), an extracellular lipase *(Olivecrona et al 1993)*, functions to hydrolyse triglycerides in TRLs (both chylomicrons and VLDL) *(Eckel 1989, Goldberg 1996)* circulating in the blood and requires apoCII as a cofactor *(Goldberg et al 1990)*. Fatty acids can thus be released from either dietary or endogenous lipoproteins and delivered to adipose tissue for storage or to skeletal muscle and the liver for their metabolic requirements (figure 1.2). The dominant tissues in LPL production are the muscle and adipose tissues, where it is attached by heparan sulphate

proteoglycans to the endothelial lining of the capillaries perfusing these tissues. LPL *(Nilsson-Ehle, Tornquist & Belfrage 1972)* is of prime importance for the regulation of fat deposition in adipose tissue. Its activity *(Braun & Severson 1992)* can be altered in a tissue specific manner, which is important because it directs fatty acid utilisation according to the metabolic demand of the individual tissues *i.e.* the degradation of TRL can be targeted to specific sites. Multiple factors seem to be involved in the regulation of LPL activity. LPL is bound to the endothelial surface where it acts, and its binding appears to be weakened by the fatty acids released by the enzymatic hydrolysis of triglyceride *(Saxena, Witte and Goldberg 1989, Olivecrona et al 1993, Coniglio 1994)*, thus feedback control of LPL by released fatty acids may be one factor involved in LPL regulation.

On fasting and feeding *(Doolittle et al 1990)*, LPL activity alters in adipose tissue compared to muscle and heart tissue. In the latter state, LPL activity increases in adipose tissue compared to heart and muscle, resulting in a channelling of circulating triglyceride-fatty acids into lipid depots. However during fasting the inverse is true. Thus the co-ordinated regulation *(Sugden, Holness and Howard 1993)* of LPL in adipose tissue and muscle during fasting and feeding is critical for maintaining triglyceride levels. LPL is bound to the vascular endothelial cell surface via attachment to heparan sulphate proteoglycans *(Goldberg et al 1990)* which provide high affinity binding sites on the endothelium for LPL *(Cisar et al 1989)*. LPL is thought to mediate binding of lipoproteins *(Eisenberg et al 1992)* to the heparan sulphate proteoglycans on the cell surface. This interaction brings the lipoproteins into close proximity with the cell surfaces and may promote metabolic events that occur at the cell surface. LPL can be released from the endothelial cell surface and removed from the circulation *(Saxena et al 1989)* as part of an LPL remnant particle complex *(Vilella et al 1993)*. Active LPL is a non-covalent homodimer *(Vilella et al 1993)* which, when converted to the monomeric form, loses its catalytic activity. Once bound to the endothelial cells, LPL activity must be modulated by cellular uptake or release of LPL protein from the cell surface into the circulation *(Cisar et al 1989)*.

Studies *(Olivecrona et al 1993)* suggested that adipocytes contain inactive forms of LPL which can be recruited and released into the medium as active LPL by incubating cells with appropriate signals *e.g.* insulin, under conditions such that synthesis of the new enzyme molecules was blocked, concluding that insulin increases LPL active secretion. It appears that newly synthesised LPL is either released rapidly or degraded within the cells. The release of LPL from the cell surface of the adipocytes *(Cisar et al 1989)* prevents its degradation. Binding of LPL to the cell surface can be

eliminated by two treatments either addition of heparin or removal of heparan-sulphate with endoglycosidases, both treatments preventing LPL degradation. Cells appear to produce LPL in excess so that the tissue is able to respond quickly to meals and during food deprivation most of the newly synthesised LPL is degraded. LPL activity may also be regulated post-translationally *(Ashby et al 1978)* and these mechanisms appear to be involved in the acute changes of LPL activity in adipose tissue. Activation of LPL is an intracellular event. It seems enzyme activity can be either protein synthesis dependent (synthesis of LPL, control at a pre-translational mRNA level) or protein synthesis independent (post-translational).

2.4.2 Hormone-sensitive lipase.

Hormone-sensitive lipase (HSL) catalyses the mobilisation of fatty acids from adipose tissue *(Fredrickson et al 1981, Yeaman 1990, Frayn et al 1993 and Yeaman et al 1994)*, thereby generating substrate for hepatic VLDL-triacylglycerol synthesis *(Lewis 1997)*. HSL catalyses the rate limiting step in the breakdown of triglyceride and its activity is regulated in the short term via reversible phosphorylation, involving a signal transduction pathway. HSL is phosphorylated and activated in response to a variety of lipolytic hormones *(Yeaman 1990)* with insulin *(Egan et al 1990)* exerting a major antilipolytic action. HSL activity is under both neural and hormonal control and stimulatory or inhibitory agents exert their effects via the adenylate cyclase-A-kinase signal transduction pathway (figure 1.8). After fasting (prolonged) HSL activity in adipose tissue increases *(Sztalryd and Kraemer 1994 a)*.

The stimulatory/ inhibitory compounds bind to receptors on the adipocyte cell membrane. These receptors are coupled to either stimulatory (G_S) or inhibitory (G_i) G proteins which in turn activate/ inhibit adenylate cyclase. Adenylate cyclase activation is reflected in intracellular levels of cyclic adenosine monophosphate (cAMP), the second messenger which activates cAMP protein dependant kinase A *(Stralfors and Belfrage 1983)* which in turn catalyses the phosphorylation and resulting activation of HSL at the regulatory site (serine 563). HSL phosphorylation is paralleled by an enhanced triglyceride lipase activity *(Fredrickson et al 1981)*, while the antilipolytic effect of insulin *(Yeaman 1990)* involves the dephosphorylation of HSL. Two serine residues are phosphorylated on HSL, cAMP-dependent protein kinase acts at the regulatory site (serine 563) of HSL. The basal site (serine 565) has no apparent direct effect on the enzyme activity but inhibits subsequent phosphorylation of the 'regulatory site'. The basal site is phosphorylated by adenosine monophosphate (5'AMP) activated protein kinase *(Garton and Yeaman 1990, Hardie 1992)* under basal conditions of

lipolysis *(Okuda, Morimoto and Tsujita 1994)*. These two phosphorylations on the regulatory (serine 563) and basal (serine 565) sites are mutually exclusive *(Garton et al 1989)* meaning that phosphorylation by AMP kinase on the basal site would prevent further phosphorylation on the regulatory site and HSL activation.

The dephosphorylation and inactivation of HSL in adipocytes in response to insulin has been proposed to involve the action of protein phosphatases *(Wood et al 1993)* upon HSL. Protein phosphatases (PP2A and 2C) were the only protein phosphatases to display activity towards HSL phosphorylated at the regulatory site. PP1, PP2A and PP2C were detected within rat adipocytes at significant levels.

Rat HSL preferentially releases polyunsaturated FA from triglyceride *(Gavino and Gavino 1992)*. Rat HSL structure *(Fredrickson et al 1981, Smith et al 1993)* has been elucidated *(Gabriele et al 1993)*. It is an 84 kDa polypeptide of 768 amino acids, with an active site at serine 423.

2.4.3 Hepatic lipase.

Hepatic lipase (HL) is involved in the catabolism of chylomicron and VLDL remnants (IDL). Hepatic lipase hydrolyses triglyceride and phospholipids in HDL_2 and in doing so mediates the conversion of HDL_2 to HDL_3. Additionally, hepatic lipase delipidates IDL to form LDL and is thought to be involved in the interconversion of LDL subclasses. Hepatic lipase mRNA is detected in the liver only. Unlike LPL, this enzyme does not require a cofactor.

2.4.4 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMG Co A-reductase) *(Feher and Richmond 1991)*.

Most of the cholesterol present in tissues is either absorbed from the diet or generated by *de novo* synthesis which occurs in many tissues. Cholesterol is derived from acetate, with the rate limiting step in the sequence catalysed by HMG CoA-reductase; this enzyme is suppressed by cholesterol, the end product, via feedback inhibition.

2.4.5 Lecithin cholesterol acyl-transferase (LCAT). *(Glomset & Verdery 1977)*

LCAT is synthesised in the liver and circulates in plasma. The enzyme requires apoAI as cofactor, and esterifies free cholesterol acquired by HDL_2 and HDL_3 particles by transfer of a fatty acid from lecithin. The esterified cholesterol is transferred to the core of the particle, forming mature, cholesteryl ester rich HDL_2.

2.4.6 Cholesterol ester transfer protein (CETP).

CETP transfers a proportion of cholesteryl esters from mature HDL_2 to TRL particles. Triglyceride is transferred by the same protein simultaneously in the reverse direction to modify HDL_2. Both CETP and LCAT are involved in reverse cholesterol transport.

2.5 Receptors *(Feher and Richmond 1991).*

2.5.1 The LDL (apoB/ E) receptor.

The LDL receptor recognises as ligands both apoB100 and apoE, and will thus bind LDL and VLDL remnants (IDL). The role of the LDL receptor is to provide cholesterol to cells throughout the body and to deliver excess cholesterol to the liver for recycling or excretion as bile acids. Receptors are synthesised in response to a fall in cellular free cholesterol concentration.

2.5.2 The LDL receptor-related protein (LRP).

LRP in its function as a lipoprotein receptor, appears to bind mainly chylomicron remnants and ß-VLDL, through apoE and lipases; LPL and HL, which mediate binding of chylomicrons to LRP. The liver expresses high levels of LRP; appropriate for the main site of chylomicron remnant catabolism *(Beisiegel 1995)*. Expression of LRP in other tissues might indicate an additional non-hepatic removal of TRLs. LRP in macrophages and smooth muscle cells might be important in the clearance of lipoproteins in the arterial wall, thus contributing to the development of atherosclerotic plaques *(Lupu et al 1994)*.

2.5.3 The VLDL receptor *(Beisiegel 1995).*

The VLDL receptor is most abundant in muscle and adipose tissue, suggesting that it may be involved in delivering endogenous triglycerides carried in VLDL or IDL from the liver to sites of fatty acid metabolism. As a result the VLDL receptor might play a major role in VLDL catabolism.

3 Postprandial lipoprotein metabolism in normal subjects.

Plasma triglyceride is gaining increased recognition as a risk factor for CHD, although the mechanisms underlying this association have not been settled *(Austin 1991).* Lipid and lipoprotein studies have traditionally been confined to the post-absorptive (overnight fasting) state, but interest in analysing lipoproteins in the postprandial state has increased since it has been demonstrated that triglycerides in the postprandial state can serve as an independent predictor/ risk factor for CHD *(Patsch et al 1983, Patsch et al 1992).* It was *Zilversmit (1979)* who first hypothesised that postprandial lipoproteins (chylomicrons and their remnants) *(Zilversmit 1995)* were important in atherogenesis in humans. Both chylomicron and VLDL levels have been shown to increase after a fat intake *(Cohn et al 1988 b, Karpe et al 1993 and Schneeman et al 1993).* The increment in TRL particle number is mainly accounted for by VLDL *(Schneeman et al 1993),* whereas 80% of postprandial triglyceride is carried by lipoprotein particles containing apoB48 (chylomicrons).

Karpe and Hultin (1995) reaffirmed in rats that accumulation of endogenous VLDL in the plasma was due to competition (for a common lipolytic pathway) between chylomicrons and VLDL *(Brunzell et al 1973),* and not due to increased synthesis of VLDL. As a result of delayed clearance of VLDL, the presence of chylomicrons and their remnants may lead to increased risk of atherogenesis. When considering the direct atherogenic potential of TRLs the question as to whether they can enter the artery wall is of importance, with lipoprotein size an important determinant of arterial wall flux. *Rapp et al (1994)* showed that VLDL and remnants can indeed cross the endothelial wall, but these apoB containing lipoproteins differed in size from plasma lipoproteins. Thus the postprandial state has been postulated to be atherogenic on the basis that intestinal chylomicrons and their remnants have the potential to cause lipid deposition in the cells of the artery wall *(Zilversmit 1979).* There is evidence that apoE is involved in the metabolism of TRLs. It is a key protein in the modulation of the metabolism of the highly atherogenic apoB containing lipoproteins. The findings of *Brown and Roberts (1991)* suggested that controlling for apoE phenotype may help to decrease inter-

individual variation in the postprandial response to a fat meal. Possible genetic variation in apoE could be a major component in the accumulation of chylomicron and VLDL remnants related to triglyceride metabolism and is associated with increased risk of atherosclerosis *(Davignon et al 1988)*.

4 Diabetes mellitus and triglyceride metabolism.

The current classification and diagnosis of diabetes was developed by the National Diabetes Data Group (NDDG) and published in 1979, which recognised 2 major forms of diabetes, termed insulin-dependent (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM), now termed type 1 and type 2 diabetes respectively. It is considered to be important to move away from a system that appears to base its classification of the disease, in large part, on the type of pharmacological treatment used in its management toward a system based on the disease etiology where possible. The vast majority of cases of diabetes fall into 2 broad categories. In one category (type 1 diabetes), the cause is an absolute deficiency of insulin secretion resulting from a cellular-mediated autoimmune destruction of the ß-cells of the pancreas. In the other, (type 2 diabetes), the cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response (relative insulin deficiency). The current expert committee (1997) have now proposed changes and modifications to the NDDG diagnosis and classification scheme (Report Committee 1997). The revised criteria for the diagnosis of diabetes mellitus are a casual plasma glucose (PG) (casual is defined as any time of the day without regard to time from last meal) concentration $\geq$ 200 mg/dl (11.1 mmol/L, or a fasting (fasting defined as no calorie intake for 8 hours) PG $\geq$ 126 mg/dl (7.0 mmol/L) or a 2 hour PG $\geq$200mg/dl during an OGTT (oral glucose tolerance test). This new criteria would not alter the status of the diabetic patients recruited here (appendix 35).

4.1 Insulin resistance.

Insulin is the major antilipolytic hormone *(Coppack et al 1994)* functioning primarily in the postprandial state. The normal effects of insulin on lipid metabolism are *(Frayn 1993)*: inactivation of HSL resulting in suppression of NEFA release from adipose tissue, suppression of hepatic VLDL triglyceride secretion and activation of LPL in adipose tissue to clear triglyceride from the circulation.

In states of insulin resistance (NIDDM, obesity), described by *Reaven (1988)* subjects have impaired glucose tolerance and the antilipolytic effects of insulin are

diminished, with excess circulating fatty acids released from adipose tissue resulting in increased hepatic triglyceride-rich VLDL synthesis and with reduced LPL activity impeding the removal of triglyceride from the circulation. Insulin is overproduced by the pancreas to try to compensate for the insulin resistance.

4.2 Non insulin dependent diabetes mellitus (NIDDM) (Type 2 diabetes).

Individuals with NIDDM are insulin resistant and often have high concentrations of insulin, both fasting and postprandially. FFA are also elevated in these individuals, although it is not certain if this is as a result of their insulin resistance or a cause of it *(Boden 1996)*. Individuals with NIDDM are not dependent on insulin therapy, allowing it to be possible to study lipoprotein metabolism in NIDDM in the absence of hormone therapy.

4.3 Lipid and lipoprotein abnormalities in Type 2 diabetes (NIDDM).

The basic dyslipidaemia that accompanies diabetes was described several years ago *(Howard 1987)*. Recent work has focussed on abnormalities in lipoprotein alterations, composition and apolipoprotein content and the metabolic mechanisms that govern these changes. In states of insulin resistance (NIDDM); first described by *Reaven (1988)* the antilipolytic effects of insulin are diminished *(Frayn 1993)*. Indeed the most common abnormality of lipid metabolism in patients with diabetes is hypertriglyceridaemia with an elevation in VLDL *(Laws 1996)*, accompanied by a decrease in HDL cholesterol concentrations. Abnormalities in both production and clearance of VLDL triglyceride have been reported in NIDDM. Several studies *(Abrams, Ginsberg and Grundy 1982, Kissebah et al 1982, Reaven 1987)* have reported an over-production of VLDL triglyceride. Defective clearance of VLDL triglyceride, with decreased fractional catabolic rates for triglyceride is seen in more severe hypertriglyceridaemia *(Abrams et al 1982, Kissebah et al 1982, Howard et al 1983, Taskinen et al 1986)*. *Kissebah (1982)* also found that the hypertriglyceridaemic diabetics had elevated rates of VLDL apoB production but there also appeared to be changes in the composition of VLDL *(Schonfeld et al 1974 a, Taskinen et al 1986)*, suggesting that diabetics had larger, triglyceride-rich VLDL, as indicated by a higher triglyceride: apoB ratio which reverts to normal on sulphonylurea therapy. The driving force for the increased synthesis of VLDL particles seems to be an increased substrate flux of FFA *(Bjorntop 1994)*.

The magnitude of postprandial lipaemia is known to be highly dependent on the fasting plasma triglyceride concentration *(Patsch 1987)*; since NIDDM patients generally have a higher fasting plasma triglyceride, a disturbed postprandial triglyceride metabolism would be expected.

5 The Zucker (*fa/fa*) rat.

Humans and rats share similar triglyceride metabolism pathways. Both produce apoB48 containing chylomicrons from the intestine and liver synthesised VLDL. Human livers only make apoB100 containing VLDL particles. However, the rat liver, unlike the human liver, secretes apoB48 VLDL containing particles. Rats also have decreased levels of CETP, resulting in increased HDL levels. These differences limit the amount of extrapolation that is possible between rat experimental work and humans. However, as yet there appear to be no substantive differences in FFA metabolism between humans and rats that would invalidate the use of the rat model for the studies described here.

The two strains of rat used in the current work were the Alderley Park and Zucker rat. The Alderley Park rat is Wistar-derived and is considered to have a normal genetic make up. Some studies have shown the genetically obese Zucker (*fa/fa*) rat to have increased levels of leptin due to a defect in the leptin receptor *(Zimanyi IA, Fathi Z, Parker E 1997)*.

The Zucker rat inherits obesity as an autosomal Mendelian recessive trait *(Bray 1977)*. This characteristic autosomal recessive obesity is linked to an allele termed *fa* which has been mapped to chromosome 5. The Zucker rat is homozygous for this trait, and cannot be distinguished from normal animals until the 3rd or 4th week of life. By 5 weeks of age, there are visible differences in body fat content and shape *(Zucker 1965)*. The genetically obese Zucker rats *(fa/fa)* were first described by *Zucker and Zucker in 1961* and are frequently used as models of early onset human obesity and NIDDM. An increase in plasma triglyceride and lipoproteins is one of the earliest abnormalities described in the Zucker rat *(Barry and Bray 1969)*. The model displays hyperinsulinaemia and hyperlipidaemia; in contrast blood glucose levels are normal. Livers from these mutants are characterised by an increased glycolytic flux, abnormally high fatty acid synthesis and esterification, triglyceride accumulation and an excessive output of VLDL. It has been suggested that the hypertriglyceridaemia which characterises these animals is due to hypersecretion of hepatic VLDL *(Schonfeld, Felski and Howald 1974b, Wang, Fukuda and Oruko 1984)* without any defect in clearance

(Bray 1977); plasma levels of all lipids were elevated including LDL, HDL and FFA. Attempts to alter plasma lipids in Zucker rats have produced variable results, as summarised by *Cleary et al (1987)* and *Fukuda, Azain and Ontto (1982)* who have suggested that the altered hepatic metabolism of FFA in this strain of rat was an underlying factor in the hypersecretion of VLDL. Adipocytes appear to be increased in size *(Johnson et al 1971)* and number, with the subcutaneous fat depot showing the largest increase in the number of fat cells. Both *in vitro* and *in vivo* studies are consistent with the conclusion that Zucker rats mobilise fat at an above normal rate with a greater output of FA from the adipose tissue and in terms of cellularity of the tissue there is also increased fat mobilisation per cell *(Zucker 1972)*. The increased flux in FFA may come from the adipose tissue since these rats are hyperinsulinaemic; *i.e.* HSL activity in the adipose tissue may be elevated. *Bray, Mothon and Cohen (1970)* showed that mobilisation of FFA from adipose tissue was normal or increased both *in vivo* and *in vitro* in Zucker rats. In a recent study *(Kasim et al 1992)* it was shown that in the Zucker rat model, HMG CoA-reductase inhibitors can reduce the rate of secretion of VLDL. The disturbed metabolism displayed by the Zucker rat may provide useful information on the mechanism of action of hypolipidaemic drugs, in particular on FFA metabolism.

6 Aims and objectives.

The aim of this thesis was to better understand the role of the regulation of triglyceride metabolism in both man and rat models. Triglyceride metabolism is controlled by two enzymes, LPL and HSL whose activity is regulated by insulin. Most of the research concentrated on two questions; the first to study the effect of postprandial chylomicronaemia on the metabolism of VLDL subfractions in both normal and diabetic patient groups, the second to investigate the antilipolytic mechanisms of action of selected agents on HSL.

(1) In human subjects, the study of triglyceride metabolism focussed upon postprandial metabolism, more specifically the effect of postprandial chylomicronaemia upon the metabolism of VLDL subfractions (large VLDL, ($VLDL_1$) and small VLDL, ($VLDL_2$)). This was to be investigated in both normal and diabetic (NIDDM) subjects. NIDDMs are insulin resistant, and as such have elevated triglyceride levels due to increased VLDL levels, so it would be of interest to observe the reaction of this system when it was challenged with chylomicrons (a naturally occurring postprandial phenomenon). In order to do this, a method to separate chylomicrons and VLDL had to be developed. Chylomicrons and VLDL share many structural similarities, size and

density, making their separation by sequential ultracentrifugation, particularly in the postprandial state, difficult. Thus an immunoaffinity chromatography method for the separation of chylomicrons and VLDL on the basis of their differing apoB proteins had to be developed (see chapter III).

(2) HSL is the rate limiting enzyme in adipocyte lipolysis. In normal individuals insulin exerts an antilipolytic effect on HSL, which is lost in insulin resistant disease states. As a result, subjects have increased triglyceride levels due to increased VLDL synthesis and secretion which is driven by increased levels of FFA the product of HSL lipolysis. This makes HSL an attractive target for drugs to help treat this and other conditions characterised by insulin resistance. Abnormally high rates of adipocyte lipolysis are thought not only to result in elevated plasma lipid levels linked to an increased risk of atherosclerosis and the development of CHD, but are closely linked to the metabolic disturbances characterised by insulin resistance. Thus the selective inhibition of HSL which catalyses the rate limiting step of triglyceride hydrolysis may contribute towards the therapeutic control of excessive adipose tissue lipolysis in various disease states. The antilipolytic mechanism of action of a series of compounds was investigated in isolated rat adipocytes *in vitro*, with *in vivo* dose response experiments using nicotinic acid carried out in Zucker rats after initial experiments in Alderley Park rats.

The objectives of the research were therefore

- To develop a method for the separation of chylomicrons and VLDL.
- To use this method to investigate VLDL subfraction metabolism in the postprandial state in both normal and diabetic subjects.
- To investigate the antilipolytic mechanism of action of selected compounds on HSL.
- To investigate and construct dose response curves for nicotinic acid in both Alderley Park and Zucker rats (NIDDM model).

Figure 1.1: ***The four major sources of free fatty acids for VLDL synthesis.*** *The circled numbers correspond to the four major sources of free fatty acids for VLDL synthesis, (1) hepatic de novo lipogenesis (DNL), (2) cytoplasmic triglyceride stores, (3) fatty acids derived from lipoproteins taken up directly by the liver and (4) exogenous (plasma) fatty acids.*

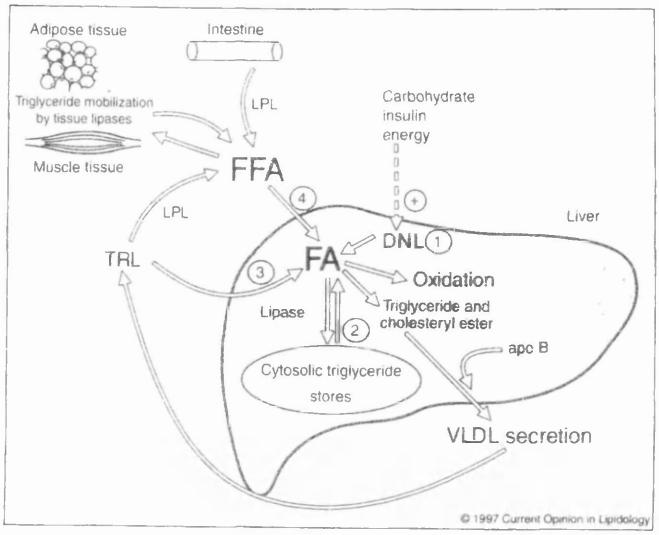


Figure 1.2: ***The possible fates of fatty acids.***

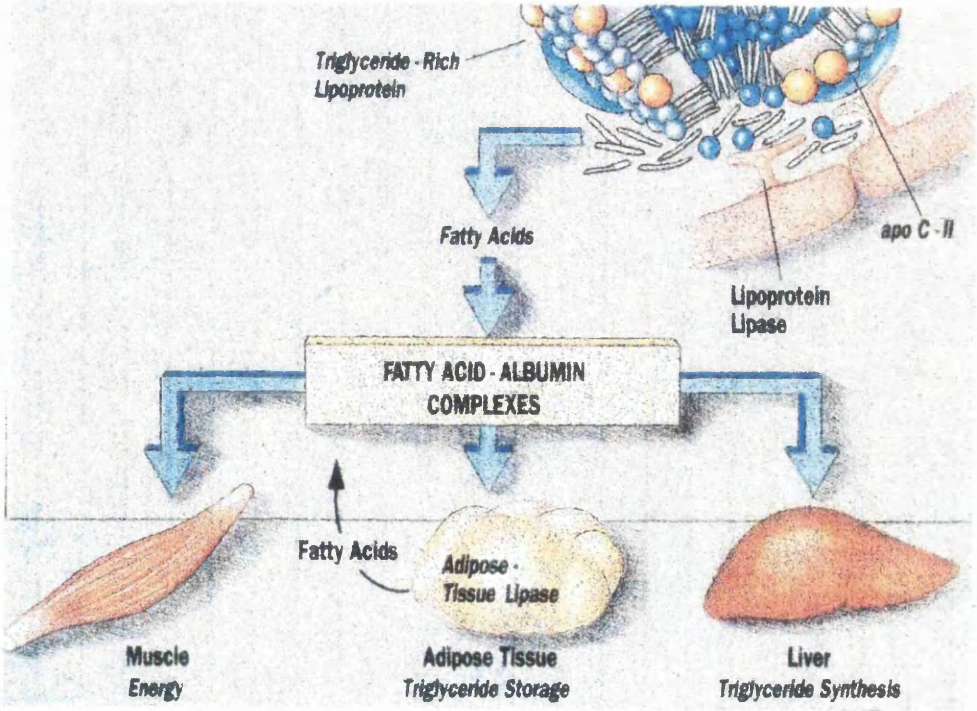


Figure 1.3: *General lipoprotein structure.*

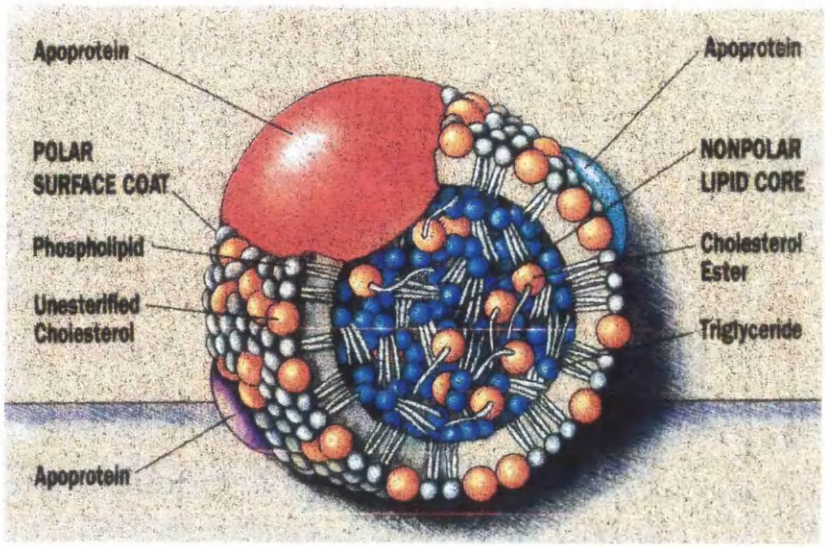


Figure 1.4: *Structure of chylomicrons.*

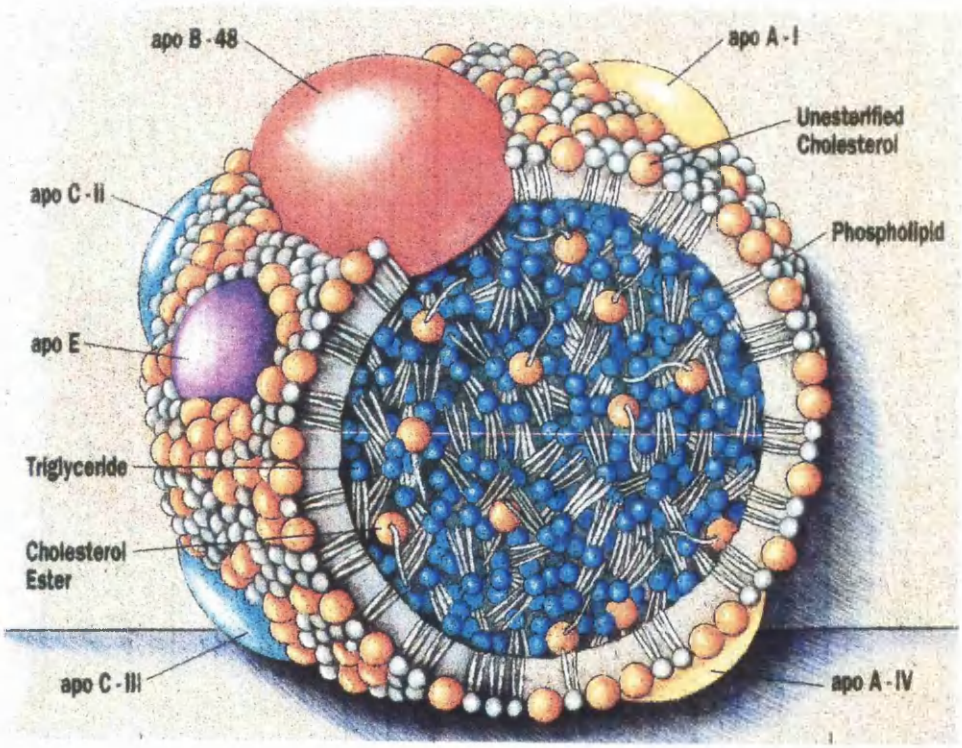


Figure 1.5: *Basic steps in the catabolism of chylomicrons - exogenous transport system.*

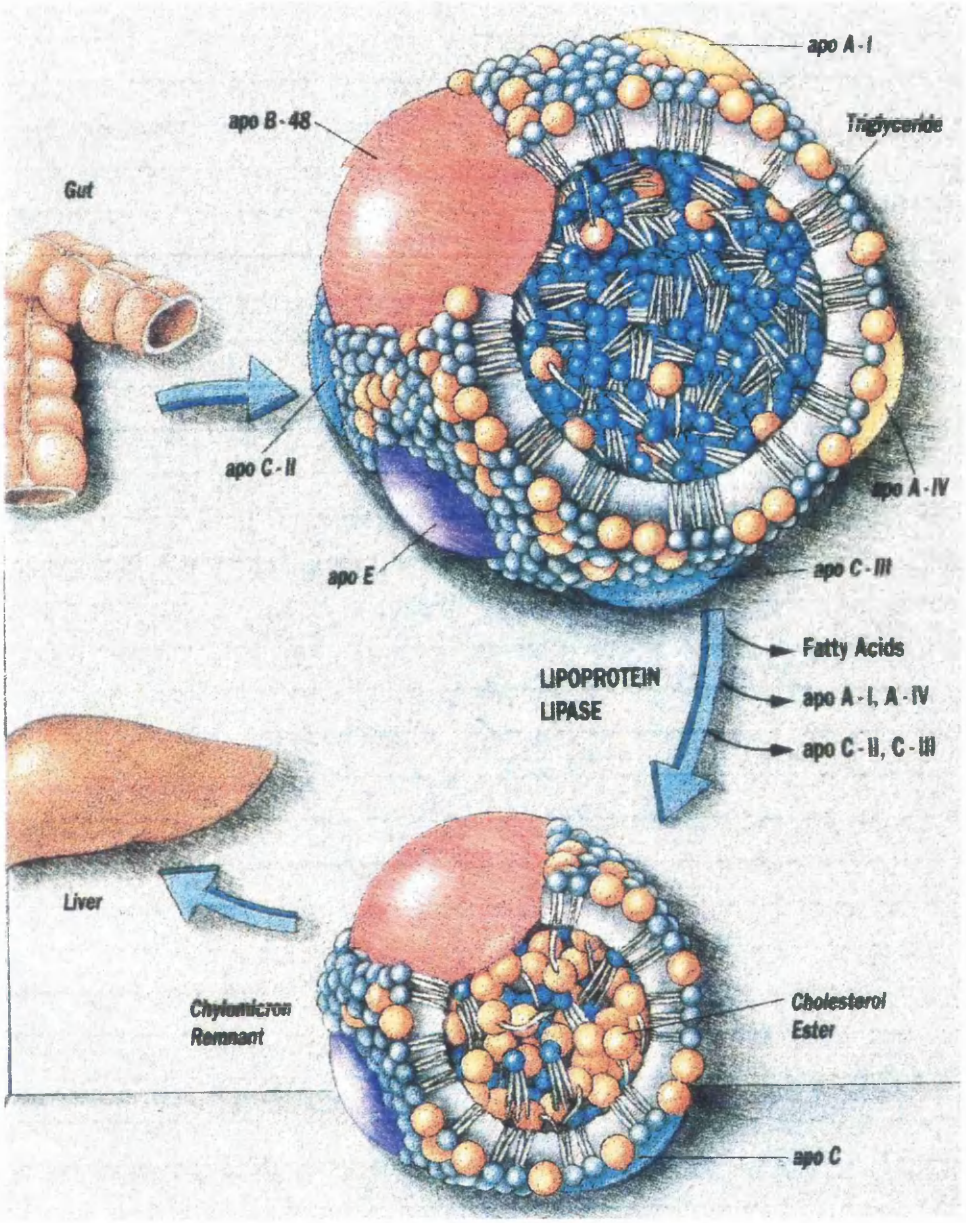


Figure 1.6: Structure of VLDL.

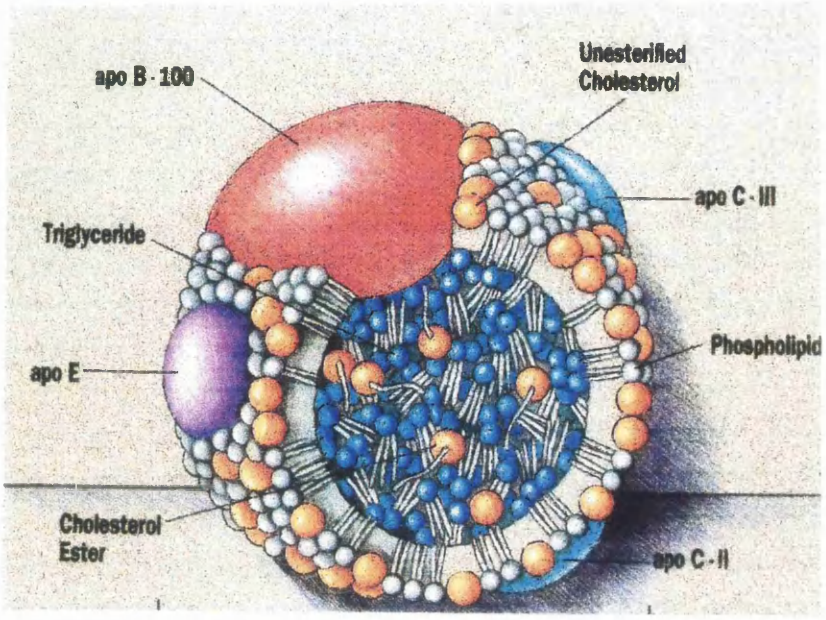


Figure 1.7: Basic steps in VLDL catabolism - endogenous transport system.

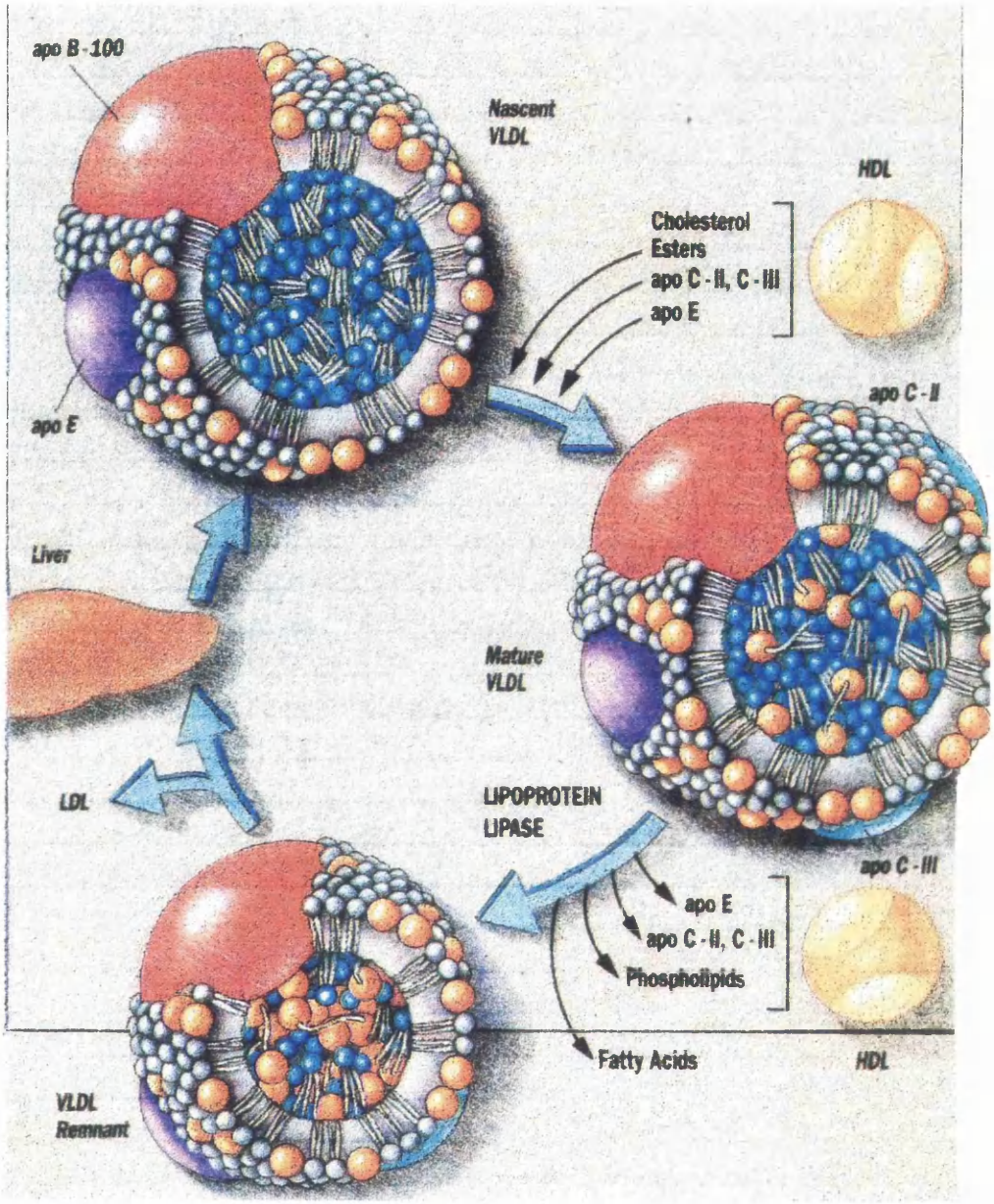
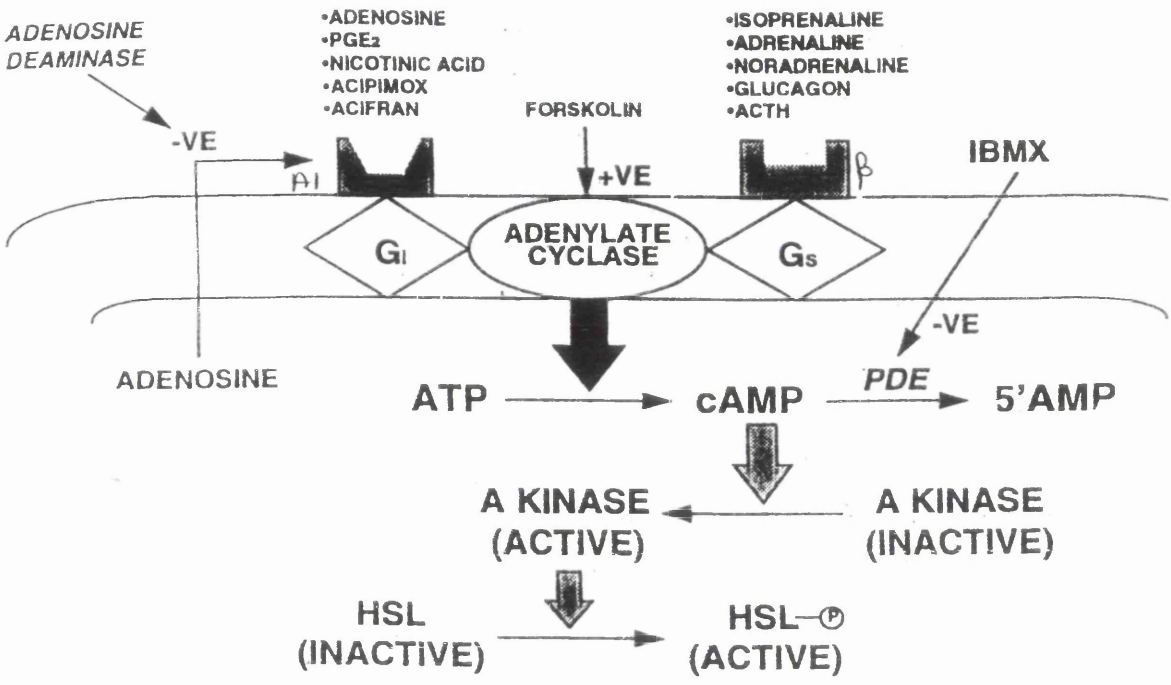


Figure 1.8: The adipocyte signal transduction pathway.



Chapter II General methods.

1 Materials.

Names and addresses of manufacturers and suppliers of reagents, hardware and software used for this thesis are given in appendix 1.

2 Laboratory methods.

2.1 ß-Quantification of lipids and lipoproteins.

Blood was collected into Na_2EDTA tubes (final concentration 1 mg/ml), plasma was separated by centrifugation (3000 rpm (1300 g), 4°C, 15 minutes), and kept at 4°C until analysis. Plasma cholesterol and triglyceride concentrations were measured using reagent kits (704121 and 704113 Boehringer Mannheim GmbH) on an automated discrete analyser (BM/Hitachi 717). Quality control was monitored using Seronorm lipid (Nycomed Pharma AS) and Precipath U (Boehringer Mannheim GmbH). Plasma cholesterol, VLDL, LDL and HDL cholesterol concentrations were analysed according to the Lipid Research Clinics Program Manual of Laboratory Operation (1975), by a combined ultracentrifugation/ precipitation technique. Five ml of plasma was placed in a 6.5 ml thermoplastic ultracentrifugation tube (Ultraclear Beckman Instruments, Inc) and overlayered with normal saline until a meniscus appeared at the top. The tubes were sealed, placed in a 50.4 rotor (Beckman Instruments, Inc) and centrifuged (35000 rpm (130000 g), 4°C, 18 hours). The supernatant was then removed by tube slicing and transferred to a 3 ml volumetric flask, the cutter was washed with normal saline and the washings used to make up 3 ml. The infranatant was transferred to a 5 ml volumetric flask and made up to volume with normal saline. One ml of the infranatant was then transferred to a second ultracentrifuge tube and the apoB containing lipoproteins were precipitated by adding 50 μl of sodium heparin ($5x10^5$ units)-manganese chloride reagent (92 mM). This was made up by mixing 9.56 grams $MnCl_2.4H_20$ in 6 ml dH_2O with 1.05 grams heparin ($5x10^5$ units; Sigma H - 3125) dissolved in 12.5 ml normal saline, made up to 25 ml with dH_2O and stored at 4°C.

The tubes were mixed, vortexed and incubated (4°C, 15 minutes), then spun (10000 rpm (11000 g), 30 minutes) in the Ti 50.4 rotor with the vacuum off to pellet the precipitate. Aliquots of the VLDL fraction, the original infranatant (LDL and HDL) and the second supernatant (HDL) were transferred to autoanalyser cups and their cholesterol concentrations determined as above and in section 2.2.1. The LDL cholesterol concentration was calculated as the difference between that in the infranatant and the second supernatant.

This routine analysis is a globally accepted standard method used to produce patient lipid profiles. It was kindly performed by staff of the Routine Lipid Section of the Biochemistry Department of the G.R.I.

2.2 Compositional analysis.

2.2.1 Cholesterol.

Cholesterol was determined using a Boehringer kit (kit number 704121) in which cholesteryl esters in the sample were converted to cholesterol by an esterase. The enzyme cholesterol oxidase then oxidised all the cholesterol to form 4-cholesterenone and hydrogen peroxide, which is then converted in the presence of the enzyme peroxidase, phenol and 4-aminophenazone to produce 4-(p-benzoquinone-mono-imino)-phenazone. Absorbance of this product was measured at 505 nm on a Hitachi 704 or 717 autoanalyser. Coefficient of variation was 1.6%.

2.2.2 Triglyceride.

Triglyceride was determined using a Boehringer kit (kit number 704113). The assay was performed on the Hitachi 717. Triglyceride is converted to glycerol by lipase enzyme. In the presence of ATP, glycerol kinase further converts glycerol to glycerol-3-phosphate which is oxidised by a third enzyme, glycerol phosphate oxidase to form dihydroxyacetone phosphate and hydrogen peroxide which is converted in the presence of the enzyme peroxidase, phenol and 4-aminophenazone to produce 4-(p-benzoquinone-mono-imino)-phenazone. Absorbance of this product was measured at 505 nm on a Hitachi 704 or 717 autoanalyser. Coefficient of variation was 2.1%.

Both triglyceride and cholesterol assays were performed by the staff of the Routine Lipid Section of the Biochemistry Department of the G.R.I.

2.3 Protein (modified Lowry) assay.

The measurement of protein was performed on lipoprotein fractions using a modification of the method of *Lowry et al (1951),* involving measurement of proteins with the Folin phenol reagent (BDH 19058B 3Q) after alkaline copper treatment. Biuret reagent was made freshly from stock solutions; 100 ml 2% Na_2CO_3 (BDH 10240) in 0.1 M NaOH (BDH 10252), 1 ml 2% NaK tartrate in deionised water and 1 ml 1% $CuSO_4$ (BDH 10091), with 1 ml SDS (Bio-Rad 161-0416) (added if sample to be assayed was turbid). Stock Folin Ciocalteu was diluted 1:1 with deionised water. A stock solution of human albumin (Fraction V, Sigma) 1 mg/ml, stored at -70°C, was used to prepare a working standard curve in the range of 0 - 50 μg protein, by taking volumes of stock standard (0 - 50 μl) then adjusted to a final volume of 400 μl. Volumes of two stock (quality control) solutions, (15 and 30 μg/ 100 μl) of bovine serum albumin (BSA) (Fraction V, Sigma) stored at -70°C, were adjusted to a volume of 400 μl. Samples requiring dilution should be adjusted to a final volume of 400 μl with deionised H_2O. Two ml Biuret reagent was added to 400 μl standard, quality control and sample, tubes were vortexed and left to stand for 10 minutes. Tubes were mixed immediately after addition of 200 μl Folin Ciocalteu reagent, and left to stand for 30 minutes. The optical density at 750 nm was read within two hours. The within batch coefficient of variation was 1.9% and between batches was 2.4%.

2.4 Measurement of apolipoprotein B and AI.

Apolipoprotein B and AI in plasma and the TRL ($d < 1.006$g/ml) fraction was quantified by immunonephelometry using kits from Orion Diagnostica (catalogue numbers 67249 for apolipoprotein B and 67265 for apolipoprotein AI). The method is based on the measurement of immunoprecipitation in the liquid phase at 292 nm on the Encore clinical chemistry centrifugal analyser (Baker instruments). In conditions of antibody excess, the amount of precipitate is proportional to the apolipoprotein concentration *(Riepponen, Marniemi, Rautaoja 1987).* The sensitivity of the assay was 0.3 g/L with a coefficient of variation of 5%. Quality control was monitored using lyophilised materials from Immuno (code nos 4977006) and Behring (catalogue number OUPG).

This assay was performed on a semi-routine basis by Mr Michael McConnell of the Lipid Research Group.

2.5 Apolipoprotein E phenotyping.

Three isoforms of apoE (E2, E3 and E4) can be separated by isoelectric focussing gel electrophoresis. These are then transferred to nitrocellulose by Western blotting. The apoE bands are visualised using a monoclonal antibody to apoE followed by an anti-IgG antibody linked to horse-radish peroxidase (HRP) and completed by the addition of 4-chloronaphthol which produces an insoluble dark blue stain.

ApoE phenotypes were determined by isoelectric focussing followed by Western blotting using an adaptation of the method of *Havekes et al (1987).* Ten μl of plasma was incubated with 28.5 mU neuraminidase (Sigma N-3001) in 20 mmol/L sodium acetate buffer, pH 5.1 (BDH 10235), then delipidated overnight with pre-cooled ethanol: ether (3:1, v/v) at -20^{o}C. The mixture was precipitated (3000 rpm, 30 minutes) and further washed with ether and incubated for a minimum of one hour at -20^{o}C. Again the mixture was precipitated as before. The precipitate was next dissolved in solubilising buffer (0.1 M Tris base (Sigma T-1503), 1% SDS, 8 M Urea, pH 10.0 (Pierce Chem Company 29700)) to which 10 μl ß-mercaptoethanol (Sigma M 7154) was added and incubated at 4^{o}C for 30 minutes. Thirty μl of sample was applied to a vertical slab gel of 5% polyacrylamide in 8 M urea containing 1% (v/v) ampholines pH 4 - 6 (Serva). Electrophoresis was carried out in a Hoefer SE 600 tank (Pharmacia) at 250 V, 12 mA per gel overnight at room temperature, with water cooling and then at 500 V for 60 minutes. The upper buffer was 0.08% NaOH (w/v), pH 10.1 and lower buffer 0.1 M sodium phosphate, pH 2.3 (BDH 10248). Transfer from acrylamide gel to nitrocellulose was carried out at room temperature with water cooling at 100 V for 3 hours in 0.2 M glycine (BDH 101196X), 0.02 M Tris base, 20% (v/v) methanol, pH 9.0 in a Biorad transblot cell (figure 2.1). The nitrocellulose strips were first soaked in blocking buffer (0.01 M Tris HCl, 0.05% (v/v) Tween 20 (Sigma P1379), 0.15 M NaCl (BDH 10241), pH 7.4) containing 5% (w/v) milk powder (Marvel) as a blocking agent. The nitrocellulose was washed for 20 minutes with 3 changes of buffer. Incubation with the first antibody (mouse monoclonal anti-apoE) was overnight at room temperature with slow shaking. The nitrocellulose was washed for 20 minutes with buffer and incubated with the second antibody (goat anti-mouse horseradish peroxidase conjugate) for 2 hours at room temperature with gentle shaking. After a final 20 minute wash with buffer, the nitrocellulose was developed by incubation in the dark, at room temperature, with 4-chloro-naphthol and hydrogen peroxide (BDH 101284N) in 0.9% saline with buffer. ApoE isoforms were identified by reading against a known apo E2/ E2 phenotype.

ApoE phenotyping was carried out on a semi-routine basis by Mrs Dorothy Bedford or Mrs Liz Murray.

2.6 Free fatty acid (FFA) assay .

FFAs were quantitatively determined using a Wako NEFA C (enzymatic colorimetric) kit (code no. 994-75409 E). The principle of the assay is described below. When FFA in plasma are treated with acyl-Co A synthetase (ACS) in the presence of ATP, magnesium cations and Co A, thiol esters of CoA known as acyl CoA are formed. The by-products AMP and pyrophosphate (PPi) are also formed. In the second part of the procedure, the acyl Co A is oxidised by added acyl CoA oxidase to produce hydrogen peroxide which in the presence of added peroxidase allows the oxidative condensation of 3-methyl-N-ethyl-N-(ß-hydroxy-ethyl)-aniline with 4-aminoantipyrine to form a purple coloured adduct with an absorption at 550 nm. The amount of FFA in the sample can be determined from the optical density measured at 550 nm. A standard curve from 0 - 1 mmol/L was prepared. Coefficient of variation was $<5\%$.

2.7 Sequential flotation ultracentrifugation for isolation of chylomicrons, VLDL, IDL and LDL.

Since plasma lipoproteins have lower hydrated densities than that of other plasma proteins and since the densities of each class are discrete from one another, progressively raising the solvent density of plasma between ultracentrifugation steps allows the isolation of defined lipoprotein classes. This provides the simplest method of simultaneously isolating lipoproteins from a number of samples in a form that does not require further concentration.

Chylomicrons and VLDL, IDL, and LDL were isolated at the density limits of 1.006, 1.019 and 1.063g/ml as originally described by *Havel, Eder and Bragdon (1955)*. The density solutions were prepared from stock solutions at d=1.006g/ml (0.195 M NaCl (BDH 10241), 0.001% Na_2EDTA (BDH 10093)) and d=1.182g/ml (0.195 M NaCl, 2.44 M NaBr (BDH 3O11645), 0.001% Na_2EDTA). Densities were measured to 3 decimal places on a Paar scientific (DMA 35) density meter.

For fed samples, 4 ml of plasma was placed in a 6.5 ml thermoplastic ultracentrifuge tube (Ultraclear; Beckman Instruments, Inc) overlayered with 2 ml normal saline and centrifuged in a Beckman Ti 50.3 rotor (10000 rpm, (7100 g) 4^{o}C, 30 minutes). The top 2 ml containing chylomicrons was aspirated using a finely drawn

glass Pasteur pipette. The remaining 4 ml was overlayered with 2 ml d=1.006g/ml solution and centrifuged overnight in a Beckman Ti 50.3 rotor (39,000 rpm (108000 g), 16 hours, 15°C). The top 2 ml containing VLDL was aspirated as previously. The remaining 4 ml was adjusted to d=1.019g/ml by the addition of 0.32 ml d=1.182g/ml solution and overlayered with 1.68 ml d=1.019g/ml. After an overnight centrifugation (39000 rpm (108000 g), 16 hours, 15°C), IDL was removed in the top 2 ml. The infranatant (4 ml) was adjusted to d=1.063g/ml by the addition of 1.47 ml d=1.182g/ml and overlayered with 0.5ml d=1.063g/ml. After an overnight centrifugation (39000 rpm (108000 g), 16 hours, 15°C), LDL was harvested in the top 1 - 2 ml. Note in fasting samples the chylomicron ultracentrifugation step is not necessary. Alternatively after feeding, chylomicrons and VLDL can be isolated in the same fraction by overlayering 4 ml plasma with 2 ml d=1.006g/ml solution, and after overnight centrifugation (39000 rpm (108000 g), 16 hours, 15°C) removing the top 2 ml.

2.8 Cumulative flotation for isolation of $VLDL_1$ *(Sf 60 - 400)* and $VLDL_2$ *(Sf 20 - 60).*

This method allows the fractionation of $VLDL_1$, $VLDL_2$, IDL and LDL from plasma by a modification of the cumulative ultracentrifugation density gradient technique described by *Lindgren and Jensen (1972)*, using a Beckman SW40 rotor and tubes pre-coated with polyvinyl alcohol. This method was used to separate $VLDL_1$ and $VLDL_2$ from a total VLDL sample previously isolated by immunoaffinity chromatography (chapter III). Density solutions were prepared from stock solutions at d=1.006g/ml (0.195 M NaCl, 0.001% Na_2EDTA) and d=1.182g/ml (2.44 M NaBr, 0.195 M NaCl, 0.001% Na_2EDTA). The densities were measured to 3 decimal places on a Paar scientific (DMA 35) density meter.

The density of 2 ml of 1 M NaSCN (Sigma S 7757) eluted VLDL, obtained by immunoaffinity chromatography, was adjusted to d=1.118g/ml by the addition of 0.224 grams NaCl, calculated from the equation below;

$$NaCl\ (g) = 2(1.118 - x)/ (1 - 0.34) \text{ at } 20^{o}C, \text{ where } x = \text{density of NaSCN (0.4)}$$

and this was carefully layered over 0.5 ml d=1.182g/ml NaBr solution in an ultraclear Beckman SW40 ultracentrifuge tube which had been coated with polyvinyl alcohol *(Holmquist 1982)*. A discontinuous salt gradient from 1.0988 - 1.0588g/ml

(d=1.0988g/ml (1 ml), d=1.0860g/ml (1 ml), d=1.0790g/ml (2ml), d=1.0722g/ml (2 ml), d=1.0641g/ml (2 ml), d=1.0588g/ml (2 ml)) was carefully constructed above it (figure 2.2). Tubes were centrifuged in a Beckman SW40 rotor in a Beckman L8 centrifuge, (39,000 rpm, 23°C, 1 hour 38 minutes). One ml containing large $VLDL_1$ (*Sf 60 - 400*) was aspirated using a finely drawn glass Pasteur pipette and replaced with 1 ml d=1.0588g/ml solution. $VLDL_2$ (*Sf 20 - 60*) was removed in the top 0.5 ml following a second centrifugation (18,500 rpm, 15 hours 41 minutes, 23°C). Rotors were decelerated without the brake.

2.8.1 Surface modification of Beckman ultraclear centrifuge tubes.

The procedure for coating the interior of Beckman ultraclear centrifuge tubes with polyvinyl alcohol to allow salt solutions to gravity feed smoothly down the sides of the tubes was described by *Holmquist (1982).* It involves coating Beckman ultraclear tubes with a polyvinyl alcohol solution containing propan-2-ol for 15 minutes, the solution is removed and tubes left to dry overnight. The tubes were washed overnight with distilled H_2O, then flushed once with H_2O and left to dry.

2.9 Apolipoprotein identification.

The apolipoprotein content of the isolated lipoproteins was resolved by sodium dodecyl sulphate glycerol polyacrylamide slab gel electrophoresis (SDS PAGE) *(Maguire, Lee and Connelly 1989).* Electrophoresis was performed using vertical slab gel apparatus (SE 600; Hoefer Scientific Instruments), 1.5 mm thick gels and a 15 tooth well former. The gel consisted of 3.6% acrylamide, with an acrylamide (BDH 44353 5P)-bisacrylamide (BDH 44355 5R) ratio of 20:1, 19% glycerol (v/v) (BDH 101186M), 1.2 % SDS, 0.08% TEMED (Electran (BDH) 44308), 0.019% ammonium persulphate (BDH 44307) in 0.8 M Tris/phosphate pH 6.8 and was made up according to the author's protocol.

Samples were prepared according to the method of *Mindham and Mayes, 1992.* One ml of lipoprotein fraction, obtained by sequential flotation ultracentrifugation (section 2.7) and containing up to 1 mg protein was shaken with 10 ml diethyl ether (BDH 10094) for 2 minutes, after which 3.6 mM sodium deoxycholate (0.2 ml) (Sigma D6750) and 4.9 M trichloroacetic acid (0.2 ml) (BDH 10286) were added, samples were vortexed briefly and the top layer of diethyl ether was removed by aspiration and a stream of nitrogen. The bottom layer was centrifuged at 6500 rpm, 10 minutes, 4°C, to pellet the precipitated apolipoproteins. The supernatant was aspirated with a fine tipped

Pasteur pipette (Alpha Laboratories Ltd LW 4233). The pelleted apolipoproteins were dissolved in sample buffer (containing 0.174 M Tris base pH 6.8 (25 ml) (Sigma T1503), 20% SDS solution (10 ml) (Biorad 161-0418), 10% bromophenol blue (10 μl) (BDH 44305), glycerol (10 ml) (BDH 10118), 2-mercaptoethanol (5 ml) (Sigma M 7154)), and heated (80^{o}C, 5 minutes) in a Techne heating block. Where necessary 5 M NaOH (BDH 10252) was added to neutralise samples. The samples were applied to gels. The lower buffer chamber was filled with 4 litres of 0.1 M sodium dihydrogen phosphate buffer (BDH 307165 U) pH 7, and the upper chamber with the same buffer containing 0.1% SDS. Electrophoresis was carried out at a constant current of 20 mA for 30 minutes and then at 60 mA for 4.5 hours at room temperature, water cooled. Gels were fixed in 4% formaldehyde (BDH 10113) for 30 minutes, stained overnight in 0.023% Coomassie Brilliant Blue G-250 (Bio-Rad 161-0406) in 45.4% methanol (Hayman Limited SIN 1230), 9.2% acetic acid (BDH 45001), and then destained with 7.5% acetic acid, 5% methanol and stored in 5% acetic acid.

High and low molecular weight markers (LMW and HMW-SDS Pharmacia AB (Lot nos. 6080446011. 6010445011, 6080615011) were included in each run. Apo B100 and apoB48 peptides were identified using molecular weight markers and LDL as an apoB100 marker (prepared as samples) and also by Western blot analysis. The method was used to identify the presence of apoB48 and apoB100 in lipoprotein fractions isolated by immunoaffinity chromatography and in Western blotting of apoB for screening antibodies to generate the immunoaffinity chromatographic column.

2.10 Staining methods.

2.10.1 Coomassie Brilliant Blue stain.

Coomassie Brilliant Blue staining is based on non-specific binding of Coomassie Blue dye to proteins. Separated proteins are simultaneously fixed and stained in the gel, and then destained to remove the background prior to storage and photocopying. The proteins are detected as blue bands on a clear background *(Wilson 1983)*. For Coomassie Blue staining the gels were fixed in 4% formaldehyde for 30 minutes, stained overnight in 0.023% Coomassie Brilliant Blue G-250 in 45.4% methanol, 9.2% acetic acid, and then destained with 7.5% acetic acid, 5% methanol and stored in 5% acetic acid. The detection limit of Coomassie Blue was approximately 0.3 - 1.0 μg protein. Coomassie Blue staining is semi-quantitative, with different proteins having different dye binding characteristics.

2.10.2 Ponceau S stain.

This was a rapid reversible staining method, which was used to verify transfer efficiency after Western blotting before proceeding with immunodetection *(Salinovich and Montelano 1986)*. The protein bands appeared after the membrane was stained with Ponceau S with a detection limit of 1 - 2 μg. Protein on nitrocellulose (after Western blotting) was stained with Ponceau Red S (Sebia) (0.1% w/v Ponceau S, 5% acetic acid) for 5 - 30 minutes. The nitrocellulose was washed in distilled H_2O to remove background staining, colour photocopied and was then completely destained with distilled H_2O allowing immunodetection to proceed.

2.11 Preparation of lipoprotein deficient plasma (LPDP).

Blood was collected into Na_2EDTA tubes, and placed on ice. Plasma was separated from red cells after centrifugation (3000 rpm, 15 minutes, 4°C). The plasma density was raised to 1.225g/ml by adding 0.35 grams KBr (BDH 10195)/ ml of plasma, and the density checked using a Paar DMA 35 densitometer (Stanton Redcroft), placed in 26 ml polycarbonate ultracentrifuge tubes (Beckman instruments Inc.) and overlayered with 2 ml of KBr solution d=1.225g/ml (approximately 3.49 grams KBr in dH_2O). The tubes were centrifuged (48 hours, 50000 rpm, 4°C) in a Ti 60 rotor on a L5 ultracentrifuge (Beckman instruments). The lipoproteins were removed in the top 2 ml and the remaining infranatant, the LPDP, dialysed overnight against 0.15 M NaCl, 10 mM Tris base, 1 mM EDTA, 0.05% NaN_3 pH 7.4 at 4°C. A sample of dialysed LPDP was prepared and run on SDS-PAGE (section 2.9) to check it was free of lipoproteins.

3 Statistical analysis.

3.1 Human studies.

Mean and standard deviation of means for plasma parameters (lipid, apolipoproteins and insulin) were reported. Paired t-tests were used to determine the statistical significance for the plasma parameters between measurements obtained from fasting (0 hour) and postprandial samples. Unpaired t-tests were used to determine statistical significance between triglyceride response curves and for area under the curves obtained from normal and diabetic subjects, where the baseline was the fasting 0 hour concentration. Area measurements were expressed as mmol.hour. The Minitab 10

statistical package was used to calculate regression and p values for correlations. The Microsoft Excel package was also used for statistical analysis.

3.2 Animal studies.

For the Zucker rats, the mean and standard deviation of means (before log transformation) for each plasma parameter were reported. An Anderson-Darling test for normal distribution was carried out on the data. Where data was not found to be normally distributed, it was log transformed. An analysis of variance (ANOVA) was performed on the log transformed data. The significance level was set at $p<0.05$. When overall significant differences were observed a Fisher's pairwise comparison was carried out to identify the groups that contributed to the significant difference. Minitab 10 and Microsoft Excel packages were used for statistical analysis.

Figure 2.1: *Diagram of the assembly of an immunoblot sandwich for Western blot transfer.*

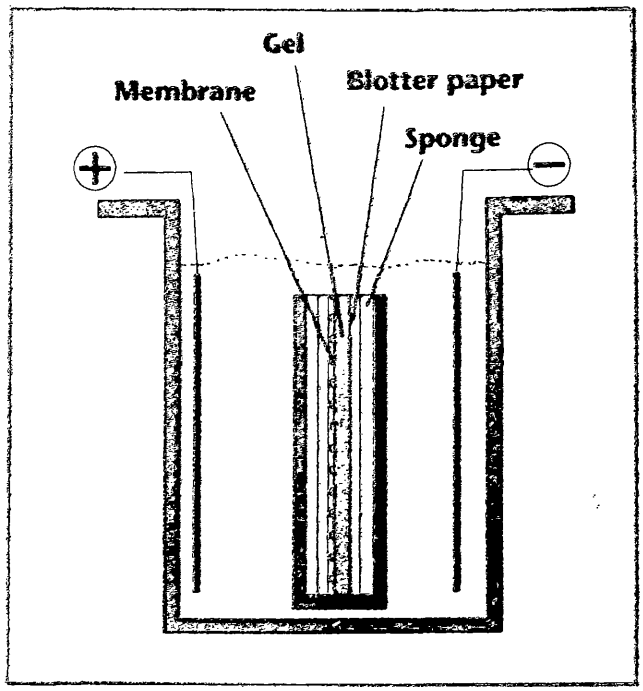
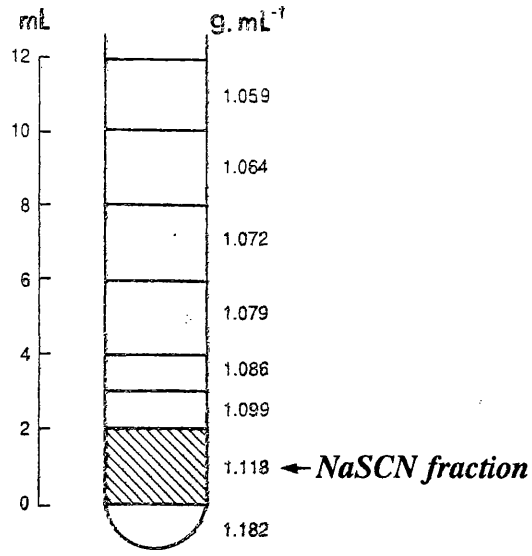


Figure 2.2: *Discontinuous NaBr gradient as used for the subfractionation of VLDL lipoproteins by ultracentrifugation.*



Chapter III Development of an immunoaffinity chromatography method to separate triglyceride-rich lipoproteins (TRL) into apoB48 (chylomicrons) and apo B100 (VLDL) TRL species.

1 Introduction.

The postprandial state has been postulated to be potentially atherogenic on the basis that intestinal chylomicrons and their remnants have the potential to cause lipid deposition in the cells of the artery wall *(Zilversmit 1979)*. The long term effects of repeated and prolonged alimentary lipaemia may also be derangements in the metabolism of endogenous lipoproteins that are atherogenic. When considering a direct atherogenic potential for TRL the question as to whether they can directly enter the artery wall due to lipoprotein size is of importance. A recent study used pauciclonal sheep anti-human apoB100 antibodies to isolate and characterise apoB containing lipoproteins from human atherosclerotic plaques and plasma *(Rapp et al 1994)*. Their results support the view that VLDL and VLDL remnants are potentially atherogenic and can indeed cross the endothelium and enter both human atherosclerotic plaques and the arterial wall, but these apoB containing lipoproteins differ in size from their plasma counterparts. With chylomicrons and VLDL (and their remnants) being potentially atherogenic, it is of importance to study both chylomicron and VLDL subfraction metabolism during alimentary lipaemia, since we spend most of our time in the postprandial state. A recent study by *Bjorkegren et al (1996)* concluded that chylomicrons (and their remnants) impede the normal lipolytic degradation of VLDL and could be indirectly implicated in the generation of atherogenic remnant lipoproteins after a 60 minute intravenous triglyceride infusion.

Postprandial lipoprotein metabolism has traditionally been evaluated by measuring triglyceride concentrations in whole plasma and in lipoprotein fractions after an oral fat load *(Foger and Patsch 1993)*. A few studies have investigated the changes in concentration of apoB48 and apoB100, the main structural components of TRL of

both intestinal and hepatic origin respectively *(Scott 1990)*. These are quantified after ultracentrifugation and separation of TRL, by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS PAGE) and subsequent densitometric scanning of the protein bands *(Poapst, Uffelman and Steiner 1987, Zilversmit and Shea 1989, Karpe and Hamsten 1994, Kotite, Bergeron and Havel 1995)*. Quantification of apoB48 by immunological methods has proved to be very difficult because apoB48 is a fragment of apoB100 *(Scott 1990)* and only reaches postprandial peak concentrations in plasma of approximately 2% of those of apoB100. Also the expression of apoB epitopes may vary depending on the lipid content of the particle, thus restricting the use of immunochemical techniques to quantify the two apoB species.

Postprandial triglyceridaemia may represent the presence in the plasma of large apoB48 TRLs (chylomicrons) from the intestine, but also reflects the plasma accumulation of apoB100 TRLs of hepatic origin (VLDL). In order to investigate this behaviour, more detailed analysis using immunoaffinity chromatographic techniques was required. Several groups have already devised methods using monoclonal antibodies to separate TRL into apoB48 and apoB100 TRL species. *Peel et al (1992, 1993)* reported using an antibody specific for apoB48 to develop an immunologically-based assay for the routine detection of lipoproteins of dietary origin. *Schneeman et al (1993)* used an antibody which specifically bound to apoB100 but not to apoB48 or apoB100 VLDL rich in apoE. The monoclonal antibody J1-H, has been used by this group to study postprandial levels of apoB100 and apoB48 fractions *(Campos et al 1992)*. The antibody binds to VLDL, IDL and LDL but fails to recognise a minor fraction of VLDL that is apoE rich and it also fails to bind apoB48 particles. *Milne et al (1984)* developed a series of monoclonal antibodies raised to apoB100 which were used to separate apoB48 and apoB100 TRL fractions in subjects with Type III hyperlipoproteinaemia.

Cohn et al (1988 a and b) initially studied TRLs collectively and then moved on to study apoB48 and apoB100 associated triglyceride separately. Separation of TRL fractions by immunoaffinity chromatographic techniques allowed the quantification and contribution of both apoB48 and apoB100 fractions during postprandial lipaemia to be determined. More recently, *Bjorkegren et al (1997)* used two monoclonal antibodies that recognised apoB100 and not apoB48 epitopes to separate VLDL from chylomicrons (and their remnants) after an oral fat load, to allow the study of VLDL composition, to see if its composition influenced its metabolic behaviour.

2 Principle.

The aim was to establish a method to separate the heterogeneous population of TRL particles (freshly isolated by sequential flotation ultracentrifugation at a density of $d < 1.006$g/ml, chapter II, section 2.7), into apoB48 and apoB100 TRL fractions (chylomicrons and VLDL respectively) using immunoaffinity chromatography. The approach was to develop an immunoaffinity chromatographic technique that would allow their separation, by passage through an immunoaffinity column which retains VLDL but not chylomicrons. The former are dissociated from the column in a second step. This would allow further isolation of large $VLDL_1$ and small $VLDL_2$ by cumulative flotation from the apoB100 TRL species eluted from the immunoaffinity column.

3 Method development.

A collection of rabbit and sheep polyclonal LDL antisera and mouse monoclonal antibodies raised to human LDL were available. These had been prepared previously in the laboratory.

3.1 Preparation of sheep and rabbit polyclonal LDL antisera.

Production of both rabbit and sheep polyclonal LDL antisera was carried out by Dr. Muriel Caslake and Prof. James Shepherd. LDL was purified from normal volunteers by rate zonal ultracentrifugation, as described by *Patsch et al 1974*. Purified LDL was used to raise antisera in New Zealand White rabbits and sheep. One mg of apo LDL was emulsified with complete Freunds adjuvant using ultrasonification, prior to subcutaneous injection in multiple sites. Injections were repeated at monthly intervals using incomplete Freunds adjuvant until an adequate titre was obtained (usually 3 to 4 injections). The antisera obtained gave a single immunoprecipitin arc with plasma and LDL, but did not react with albumin, HDL or purified apoAI and AII. Antisera were stored in 1 ml aliquots at -70°C.

3.2 Preparation of mouse monoclonal antibodies to human LDL.

Production of mouse monoclonal antibodies was carried out by Mrs. Isobel Cameron. LDL was prepared according to the method described in chapter II, section 2.7. Five mice were injected intraperitoneally with 10 μg human LDL emulsified with complete Freund's adjuvant. Booster injections were applied every 4 weeks using dosage and route of injection identical to the first injection, until an adequate titre was

obtained (usually two to three injections). Two weeks later the animals were anaesthetised with ether and 1 - 2 blood spots from the tail vein were collected on filter paper for antibody binding tests.

A 6 mm disc was punched from the filter paper dried bloodspot and incubated overnight in 300 μl saline. Plasma was eluted with 0.9% saline at an estimated dilution of 1:500. A further dilution of 1:1000 was prepared using the same buffer. Aliquots (100 μl) were taken in duplicate for each dilution and 200 μl PBS containing ^{125}I LDL (about 10000 cpm) added. Samples were mixed thoroughly and incubated at room temperature overnight. Then 200 μl solid phase sheep anti-mouse immunoglobulin (prepared using carbonyldiimidazole activated Sepharose-CL 4B) was added and incubated on an orbital shaker for another 1 hour. After 3 washes with saline plus 0.2% Tween 20, radioactivity in the Sepharose pellet was determined and expressed as percentage of initial radioactivity. Non-specific binding of ^{125}I LDL was determined in controls containing saline instead of plasma.

Murine immortalised myeloma cells (ICN) and spleen lymphocytes obtained after LDL immunisation were fused to form hybridoma cells. Metabolic selection of such hybrids then followed favouring their growth at the expense of unfused cells. Hybridoma cell lines were screened for secretion of LDL-specific antibodies by a qualitative enzyme-linked immunosorbent assay (ELISA) using human LDL-coated cell culture plates.

Two ml LDL was dissolved in 0.3 ml of 0.05 M sodium carbonate buffer, pH 9.6 and protein concentration was measured by the Lowry method *(Lowry et al 1951)* (chapter II, section 2.3). Protein concentration was adjusted to 2 μg/ml and aliquots of 150 μl per well were dispensed on 96 well culture plates. Plates were incubated for 4 hours at room temperature, emptied and filled with 3% BSA, in 0.05 M sodium carbonate buffer, pH 9.6. After another 3 hour incubation, plates were again emptied and covered with transparent foil and stored at -20^{o}C until required. Screening for anti-LDL antibodies involved the displacement of 5 μg/ml cold LDL (see previously).

Cloning anti-LDL producing hybridoma cell lines.

Cell lines secreting anti-LDL antibodies had to be cloned to ensure that the antibody originated from a single hybridoma cell. Cell lines which were found to secrete anti-LDL antibodies (200 μl from a 96 well plate) together with 2 ml RPMI-1640, containing peritoneal macrophages (acting as feeders) were placed into 2 ml wells

(24 well plate) and grown to 50% confluence. One hundred μl of this cell suspension was transferred into each well of the eight wells of the far left row of a 96 well plate which had previously been primed with 100 μl RPMI-1640 per well. A serial dilution of cells ranging from 1:2 to $1:2^{12}$ was carried out by subsequent transfer of 100 μl aliquots per well from one vertical row to the next, working left to right. After addition of another 100 μl of RPMI-1640 containing peritoneal macrophages, the plate was sealed and incubated for 7 - 14 days at 37°C. Then 6 vertical rows with the lowest cell concentrations were screened for anti-LDL antibodies. Cells in the highest dilution still positive for anti-LDL antibodies were assumed to be monoclonal.

3.2.1 Production of murine ascites containing monoclonal anti-LDL antibodies.

A hybridoma clone producing an anti-LDL antibody was transferred from a cloning plate into a 25 cm^2 flask and grown up in 5 ml RPMI-1640 in the presence of peritoneal macrophages. After 7 - 14 days, cells were centrifuged (800 rpm, 10 minutes) and resuspended in six 0.5 ml aliquots RPMI-1640. Five female mice (8 - 10 weeks old) who had been primed by an intraperitoneal injection of 0.5 ml pristane (Sigma) 1 - 3 weeks earlier were injected with 0.5 ml of the above cell suspension ($5x10^5$ to $8x10^6$ hybridoma cells) into the peritoneal cavity. Thereafter their weight was checked every second day. When weight increased by 5 - 10 grams, ascites fluid was collected via a peritoneal cannula. This procedure was repeated a maximum of two times. The ascitic fluid was centrifuged (3000 rpm, 10 minutes), aliquoted and stored at -20°C. Hybridoma cell lines not used for intraperitoneal re-injection directly after cloning were resuspended in FCS, 10% DMSO at a concentration of 10^4 cells per 0.5 ml and stored in liquid nitrogen.

3.3 Immunodetection of apoB, by Western blotting.

To assess the binding specificity of the rabbit/ sheep polyclonal and mouse monoclonal antibodies prepared previously, to apoB48 or apoB100, epitopes the antibodies were screened by Western blotting. TRL particles, (chylomicrons and VLDL) were isolated separately from plasma samples by sequential flotation ultracentrifugation (chapter II, section 2.7). Chylomicrons were isolated from postprandial plasma samples and VLDL from fasted plasma samples. The apolipoproteins of freshly isolated chylomicron and VLDL samples were prepared for SDS gel electrophoresis according to the method of *Mindham and Mayes (1992)* (chapter II, section 2.9). Apolipoproteins present were resolved by SDS PAGE

(Macguire, Lee and Connelly 1989). Electrophoresis was performed using vertical slab gel apparatus (SE 600; Hoefer Scientific Instruments), 1.5 mm thick gels and a 15 tooth well former. The gel consisted of 3.6% acrylamide, with an acrylamide (BDH 44353 5P) - bisacrylamide (BDH 44355 5R) ratio of 20:1, 19% glycerol (v/v) (BDH 101186M), 1.2% SDS, 0.08% TEMED (Electran (BDH) 44308), 0.019% ammonium persulphate (BDH 44307) in 0.8 M Tris/phosphate pH 6.8 and was made up according to the author's protocol. The samples were applied to gels (up to 1500 μg of chylomicron protein and approximately 500 μg VLDL protein). The lower buffer chamber was filled with 4 litres of 0.1 M sodium dihydrogen phosphate buffer (BDH 307165 U) pH 7, and the upper chamber with the same buffer containing 0.1% SDS. Electrophoresis was carried out at a constant current of 20 mA for 30 minutes and then at 60 mA for 4.5 hours at room temperature, water cooled. ApoB100 and apoB48 peptides were identified using molecular weight markers and LDL as a apoB100 marker (prepared as samples in chapter II, section 2.9) and also by Western blot analysis.

The method was used to identify the presence of apoB48 and apoB100 in lipoprotein fractions isolated by immunoaffinity chromatography and for Western blotting of apoB for screening antibodies to generate the immunoaffinity chromatographic column.

3.3.1 Western blotting.

Samples and gels were prepared and gel electrophoresis carried out as above. Antibodies raised to apoB to be used for screening were prepared as in sections 3.1 and 3.2. The efficiency of transfer was assessed by staining blots with Ponceau S (chapter II section 2.10.2), a total protein stain that does not interfere with subsequent immunodetection.

For blotting SDS PAGE gels, nitrocellulose membranes, blotting paper (Whatman 3MM) and Scotch-brite pads were soaked in transfer buffer (192 mM glycine, 25 mM Tris base, 20%(v/v) methanol, pH 7.9) for 30 minutes, assembled (chapter II, figure 2.1) and blotted overnight (4^{o}C, at 10 - 14 V) using the Transphor transfer cell (Hoefer Scientific Instruments). The blots were stained with Ponceau S (0.1% (w/v), 5% acetic acid) for 5 - 30 minutes to visualise the transferred peptides, and destained with distilled H_2O until the background was white, (the gel was also stained with Coomassie Brilliant Blue, see chapter II, section 2.10.1 to verify that transfer had occurred). The blot was colour photocopied and then completely destained with distilled H_2O. The blot was then incubated with blocking buffer (0.01 M Tris

HCl, 0.055% (v/v) Tween 20, 0.15 M NaCl, pH 7.4 containing 5% (w/v) milk powder (Marvel)) (as in apoE phenotyping chapter II, section 2.5) for 60 minutes, on an orbital mixer (Denley-Tech Ltd) and washed in two changes of buffer A (10 mM Tris HCl, 0.055% (v/v) Tween 20, 0.15 M NaCl pH 7.4). The blot was incubated with the primary antibody (either the polyclonal sheep or rabbit anti-apoB antibody or the monoclonal murine anti-LDL antibody (LDL A4) diluted in buffer A (20 μl serum/ ascites in 20 ml buffer A) overnight at room temperature and washed as before. It was then incubated with the secondary antibody (either anti-sheep IgG HRP, anti-rabbit IgG HRP or goat-anti-mouse IgG HRP, depending on the chosen primary antibody) for 2 hours. The blot was washed as before then developed for 45 minutes in a substrate solution containing 60 mg 4-chloronaphthol (Sigma 8890) in 10 ml methanol, gently mixed with 60 ml saline and 30 μl hydrogen peroxide (30%) in the dark, washed with distilled water, dried at room temperature, photocopied and stored in the dark.

3.3.2 Results.

Lanes 1 - 11 of figure 3.1, contain the apolipoprotein content resolved from freshly isolated TRL ($d < 1.006$g/ml) fractions. Several repetitions of the Western blotting procedure were carried out using these antibodies to ensure that the results obtained were reproducible. The immunoblots showed that the sheep and rabbit polyclonal antibodies bound to both apoB100 or apoB48 isolated from TRL ($d < 1.006$ g/ml) samples. Figure 3.1 shows examples of rabbit (lanes 6 - 9)/ sheep (lanes 1 - 5) polyclonal antibodies and a mouse monoclonal LDL A4 antibody (lanes 10, 11) binding to apoB, with the polyclonal antibodies binding to all apoB fragments on the Western blot, and the mouse monoclonal antibody seeming to be more specific in its binding. As a result the sheep and rabbit polyclonal antibodies were discounted as potential tools for separation, although their lack of specificity made them useful for showing the presence of both apoB48 and apoB100 protein bands previously isolated from chylomicron and VLDL fractions respectively, thus allowing the specificity of other antibodies for either apoB48 or apoB100 to be clearly determined.

Figure 3.2 shows Ponceau S staining of proteins after transfer to a nitrocellulose membrane before antibody incubations. High and low molecular weight markers are present at either side of the nitrocellulose membrane. Lanes 1, 2, 3, 9, 10, and 11 contain chylomicron apolipoproteins (apoB48) and lanes 4 - 8 contain VLDL apolipoproteins (apoB100). The presence of apoB100 protein bands can be clearly seen in lanes 4 - 8, with faint apoB100 protein bands also present in the lanes containing chylomicron samples. ApoB48 protein bands can be clearly seen in lanes 1, 2, 3, 9, 10

and 11. After complete destaining, the blot was cut in half with lanes 1 - 5 in one half and incubated with the primary mouse monoclonal (LDL A4) antibody followed by a secondary antibody goat-anti-mouse IgG HRP. The other half containing lanes 6 - 11 was incubated with the primary polyclonal rabbit antibody, followed by the secondary antibody anti-rabbit IgG HRP.

Figure 3.3 shows immunoblot results after above incubation with both primary and secondary antibodies. In lanes 1 - 5 there is an apoB100 protein band (faint in lanes 1 - 3) with no detectable apoB48 protein band present in any the lanes which from figure 3.2 we know should be present in at least lanes 1 - 3 (apoB48 containing fractions). This indicates that the monoclonal antibody (LDL A4) is specific for apoB100 with no reaction to apoB48. In lanes 6 - 11, incubated with the non-specific polyclonal rabbit antibody, there were clearly detectable levels of both apoB100 (in VLDL and chylomicron samples) and apoB48 (strong protein bands in chylomicron samples and faint apoB48 protein bands in VLDL samples) present.

It was concluded from Western blotting results that the mouse monoclonal antibody LDL A4, binds selectively to apoB100 (present on VLDL) and not to apoB48 (present on chylomicrons). On the basis of these results it was decided to set up a monoclonal antibody column, using LDL A4 to attempt to physically separate TRL particles into apoB48 and apoB100 TRL species (chylomicrons and VLDL) by immunoaffinity chromatography.

3.4 Preparation of an immunoaffinity chromatography column.

The total protein concentration of the ligand (LDL A4) to be coupled to the CNBr A Sepharose gel was measured and calculated by Lowry assay (chapter II, section 2.3) and was found to be 25 mg/ml.

Lipoprotein fractions were prepared for immunoaffinity chromatography by sequential flotation ultracentrifugation according to the method described in chapter II, section 2.7. The required amount of freeze dried CNBr A Sepharose (1 gram = 3.5 ml final volume of swollen gel, a final volume of 10 ml of gel = 2.86 grams) (Pharmacia Biotech code no. 17-0430-01) was weighed out and suspended in 1 mM HCl (Sigma H 7020). The gel swells immediately and was washed for 15 minutes with 1 mM HCl on a sintered glass filter (approximately 200 ml 1 mM HCl/ gram freeze dried powder).

3.4.1 Coupling the ligand.

The ligand (LDL A4 antibody - 2 ml as determined by Lowry assay, chapter II, section 2.3) was dissolved in coupling buffer, (0.1 M sodium hydrogen carbonate ($NaHCO_3$) pH 8.3 containing 0.5 M NaCl, 5 ml coupling buffer/ gram freeze dried powder). About 5 - 10 mg protein per ml gel was used. The swollen gel and coupling buffer containing the ligand were placed in a stoppered vessel and the mixture rotated overnight at 4°C on a spiramixer (Denley). Excess ligand was removed by washing on a sintered filter with 5 gel volumes of coupling buffer (70 ml). Protein levels in the washings were determined by Lowry assay, (chapter II, section 2.3) to allow determination of % ligand bound to the gel, using the equation below;

% Ligand bound = (total ligand (protein before coupling) - total unbound ligand (protein in washings x volume of washings)/ total ligand protein) x100.

The column was prepared and Lowry assays performed to determine the total protein concentration of the ligand and the washings after coupling of ligand to resin, in order to estimate the amount of unbound ligand. This value was used to calculate % of ligand bound to resin. The calculation used is below.

Total ligand protein concentration = 25098 μg/ml x 2 ml (volume of ligand coupled)
= 50196 μg
= 50.2 mg
(10 ml column, 5.2 mg/ml ligand)

Total unbound ligand protein concentration = 43.7 μg/ml x 75 ml (total volume of washings)
= 3277 μg
= 3.28 mg.

Ligand bound to resin = 50.2 - 3.28
= 46.92 mg

% ligand bound to resin = (46.92/ 50.2) x 100
= 93.5%

i.e. 93.5 % of ligand (LDL A4) was coupled to the gel.

The coupled gel was suspended in 0.1 M Tris HCl (Sigma T-3253) buffer pH 8 for 2 hours to block any remaining active groups and washed on a sintered filter with 3 cycles of alternating pH. Each cycle consisted of a wash with 0.1 M acetate buffer (BDH 10235) pH 4.0 containing 0.5 M NaCl followed by 0.1 M Tris HCl buffer pH 8.0 containing 0.5 M NaCl.

The gel slurry was packed in a column and equilibrated with 10 column volumes of phosphate buffer (0.0675 M sodium dihydrogen orthophosphate (NaH_2PO_4), 0.01% Na_2EDTA, pH 7.3). A 10% LPDP solution (prepared according to the method in chapter II, section 2.11) was passed down the column to block any remaining non-specific binding sites followed by a further 10 column volumes of phosphate buffer. Two ml fractions were collected and the absorbance at 280 nm measured. The absorbance increased and then decreased to starting levels as the unbound LPDP was washed from the column. The column was stored at 4^{o}C until required for use.

3.5 Testing of the LDL A4 monoclonal antibody immunoaffinity chromatography column.

Once the LDL A4 monoclonal antibody immunoaffinity column was equilibrated with phosphate buffer, samples of both human VLDL (as a check of Western blotting results) and LDL (to which to antibody was originally raised) were applied to the resin on separate occasions. Once the sample was bound to the resin, an agent for elution had to be decided upon.

Initially the flow rate was set at 1 ml/ minute. One ml of VLDL isolated from a fasted plasma sample (according to the method in chapter II, section 2.7) was applied to the top of the column using a plastic pipette. The sample was allowed to run into the resin and the resin overlayered (in the same manner as the sample application) with phosphate buffer and left to stand (1 hour) to allow binding to occur. Five column volumes (50 ml) of phosphate buffer were passed through the column, with 2 ml eluant fractions collected. The absorbance of these fractions was read at 280 nm to monitor protein (sample) elution (figure 3.4).

The absorbance at 280 nm did not alter, suggesting that the VLDL remained bound to the column. Several agents for elution of VLDL from the immunoaffinity column were tested. The possibilities included and tried were solutions with

- high pH,
- high salt concentrations and
- chaotropic agents.

The immunoaffinity column was equilibrated with phosphate buffer, 2 ml fractions were collected and the absorbance read at 280 nm. Next, 5 column volumes (50 ml) of phosphate buffer (containing 0.5 M NaCl), followed by 5 column volumes (50 ml) of phosphate buffer (this time containing 1 M NaCl) were passed through the column. As previously, 2 ml fractions were collected and their absorbance read at 280 nm. There was no change in the absorbance at 280 nm in the fractions eluted using either phosphate buffer, phosphate buffer containing 0.5 M or 1 M NaCl (figure 3.4) detected. That is high salt concentrations did not dissociate VLDL from the affinity column.

The immunoaffinity column was pre-washed with phosphate buffer as before. glycine buffer pH 10.3 (15 ml) was passed through the column. Again, 2 ml fractions were collected and their absorbance read at 280 nm. Phosphate buffer was washed through the immunoaffinity column until washings returned to pH 7, as determined with pH paper. There was no alteration in the absorbance at 280 nm (figure 3.4) suggesting that the VLDL remained bound to the column, thus high pH conditions did not release VLDL from the immunoaffinity column.

The column was pre-washed with phosphate buffer. It was decided to use 1 M sodium thiocyanate (NaSCN) to try and dissociate VLDL from the column. Two and a half column volumes (25 ml) of 1 M NaSCN buffer were passed through the column. One ml fractions were collected and the absorbance at 280 nm measured. The absorbance at 280 nm increased, peaked between fractions numbers 4 - 15, then decreased (figure 3.4). This indicated that VLDL was being eluted from the column. The eluted fractions (4 - 15) with the highest absorbances at 280 nm, were pooled and concentrated using the Amicon concentration system in a Ti 60 rotor by centrifugation (5000 rpm, 20 minutes then 2000 rpm, 2 minutes). The resulting concentrated fraction was then prepared for gel electrophoresis to determine if there was any apoB100 present in the fraction to indicate the presence of VLDL. A pooled, concentrated sample of the phosphate buffer eluted fractions was also prepared for gel electrophoresis.

An faint apoB100 protein band was detectable in the NaSCN eluted fraction. No apoB100 protein band was detectable in the phosphate buffer eluted fraction. This suggested that VLDL had been bound to the monoclonal LDL A4 antibody and could be dissociated from the column using 1 M NaSCN. For future reference, fractions eluted using phosphate buffer were designated the apoB48 (unbound) fraction while fractions eluted with 1 M NaSCN were designated the apoB100 (bound) fraction.

3.5.1 Testing of LDL A4 monoclonal antibody immunoaffinity column using VLDL and LDL isolated from a fasted plasma sample.

VLDL and LDL fractions were prepared from fasted plasma samples by sequential flotation ultracentrifugation (chapter II, section 2.7). Both VLDL (1 ml) and LDL (1 ml) were applied to the immunoaffinity column on separate occasions. On each occasion, the column was washed with phosphate buffer (2 column volumes) (20 ml) followed by 2 column volumes (20 ml) of 1 M NaSCN. Eluted fractions (0.5 ml) were collected and their absorbances read at 280 nm.

In both VLDL and LDL elution profiles, measurement of the absorbance at 280 nm (figure 3.5) detected the presence of 2 elution peaks. The first elution peak was detected in the apoB48 (unbound) fraction in fraction numbers 10 - 20. The second elution peak, as expected was detected in the apoB100 (bound) fraction over fraction numbers 40 - 80.

The absorbance of all eluted fractions was also measured at 500 nm (figure 3.6). Absorbance at 500 nm is a measure of sample turbidity; lipoproteins scatter light at this wavelength but free proteins do not, they only absorb at 280 nm. As a result the elution peak present in the apoB48 (unbound) fraction all but disappeared, while the apoB100 (bound) fraction elution peaks were still detectable. These results indicate that free proteins, maybe plasma proteins, but not lipoproteins, were responsible for the elution peak detected in the apoB48 (unbound) fraction. The lipoproteins, VLDL and LDL were responsible for the elution peak present in the apoB100 (bound) fraction only.

The eluted fractions with the highest absorbances at 280 nm, fraction numbers 10 - 20 (apoB48 (unbound) fraction) and fraction numbers 40 - 80 (apoB100 (bound) fraction) were pooled, concentrated and any apolipoproteins present resolved by gel electrophoresis. SDS glycerol gel electrophoresis results confirmed previous results (figures 3.7, 3.8). ApoB100 protein bands were detectable in the apoB100 (bound)

fractions from both VLDL (figure 3.7) and LDL (figure 3.8) eluted fractions, with no apoB100 protein bands detectable in either of the apoB48 (unbound) fractions.

In conclusion, both VLDL and LDL bind to the monoclonal antibody, LDL A4, which is coupled to activated CNBr Sepharose resin. Elution from the column is accomplished with the use of 1 M NaSCN. Measurement of absorbance at 500 nm is now no longer required since the presence of the peak in the apoB48 (unbound) fraction was determined and found to be of no importance in that it was not produced by the lipoproteins and did not contain detectable levels of triglyceride.

Once the basic immunoaffinity chromatographic methodology was set up, the next step was to use the immunoaffinity column, now known to bind VLDL, to test whether physical separation of TRL fractions into apoB48 and apoB100 TRL containing fractions is possible from a postprandial plasma sample.

3.5.2 Method for elution of lipoprotein samples from the monoclonal antibody immunoaffinity column.

One ml of lipoprotein sample was applied to the column and overlayered with phosphate buffer and left to stand for 1 hour. The apoB48 (unbound) fraction was washed from the column in a first step with 2 column volumes of phosphate buffer. The apoB100 (bound) fractions were dissociated using 2 column volumes 1 M NaSCN, 0.0675 M NaH_2PO_4, 0.01% Na_2EDTA buffer, pH 7.3 and eluted fractions collected. The column was finally washed with phosphate buffer and stored at 4°C. Triglyceride and cholesterol levels were measured in eluted fractions by the methods described in chapter II, sections 2.2.1 and 2.2.2.

3.5.3 Testing of the monoclonal antibody immunoaffinity column using postprandial plasma samples.

A subject was given a fat rich meal. Blood (40 ml) was collected into Na_2EDTA tubes 3 - 4 hours after ingestion of the meal. Plasma was harvested by centrifugation (3000 rpm, 15 minutes, 4°C). The TRLs (chylomicrons and VLDL) were isolated together from plasma by a single sequential flotation centrifugation step (39000 rpm (108000 g), 16 hours, 4°C) at a density of $d < 1.006$g/ml and removed in the top 2 ml.

After washing the immunoaffinity column with 2 column volumes (20 ml) of phosphate buffer, 1 ml of the freshly prepared TRLs was applied to the column and left to stand for 1 hour. Phosphate buffer was passed through the column (2 column volumes - 20 ml) followed by 1 M NaSCN (2 column volumes - 20 ml) followed by further washing with phosphate buffer (10 column volumes - 100 ml). The flow rate was as previously. One ml fractions were collected with the absorbance measured at 280 nm (figure 3.9). Fractions with the highest absorbances were pooled and (for both unbound and bound fractions) concentrated using the Amicon Centrificon method as previously. Triglyceride and cholesterol concentrations were measured in pooled fractions (according to the method in chapter II, section 2.2)

Results.

Absorbance at 280 nm (figure 3.9) showed the presence of 2 peaks. The first large peak appears during washing with phosphate buffer (fraction numbers 1 - 8) in the apoB48 (unbound) fraction, suggesting the elution of light scattering chylomicrons from the immunoaffinity column. The second smaller peak (fraction numbers 24 - 37) appears during column washing with 1 M NaSCN, in the apoB100 (bound) fraction, suggesting dissociation of VLDL from the column. The absorbance peak for chylomicrons is large since these particles scatter more light than VLDL because of their size. The fractions contributing to both peaks (fraction numbers 1 - 8 and 24 - 37 for the apoB48 (unbound) and apoB100 (bound) fractions, respectively) were pooled and concentrated using the Amicon Centrificon system. SDS gel electrophoresis detected the presence of an apoB100 protein band in the apoB100 (bound) fraction. There was no apoB48 protein band visible in the apoB48 (unbound) fraction. Triglyceride and cholesterol levels were measured in the pooled samples and adjusted to plasma levels to account for any dilutions (chapter II, section 2.2) (table 3.1).

Table 3.1: ***Triglyceride and cholesterol levels in pooled samples isolated from postprandial samples by immunoaffinity (LDL A4 monoclonal antibody) chromatography.***

Sample	Triglyceride (mmol/L)	Cholesterol (mmol/L)
apoB48 (unbound) fraction	8.27*	0.09
apoB100 (bound) fraction	>11*	0.32

an early problem that was encountered was the very high triglyceride levels, found to be due to the presence of glycerol, which is measured by the triglyceride assay, in the Amicon Centrificon concentration filters. This difficulty was overcome by thorough pre-rinsing of filters with distilled water.

Although triglyceride levels were high and not representative of triglyceride present in the eluted fractions, the cholesterol levels were of use. The cholesterol concentrations appear consistent with concentrations found in chylomicron and VLDL fractions. On the basis of these results it seems possible that TRLs could be physically separated into apoB48 and apoB100 TRL species (chylomicrons and VLDL respectively) by immunoaffinity chromatography with further testing required.

3.5.4 Further Testing.

Chylomicron, VLDL and chylomicron and VLDL mixed (TRL $d < 1.006$g/ml) fractions were prepared by sequential flotation ultracentrifugation (chapter II, section 2.7) from postprandial plasma. Chylomicron and VLDL samples were isolated separately and also together at $d < 1.006$g/ml. Each sample (1 ml) underwent immunoaffinity chromatography. Absorbances of eluted 0.5 ml fractions were measured at 280 nm. Those fractions with increased absorbance at 280 nm were pooled and triglyceride and cholesterol levels measured and adjusted back to plasma levels (to correct for dilution factors)(chapter II, section 2.2).

Results.

The chylomicron sample produced a single elution peak in the apoB48 (unbound) fraction (figure 3.10) (fraction numbers 4 - 22) while the VLDL sample produced 2 elution peaks (figure 3.11). The first was in the apoB48 (unbound) (fraction numbers 4 - 20) and the second was in the apoB100 (bound) (fraction numbers 45 - 80) fractions. The TRL ($d < 1.006$g/ml) fraction also produced 2 peaks (figure 3.12), the first in the apoB48 (unbound) fraction (over fraction numbers 3 - 20) and the second after elution with 1 M NaSCN in the apoB100 (bound) fraction (over fraction numbers 45 - 70).

The peak detected in the apoB100 (bound) fraction (in fraction numbers 45 - 70/ 80) from both the VLDL and the TRL ($d < 1.006$g/ml), that were originally applied to the immunoaffinity column, is due to the dissociation of VLDL from the affinity column by 1 M NaSCN, a phenomenon that has been previously noted and confirmed by gel electrophoresis results. The elution peak present in the apoB48 (unbound) fraction of

chylomicron, TRL ($d < 1.006$ g/ml) and VLDL samples, eluted over fraction numbers 4 - 20 in each case, was suggested to be a result of washing of chylomicrons from the immunoaffinity column, with phosphate buffer. The presence of such a peak in the original VLDL sample initially applied to the immunoaffinity column could be due to contamination of the VLDL fraction with chylomicrons during the isolation by ultracentrifugation, or to the presence of small chylomicron remnants in plasma. This peak contained low levels of triglyceride. It is very difficult to separate chylomicrons and VLDL from a postprandial plasma sample by ultracentrifugation alone, which is the reason for establishing the immunoaffinity column. During centrifugation, large VLDL may spin up into the density fraction containing chylomicrons, with some of the small chylomicron remnants spinning down into the VLDL density fraction.

SDS gel electrophoresis (figure 3.13, lanes 1 and 2) showed detectable apoB100 protein bands in the apoB100 (bound) fractions, but no apoB48 protein bands were detectable in the apoB100 (bound) fractions. Faint apoB48 protein bands were detected in the apoB48 (unbound) fractions. The immunoaffinity column provides very adequate separation of chylomicrons and VLDL. The triglyceride and cholesterol concentrations (table 3.2) reaffirm the results from the elution profiles and SDS gels. However recovery of initial sample added to the immunoaffinity column was 33 - 47% as estimated by measuring the recovered triglyceride in the bound and unbound fractions, adjusted back to plasma levels (to correct for dilution factors). This value was low and needed to be improved.

Conclusions.

From the triglyceride and cholesterol results, elution profiles and SDS gel electrophoresis results, the immunoaffinity column appears to be giving adequate separation of apoB48 and apoB100 TRL species (chylomicrons and VLDL). The chylomicrons are eluted in a first step using phosphate buffer with VLDL dissociated from the column in a second step with 1 M NaSCN. However the % recovery of initial TRL added to the column is low, ranging from 33 - 47%. It is clear that the methodology is working but modifications are required to improve the recovery in order to use the method for quantification of postprandial responses.

From the elution profiles, chylomicrons are eluted in approximately the first 11 ml phosphate buffer and VLDL dissociated in approximately the first 15 ml NaSCN (or in 20 - 35 ml of total volume), therefore instead of collecting all fractions and measuring absorbances and pooling the relevant fractions, which is very time

consuming, the stated volumes of buffers representative of the apoB48 (unbound) and apoB100 (bound) fractions were collected and then concentrated.

Table 3.2: Triglyceride (TG) and cholesterol (Chol) levels in samples separated by immunoaffinity (LDL A4 monoclonal antibody) chromatography.

Samples applied to column and eluted fractions	**TG (mmol/L)**	**Chol (mmol/L)**	**TG adjusted to plasma levels (mmol/L)**	**Chol adjusted to plasma levels (mmol/L)**	**Lowry (μg/ml) adjusted to plasma levels**	**% TG recovery**
Chylomicrons	1.04	0.13	0.52	0.065	523	-
apoB48 (unbound)*	0.3	0.03	0.24	0.024	102.5	**46**
apoB100 (bound)*	0	0	0	0	136.7	-
VLDL	2.09	0.7	1.045	0.35	347.5	-
apoB48 (unbound)*	0.25	0.04	0.125	0.02	119.7	**33**
apoB100 (bound)*	0.3	0.07	0.225	0.0525	276.71	-
TRL d < 1.006g/ml	1.75	0.45	0.875	0.225	660.5	-
apoB48 (unbound)*	0.29	0.04	0.33	0.046	362.5	**47**
apoB100 (bound)*	0.11	0.14	0.0825	0.105	167.6	-

**Samples of chylomicrons, VLDL and TRL (d < 1.006g/ml) were initially applied to the resin and separated into apoB48 (unbound) and apoB100 (bound) fractions by immunoaffinity chromatography with the LDL A4 monoclonal antibody.*

3.5.5 Separation of chylomicrons and VLDL by immunoaffinity chromatography from postprandial samples (Pilot Study) as a check of method reproducibility.

TRL ($d < 1.006$g/ml) fractions (chylomicron and VLDL mix) were prepared from 16 postprandial plasma samples by sequential flotation ultracentrifugation (chapter II, section 2.7). The freshly isolated TRL fractions were subjected to immunoaffinity chromatography (section 3.5.2). Triglyceride and cholesterol concentrations were measured in the TRL ($d < 1.006$g/ml), the apoB48 (unbound) and apoB100 (bound) fractions (chapter II, section 2.2) then concentrations were adjusted back to plasma levels (to correct for dilution factors). SDS gel electrophoresis was performed to check for adequate separation.

Results.

SDS gels show the presence of apoB100 protein bands in the apoB100 (bound) fractions with no detectable apoB100 and faint apoB48 protein bands in the apoB48 (unbound) fractions. There were no detectable apoB48 bands in the apoB100 (bound) fractions.

The recovery of initial TRL added to the immunoaffinity column was variable (table 3.3), ranging from 33 - 88%, with a mean of 58 $\pm$ 17% (mean $\pm$ SD). Further individual recoveries of TRL added to the immunoaffinity column were not very consistent. A more reproducible method was required to maintain individual sample consistency of recovery of initial TRL added to the immunoaffinity column.

The major causes of failure to recover triglyceride from the immunoaffinity column is likely to be the multiple manipulations and concentration step in which some VLDL and chylomicrons may stick to the filter.

Table 3.3: ***Recoveries of initial TRL (d < 1.006g/ml) added to the LDL A4 monoclonal antibody immunoaffinity column, as estimated by measuring recovered triglyceride (TG) concentration (mmol/L) in the TRL (d < 1.006g/ml) apoB48 (unbound) and the apoB100 (bound) fractions.***

Sample Number	Fasting Plasma TG (mmol/L)	TRL d < 1.006g/ml TG (mmol/L)	ApoB48 (unbound) fraction TG (mmol/L)	ApoB100 (bound) fraction TG (mmol/L)	TG recovered (%)
1	1.94	1.34	0.12	0.33	**33**
2	2.02	1.265	0.31	0.12	**34**
3	2.43	1.34	0.37	0.13	**37**
4	2.28	1.29	0.43	0.16	**46**
5	1.7	1.04	0.18	0.36	**52**
6	1.63	1.05	0.34	0.27	**58**
7	1.83	1.03	0.48	0.22	**68**
8	2.43	1.35	0.57	0.19	**56**
9	1.68	0.77	0.31	0.23	**70**
10	1.22	0.57	0.18	0.21	**68**
11	1.07	0.45	0.17	0.23	**88**
12	0.99	0.48	0.11	0.17	**58**
13	1.08	0.58	0.13	0.17	**51**
14	1.74	0.93	0.47	0.21	**73**
15	1.37	0.8	0.47	0.22	**86**
16	1.18	0.69	0.26	0.19	**65**

3.6 Immunoaffinity chromatography, modified method *(Cohn et al 1993).*

In the modified approach, separation of apoB48 TRL and apoB100 TRL fractions was carried out in 1.5 ml Eppendorf tubes. Antibody containing sepharose gel (section 3.4) in phosphate buffer pH 7.3 (approximately 1.4 ml) was aliquoted into the eppendorf tubes and centrifuged (5 minutes, 1000 rpm). The supernate was aspirated and TRL ($d < 1.006$g/ml) (0.5 ml) added to the remaining 0.6 ml of packed sepharose gel. Tubes were mixed for 60 minutes at room temperature on a roller mixer, centrifuged and the supernate was collected (approximately 0.5 ml was retrieved). The gel was washed with 1 ml phosphate buffer, rocked for 20 minutes, and after centrifugation, the supernate was aspirated and added to the first supernate. The washing procedure was repeated with a second volume of phosphate buffer. This sample, termed the apoB48 (unbound) fraction contained the apoB48 TRL fraction (chylomicrons) (final volume = 2.5 ml). Lipoproteins bound to the gel were dissociated by the addition of 1 M NaSCN buffer (1.0 ml). Samples were rocked for an hour at room temperature, centrifuged, and the supernate was collected. The gel was washed with 2 further 1.0 ml aliquots of 1 M NaSCN, and these supernates were added to the first NaSCN supernate. This sample the apoB100 (bound) fraction (final volume = 3.0 ml) contained the apoB100 (VLDL) TRL fraction. Recovery of initial TRL fractions added to the immunoaffinity gel (using Eppendorfs) was estimated by measuring the recovered triglyceride in the bound and unbound fractions. The identity of the apoB protein bands was confirmed by either SDS gel electrophoresis or by Western blotting analysis using a polyclonal antibody, previously shown to react with both apoB48 and apoB100. Triglyceride and cholesterol concentrations in the TRL ($d < 1.006$g/ml), apoB48 (unbound) and apoB100 (bound) fractions were measured (chapter II, section 2.2), and adjusted to plasma levels to allow calculation of the recovery of initial TRL added to the immunoaffinity gel.

Results and discussion.

Specific monoclonal antibodies for apoB100 have been used previously *(Milne et al 1984, Terce et al 1985)* to isolate and characterise the very low density lipoprotein (VLDL) fraction of dyslipidaemic subjects in the fasted state. *Cohn et al (1993)* used the same antibodies to isolate the apoB48 and apoB100 containing TRL from plasma obtained from subjects in a fed state. In order to obtain reliable quantitative data, Cohn separated single sample aliquots in individual Eppendorf tubes, containing the antibodies bound to sepharose, rather than eluting fractions from immunoaffinity chromatography columns *(Milne et al 1984)*. With certain modifications *Cohn et al (1993)* achieved an

acceptable recovery (85 - 95%) of TRL and allowed for the plasma concentration of different fractions to be estimated. Some contamination of the apoB48 (unbound) fraction with apoB100 was noted. The adequacy of separation of chylomicrons and VLDL was monitored by SDS polyacrylamide gel electrophoresis.

In the present work, the method of *Cohn et al (1993)*, which used separate single sample aliquots in individual Eppendorf tubes containing LDL A4 antibody bound to sepharose, rather than eluting fractions from the immunoaffinity chromatography columns (as previously described chapter III, section 3.5.2), was followed. However, instead of using 3 M NaSCN, 1 M NaSCN was used to dissociate the apoB100 containing TRL (VLDL) fraction from the immunoaffinity gels.

This method was thoroughly and repeatedly tested to ensure reproducibility of results and of TRL recoveries. Several VLDL (isolated from fasted plasma samples), chylomicron and TRL ($d < 1.006$g/ml) (isolated from postprandial plasma) samples were prepared by sequential flotation ultracentrifugation (chapter II, section 2.7) and were subjected to immunoaffinity chromatography in Eppendorf tubes to separate the TRL into apoB48 and apoB100 TRL fractions.

There was an apoB100 protein band present in the apoB100 (bound) fraction. A faint apoB48 protein band was detected in the apoB48 (unbound) fraction (figure 3.13, lanes 3 and 4), with no apoB48 protein detectable in the apoB100 (bound) fraction, however there was some contamination of the apoB48 (unbound) fraction with apoB100 noted, but this would only lead to an underestimation of apoB100 TRL lipid concentrations. This problem was also noted by *Cohn et al (1993)*. Additional chromatography was not carried out to remove contaminating apoB100 material, as this would adversely effect recovery.

Triglyceride and cholesterol concentrations in each of the fractions were measured and results were as expected. In the VLDL samples, the majority of triglyceride was in the apoB100 (bound) TRL fraction (table 3.4). In the chylomicron samples (table 3.5), the majority of triglyceride was detected in the apoB48 (unbound) TRL fraction; and finally in the postprandial TRL ($d < 1.006$g/ml) samples the triglyceride concentrations were split between the apoB48 (unbound) and apoB100 (bound) containing TRL fractions, with levels varying between samples (table 3.6).

Table 3.4: ***Triglyceride and cholesterol concentrations (mmol/L) (adjusted to plasma levels) and triglyceride recoveries in the apoB48 (unbound) and apoB100 (bound) fractions separated from VLDL samples were mixed with Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes, based on the method of Cohn et al (1993).***

Sample applied	**Eluted fractions**	**TG (mmol/L)**	**Chol (mmol/L)**	**TG adjusted to plasma levels (mmol/L)**	**Original TRL (d < 1.006 g/ml) TG (mmol/L) adjusted to plasma levels**	**% TG recovery**
VLDL	apoB48 (unbound)*	0.06	0	0.15	0.75	68
	apoB100 (bound)*	0.12	0.05	0.36		
VLDL	apoB48 (unbound)*	0.08	0.03	0.20	0.62	85.5
	apoB100 (bound)*	0.11	0.06	0.33		

****Samples of VLDL were initially mixed with Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes and separated into apoB48 (unbound) and apoB100 (bound) fractions.***

Table 3.5: ***Triglyceride and cholesterol concentrations (mmol/L) (adjusted to plasma levels) and triglyceride recoveries in the apoB48 (unbound) and apoB100 (bound) fractions separated from chylomicron samples were mixed with Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes, based on the method of Cohn et al (1993).***

Sample applied	Eluted fractions	TG (mmol/L)	Chol (mmol/L)	TG adjusted to plasma levels (mmol/L)	Original (d < 1.006 g/ml) TG (mmol/L) adjusted to plasma levels	% TG recovery
Chylomicron 1	apoB48 (unbound)*	0.28	0.06	0.7	0.76	100
	apoB100 (bound)*	0.03	0.04	0.09		
2	apoB48 (unbound)*	0.28	0.03	0.7	0.75	97
	apoB100 (bound)*	0.01	0	0.03		
3	apoB48 (unbound)*	0.27	0.02	0.675	0.75	97
	apoB100 (bound)*	0.02	0	0.05		
4	apoB48 (unbound)*	0.26	0	0.60	0.76	83
	apoB100 (bound)*	0.01	0	0.03		

****Samples of chylomicrons were initially mixed with Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes and separated into apoB48 (unbound) and apoB100 (bound) fractions.***

Table 3.6: ***Triglyceride and cholesterol concentrations (mmol/L) (adjusted to plasma levels) and triglyceride recoveries in the apoB48 (unbound) and apoB100 (bound) fractions separated from postprandial TRL (d<1.006g/ml) samples were mixed with Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes, based on the method of Cohn et al (1993) .***

Sample applied	**Eluted fractions**	**TG (mmol/L)**	**Chol (mmol/L)**	**TG adjusted to plasma levels (mmol/L)**	**Original TRL (d<1.006 g/ml) TG adjusted to plasma levels**	**% TG recovery**
TRL (d<1.006 g/ml) 1	apoB48 (unbound)*	0.29	0.04	0.725	1.435	90.2
	apoB100 (bound)*	0.19	0.05	0.57		
2	apoB48*	0.66	0.17	1.65	2.8	94
	apoB100*	0.33	0.12	0.99		
3	apoB48*	0.15	0.04	0.375	0.88	90
	apoB100*	0.14	0.10	0.42		
4	apoB48*	0.11	0.03	0.275	0.73	91
	apoB100*	0.13	0.06	0.39		
5	apoB48*	0.07	0	0.175	0.475	93.7
	apoB100*	0.09	0	0.27		
6	apoB48*	0.06	0	0.15	0.48	87.5
	apoB100*	0.09	0	0.27		
7	apoB48*	0.05	0.01	0.125	0.475	89
	apoB100*	0.10	0.03	0.30		
8	apoB48*	0.05	0	0.125	0.48	88.5
	apoB100*	0.10	0.02	0.30		
9	apoB48*	0.44	0.08	1.1	2.075	86
	apoB100*	0.23	0.09	0.69		

Samples of TRL (d<1.006gml) were initially mixed with Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes and separated into apoB48 (unbound) and apoB100 (bound) fractions.

The individual % recoveries of initial TRL samples added to the immunoaffinity gel show little variation indicating the method was reproducible. The mean recovery of the initial TRL added to the immunoaffinity gel was calculated to be 89 ± 7% (mean ± SD) based on triglyceride concentration. This value was similar to that obtained by *Cohn et al (1993)* of 92 ± 4% (based on protein concentration). The individual recoveries showed the method to be very reproducible, however some minor contamination of the apoB48 fraction was noted, which prompted a further modification. Additional chromatography was not carried out to remove contaminating apoB100 material, as this was found to adversely affect recovery. Immunoaffinity chromatography(using a Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes) was repeated on several occasions using the same sample to check for reproducibility. Chylomicron samples 3 and 4 were repetitions of chylomicrons samples 1 and 2, while postprandial samples 7 and 8 were repetitions of postprandial samples 5 and 6. From the % recoveries of initial triglyceride added to the immunoaffinity gel; 97%, 83%, 100%, 97%, 89%, 88.5%, 93.7% and 87.5% respectively (tables 3.5 and 3.6), it can be concluded that, using this modified method, recoveries are very consistent and reproducible between samples.

The modified immunoaffinity chromatography method using single sample aliquots in individual Eppendorf tubes increased the recovery of initial TRLs added to the immunoaffinity gel to 89 ± 7%, while the previous method in which fractions were eluted from an immunoaffinity column had a recovery of 58 ± 17%. Analysis using a 2 sample t-test showed that on using individual sample recoveries for both methods that recovery of initial TRL added to the immunoaffinity gel was highly significantly *($p<0.0001$)* improved using the new modified method. The modified method has several benefits; increased recovery levels, yet using a smaller volume of sample with none of the sample being lost by concentration steps which were both time consuming and necessary when fractions were eluted from the immunoaffinity column. This new method is better for analysis and provides the increased recovery that was necessary to make using the method feasible. This methodology was used to analyse samples from oral fat load experiments in normal and diabetic (NIDDM) subjects.

3.7 Validation of modified Sepharose bound LDL A4 monoclonal antibody immunoaffinity chromatography method (in Eppendorf tubes).

Due to the noted contamination of the apoB48 (unbound) fraction, a time course experiment using TRL fractions isolated from plasma from both postprandial (5 hours postprandially) and fasted (12 hour overnight fast) samples (by sequential flotation ultracentrifugation (chapter II, section 2.7)) were used to assess the optimum initial incubation time for samples without affecting recoveries of the initial TRL added to the immunoaffinity gel.

The time points for initial incubation were 30, 60, 90, 120 and 180 minutes with no other changes to the methodology. TRL fractions were freshly prepared by sequential flotation ultracentrifugation (chapter II, section 2.7) and then subjected to immunoaffinity chromatography, the only change to the methodology being the different initial incubation times. Triglyceride and cholesterol concentrations in the TRL ($d < 1.006$g/ml), apoB48 (unbound) and apoB100 (bound) fractions were measured (chapter II, section 2.2), and adjusted back to plasma levels (to correct for dilution factors), to allow calculation of the recovery of initial TRL added to the immunoaffinity gel.

Results.

In the TRL fractions isolated from both the postprandial (table 3.7, figure 3.14) and fasted (table 3.8, figure 3.15) plasma samples, maximal binding of apoB100 containing lipoprotein fractions to the Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes without affecting recovery levels was achieved with an initial incubation period of 90 minutes, after which time binding of the apoB100 containing lipoprotein fractions to the immunoaffinity gel seems to decrease with recovery rates also falling slightly. The binding period was previously 60 minutes.

SDS gel electrophoresis results detected an apoB100 protein band present in the apoB48 (unbound) fraction even after an initial incubation step of 90 minutes, but this was reduced to minor levels.

Table 3.7: *Triglyceride concentrations (mmol/L) and recoveries in apoB48 (unbound) and apoB100 (bound) fractions. TRL fractions isolated from postprandial plasma samples, with varied initial incubation times, were mixed with Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes .*

Sample and eluate fractions	**Time of initial incubation (minutes)**	**TG (mmol/L)**	**Chol (mmol/L)**	**TG adjusted to plasma levels (mmol/L)**	**% TG recovery**
Plasma		1.62	4.52		
TRL (d < 1.006g/ml) originally applied		2.06	0.41	1.03	
apoB48 (unbound)*	30	0.28	0.03	0.70	91
apoB100 (bound)*		0.08	0.03	0.24	
apoB48 (unbound)*	60	0.27	0.00	0.67	88
apoB100 (bound)*		0.08	0.02	0.24	
apoB48 (unbound)*	**90**	**0.26**	**0.01**	**0.65**	**92**
apoB100 (bound)*		**0.10**	**0.03**	**0.30**	
apoB48 (unbound)*	120	0.25	0.01	0.62	86
apoB100 (bound)*		0.09	0.01	0.27	
apoB48 (unbound)*	180	0.27	0.01	0.67	94
apoB100 (bound)*		0.10	0.03	0.30	

**TRL fractions, isolated from postprandial plasma samples, were initially mixed with Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes and separated into apoB48 (unbound) and apoB100 (bound) fractions.*

Table 3.8: ***Triglyceride concentrations (mmol/L) and recoveries in apoB48 (unbound) and apoB100 (bound) fractions. TRL fractions isolated from fasted plasma samples, with varied initial incubation times, were mixed with Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes.***

Samples and eluate fractions	**Time of initial incubation (minutes)**	**TG (mmol/L)**	**Chol (mmol/L)**	**TG adjusted to plasma levels (mmol/L)**	**% TG recovery**
Plasma		0.74	4.46		
TRL (d < 1.006g/ml) originally applied		0.95	0.46	0.475	
apoB48 (unbound)*	30	0.09	0.01	0.225	100
apoB100 (bound)*		0.10	0.04	0.30	
apoB48 (unbound)*	60	0.07	0.02	0.175	100
apoB100 (bound)*		0.10	0.03	0.30	
apoB48 (unbound)*	**90**	**0.07**	**0.00**	**0.175**	**100**
apoB100 (bound)*		**0.10**	**0.04**	**0.30**	
apoB48 (unbound)*	120	0.06	0.01	0.15	88.4
apoB100 (bound)*		0.09	0.03	0.27	
apoB48 (unbound)*	180	0.06	0.00	0.15	88.4
apoB100 (bound)*		0.09	0.04	0.27	

****TRL fractions, isolated from fasted plasma samples were mixed with Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes and separated into apoB48 (unbound) and apoB100 (bound) fractions.***

The binding capacity of the gel was also determined experimentally to make sure that the Eppendorfs were not being overloaded as this could be a reason for the presence of a faint apoB100 protein band in the apoB48 (unbound) fraction. A saturation curve was prepared using fasting plasma samples from which VLDL was isolated by sequential flotation ultracentrifugation (chapter II, section 2.7), the new initial incubation time was used to ensure maximal binding conditions. Once isolated, the concentrated VLDL samples were diluted using phosphate buffer to give samples with triglyceride concentrations (adjusted to plasma levels) of 2.43, 1.23, 0.6, 0.3, 0.125 and

0.08 mmol/L. Triglyceride and cholesterol concentrations were measured and adjusted back to plasma levels (to correct for dilution factors), in both the apoB48 and apoB100 containing fractions separated by immunoaffinity chromatography, for each triglyceride dilution (table 3.9). A saturation curve was plotted for fasted VLDL triglyceride dilutions (mmol/L) vs actual triglyceride concentrations in the apoB48 and apoB100 TRL fractions (mmol/L) (figure 3.16) (table 3.9).

At the triglyceride dilution concentration of 2.48 mmol/L the triglyceride concentration in the apoB100 containing fraction is the highest, indicating binding of VLDL to the immunoaffinity gel is saturated (0.57 mmol/L). The binding levels may be maximal but the immunoaffinity resin is clearly overloaded with the presence of triglyceride in the apoB48 (unbound) fraction (2.05 mmol/L). To achieve a triglyceride concentration for maximal binding, a triglyceride concentration that will give as near maximal VLDL binding in the apoB100 (bound) fraction with minimal triglyceride levels present in the apoB48 (unbound) fraction was required.

It is evident from figure 3.16 that as triglyceride concentrations in the apoB48 containing fraction decrease (present as a result of overloading), they were also decreased in the apoB100 containing fraction, a minor amount of triglyceride being detectable in the apoB48 fraction even although the immunoaffinity gel was not always at maximal binding of VLDL. The binding capacity of the gel was determined by extrapolation to be 0.5 - 0.7 mmol/L VLDL associated triglyceride/ 0.6 ml of gel. This is the point where near-maximal levels of VLDL appear to have bound to the immunoaffinity gel, with the least amount present in the apoB48 (unbound) fraction, without affecting triglyceride recovery levels. There was still minor contamination of the apoB48 (unbound) fraction.

Table 3.9: ***Triglyceride concentrations (mmol/L) in apoB48 (unbound) and apoB100 (bound) fractions. VLDL fractions isolated from fasted plasma samples, with various concentrations of apoB100 VLDL TRL triglyceride, were mixed with Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes.***

Eluate Fraction	**VLDL triglyceride dilution applied to column (mmol/L)**	**Triglyceride (mmol/L)**	**Cholesterol (mmol/L)**	**Triglyceride adjusted to plasma levels (mmol/L)**	**% recovery of triglyceride**
apoB48 (unbound)*	2.48	0.82	1.31	2.05	100
apoB100 (bound)*		0.19	0.3	0.57	
apoB48 (unbound)*	1.235	0.36	0.5	0.90	100
apoB100 (bound)*		0.16	0.23	0.48	
apoB48 (unbound)*	0.6	0.14	0.04	0.35	100
apoB100 (bound)*		0.14	0.06	0.42	
apoB48 (unbound)*	0.3	0.07	0.01	0.175	100
apoB100 (bound)*		0.10	0.03	0.30	
apoB48 (unbound)*	0.125	0.04	0.01	0.10	100
apoB100 (bound)*		0.06	0.02	0.18	
apoB48 (unbound)*	0.08	0.05	0.01	0.125	100
apoB100 (bound)*		0.06	0.02	0.18	

**Samples of VLDL were mixed with Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes.*

4 Overall conclusions and future prospects.

The monoclonal antibody LDL A4 showed binding specificity for apoB100 but not apoB48. TRL ($d < 1.006$g/ml) fractions isolated by sequential flotation ultracentrifugation can be separated into apoB48 and apoB100 TRL species by immunoaffinity chromatography, using the monoclonal antibody LDL A4. Good separations were achieved. ApoB48 protein was not detectable in the apoB100 (bound) fraction, with apoB48 protein sometimes difficult to visualise in the apoB48 (unbound) fraction (only accounting for 2% of protein at the peak of postprandial lipaemia). A faint apoB100 band was often observed in the apoB48 (unbound) fraction and this contamination was noted, but to proceed with additional chromatography would adversely affect recovery of the initial TRL added to the immunoaffinity gel. The modified method using Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes, based on the technique of *Cohn et al (1993)* provided a highly significant increase in recovery to 89 $\pm$ 7% compared to the original method of eluting fractions from the immunoaffinity chromatography column (58 $\pm$ 17%). The method has been thoroughly tested and has produced satisfactory results, it provided a much improved recovery of the apoB48 TRL species, although visualisation of the apoB48 protein band in the apoB48 (unbound) fraction by SDS gel electrophoresis could be further improved. The LDL A4 monoclonal antibody coupled to the gel binds VLDL and not chylomicrons, giving a clean separation of the apoB100 TRL species from the apoB48 TRL species. The contamination of the apoB48 (unbound) fraction with apoB100 protein was noted, this would lead to a small underestimation of apoB100 TRL lipid concentrations but this underestimation would only increase the contribution of apoB100 TRL and as such enhance the results which would thus be representative of what was actually happening to VLDL postprandially. In Eppendorfs recovery at 89% is virtually complete. The inability to recover 100% of triglyceride is due in part to manipulation loses and the measurement of low concentrations of triglyceride in the washings but mainlyto the fact that there is a void volume around the gel that accounts for appoximately 10% of starting volume. Washing the gel reduced this soucre of contamination to 5%.

This methodology was used to study the effect of postprandial chylomicronaemia of the metabolism of VLDL subfractions after an oral fat load in two different subject groups; normals and diabetics (see chapters VI and VII).

Figure 3.1: *A Western blot showing the binding specificities of the tested polyclonal sheep and rabbit antibodies and the mouse monoclonal LDL A4 antibody to apoB.*

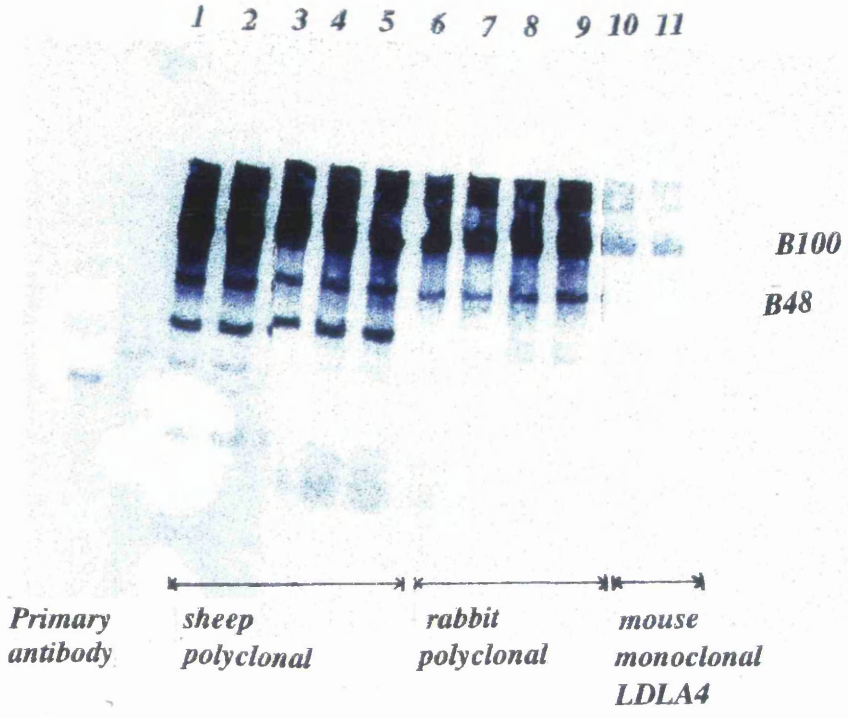


Figure 3.2: ***Ponceau S stain of apoB Western blot.*** *It shows the presence of all proteins with a sensitivity of 1-2μg. ApoB48 and apoB100 can be clearly seen in the chylomicron and VLDL fractions respectively.*

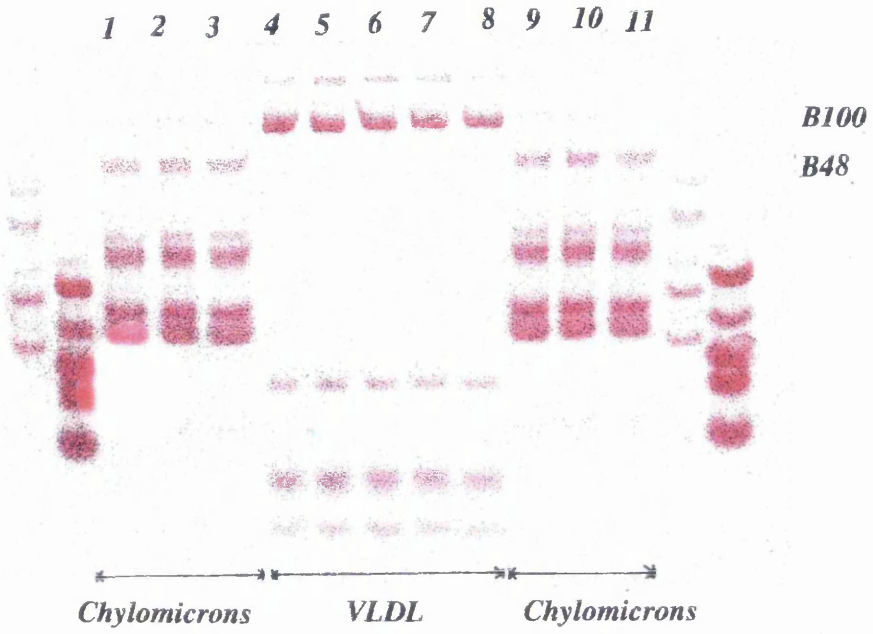


Figure 3.3: ***Western blot of apoB.*** *Half of the blot was incubated with the mouse monoclonal antibody LDL A4 and the other half with the rabbit polyclonal apo B antibody (used as a control). There is a total absence of an apoB48 band in the chylomicron fraction after incubation with LDL A4, but an apoB100 band is present in the VLDL fraction. There are apoB48 and apoB100 bands present in the chylomicron fraction, and an apoB100 band present in the VLDL fraction after incubation with the rabbit polyclonal antibody. It was concluded that only the mouse monoclonal antibody LDL A4 shows a binding that is specific to apoB100 and not apoB48.*

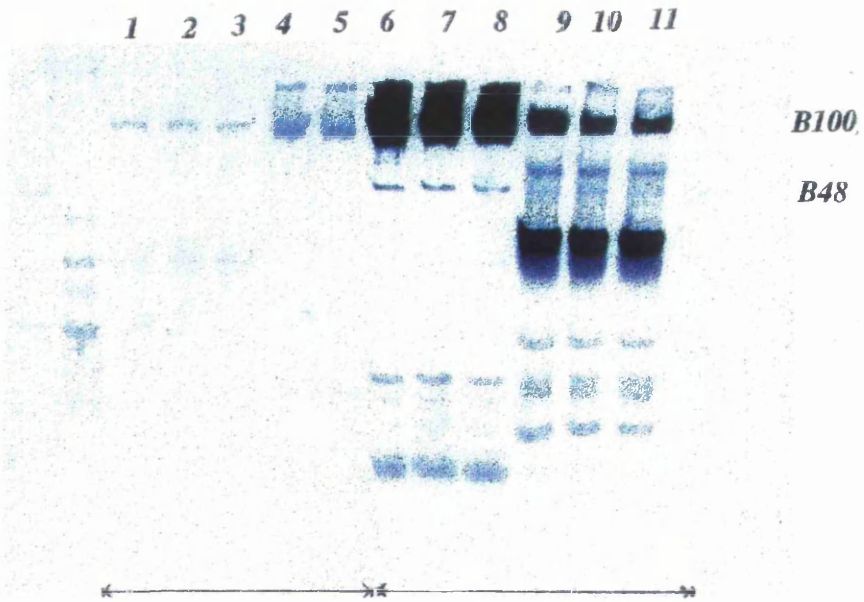


Figure 3.4: ***Elution profiles for dissociation of VLDL from an LDL A4 monoclonal antibody immunoaffinity column.*** *Testing a series of agents: 0.5M and 1M NaCl, Glycine buffer pH 10.3 and 1M NaSCN buffer, absorbance measured at 280nm.*

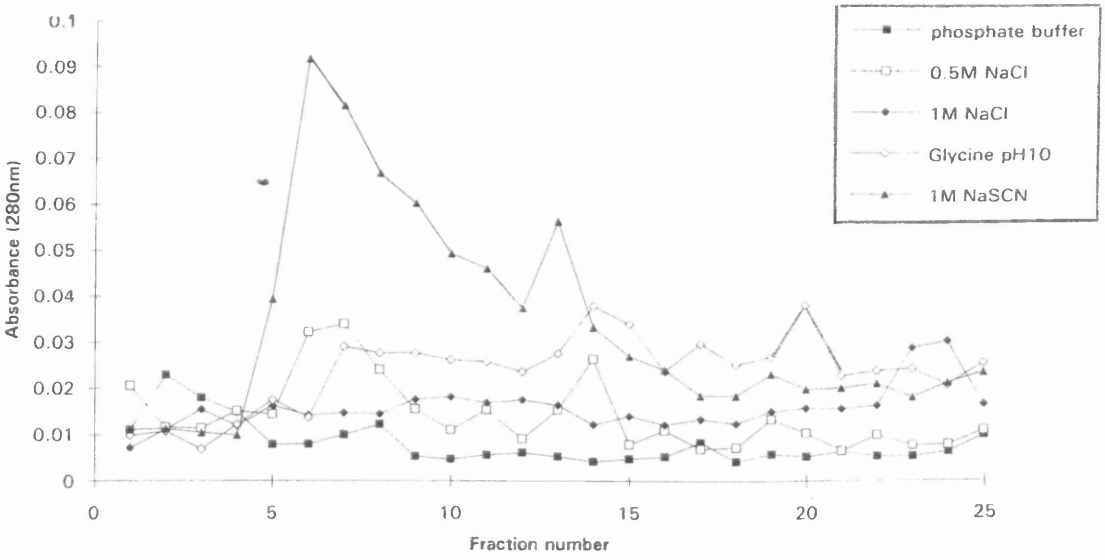


Figure 3.5: ***Elution profile of VLDL and LDL.*** *Lipoprotein samples were prepared from a fasted plasma sample by ultracentrifugation, followed by elution from an LDL A4 monoclonal antibody immunoaffinity column, with absorbance measured at 280nm.*

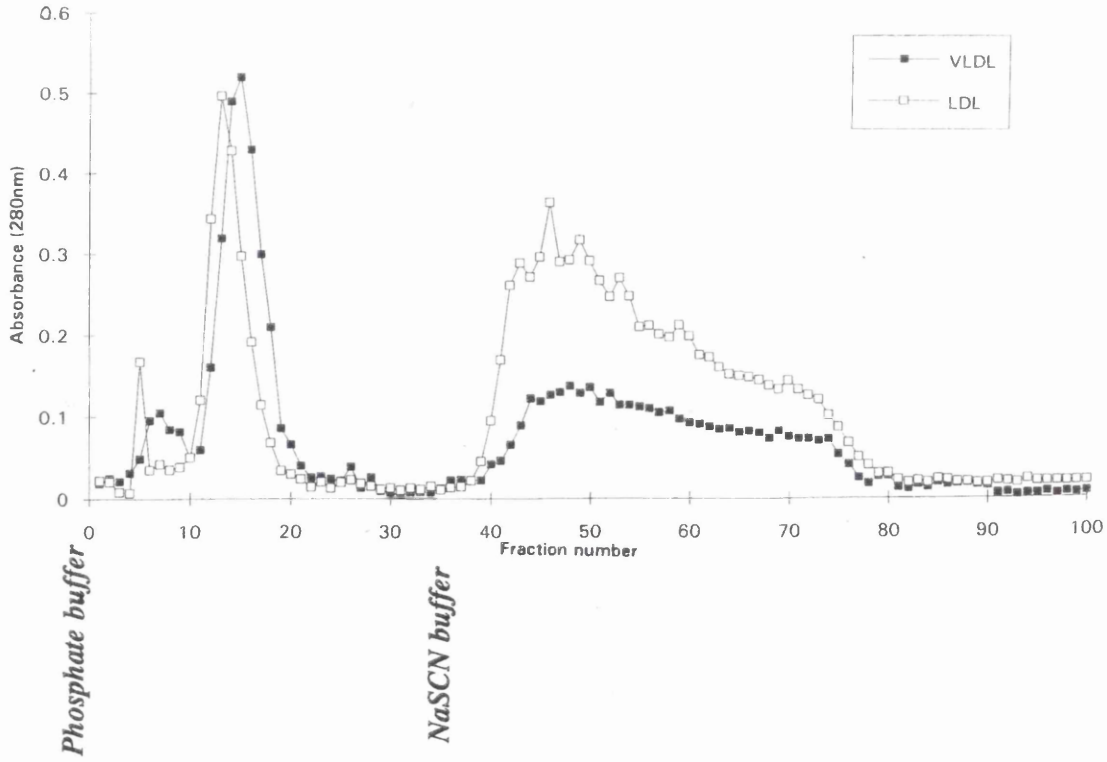


Figure 3.6: ***Elution profile of VLDL and LDL.*** *Lipoprotein samples were prepared from a fasted plasma sample by ultracentrifugation, followed by elution from an LDL A4 monoclonal antibody immunoaffinity column, with absorbance measured at 500nm.*

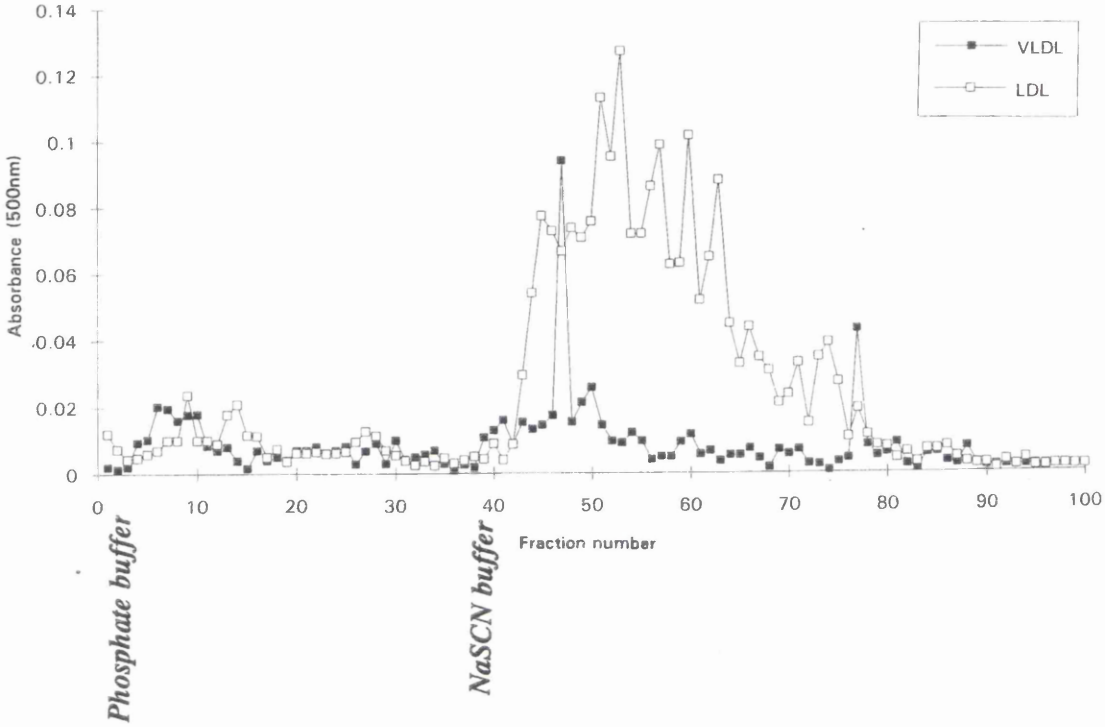


Figure 3.7: ***SDS gel electrophoresis of VLDL.*** *VLDL samples were prepared by ultracentrifugation followed by elution from an LDL A4 monoclonal antibody immunoaffinity column using 1M NaSCN, showing the presence of an apoB100 protein band in the apoB100 (bound) fraction.*

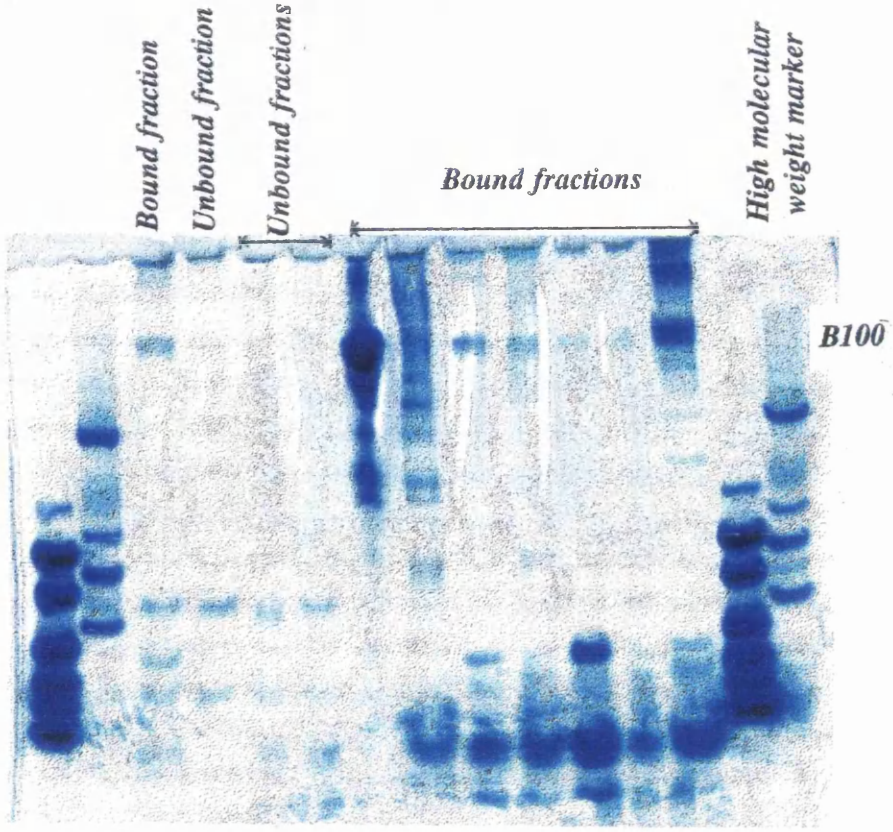


Figure 3.8: ***SDS gel electrophoresis of LDL.*** *LDL samples were prepared by ultracentrifugation followed by elution from an LDL A4 monoclonal antibody immunoaffinity column using 1M NaSCN, showing the presence of an apoB100 protein band in the apoB100 (bound) fraction.*

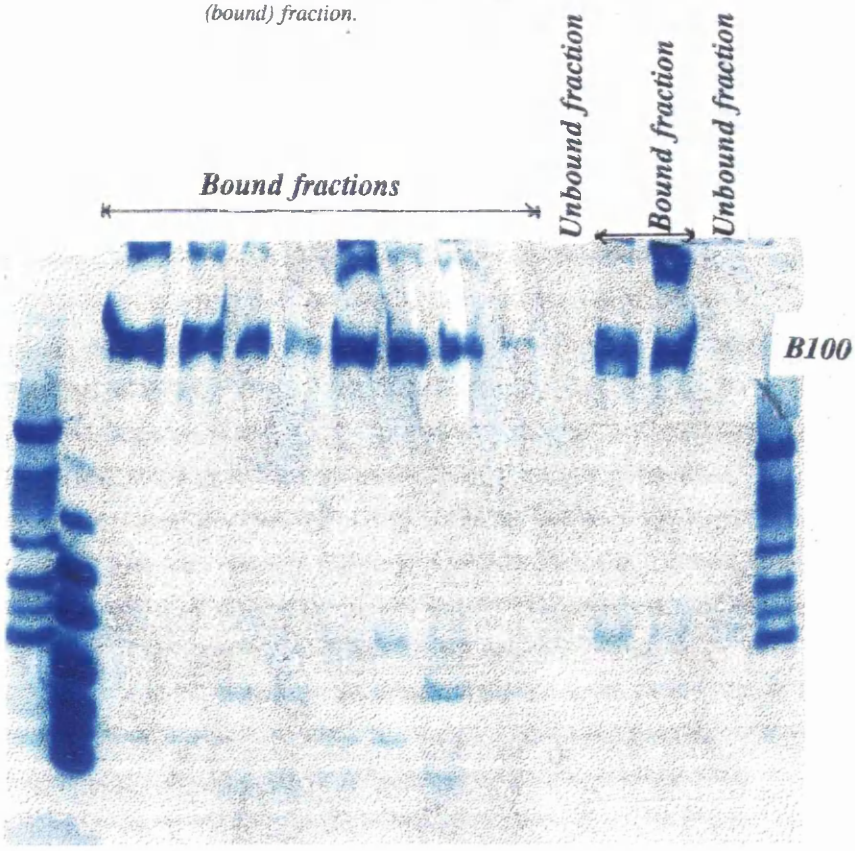


Figure 3.9: ***Elution profile for the separation of chylomicrons and VLDL from a postprandial plasma sample.*** *Lipoprotein samples were prepared by a single ultracentrifugation step, followed by elution using an LDL A4 monoclonal antibody immunoaffinity chromatography column, with absorbance measured at 280nm.*

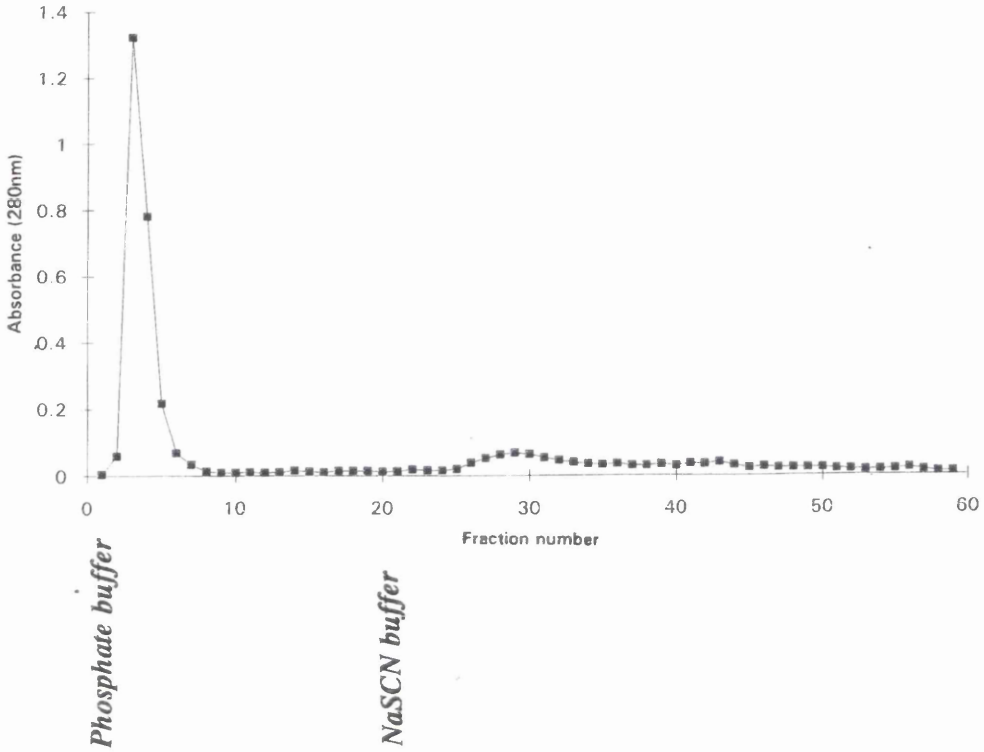


Figure 3.10: ***Elution profile for chylomicrons.*** *Lipoprotein samples were prepared from a postprandial plasma sample by ultracentrifugation, followed by elution from an LDL A4 monoclonal antibody immunoaffinity column, with absorbance measured at 280nm.*

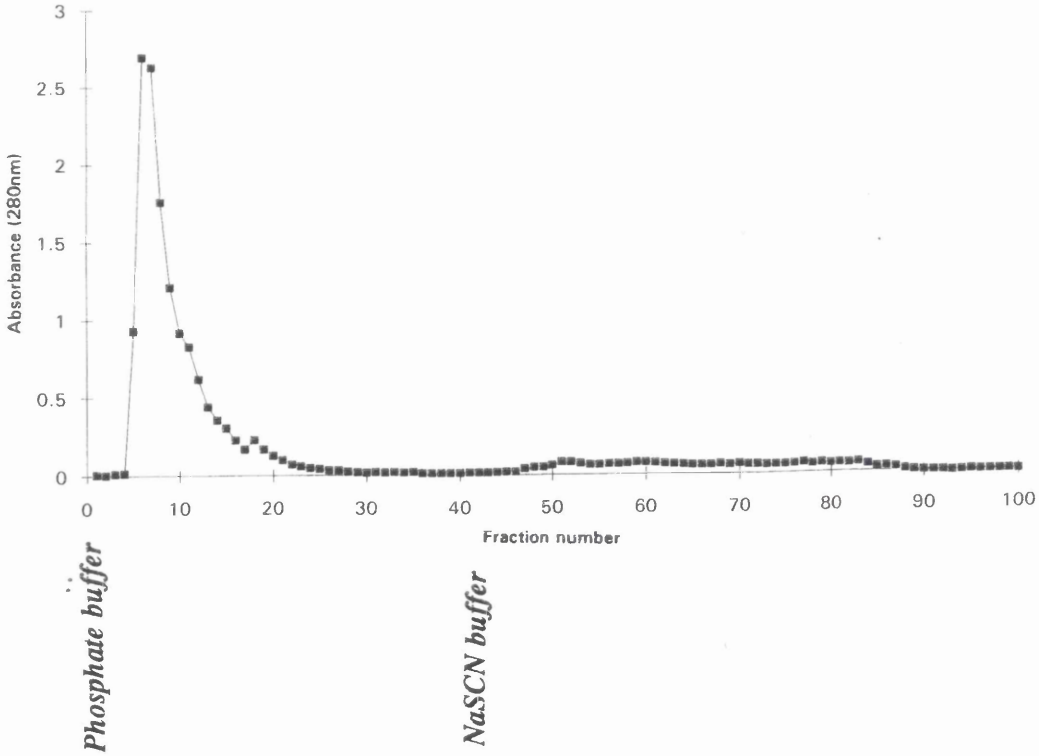


Figure 3.11: ***Elution profile for VLDL.*** *Lipoprotein samples were prepared from a postprandial plasma sample by ultracentrifugation, followed by elution from an LDL A4 monoclonal antibody immunoaffinity column, with absorbance measured at 280nm.*

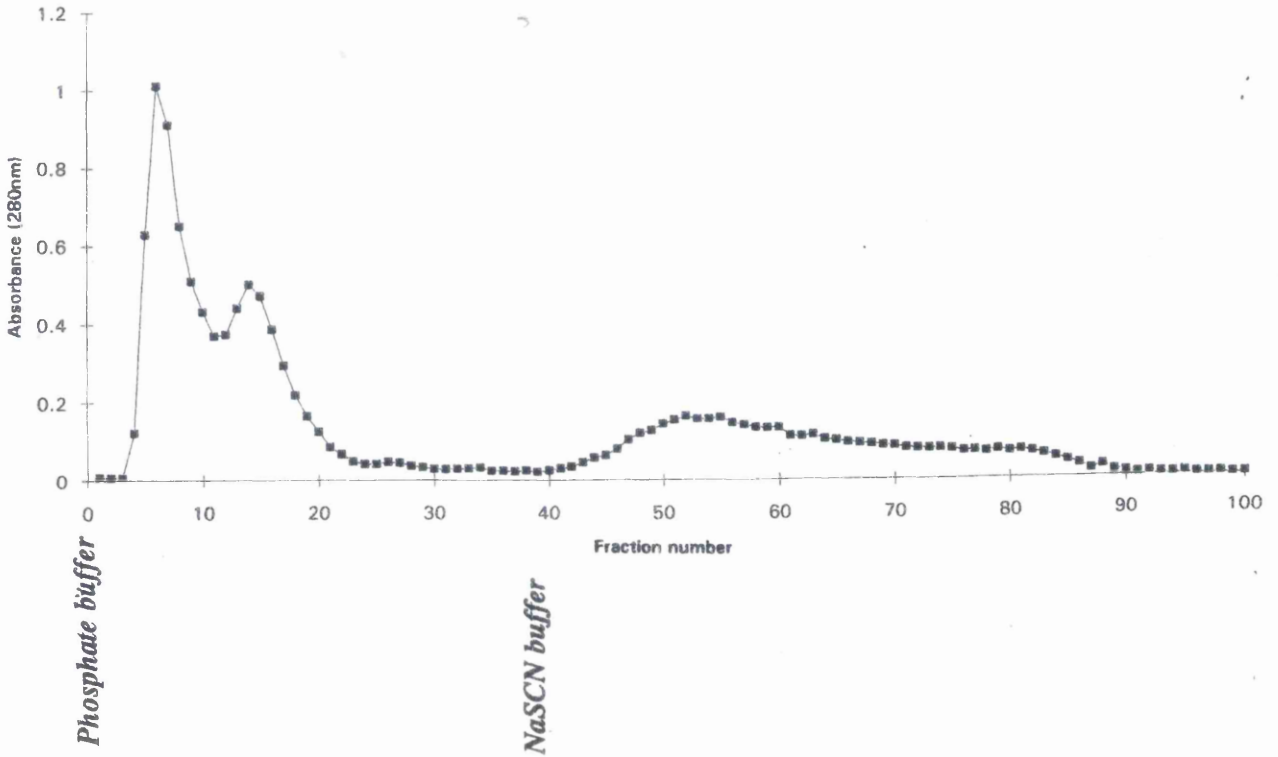


Figure 3.12: ***Elution profile for the chylomicron and VLDL mixed fraction (TRL ($d < 1.006g/ml$)).*** *Lipoprotein samples were prepared from a postprandial plasma sample by ultracentrifugation, followed by elution from an LDL A4 monoclonal antibody immunoaffinity column, with absorbance measured at 280nm.*

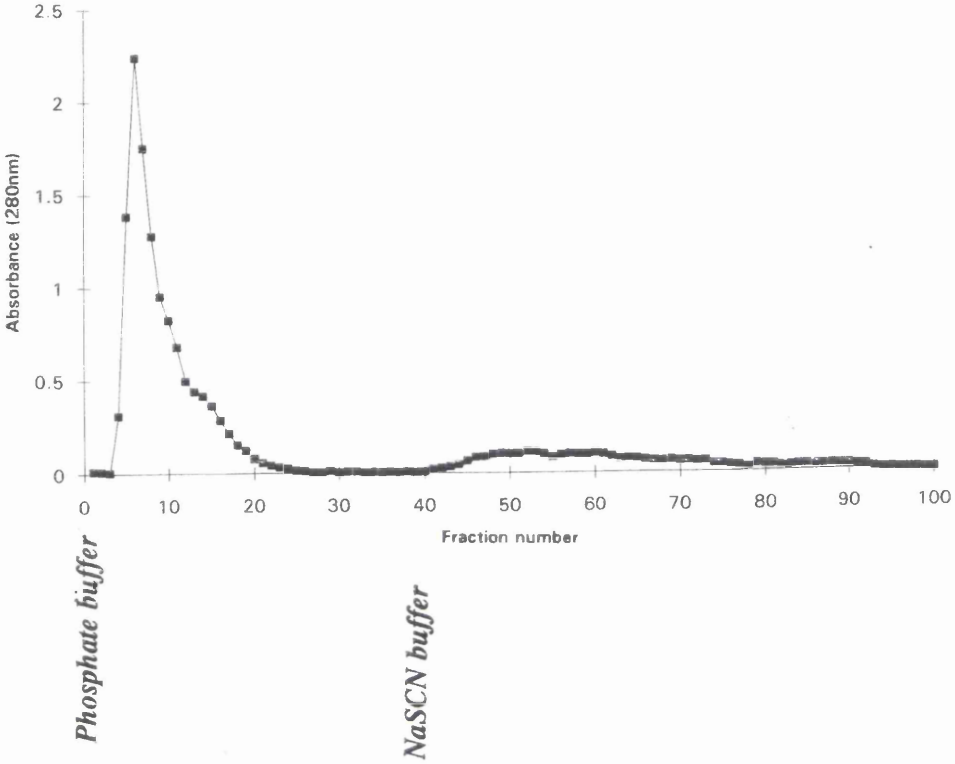


Figure 3.13: ***SDS gel electrophoresis of chylomicrons and VLDL.*** *The lipoprotein fractions were prepared together by a single ultracentrifugation step followed by the separation into apoB48 and apoB100 TRL fractions by a Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes , showing the presence of an apoB100 protein band in the apoB100 (bound) fraction and a faint apoB48 protein band in the apoB48 (unbound) fraction There were also faint apoB100 bands present in the apoB48 (unbound) fraction.*

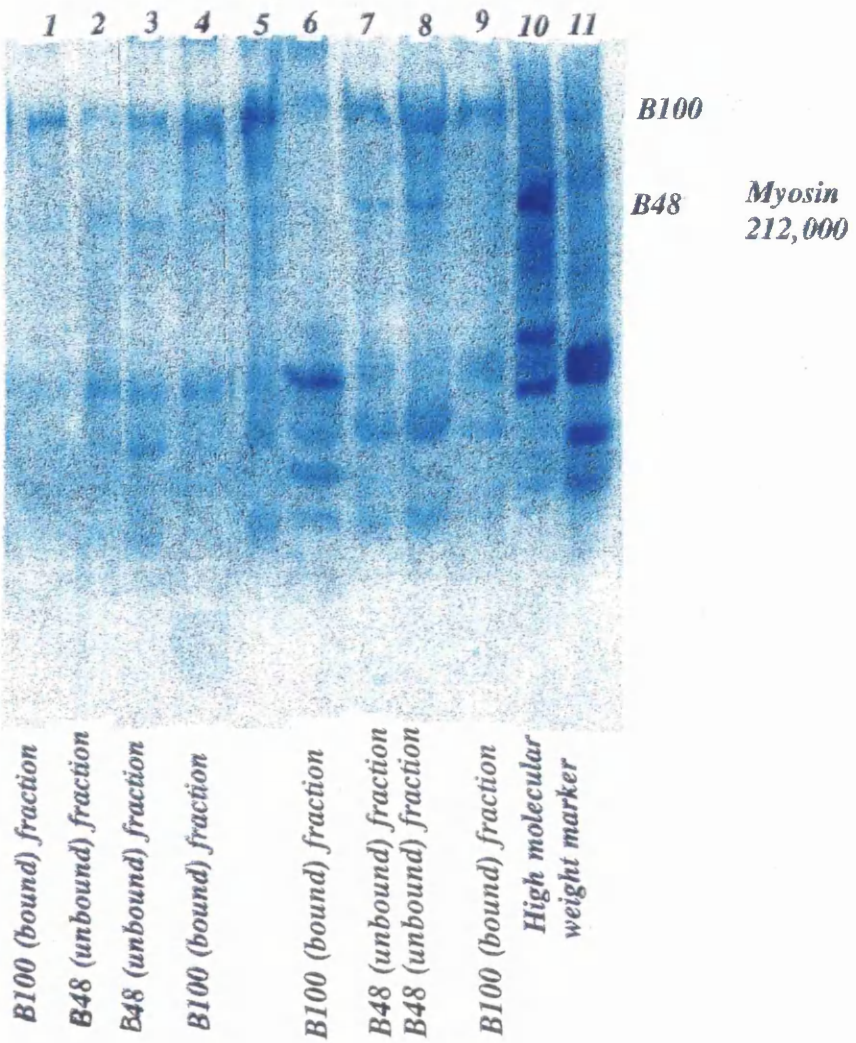


Figure 3.14: ***The effect of the duration of the initial incubation step on the binding of VLDL.*** *VLDL samples were isolated from a postprandial plasma sample by ultracentrifugation, and loaded onto an LDL A4 monoclonal antibody immunoaffinity gel in Eppendorf tubes.*

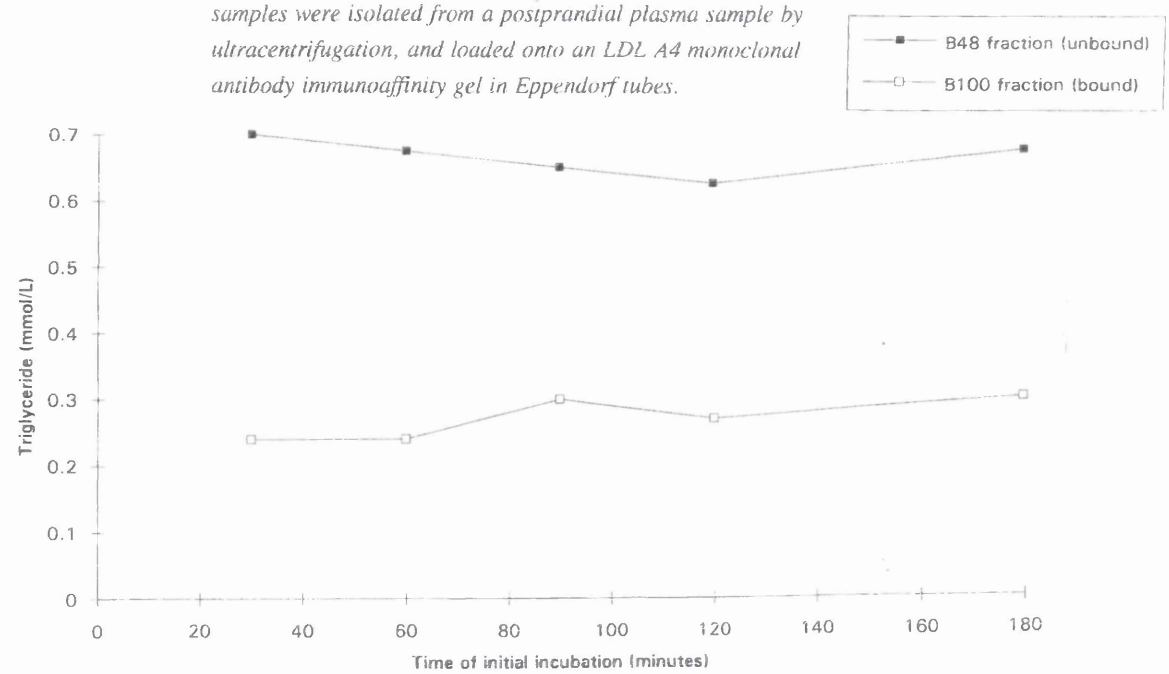


Figure 3.15: ***The effect of the duration of the initial incubation step on the binding of VLDL.*** *VLDL samples were isolated from a fasted plasma sample by ultracentrifugation, and loaded onto an LDL A4 monoclonal antibody immunoaffinity gel in Eppendorf tubes.*

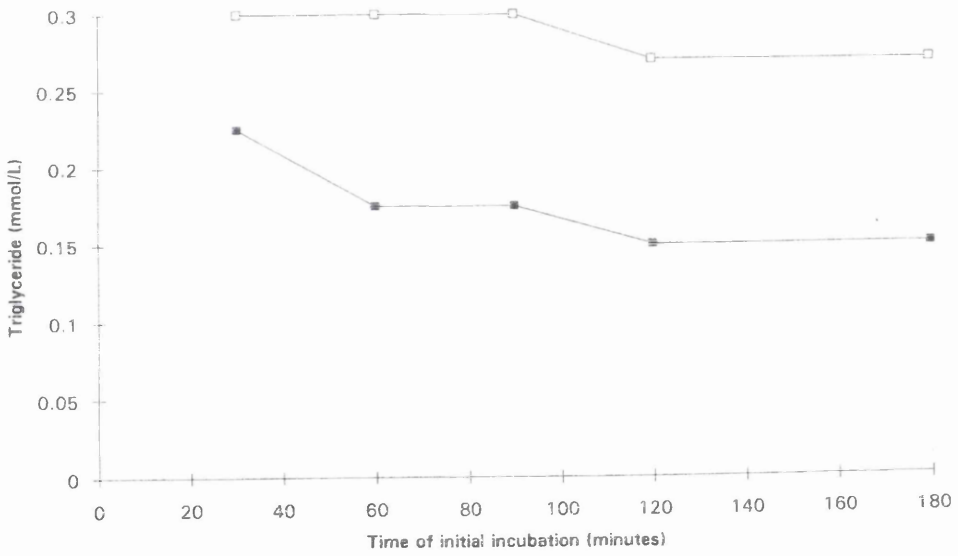
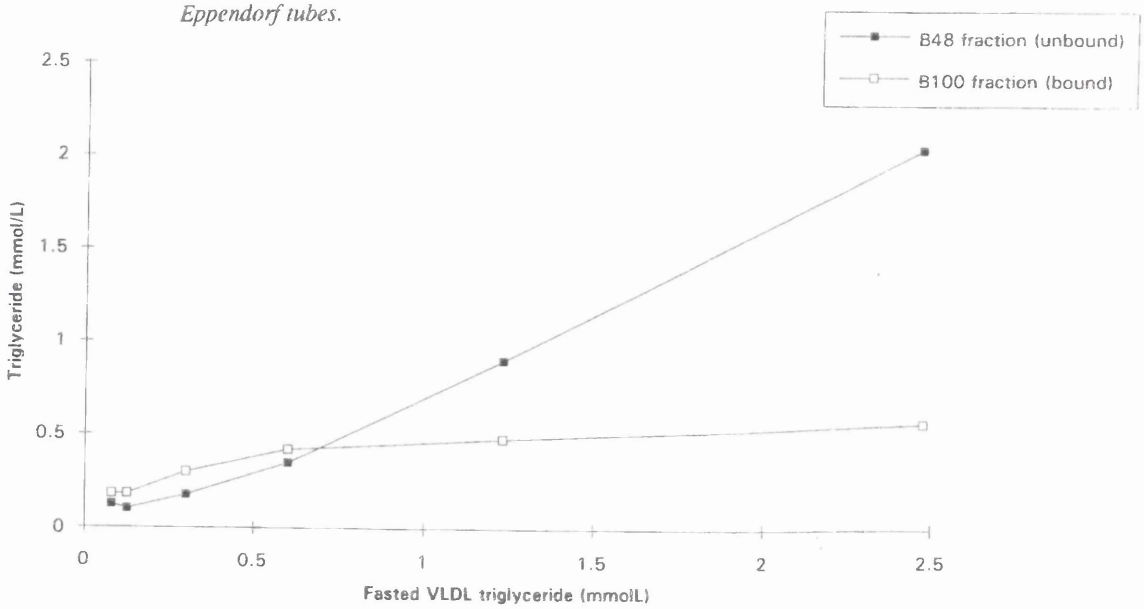


Figure 3.16: ***A saturation curve for the binding of various concentrations of VLDL.*** *VLDL samples were isolated from a fasted plasma sample by ultracentrifugation, and loaded onto an LDL A4 monoclonal antibody immunoaffinity gel in Eppendorf tubes.*



Chapter IV Inhibition of adipocyte lipolysis.

1 Introduction.

Fatty acid metabolism *(Arner 1990)* plays a key role in the regulation of the energy balance of mammals. The major pathways for FFA metabolism are mobilisation through lipolysis at a time of energy deficit, and esterification of both exogenous and endogenous fatty acids to triglycerides when energy needs are met. In anabolic situations fatty acids are stored in various tissues as triglyceride. During prolonged physical work or in catabolic states, fatty acids are mobilised by the action of HSL *(Frayn et al 1995)* and used as fuel. Disturbances in fatty acid metabolism are involved in the pathogenesis of common diseases such as obesity, diabetes *(Boden 1996, Durrington 1996, Lewis and Steiner 1996)* and atherosclerosis *(Frayn, Williams and Arner 1996)*. Some FFA never leave the fat cells, instead they are re-esterified to triglyceride. FFA can be taken up by the fat cell directly from the albumin bound fatty acids in the blood stream or from lipoprotein triglyceride which is hydrolysed outside the fat cell through the action of LPL *(Goldberg 1996)*. FFA can also be produced through *de novo* synthesis from carbohydrates and used for esterification to triglyceride *(Saloranta and Groop 1996)*.

Adipocyte lipolysis *(Coppack, Jensen and Miles 1994)* is regulated through a signal transduction pathway *(Yeaman 1990, Yeaman et al 1994)* (chapter I, figure 1.8) and proceeds by the action of 2 enzymes, HSL (hydrolysis of triacylglycerol (TAG) to diacylglycerol (DAG)) and monoacylglycerol lipase (hydrolysis of DAG to monoacylglycerol (MAG)). It is HSL that regulates the mobilisation of fatty acids from adipose tissue *(Fredrickson et al 1981, Yeaman 1990, Frayn 1993 and Yeaman et al 1994)*. In the short term, activity of the enzyme is controlled via reversible phosphorylation, involving a signal transduction pathway. HSL is phosphorylated and activated in response to a variety of lipolytic hormones *(Yeaman 1990)*, with insulin *(Egan et al 1990)* exerting a major antilipolytic action. Activation by catecholamines occurs through an increase in cAMP levels and results in phosphorylation of HSL by cAMP dependent protein kinase on a single serine residue, termed the regulatory site (serine 563). In more detail, HSL lipolysis is under neural and hormonal control and stimulatory or inhibitory agents exert their effects via the adenylate cyclase-A-kinase

signal transduction pathway (chapter I, figure 1.8). The stimulatory/ inhibitory compounds bind to receptors on the adipocyte cell membrane. These receptors are coupled to either stimulatory (G_S) or inhibitory (G_i) G proteins which in turn activate/ inhibit adenylate cyclase. HSL phosphorylation is paralleled by an enhanced triglyceride lipase activity *(Fredrickson et al 1981)*. Two serine residues are phosphorylated on HSL. Phosphorylation on the basal site (serine 565) has no apparent direct effect on the enzyme activity but inhibits subsequent phosphorylation of the regulatory site (ser 563). The basal site is phosphorylated by 5'AMP activated protein kinase *(Garton and Yeaman 1990, Hardie 1992)* under basal conditions of lipolysis *(Okuda, Morimoto and Tsujita 1994)*. These two phosphorylations on the regulatory (serine 563) and basal (serine 565) sites are mutually exclusive *(Garton et al 1989)* meaning that phosphorylation by AMP kinase on the basal site would prevent further phosphorylation on the regulatory site and HSL activation.

The antilipolytic effect of insulin *(Yeaman 1990)* involves the dephosphorylation and hence inactivation of HSL in adipocytes. This action has been attributed to protein phosphatases *(Wood et al 1993)*. Protein phosphatases (PP2A and 2C) are the only protein phosphatases to display activity towards HSL phosphorylated at the regulatory site and PP1, PP2A and PP2C have been detected within rat adipocytes at significant levels.

Rat HSL preferentially releases polyunsaturated fatty acids from triglycerides *(Gavino and Gavino 1992)*. Rat HSL *(Fredrickson et al 1981, Smith et al 1993)* structure has been elucidated recently *(Gabriele et al 1993)*. It is an 84 kDa polypeptide of 768 amino acids, with an active site at serine 423. After fasting (prolonged), HSL activity in adipose tissue increases *(Sztalryd and Kraemer 1994 a)*.

1.1 *In vitro* stimulation of lipolysis.

Lipolysis can be stimulated experimentally by incubating rat adipocytes with either adenosine deaminase (ADA)/ 3-isobutyl-1-methylxanthine (IBMX), forskolin or isoprenaline (chapter I, figure 1.8).

ADA/IBMX stimulated lipolysis.

ADA is an enzyme which converts adenosine to inosine. Inosine is inactive at the adenosine A_1 receptor; ADA thus removes the inhibition of adenylate cyclase caused by any endogenously released adenosine. IBMX is a purine analogue, and as such is a phosphodiesterase inhibitor, which prevents the breakdown of cAMP to AMP. With no breakdown of cAMP, HSL can be repeatedly phosphorylated and therefore activated. Using ADA and IBMX in combination means that HSL is constantly being activated, so if any inhibition of HSL activity occurs it is most probably due to the antilipolytic action of the drug present in the incubate.

Forskolin stimulated lipolysis. ***(Allen, Ahmed and Naseer 1986)***

Forskolin activates adenylate cyclase directly, leading to an increase in intracellular levels of cAMP, phosphorylation and activation of HSL.

Isoprenaline stimulated lipolysis.

Isoprenaline binds to the ß-receptor which is coupled to the G_S protein which results in the activation of adenylate cyclase, increasing intracellular levels of cAMP leading to the activation of HSL. Glycerol is a better indicator of lipolysis than FFAs since glycerol can be re-utilised by fat cells only to a minor extent because of low glycerol kinase activity in these cells.

1.2 Aim.

The objective of the present study was to investigate the effect and mechanism of action of a number of agents - nicotinic acid (and analogues: acipimox, 3-methyl-5-pyrazole carboxylic acid (3M5P), 3-pyridyl acetic acid hydrochloride (3PAA)), clofibrate, a thiazolidione (TZD), 5-amino-4-imidazole carboximide ribonucleoside (AICAR), a $ß_3$ agonist, phenyl isopropyl adenosine (PIA), acifran, insulin and p-nitrophenylethylhexyl phosphonate (304205) - on HSL activity and adipocyte lipolysis in isolated rat adipocytes from fasted male Alderley Park rats. Figure 4.1 shows their structures. The overall aim was to understand how control of HSL activity by drugs could effect the flux of triglyceride postprandially and change the liver-adipose tissue relationship in fatty acid metabolism.

2 Methods.

Male Alderley Park rats (150 - 200 grams) were fasted overnight and killed by exsanguination, before the immediate removal of the epididymal fat pads *(Sztalryd and Kraemer 1994 b)* for isolation of adipocytes by collagenase digestion.

2.1 Isolation of primary rat adipocytes from the epididymal fat pads of Alderley Park rats.

Primary rat adipocytes were isolated from the epididymal fat pads of fasted male Alderley Park rats. The cells were liberated by collagenase digestion (1 mg/ml, 120 rpm, 60 minutes, 37°C) (Worthington Biochemical Corp.) *(Rodbell 1964)*, washed, diluted and resuspended (cell density = $4.7x10^5$ cells/ml) in 1% bovine serum albumin (BSA) (Sigma A7030) pH 7.4 - 7.5 in polyethylene tubes.

2.2 Preparation of compounds.

Test compounds were dissolved in neat dimethyl sulphoxide (DMSO) to give a 5 mM stock solution, then serially diluted (see appendix 2) to give a final concentration range in the incubation media of 100 μM - $3x10^{-6}$ μM, with the exception of AICAR and insulin. AICAR was dissolved in neat DMSO to give a stock solution of 250 mM and serially diluted as for all other agents, to give a final concentration range in the incubation media of $5x10^3$ μM - $1.5x10^{-4}$ μM. Insulin was prepared as follows; 1.0 mg insulin was dissolved in 1.0 ml 0.03 M HCl. The solution was made up to 3.0 ml with de-ionised H_2O, 100 μl was diluted to 5 ml with 1% BSA buffer. This gives a stock solution of insulin of 1 μM and after serial dilution a final concentration range in the incubation media of $2x10^{-2}$ μM - $6x10^{-10}$ μM.

2.3 Measurement of lipolysis.

Lipolysis was measured by quantitative enzymatic determination of glycerol (Sigma GPO-Trinder Reagent A kit) and non-esterified fatty acids (NEFA) (enzymatic colorimetric method) (Wako NEFA C kit). Concentrations of glycerol were determined spectrophotometrically at a wavelength of 540 nm with reference to a standard curve over a range 0 - 1.25 mg/ml. The assay was performed in triplicate. Concentrations of NEFA were also determined spectrophotometrically at a wavelength of 540 nm with reference to a standard calibration curve using oleic acid over a range 0 - 1 mEq/L (1 mmol/L = 1 mEq/L). The assay was performed in triplicate.

2.4 HSL assay in isolated rat adipocytes.

Triplicate aliquots of adipocytes were pre-incubated for 10 minutes at 37°C, (AICAR was preincubated with the adipocytes for 1 hour instead of 10 minutes, as it is an inactive cell permeable precursor of 5-amino-4-imidazolecarboxamide ribotide (ZMP)) with compounds (concentration range of 100 μM - $3x10^{-6}$ μM, $5x10^{3}$ μM - $1.5x10^{-4}$ μM for AICAR and $2x10^{-2}$ μM - $6x10^{-10}$ μM for insulin). One percent (1%) BSA containing ADA/IBMX (2 μM) was then added and adipocytes incubated for a further 2 hours at 37°C, 65 rpm. After the incubation was complete, an aliquot of medium was removed and assayed for glycerol and NEFA (chapter IV, section 2.3).

2.5 Alternative preparation of stimulatory buffers.

Adipocyte lipolysis was stimulated experimentally by either ADA/IBMX (2 μM), forskolin (10 μM) (Sigma F 6886) or isoprenaline (10 and 100 nM) in 1% BSA buffer pH 7.4 - 7.5 to study lipolysis (chapter IV, section 2.3) and HSL activity (chapter IV, section 2.6).

2.6 HSL assay in isolated primary rat adipocytes using ^{3}H MOME as a triglyceride substrate.

Monoacyl monoalkylglycerol (MOME) or 1(3)oleoyl-(2)-oleyl-glycerol (figure 4.2) is a triglyceride analogue specific for the conversion of DAG to MAG, and was therefore used to measure HSL activity. A radioactive emulsion was prepared, 5 mg cold MOME (Servicon) was added to 62 mg/ml ^{3}H MOME (Servicon) and evaporated using N_2 gas. Two ml of ice cold gum arabic (5 mg/ml) (Sigma G 9752) was added and the mixture sonicated for 5 minutes (10x30 secs) using a MSE soniprep 150 at maximum amplitude. The preparation was used and could be stored for up to 2 - 3 hours at 4°C. ^{3}H MOME was diluted 1:7 with 72 mM sodium phosphate pH 6.8 (plus 1.43 mM EDTA, 115 mM KCl, 29 mM NaCl, 67 mg/ml BSA).

Triplicate aliquots of adipocytes (prepared in section 2.1), compounds and 1% BSA buffer containing ADA/IBMX (2 μM) (all BSA buffers contained 0.005% adenosine (Sigma A 9876) were incubated (2 hours, 37°C, 65 rpm) and the medium was removed and assayed for glycerol and NEFA levels. ^{3}H MOME substrate emulsion was prepared and added directly to the adipocytes, which were vortexed and incubated (10 minutes, 37°C). The reaction was stopped by the addition of stop solution (methanol: chloroform: heptane (1.41: 1.25: 1.00)) and the tubes again vortexed. Carrier oleate

(200 μg, from a 20 mg/ml stock solution stored at -20^{o}C) and 0.1 M sodium carbonate, pH 10.5, were added and tubes centrifuged (5 minutes, 13500 rpm). The top aqueous layer was aspirated and ^{3}H dpm counted.

2.7 *In vitro* HSL assay using isolated, partially purified HSL.

Partially purified HSL was prepared by Mr Robert Forder of Zeneca Pharmaceuticals *(Egan et al 1992)*.

The compounds, ^{3}H MOME emulsion and partially purified HSL preparation (at a concentration to allow 5% hydrolysis of ^{3}H MOME) *(Egan et al 1992)* were incubated in a glass tube (60 minutes, 30^{o}C). The reaction was terminated by the addition of stop solution, with 200 μg carrier oleate and 0.1 M sodium carbonate, pH 10.5, also added. The tubes were vortexed and centrifuged (2000 rpm, 15 minutes) and the upper aqueous layer removed and counted for ^{3}H dpm. Blank tubes received enzyme diluent (50 mM Tris pH 7, 2 mM EDTA, 0.2 mM benzamidine diluted 50: 50 with ethylene glycol).

2.8 Statistical analysis.

Statistical analysis was not performed on the data presented in the results section as experiments were performed only once or twice and only used triple replicates. The results presented in sections 3 and 3.1 were confirmed by the experiments whose results are presented in section 3.2.

3 Results.

Direct inhibition of HSL.

Compound 304205 was the only agent to inhibit the activity of partially purified isolated HSL *in vitro*. The other compounds showed no inhibitory effects suggesting their antilipolytic mechanism of action is exerted on the adipocyte signal transduction pathway.

3.1 Effects of selected agents on lipolysis and HSL activity in ADA/IBMX stimulated primary rat adipocytes.

Adipocytes were incubated with selected agents over the previously stated concentration ranges. NEFA and glycerol dose response curves were constructed for each agent. A characteristic dose response curve for nicotinic acid is shown in figure 4.3.

The agents inhibited ADA/IBMX stimulated adipocyte lipolysis in a concentration dependent manner, with NEFA and glycerol concentrations reaching basal levels or below at 3 μM for nicotinic acid, 3 μM for compound 304205, 5000 μM for AICAR, 10 μM for acipimox, 0.003 μM for PIA, 30 μM for 3PAA, 10 μM for 3M5P, $6x10^{-4}$ μM for insulin and 10 μM for acifran.

Nicotinic acid (and analogues; acipimox, 3M5P, 3PAA), acifran, PIA, insulin, AICAR and 304205 all inhibited ADA/IBMX stimulated lipolysis in the isolated rat adipocyte. NEFA and glycerol IC_{50} concentrations were estimated for each agent (table 4.1) from the dose response curves. Insulin was the most potent antilipolytic agent (IC_{50} = $5x10^{-5}$ μM). The order of potency was determined as (most potent first) insulin > PIA > 304205 > 3M5P > acifran/ nicotinic acid/ acipimox > 3PAA > AICAR.

TZD, clofibrate and the β_3 agonist failed to inhibit both ADA/IBMX stimulated lipolysis and HSL activity in isolated primary rat epididymal adipocytes.

Table 4.1: *NEFA and glycerol IC_{50} values (μM) for selected agents determined as a result of their antilipolytic mechanism of action in ADA/IBMX stimulated primary rat adipocytes isolated from fasted male Alderley Park rats.*

Name	NEFA IC_{50} (μM)	Glycerol IC_{50} (μM)
Nicotinic acid	2	2
Acipimox	4	2
Acifran	2	1.38
3-pyridyl acetic acid hydrochloride (3PAA)	9	4
3-methyl-5-pyrazole carboxylic acid (3M5P)	0.74	1
Phenyl isopropyl adenosine (PIA)	$1.5x10^{-3}$	$2.7x10^{-3}$
Insulin	$5.8x10^{-5}$	$5.4x10^{-5}$
AICAR	950	700
p-nitrophenylethyl hexylphosphonate (304205)	0.16	0.39
TZD (263423)	Non active	Non active
Clofibrate	Non active	Non active
ß3 agonist (190483)	Non active	Non active

Table 4.2: Effect of agents on HSL activity (% inhibition) in ADA/IBMX stimulated rat adipocytes using ^{3}H MOME.

Agent	Concentration (μM)	Inhibition of HSL activity (%)
Nicotinic acid	100	41
	30	37
	3	43
Acipimox	100	36
	10	32
	1	21
Acifran	100	13
	10	59
	3	36
3PAA	100	6
	30	24
	3	9
3M5P	30	16
	10	17
	1	16
PIA	1	24
	0.03	42
	0.003	26
Insulin	$6x10^{-3}$	7
	$6x10^{-4}$	7
	$6x10^{-5}$	1
AICAR	5000	37
	500	28
304205	30	99
	3	89
	0.3	44
TZD (263423)	100	-21
	0.003	-40
Clofibrate	100	16
	0.003	-28
ß3 agonist (190483)	0.3	-142
	0.0003	-73
	3x10-6	-30

Using the foregoing dose response data, the concentration of agents closest to the estimated ED_{95} and IC_{50} concentrations were selected for incubation with ADA/IBMX stimulated rat adipocytes and ^{3}H MOME substrate emulsion was used for the measurement of HSL activity.

Inhibition of HSL activity was concentration dependent for compound 304205 (table 4.2). Compound 304205 inhibited HSL activity >90% at concentrations >3 μM with the other agents also inhibiting HSL activity: 40% at concentrations >3 μM nicotinic acid, 37% at 5000 μM AICAR, 32 - 36% at concentrations >10 μM acipimox, 24 - 42% at concentrations >0.003 μM PIA, 24% for concentrations above 30 μM for 3PAA, 16 - 17% for concentrations >10 μM 3M5P, 7% for concentrations $>6x10^{-4}$ μM insulin and 13 - 59% at concentrations >10 μM acifran (table 4.2), yet in contrast NEFA and glycerol levels were inhibited to basal levels at these concentrations. Thus inhibition of HSL activity did not appear to be concentration dependent and lipolysis measurements indicated almost total inhibition of triglyceride hydrolysis, yet levels of HSL inhibition appeared to be lower than that expected.

3.2 Effects of selected agents on lipolysis and HSL activity in ADA/IBMX, forskolin and isoprenaline stimulated rat adipocytes.

Compound 304205, nicotinic acid and AICAR all inhibited lipolysis (both NEFA and glycerol levels) in ADA/IBMX stimulated primary rat epididymal adipocytes. Only AICAR and 304205 also inhibited lipolysis in forskolin and isoprenaline stimulated rat adipocytes. Both concentrations of isoprenaline (100 and 10 nM) gave identical results. Nicotinic acid failed to inhibit lipolysis in forskolin and isoprenaline stimulated rat adipocytes, even at the maximal concentration of 100 μM. Where inhibition of adipocyte lipolysis (by either of the stimulatory agents) was observed, NEFA and glycerol levels reached near basal levels at 3 μM for 304205, 3 μM for nicotinic acid and 5000 μM for AICAR as seen previously in section 3.1.

Compound 304205 inhibited HSL activity >90% under all modes of lipolytic stimulation. Inhibition of HSL activity by 304205 appears to be concentration dependent. Nicotinic acid inhibited HSL activity only in ADA/IBMX stimulated rat adipocytes. AICAR, like compound 304205, inhibited HSL acitivity under all modes of stimulation. Nicotinic acid and AICAR appeared to inhibit HSL activity in a non-concentration dependent manner (table 4.3). NEFA and glycerol measurements indicated almost total inhibition of triglyceride hydrolysis but the levels of inhibition of

HSL activity observed were lower than expected, confirming the results of previous sections.

Table 4.3: Effect of selected agents on HSL activity (% inhibition) in ADA/IBMX (2 μM), forskolin (10 μM) and isoprenaline (100 and 10 nM) stimulated rat adipocytes.

Agent	Concentration (μM)	ADA/ IBMX (2 μM)	Forskolin (10 μM)	Isoprenaline (100 nM)	Isoprenaline (10 nM)
304205	30 0.3	99% 79%	99% 49%	99% 60%	99% 38%
Nicotinic acid	100 3	52% 38%	- -	- -	- -
AICAR	5000 500	27% 25%	36% 28%	41% 40%	44% 19%

3.3 Effects of different lipolytic stimuli on the NEFA: glycerol ratio.

Throughout these experiments it was noted that the ratio of NEFA: glycerol was not the expected theoretical 3:1, but instead approximately 1:5. The NEFA: glycerol ratio was calculated in ADA/IBMX, forskolin and isoprenaline stimulated rat adipocytes in the absence of any antilipolytic agents. Results indicate no significant differences in NEFA: glycerol ratios (table 4.4) between the different methods of lipolytic stimulation of adipocyte lipolysis. The ratio remains largely unaltered in the presence of antilipolytic agents. The explanation for these findings is unclear requiring further investigation however these results suggest that there may be some re-esterification of NEFAs by the adipocyte. BSA, present in the incubation medium, can bind NEFAs released into the incubation medium by lipolysis making them less available for reuptake and re-esterification. If the concentration of BSA (1%) is too low and not all NEFAs are bound by it, then they are more likely to be taken up and re-esterified by the adipocyte. BSA concentration, cell concentration and variable removal of adenosine during isolation of adipocytes may have affected the 'basal' rate of lipolysis and the NEFA:glycerol ratio.

Table 4.4: The effects of different lipolytic stimuli on the NEFA: glycerol ratio in isolated primary rat adipocytes from fasted male Alderley Park rats.

Condition	NEFA: glycerol ratio
ADA/IBMX	0.152 ± 0.033
Forskolin	0.150 ± 0.017
Isoprenaline	0.165 ± 0.007
Basal	0.170 ± 0.022

4 Discussion.

In the present study, the antilipolytic mechanism of action of selected agents on rat adipose tissue lipolysis and HSL activity was investigated under three experimental conditions. Firstly it was tested in the presence of ADA/IBMX, secondly with forskolin a direct activator of adenylate cyclase and thirdly in the presence of the non-selective ß-adrenoreceptor agonist, isoprenaline.

Three agents, clofibrate, TZD and a $ß_3$ agonist failed to inhibit both lipolysis and HSL activity in ADA/IBMX stimulated rat adipocytes. All other compounds inhibited lipolysis and HSL activity in this model system.

TZDs act as insulin sensitisers *(Bressler and Johnson 1992, Hofmann and Colca 1992)* with adipose tissue being one of its target tissues. Studies in Zucker rats *(Fujiwara et al 1988)* show a lowering of FFA levels from adipose tissue which may be due to the antilipolytic action of insulin as a result of TZD increasing insulin sensitivity in the adipose tissue. Studies in NIDDM subjects *(Suter et al 1992)* show compound CS-045 (a TZD) improves insulin resistance, although the mechanism of action of TZDs remains unknown and from the results of the present study does not seem to involve the signal transduction pathway for HSL.

Lipolysis in the ADA/IBMX stimulated rat adipocytes was inhibited in a concentration dependent manner with NEFA and glycerol concentrations reaching basal

levels of lipolysis at 3 μM for nicotinic acid, 3 μM for compound 304205, 5000 μM for AICAR, 10 μM for acipimox *(Christie et al 1996)*, 0.003 μM for PIA, 30 μM for 3PAA, 10 μM for 3M5P, $6x10^{-4}$ μM for insulin and 10 μM for acifran. NEFA and glycerol IC_{50} concentrations were established for each agent allowing an order of potency for inhibition of adipocyte lipolysis in ADA/IBMX stimulated rat adipocytes to be determined. Most of the agents demonstrated equal potencies on both NEFA and glycerol concentrations. Insulin the physiological hormone was the most potent. The order of potency was insulin > PIA > 304205 > 3M5P > acifran/ nicotinic acid/ acipimox > 3PAA > AICAR.

In vitro studies showed only compound 304205 inhibited HSL activity. This suggests that while 304205 is a direct inhibitor of HSL and acts possibly by binding directly to the enzyme, the other agents exert their antilipolytic effect via the signal transduction pathway for HSL. Compound 304205 inhibits HSL activity in a concentration dependent manner. With the exception of compound 304205, inhibition of HSL activity in ADA/IBMX stimulated rat adipocytes seemed not to be concentration dependent and the levels of inhibition of HSL observed appeared to be lower in all experiments than that expected, since both NEFA and glycerol concentrations were reduced to basal levels *i.e.* NEFA and glycerol results suggest complete inhibition of triglyceride hydrolysis.

We hypothesise that there could be two forms of HSL present in the adipocyte; an active phosphorylated form and a basal unphosphorylated form. The basal unphosphorylated form may not have been translocated from the lipid droplet to its site of action and it is only when the adipocyte is lysed during the HSL assay that the unphosphorylated HSL can act on the ^{3}H MOME substrate, a triglyceride analogue, which it can freely hydrolyse. This would explain the results observed in the present study. The levels of inhibition of HSL activity produced by the agents is representative of 100% inhibition of the active phosphorylated form of HSL accounting for the observed inhibition of NEFA and glycerol concentrations to basal levels. Compound 304205, the direct inhibitor of HSL, binds to the active site on HSL thus inhibiting activity of all forms of HSL present within the adipocyte and so produces >90% inhibition of HSL activity. This increased level of inhibition of HSL activity is not observed with the other agents, which require the signal transduction pathway to inhibit HSL activity, suggesting that it is possibly binding to both the active and basal forms of HSL.

The antilipolytic mechanism of action of selected agents on HSL activity and on rat adipose tissue lipolysis was investigated using three experimental conditions in the presence of ADA/IBMX (2 μM), forskolin (10 μM) and isoprenaline (100 nM/ 10 nM).

Compound 304205 and AICAR were found to inhibit HSL activity and adipocyte lipolysis under all modes of stimulation. Nicotinic acid inhibited HSL activity and lipolysis in ADA/IBMX stimulated rat adipocytes but failed to do so in forskolin and isoprenaline stimulated rat adipocytes. As shown by the *in vitro* assay, compound 304205 is a direct inhibitor of HSL and as such its antilipolytic mechanism of action is not dependent on the stimulatory conditions present for adipocytes. Therefore as expected 304205 inhibits HSL activity and lipolysis under all of the modes of stimulation.

AICAR, the cell permeable precursor of ZMP has been shown by *Sullivan et al (1994)* to activate AMP kinase intracellularly in primary rat adipocytes resulting in the inhibition of lipolysis and HSL activity. Phosphorylation of HSL in intact rat adipocytes occurs on 2 distinct sites termed the regulatory and basal sites, although the physiological role of the basal site is not clear. Nevertheless, it has been proposed that by phosphorylating the basal site, AMP kinase plays an antilipolytic role *in vivo (Garton et al 1989)*. This is the mechanism by which AICAR exerts its antilipolytic action upon HSL activity and lipolysis. Therefore, as expected its antilipolytic mechanism of action, like compound 304205, is not dependent on the stimulatory conditions present therefore AICAR, inhibits HSL activity and lipolysis under all of the modes of stimulation. *Sullivan et al (1994)* also showed AICAR to profoundly inhibit lipogenesis through increased phosphorylation of acetyl CoA-carboxylase (ACC). It appears that in addition to regulating lipogenesis, AMP kinase also plays an important antilipolytic role by regulating HSL in rat adipocytes.

Nicotinic acid inhibits lipolysis and HSL activity in ADA/IBMX stimulated rat adipocytes but failed to inhibit lipolysis and HSL activity in forskolin and isoprenaline stimulated rat adipocytes. An inherent part of ADA stimulated lipolysis involves the removal of adenosine. ADA converts adenosine to inosine. Inosine is inactive at the adenylate cyclase linked G_i protein complex (adenosine A_1 receptor) thus removing the inhibition of adenylate cyclase and HSL activation caused by any endogenously released adenosine. However where adipocytes were stimulated with either forskolin or isoprenaline, ADA was not present in the incubation media, thus allowing endogenous and exogenous adenosine to accumulate. It is suggested that nicotinic acid (and analogues: acipimox, 3PAA, 3M5P which are pharmacologically related) and acifran

are G_i receptor linked agonists, depending on adenosine removal to exert their antilipolytic effect, resulting in suppression of cAMP levels through inhibition of adenylate cyclase *(Butcher 1971, Aktories, Jakobs and Schultz 1980, Christie et al 1996)*. This would also be true of the adenosine analogue PIA (an A_1 agonist) which appears to require identical conditions for the inhibition of HSL *(Aktories, Schultz and Jakobs 1980)*. In ADA/IBMX stimulated lipolysis, the adenylate cyclase linked G_i protein complex would be susceptible to inhibition whereas accumulation of endogenous and exogenous adenosine in forskolin and isoprenaline stimulated rat adipocytes may leave the adenylate cyclase linked G_i protein complex unresponsive to G_i linked agonists as the G_i protein complex is fully functioning and cannot be further activated by these agents. The dependence of nicotinic acid (and analogues) upon the removal of adenosine suggests that the antilipolytic mechanism used by adenosine may be similar to that of nicotinic acid. It is proposed that nicotinic acid initiates an antilipolytic effect via binding to a distinct G_i linked receptor on the plasma membrane of adipocytes.

The most important and potent antilipolytic agent is insulin. The mechanism of action of this hormone remains unclear but it is thought to exert its antilipolytic effect via a decrease in phosphorylation of HSL by inhibiting the action of protein phosphatases *(Wood et al 1993)* on HSL.

Independent of the incubation conditions of rat adipocytes, the NEFA: glycerol ratio remained largely unaltered. Glycerol was used as an index of lipolysis since it cannot be re-utilised by the adipocyte. The hypothesis is that re-esterification of NEFAs is occurring, with the possibility that the rate of cycling of the futile cycle is being increased in an attempt to increase the sensitivity of the metabolic system due to an artificial environment or to the presence of the inhibitory agents. Another possibility may be that Alderley Park rats over-express acyl coA-synthetase and as a result have increased levels of NEFA re-esterification. It would be of interest to study another breed of rats to see if this hypothesis was correct. Experiments using radiolabelled fatty acids could also be performed to detect if NEFA re-esterification is occurring.

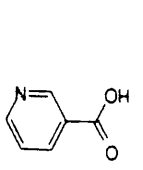
A schematic representation of the antilipolytic action of agents upon HSL activity, which includes information derived from this study, is illustrated in figure 4.4.

The main findings of this study were clofibrate, TZD and $ß_3$ agonist failed to inhibit both lipolysis and HSL activity in ADA/IBMX stimulated rat adipocytes. All other compounds inhibited ADA/IBMX stimulated lipolysis, with insulin, the physiological hormone, being the most potent. Compound 304205 is a direct inhibitor

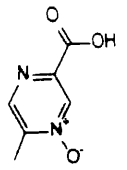
of HSL and inhibits HSL acitivity by binding directly to the enzyme, while the other agents exert their antilipolytic effect via the signal transduction pathway for HSL. It is proposed that nicotinic acid (and its analogues) initiates its antilipolytic effect via binding to a distinct G_i linked receptor on the plasma membrane of the rat adipocytes.

It is suggested that HSL is present in the isolated rat adipocyte in both an active phosphorylated form and a basal unphosphorylated form, with antilipolytic agents exerting their inhibitory effect upon the active form of HSL.

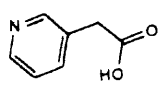
Figure 4.1: *Structures of selected agents used to investigate lipolysis and HSL activity in isolated rat epididymal adipocytes.*



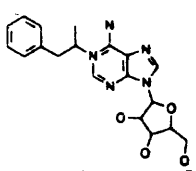
nicotinic acid



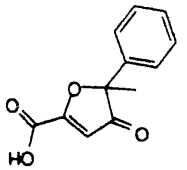
acipimox



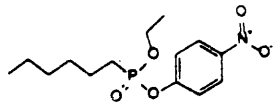
3-pyridyl acetic acid hydrochloride (3PAA)



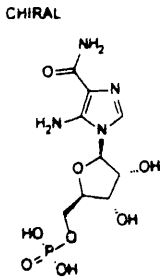
phenyl isopropyl adenosine (PIA)



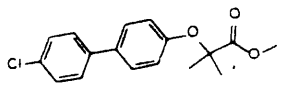
acifran



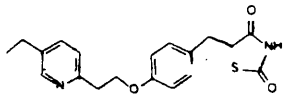
p-nitrophenylethyl hexylphosphonate (304205)



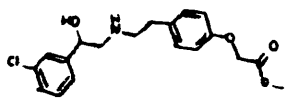
5-amino-4-imidazole carboxamide ribonculeoside (AICAR)



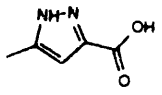
clofibrate



thiazolidione (TZD)



β3 agonist



3-methyl-5-pyrazole carboxylic acid (3M5P)

Figure 4.2: *The structure of the triglyceride analogue, ^{3}H MOME.*

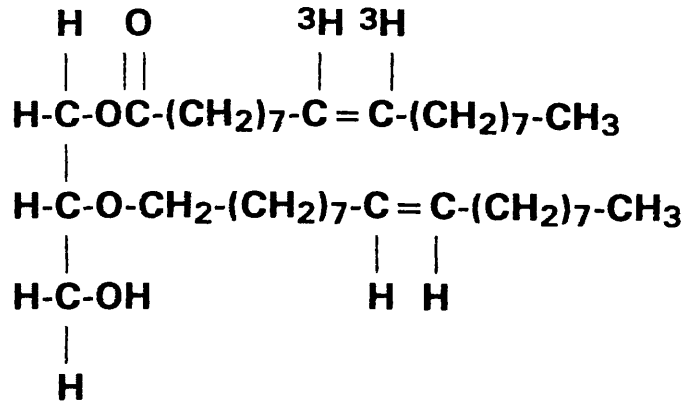


Figure 4.3: *Dose response curve for nicotinic acid in ADA/ IBMX stimulated rat epididymal adipocytes.*

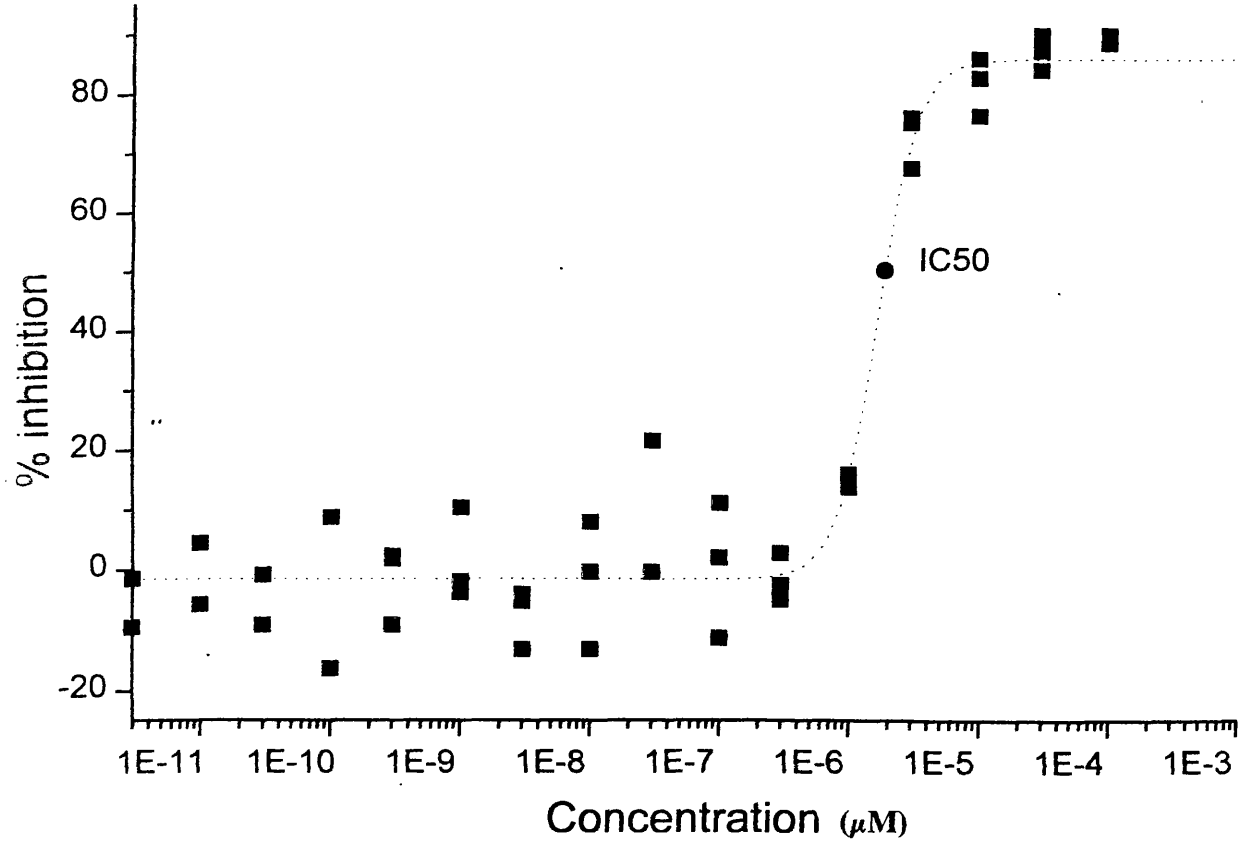
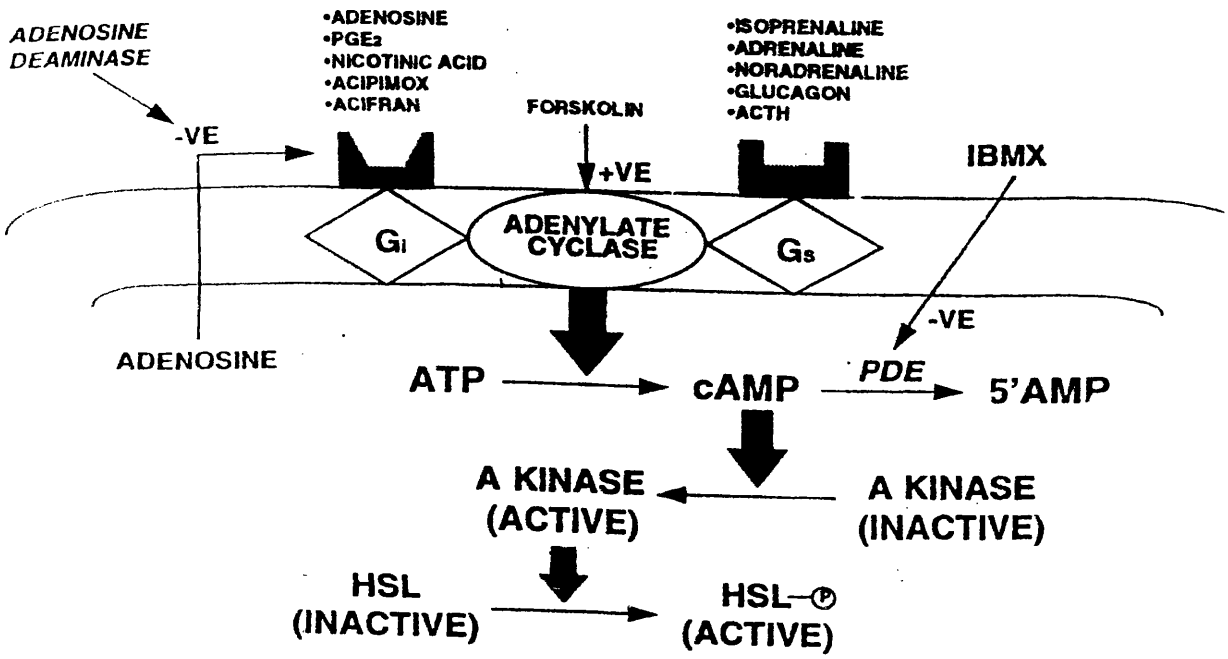


Figure 4.4: The adipocyte signal tranduction pathway.



Chapter V The Zucker rat: preliminary studies with nicotinic acid.

1 Introduction.

1.1 The Zucker rat.

The Zucker rat inherits obesity as an autosomal Mendelian recessive trait *(Bray 1977)*; this characteristic obesity is linked to an allele termed *fa* which has been mapped to chromosome 5. The genetically obese Zucker *(fa/fa)* rat was first described by *Zucker and Zucker in 1961* and is frequently used as a model of early onset human obesity and NIDDM. An increase in plasma triglyceride and lipoproteins is one of the earliest abnormalites described in the Zucker rat *(Barry and Bray 1969)*. The model displays hyperinsulinaemia and hyperlipidaemia but in contrast blood glucose levels are normal. *Schonfeld et al (1974 b)* and *Wang et al (1984)* suggested that the hypertriglyceridaemia which characterises these animals is due to hypersecretion of hepatic VLDL; plasma levels of all lipids are elevated including VLDL, LDL, HDL and FFA. The accumulation of large VLDL in plasma probably reflects increased hepatic production without any defect in clearance *(Bray 1977)*. *Cleary et al (1987)* and *Fukuda et al (1982)* have suggested that altered hepatic metabolism of FFA in this strain of rat could be an underlying factor in the hypersecretion of VLDL by the liver. The increased flux in FFA may come from the adipose tissue as these rats are hyperinsulinaemic, so conceivably HSL activity in the adipose tissue may be elevated. *Bray et al (1970)* showed that mobilisation of FFA from the adipose tissue was normal or increased both *in vivo* and *in vitro* in Zucker rats. In a recent study *(Kasim et al 1992)* it was shown that in the Zucker rat model, HMG CoA-reductase inhibitors can reduce the rate of secretion of VLDL.

Nicotinic acid (vitamin B3) *(Hotz 1983, Shepherd 1991)* (chapter IV, figure 4.1) is unique among the hypolipidaemic drugs in that it is also an important component of a healthy diet. Nicotinic acid plays a number of roles in metabolism as an essential component of the pyrimidine nucleotides, and as an electron acceptor in a variety of oxidative reactions during the dehydrogenation of specific substrates in fatty acid

oxidation, the pentose phosphate pathway and the tricarboxylic acid cycle. The pharmacological effects of nicotinic acid only manifest at high doses with no effect on lipoprotein metabolism at its recommended daily allowance of approximately 14 mg.

Nicotinic acid and its analogues have been used to treat both hypercholesterolaemia and hypertriglyceridaemia *(Gotto et al 1996)*. Their clinical efficacy was first noted by *Altshul, Hoffer and Stephen (1955)* and they have since been used with varying success in the management of all forms of hyperlipidaemia. Treatment with nicotinic acid has not been widespread due to its unpleasant side effects, namely flushing and gastrointestinal irritation.

Nicotinic acid inhibits lipolysis of triglyceride by HSL in the adipose tissue, thus diminishing the supply of FFA and the synthesis of VLDL. It is thought that nicotinic acid mediates its antilipolytic effect through its agonist action at the G_i coupled receptor resulting in a decrease in the level of intracellular cAMP, mainly due to the inhibition of adenylate cyclase (chapter IV). The direct consequence of the reduced cAMP concentration in the adipose tissue is a reduction in the activity of HSL. This is associated with a reduced enzymatic cleavage of triglycerides into fatty acids, thereby lowering the flux of plasma FFA levels to the liver and diminishing production of VLDL. This leads to alterations in LDL and HDL metabolism: LDL synthesis appears to be inhibited and HDL cholesterol concentrations increase.

1.2 Aim.

To investigate the antilipolytic effect of nicotinic acid on adipose tissue lipolysis primarily in fasted and fed Zucker rats; by measurement of plasma FFA concentrations, free glycerol, true triglyceride and total triglyceride concentrations.

2 Methods.

2.1 Animals.

Fasted male Alderley Park (Wistar-derived) rats (200 - 300 grams) were kept in reverse phase lighting conditions (figure 5.1) for 1 week to acclimatise to conditions, then fasted for 18 - 20 hours prior to commencing the experiment. Two dose response curves were constructed; over dose ranges of 5 - 200 mg/kg and 15 - 60 mg/kg nicotinic acid (appendix 3). Groups of six rats were used.

Dose response 1: 5 - 200 mg/kg nicotinic acid.

Group 1 - Control - receiving excipient only (0.5% polysorbate).
Group 2 - one dose of nicotinic acid 5 mg/kg.
Group 3 - one dose of nicotinic acid 10 mg/kg.
Group 4 - one dose of nicotinic acid 25 mg/kg.
Group 5 - one dose of nicotinic acid 50 mg/kg.
Group 6 - one dose of nicotinic acid 100 mg/kg.
Group 7 - one dose of nicotinic acid 200 mg/kg.

Bleeding for all groups was 2 hours post dose.

Dose response 2: 15 - 60 mg/kg nicotinic acid.

Group 1 - Control - receiving excipient only (0.5% polysorbate).
Group 2 - one dose of nicotinic acid 15 mg/kg.
Group 3 - one dose of nicotinic acid 30 mg/kg.
Group 4 - one dose of nicotinic acid 45 mg/kg.
Group 5 - one dose of nicotinic acid 60 mg/kg.

Bleeding for all groups was 2 hours post dose.

Fasted male obese Zucker *(fa/fa)* rats (500 - 600 grams) (appendix 4, 5) were kept in reverse phase lighting (figure 5.1) and fasted for 18 - 20 hours prior to experimentation. Two dose response curves were constructed; over dose ranges of 10 - 200 mg/kg and 1.25 - 25 mg/kg nicotinic acid. Groups of six rats were used.

Dose response 1; 10 - 200 mg/kg nicotinic acid.

Group 1 - Control - receiving excipient only (0.5% polysorbate).
Group 2 - one dose of nicotinic acid 10 mg/kg.
Group 3 - one dose of nicotinic acid 25 mg/kg.
Group 4 - one dose of nicotinic acid 50 mg/kg.
Group 5 - one dose of nicotinic acid 100 mg/kg.
Group 6 - one dose of nicotinic acid 200 mg/kg.

Bleeding for all groups was 90 minutes post dose.

Dose response 2: 1.25 - 25 mg/kg nicotinic acid.

Group 1 - Control - receiving excipient only (0.5% polysorbate).
Group 2 - one dose of nicotinic acid 1.25 mg/kg.
Group 3 - one dose of nicotinic acid 2.5 mg/kg.
Group 4 - one dose of nicotinic acid 5 mg/kg.
Group 5 - one dose of nicotinic acid 10 mg/kg.
Group 6 - one dose of nicotinic acid 25 mg/kg.

Bleeding for all groups was 90 minutes post dose.

Female Zucker rats were also used for one experiment (1.25 - 25 mg/kg nicotinic acid) and kept in the same conditions as their male counterparts.

The rats were allowed to acclimatise to their altered lighting conditions for one week before commencing experimentation. There are several advantages to keeping the rats in reverse phase lighting prior to and during the experiment: the rats were allowed to sleep uninterrupted by the working day's activities, the rats would be truly overnight fasted; under normal lighting conditions the rats are fasting during their active period, there is greater control over the fasting period ($<$24 hours) and immediate postprandial experiments can be done during working hours.

Nicotinic acid was administered orally. A 200 mg/kg stock solution of nicotinic acid (Sigma N4126) (40 mg/ ml/ 200 gram rat) was prepared and serially diluted with excipient (0.5% polysorbate) to produce solutions for the dose response experiments in the Alderley Park (5 - 200 mg/kg and 15 - 60 mg/kg nicotinic acid) and Zucker rats (10 - 200 mg/kg and 1.25 - 25 mg/kg nicotinic acid) (appendix 3).

Both Alderley Park and Zucker rats were anaesthetised by inhalation of fluothane and bled 2 hours post dose for the Alderley Park rats and 90 minutes post dose for the Zucker rats. The Zucker rats were anaesthetised earlier due to the excessive length of time taken for them to be anaesthetised as a consequence of their size and fat content. Blood samples (5 ml) were obtained by aortic bleeding and collected into Na_2EDTA tubes (final concentration of 1 mg/ml).

Rats were killed by exsanguination and disposed of by the animal unit at Zeneca Pharmaceuticals, Macclesfield.

2.2 Laboratory methods.

Plasma was harvested by centrifugation (1800 rpm, 15 minutes, 4°C) and frozen at -20°C until analysis. Lipolysis was measured primarily by quantitative enzymatic determination of plasma FFA (Wako NEFA C Kit), and plasma free glycerol (Sigma GPO-Trinder Kit). True triglyceride (total triglyceride minus free glycerol) and total triglyceride concentrations (Sigma GPO-Trinder Kit) were also determined. This kit releases glycerol from triglyceride and measures total glycerol concentrations present (including any free glycerol)). Concentrations of each variable were determined spectrophotometrically at 540 nm, with reference to standard curves. Coefficient of variation was $<3\%$, for each variable. Assays were performed in triplicate.

Only plasma FFA levels were measured in the Alderley Park rats. Plasma FFA, free glycerol, true triglyceride and total triglyceride concentrations were determined in the Zucker rats. FFA IC_{50} values for nicotinic acid, were calculated for both the Alderley Park and Zucker rats. Glycerol IC_{50} values were calculated for the Zucker rats.

2.3 Data analysis.

The mean and standard deviation of means (before log transformation) for each variable were reported. Due to large variations in plasma FFA, free glycerol, true triglyceride and total triglyceride concentrations an Anderson-Darling test for normal distribution was carried out on the data. Data was not found to be normally distributed and was thus log transformed. Analysis of variance (ANOVA) was performed (on the log transformed data) to see if there were any significant differences in the concentrations of plasma FFA, free glycerol, true triglyceride and total triglyceride concentrations in rats who were given nicotinic acid compared to the control group of rats. The significance level was set at $p<0.05$. When overall significant differences were observed, a Fisher's pairwise comparison was carried out to identify the groups that contributed to the significant difference.

3 Results.

Two experiments were carried out to establish a dose response for nicotinic acid in Alderley Park rats: the first over a wide concentration range of 5 - 200 mg/kg nicotinic acid and the second over a more specific range 15 - 60 mg/kg. The mean plasma FFA concentrations from both experiments were pooled and a dose response curve plotted from which the FFA IC_{50} value was determined. These results were used to establish a preliminary concentration range for administration of nicotinic acid to the Zucker rats. Similarly, two experiments were carried out to establish a dose response for nicotinic acid in the Zucker rats: the first over a wide concentration range of 10 - 200 mg/kg of nicotinic acid and the second over a more specific range 1.25 - 25 mg/kg of nicotinic acid. The results from both experiments were used together to produce dose response curves for plasma FFA and free glycerol from which the FFA and free glycerol IC_{50} values were determined. Free glycerol, true triglyceride and total triglyceride concentrations were measured in the Zucker rats.

3.1 Effect of administration of nicotinic acid (5 - 200 mg/kg) in fasted male Alderley Park rats.

In the fasted male Alderley Park rats, administration of nicotinic acid produced an overall significant decrease *($p<0.001$)* in plasma FFA levels compared to the control rats. Fisher's pairwise comparison showed that administration of doses of 25 mg/kg of nicotinic acid and above produced significant differences *($p<0.05$)* in plasma FFA levels from the control rats thus contributing to the overall significant decrease in

plasma FFA levels (table 5.1). Administration of a dose of nicotinic acid as low as 25 mg/kg produces more than 50% inhibition of plasma FFA levels, with doses of 100 and 200 mg/kg nicotinic acid producing almost complete inhibition of plasma FFA levels compared to the control rats, suggesting almost complete inhibition of adipocyte lipolysis.

Table 5.1: ***Mean plasma FFA concentrations in Alderley Park rats after administration of nicotinic acid over doses 5 - 200 mg/kg.*** *Significantly different from control group as assessed by ANOVA and Fisher's pairwise comparison.*

Nicotinic acid dose (mg/kg)	**FFA Mean ± SD (μmol/L)**	***p* value when log transformed**
control	1210 ± 184	-
5	1180 ± 168	NS
10	1268 ± 212	NS
25	592 ± 575	<0.05
50	369 ± 441	<0.05
100	9.80 ± 0.40	<0.05
200	22.30 ± 19.40	<0.05

**** Doses of nicotinic acid (mg/kg) administered to the rat are very large compared to those given to humans.***

3.2 Effect of administration of nicotinic acid (15 - 60 mg/kg) in fasted male Alderley Park rats.

In the fasted male Alderley Park rats administration of nicotinic acid produced an overall significant decrease *($p<0.001$)* in plasma FFA levels compared to the control rats. Fisher's pairwise comparison showed that after administration of nicotinic acid doses of 45 and 60 mg/kg, plasma FFA levels were significantly decreased compared to the control rats, thus contributing to the overall significant difference seen (table 5.2). Administration of 45 mg/kg nicotinic acid, produced approximately 44% inhibition of plasma FFA levels, as compared to the control rats, with almost complete inhibition (94%) produced by administration of 60 mg/kg of nicotinic acid compared to the control group.

Table 5.2: ***Mean plasma FFA concentrations in Alderley Park rats after administration of nicotinic acid over doses 15 - 60 mg/kg (before log transformation).*** *Significantly different from control group as assessed by ANOVA and Fisher's pairwise comparison.*

Nicotinic acid dose (mg/kg)	**FFA Mean ± SD (μmol/L)**	***p* value when log transformed**
control	695 ± 112	-
15	753 ± 76	NS
30	691 ± 259	NS
45	387 ± 437	<0.05
60	40 ± 11	<0.05

The mean plasma FFA concentrations for the various doses of nicotinic acid administered from both experiments were pooled (using data before log transformation), and a dose response curve for nicotinic acid in Alderley Park rats was plotted (figure 5.3) and the FFA IC_{50} value was estimated to be approximately 45 mg/kg.

3.3 Effect of administration of nicotinic acid (10 - 200 mg/kg) in fasted male Zucker rats.

Analysis using ANOVA (after log transformation) showed that after administration of nicotinic acid there was an overall significant decrease in plasma FFA, free glycerol, true triglyceride and total triglyceride levels compared to control rats (table 5.3).

Plasma FFA.

Administration of nicotinic acid produced an overall significant decrease ($p<0.001$) in plasma FFA concentrations compared to control rats. Fisher's pairwise analysis showed plasma FFA levels, for all doses of nicotinic acid administered, to be significantly decreased ($p<0.05$) contributing to the overall significant decrease achieved (table 5.3).

In the Zucker rats, plasma FFA concentrations were significantly decreased compared to controls, with administration of a dose of nicotinic acid as low as 10 mg/kg producing a 74% inhibition of plasma FFA concentration. The other doses of nicotinic

acid, 25, 50, 100 and 200 mg/kg produced 77%, 81%, 78% and 81% inhibition of plasma FFA levels compared to the control rats.

Table 5.3: ***Mean plasma FFA, free glycerol, true triglyceride and total triglyceride concentrations in Zucker rats after administration of nicotinic acid over doses 10 - 200 mg/kg (before log transformation).*** *Significantly different from control group (after log transformation) as assessed by ANOVA and Fisher's pairwise comparison; *$p < 0.05$.*

Nicotinic acid dose (mg/kg)	FFA Mean±SD (μmol/L)	Free glycerol Mean±SD (mmol/L)	True triglyceride Mean±SD (mmol/L)	Total triglyceride Mean±SD (mmol/L)
control	1037 ± 128	0.90 ± 0.18	2.55 ± 0.74	3.54 ± 0.805
10	270 ± 103*	0.28 ± 0.08*	2.06 ± 1.72	2.36 ± 1.76*
25	241 ± 41*	0.28 ± 0.04*	1.10 ± 0.31*	1.40 ± 0.34*
50	193 ± 53*	0.41 ± 0.21*	2.19 ± 1.64	2.62 ± 1.86
100	225 ±208*	0.25 ± 0.13*	1.04 ± 0.29*	1.31 ± 0.42*
200	193 ± 75*	0.29 ± 0.03*	1.30 ± 0.41*	1.61 ± 0.43*
Overall significant difference (p value)	<0.001	<0.001	0.013	0.02

Plasma free glycerol.

Plasma free glycerol levels behaved in a similar manner as plasma FFAs (table 5.3). Analysis using ANOVA showed there to be an overall significant decrease ($p < 0.001$) in plasma free glycerol concentrations produced by administration of nicotinic acid over the dose range of 10 - 200 mg/kg compared to control group. Fisher's pairwise analysis showed that after administration of all doses of nicotinic acid administered, plasma free glycerol levels were significantly decreased ($p < 0.05$) from the control rats contributing to the overall significance level of inhibition achieved. Plasma free glycerol concentrations were significantly decreased compared to controls, with administration of a dose of nicotinic acid as low as 10 mg/kg producing a 69% inhibition of plasma free glycerol levels. The other doses of nicotinic acid, 25, 50, 100 and 200 mg/kg produced 69%, 54%, 72%, and 68% inhibition of plasma free glycerol levels compared to the control group.

Plasma true triglyceride.

Analysis using ANOVA showed there to be an overall significant decrease ($p=0.013$) (table 5.3) in plasma true triglyceride levels after nicotinic acid administration over a dose range of 10 - 200 mg/kg, compared to the control group of rats. Fisher's pairwise analysis showed that after administration of nicotinic acid doses of 25, 100 and 200 mg/kg, plasma true triglyceride levels were significantly decreased ($p<0.05$) compared to the control group therefore contributing to the overall significant difference seen. The levels of inhibition of plasma true triglyceride concentrations were not as pronounced as those obtained for plasma FFAs. Plasma true triglyceride levels were inhibited by 19%, 57%, 14%, 59%, and 49% at the nicotinic acid doses of 10, 25, 50, 100 and 200 mg/kg respectively.

3.4 Effect of administration of nicotinic acid (1.25 - 25 mg/kg) in fasted male Zucker rats.

Analysis using ANOVA (after log transformation) showed that after administration of nicotinic acid (1.25 - 25 mg/kg), there was an overall significant decrease in plasma FFA, free glycerol and total triglyceride compared to control rats. True triglyceride levels showed no significant differences compared to the control rats (table 5.4).

Plasma FFA.

Administration of nicotinic acid (1.25 - 25 mg/kg) produced an overall significant decrease ($p<0.001$) in plasma FFA levels. Ten and 25 mg/kg doses of nicotinic acid caused a significant decrease ($p<0.05$) in levels of FFA, thus contributing to the overall significant decrease observed (table 5.4). In the Zucker rats, plasma FFA concentrations were significantly decreased compared to control rats with plasma FFA levels inhibited by 54% and 79% at doses as low as 10 mg/kg and 25 mg/kg of nicotinic acid, respectively. Doses of nicotinic acid lower than 10 mg/kg had no inhibitory effect on plasma FFA levels.

Plasma free glycerol.

Analysis using ANOVA showed there to be an overall significant difference *($p < 0.001$)* in plasma free glycerol levels after nicotinic acid administration (1.25 - 25 mg/kg), compared to the control group. Fisher's pairwise comparisons showed that after administration of nicotinic acid, 25 mg/kg was the only dose where the plasma free glycerol levels decreased significantly *($p < 0.05$)* compared to the control rats contributing to the overall significant decrease *($p < 0.001$)* seen (table 5.4). The levels of plasma free glycerol were inhibited by 17%, 9%, 17%, 40% and 84% compared to control rats at doses of 1.25, 2.5, 5, 10, and 25 mg/kg of nicotinic acid respectively.

Plasma true triglyceride.

Analysis using ANOVA showed there to be no overall significant difference in plasma true triglyceride levels (table 5.4) after administration of nicotinic acid (1.25 - 25 mg/kg) compared to the control group of rats. Plasma true triglyceride levels were inhibited by 9%, 26%, 37%, 47%, and 29% at the nicotinic acid doses of 1.25, 2.5, 5, 10 and 25 mg/kg respectively.

Table 5.4: ***Mean plasma FFA, free glycerol, true triglyceride and total triglyceride concentrations in Zucker rats after administration of nicotinic acid over doses 1.25 - 25 mg/kg (before log transformation).*** *Significantly different from control group (after log transformation) as assessed by ANOVA and Fisher's pairwise comparison; *$p < 0.05$.*

Nicotinic acid dose (mg/kg)	**FFA Mean ± SD (μmol/L)**	**Free Glycerol Mean ± SD (mmol/L)**	**True TG Mean ± SD (mmol/L)**	**Total TG Mean ± SD (mmol/L)**
control	1217 ± 400	0.70 ± 0.18	2.72 ± 1.01	3.45 ± 1.20
1.25	1184 ± 194	0.58± 0.20	2.46 ± 1.05	3.07 ± 1.24
2.5	1324 ± 264	0.64 ± 0.15	2.02 ± 0.95	2.68 ± 1.06
5	1347 ± 219	0.58 ± 0.09	1.71 ± 0.39	2.31 ± 0.43
10	557 ± 202*	0.42 ± 0.12	1.44 ± 0.26*	1.87 ± 0.25*
25	256 ± 71*	0.11 ± 0.08*	1.93 ± 0.92	2.05 ± 0.97*
Overall significant difference (*p* value)	<0.001	<0.001	0.102	0.035

The mean plasma FFA concentrations for the various doses of nicotinic acid administered from both experiments were pooled (using data before log transformation), and a dose response curve for the effect of nicotinic acid on plasma FFA levels in Zucker rats was plotted (figure 5.3) and the FFA IC_{50} value was calculated by extrapolation at 50% control and was estimated to be approximately 10 mg/kg.

Similarly the mean plasma free glycerol concentrations for the various doses of nicotinic acid administered from both experiments were pooled (using data before log transformation), and a dose response curve for the effect of nicotinic acid on plasma free glycerol levels in Zucker rats was plotted (figure 5.4) and the free glycerol IC_{50} value was calculated by extrapolation at 50% control and was estimated to be approximately 10 mg/kg.

3.5 Effect of administration of nicotinic acid (1.25 - 25 mg/kg) in fasted female Zucker rats.

Female fasted Zucker rats were used for one experiment but there was no relationship between mean plasma FFA concentrations and dose of nicotinic acid given even after log transformation (table 5.6). It was decided not to use female Zucker rats for any further experiments: plasma FFA levels were too variable and unreliable thus not giving a clear representation of the effects of nicotinic acid on plasma FFAs.

Table 5.5: Mean plasma FFA concentrations in female Zucker rats after administration of nicotinic acid over doses 1.25 - 25 mg/kg (before log transformation).

Nicotinic acid dose (mg/kg)	**FFA Mean ± SD (μmol/L)**	**% Inhibition of plasma NEFA levels**
control	719 ± 295	0
1.25	733 ± 481	-1.8
2.5	543 ± 24	24
5	860 ± 502	-20
10	326 ± 500	55
25	273 ± 311	62
Overall significant differences (*p* value)	0.069	-

4 Discussion.

It is widely believed that raised plasma FFA levels induce insulin resistance (hyperinsulinaemia) as seen for example in NIDDMs. The Zucker rat provides a good animal model of insulin resistance and if plasma FFA levels could be normalised with nicotinic acid for example over a 24 hour period, then perhaps insulin levels may also be reduced accordingly. Preliminary dose response studies were carried out in Alderley Park rats to establish a dosing range for nicotinic acid administration in the Zucker rats; FFA IC_{50} values were estimated for nicotinic acid in both Zucker and Alderley Park rats. It is known that plasma FFA levels are lower during the day when constant browsing will cause insulin to be released from the pancreas, which in turn will inhibit lipolysis and FFA production. During the evening and night however plasma FFA levels rise to almost fasting levels and it is at this time that nicotinic acid should be administered to keep FFAs low. Changing the lighting scheme should make it easier to accomplish this so experiments can be carried out in the fed state.

Nicotinic acid inhibits triglyceride lipolysis in adipose tissue, exerting its antilipolytic effect by inhibiting HSL stimulated adipocyte lipolysis. Work performed in the previous chapter using Alderley Park primary rat adipocytes isolated from fasted male rat epididymal fat pads, showed that the antilipolytic effect of nicotinic acid is exerted on the adipocyte signal transduction pathway for HSL activation (chapter I, figure 1.8). More precisely, nicotinic acid acts as a G_i-linked receptor agonist inhibiting adenylate cyclase via G_i coupled receptors and therefore decreasing intracellular cAMP levels. Plasma FFA levels were used merely as an indicator that nicotinic acid was inhibiting adipocyte lipolysis *in vivo*. Nicotinic acid clearly had an inhibitory, dose related effect in both male Alderley Park and Zucker rats. From the dose response curves for FFA and glycerol levels, fasted male Zucker rats seem to be more sensitive to nicotinic acid (FFA IC_{50} approximately 10 mg/kg) than their Alderley Park counterparts (FFA IC_{50} approximately 45 mg/kg) which could be due to either increased fasting levels of FFA in Zucker rats or a different HSL activation state. However it must be taken into account that the Zucker rats were bled 90 minutes post dose, due to the length of time taken to anaesthetise them due to their excessive amount of body fat, and to prevent excessive fasting for some of the animals, whereas the fasted male Alderley Park were bled 120 minutes post dose. The earlier bleeding of the Zucker rats would favour a lower IC_{50} value; this issue would therefore have to be resolved by repeating the Alderley Park rat dose response experiments and bleeding them 90 minutes post dose. In the fasted male Zucker rats the glycerol IC_{50} was estimated to be 10 mg/kg thus confirming the results seen in the previous chapter that

nicotinic acid appears to inhibit FFA and glycerol levels to the same extent both *in vivo* and *in vitro*.

These preliminary studies with nicotinic acid in the Zucker rat were carried out with a long term aim towards acquiring a dose of nicotinic acid which would normalise insulin levels in the Zucker rat. This would be of great use, as Zucker rats are hyperinsulinaemic and develop marked hyperlipoproteinaemia as a result of over-production of VLDL enriched in triglyceride similar to the situation in NIDDMs. It was hoped that by finding a dose of nicotinic acid that would suppress FFA levels (for example) for 24 hours or longer, this would normalise plasma insulin levels and maybe even start to correct some of the defects in the adipose tissue-liver relationship in Zucker rats.

Figure 5.1: Reverse phase lighting conditions.

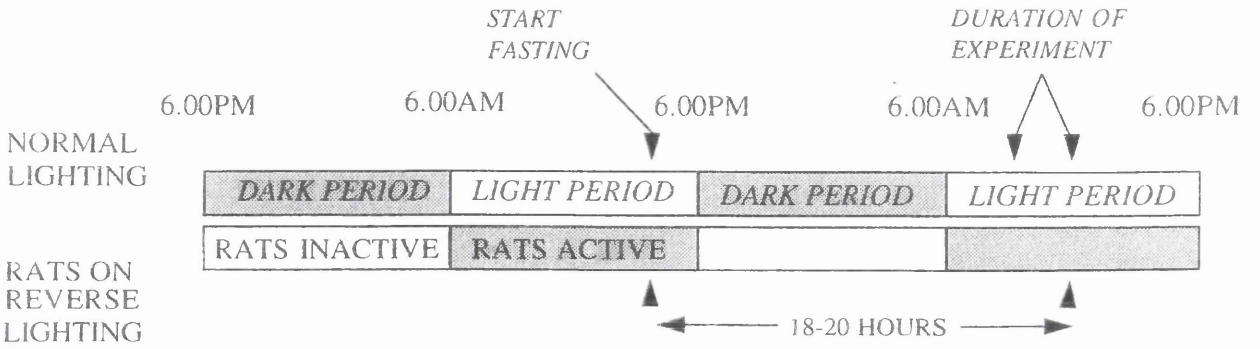


Figure 5.2: Nicotinic acid dose response curves for plasma FFA levels in the fasted male Alderley Park rats (IC_{50}=45 mg/kg) and Zucker rats (IC_{50}=10 mg/kg).

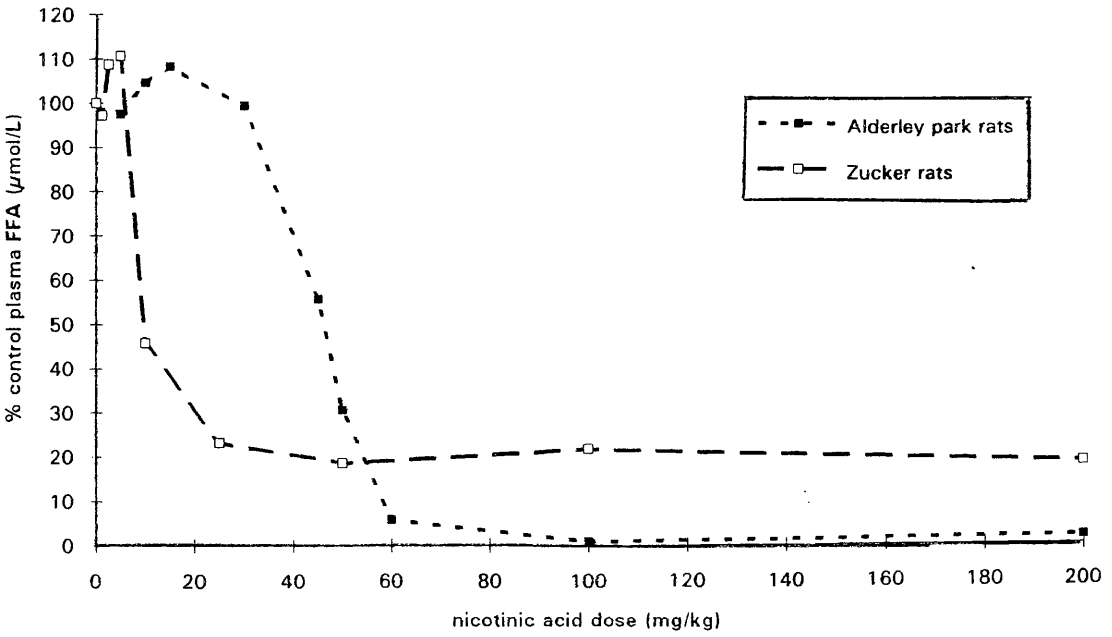
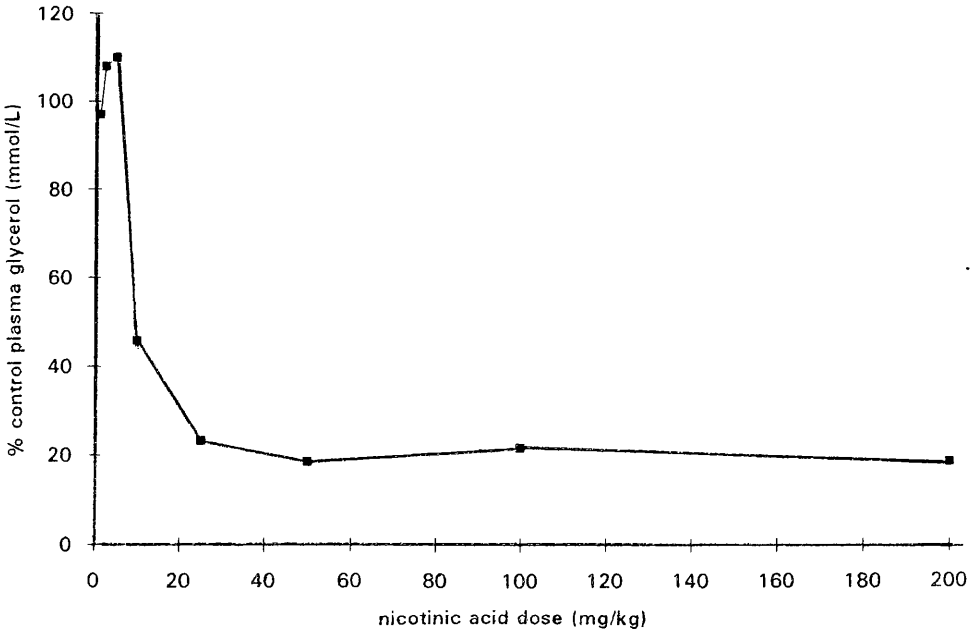


Figure 5.3: Nicotinic acid dose response curves for plasma free glycerol levels in the fasted male Zucker rats (IC_{50}=10 mg/kg).



Chapter VI The regulation of intestinal and hepatic lipoprotein synthesis in normal patients after a fat meal.

1 Introduction.

Triglyceride-rich lipoproteins (TRL) are a heterogeneous population of lipoprotein particles. VLDL are produced in the liver and contain apoB100 and endogenous lipids. In contrast, ingestion of fat leads to the formation of chylomicrons containing apoB48 in the intestine which compete for a common lipolytic pathway with VLDL *(Brunzell et al 1973)*. The lipoprotein species of primary interest in the postprandial phase are the intestinally derived chylomicrons. Also of recent interest is the effect of postprandial chylomicronaemia upon VLDL subfraction metabolism. Suprisingly, it has been shown that the postprandial increase in TRL particle numbers are mainly accounted for by VLDL, particularly large VLDL, $VLDL_1$ *(Cohn et al 1988 a and b, Karpe et al 1993, Schneeman et al 1993)* whereas 80% of postprandial triglyceride is carried by lipoprotein particles containing apoB48. Most lipoprotein studies have been carried out in the fasting state, but humans spend most of their time in the postprandial phase. Investigations in the fed state require to be carried out, to understand the changes in lipoprotein metabolism postprandially. Traditionally these studies have been performed after subjects receive either an oral fat meal or an intravenous Intralipid infusion.

1.1 Quantification of postprandial lipoprotein metabolism.

In the past, several methods have been developed and used to study postprandial metabolism. Originally, postprandial lipoprotein metabolism was evaluated by measuring triglyceride concentrations in whole plasma and in TRL ($d < 1.006$g/ml) fractions after an oral fat load *(Foger and Patsch 1993)*. Triglyceride is the chief component of chylomicrons (constituting 85%) (chapter I, table 1.2) and was used in this study to monitor chylomicron levels postprandially. Chylomicrons and VLDL were isolated together in the TRL ($d < 1.006$g/ml) fraction after a single sequential ultracentrifugation spin, following ingestion of an oral fat load. This yielded important

insights into TRL metabolism but gave no indication or quantification of which lipoprotein fractions were responsible for changes in triglyceride levels in the $d < 1.006$g/ml fraction postprandially. Indeed it was assumed that all changes occurred in apoB48 particles, so other methods for quantification were required to be used to get a clearer picture of what was happening.

Postprandial triglyceridaemia predominately represents the presence in the plasma of large TRL, of both intestinal and hepatic origin. To determine the contribution of each of these species to postprandial triglyceridaemia several methods have been developed. These were either used separately or in combination to determine the contribution of both TRL species to postprandial triglyceridaemia. Both apolipoprotein (apoB48 and apoB100) determination and retinyl esters (RE) have been used for this purpose. RE (retinyl palmitate) have been employed as a marker of cholesteryl ester transport by chylomicrons and their remnants *(Berr and Kern 1984)*. RE are incorporated into the core of chylomicrons in the intestine, following the absorption and esterification of vitamin A *(Goodman et al 1966)*, the rationale *(Berr and Kern 1984)* for this approach is based on the concept that dietary vitamin A is esterified in the intestine and is incorporated into the core of chylomicron particles. These lipoproteins are secreted into intestinal lymph and their triglyceride component hydrolysed by LPL in the circulation. It is believed that the RE remain associated with chylomicrons during lipolysis and are taken up by the liver within chylomicron remnants via a receptor-mediated process. Evidence suggests that the liver does not re-secrete these RE and they are either stored or exported as unesterified retinol bound to retinal-binding protein. As plasma exchange between lipoproteins is believed to be minimal *(Berr and Kern 1984)* and RE are not re-secreted into the circulation, it was postulated that RE in plasma were appropriate markers for lipoproteins containing apoB48 of intestinal origin. Supplementation of test fat meals with vitamin A has therefore been commonly used as a means of quantifying lipoproteins of intestinal origin in the postprandial state *(Simpson et al 1990, Karpe et al 1995)*. This method has been adopted by several groups in combination with other methods. However a recent study *(Cohn et al 1993)* found that RE within the TRL fraction were not always associated with the apoB48 containing lipoproteins and they are therefore not necessarily indicative of TRL of intestinal origin. RE were found to be regularly detected in apoB100 TRL as well as in apoB48 samples. It was demonstrated that an increase in VLDL triglyceride accounts for approximately 20% of postprandial triglyceride. A significant portion of retinyl palmitate, up to 25% is bound to apoB100 containing TRL at later time points, which suggests that it derives from transfer of core lipids between lipoproteins. Thus RE labelling of chylomicrons seems not to be a valid means over the longer term of

quantifying TRL particles of intestinal origin in the postprandial state but retinyl palmitate is a valid tracer substance of intestinal lipoproteins over the first few hours following ingestion of a fatty meal and as such, is useful in conjunction with other methods.

Concentrations of apoB48 and apoB100, the main structural components of TRL of both intestinal and hepatic origin respectively *(Scott 1990)* can be quantified after isolation of lipoproteins by ultracentrifugation, by SDS PAGE and subsequent densitometric scanning of protein bands stained with Coomassie Blue stain (chapter II, section 2.10.1) *(Poapst et al 1987, Zilversmit and Shea 1989, Karpe and Hamsten 1994, Kotite, Bergeron and Havel 1995).* One or two groups have already devised methods using monoclonal antibodies to separate TRL into apoB48 and apoB100 TRL species, as described previously in chapter III. The methodology developed in chapter III allows the separation of apoB48 (chylomicrons) and apoB100 (VLDL) TRL species, but goes on further to delineate the behaviour of VLDL subfractions (large and small VLDL, $VLDL_1$ and $VLDL_2$) during postprandial lipaemia.

1.2 Aim.

This study was undertaken to delineate the behaviour of VLDL subfractions in both normal and diabetic patients (see chapter VII) during postprandial lipaemia. These lipoprotein particles were isolated by immunoaffinity chromatography and cumulative flotation.

2 Methods.

2.1 Patient selection, clinical measurements and oral fat tolerance tests.

Nine healthy subjects (6 males and 3 females) (table 6.1) were recruited from laboratory staff, friends and family. Initially a single fasting blood sample was taken to check plasma lipid levels were within the reference range (appendix 7). All were non-smokers.

Table 6.1: Normal subject characteristics.

Subjects (n=9)	Mean (± SD)
Age (years) BMI (kg/m^2)	36 ± 10 23.03 ± 1.19
Triglyceride (mmol/L)	1.11 ± 0.45
Cholesterol (mmol/L)	4.84 ± 0.72
VLDL cholesterol (mmol/L)	0.48 ± 0.17
LDL cholesterol (mmol/L)	2.91 ± 0.65
HDL cholesterol (mmol/L)	1.28 ± 0.34

Subjects were admitted at 8 am after an overnight fast, and a 22G venous cannula was placed in one forearm. Blood samples were taken through this during the day, the cannula being kept patent by flushing with normal saline (0.9%). A standard fat milkshake was prepared, containing 137 grams fat with a total energy content of 1400 kcal *(Warwick et al 1992)*. The oral fat load consisted of 280 ml double cream, 20 grams sugar, 20 grams dried skimmed milk and 20 ml flavouring. After a 12 hour fast, subjects drank the milkshake within 15 minutes. The milkshake was well tolerated by all subjects. The subjects fasted for a further 11 hours, with free access to unlimited low calorie soft drinks.

A fasting blood sample was taken before intake of the fat meal, subsequent blood samples being drawn hourly for 11 hours after the ingestion of the fat meal. At 4 pm, the patients were given a low calorie fat free meal. In total, about 300 ml blood was taken during the turnover study. The bleeding schedule is given in appendix 8. Blood samples were collected into Na_2EDTA tubes. For every time point, plasma was harvested by centrifugation (3000 rpm (1300 g), 15 minutes, 4^oC) and stored at 4^oC until analysis. For each time point, TRL ($d < 1.006 g/ml$) were freshly isolated from plasma by sequential flotation ultracentrifugation (39000 rpm (108000 g), 15^oC, 16 hours) (chapter II, section 2.7) and subjected to immunoaffinity chromatography (using a Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes) (chapter III, section 3.6) in order to separate apoB48 and apoB100 containing TRL, *i.e.* chylomicrons and VLDL, respectively. VLDL were further separated into large

$VLDL_1$ and small $VLDL_2$ subfractions by cumulative flotation *(Lindgren and Jensen 1972)* (chapter II, section 2.8)

ß-Quantification of fasting lipids and lipoproteins, and apoE phenotyping were measured on the fasting plasma sample (0 hour). Plasma insulin concentrations were measured at 0 and 2 hours. ApoB and AI concentrations were measured in plasma and the TRL ($d < 1.006$g/ml) fractions at all time points. Triglyceride and cholesterol concentrations were measured in all plasma, TRL ($d < 1.006$g/ml), apoB48 (unbound), apoB100 (bound), large $VLDL_1$ and small $VLDL_2$ fractions at all time points. Plasma FFA concentrations were measured at all time points.

Bleeding of normal subjects was carried out by Dr. Vian Anber of the Lipid Research Group. The fat milkshakes were prepared on the morning of the oral fat loads by the hospital diet cooks, in the hospital kitchens.

2.2 Ethics approval.

Studies were approved by the Ethics Committee of Glasgow Royal Infirmary.

2.3 Statistical analysis.

Mean and standard deviation of means for plasma parameters were reported. Paired t-tests were used to determine the statistical significance for lipid, apoproteins and insulin, between measurements obtained from fasting (0 hour) and postprandial samples. Area measurements were expressed as mmol.hour. The Minitab 10 statistical package was used to calculate regression coefficients and *p* values for correlations.

3 Results.

The ß-Quantification measurements for the initial fasting plasma samples for each patient are shown in table 6.2. All fasting plasma lipid levels were within the acceptable reference range for the laboratory (appendix 7).

Triglyceride, cholesterol, apoB, and apoAI concentrations determined in the various lipoprotein fractions were adjusted back to plasma levels. Triglyceride concentration was used to study the metabolism of postprandial lipoproteins. Individual plasma, TRL ($d < 1.006$g/ml), apoB48, apoB100, $VLDL_1$ and $VLDL_2$ triglyceride

concentrations before and after ingestion of a fat load are shown in tables in the appendices (9 - 14). Individual plasma cholesterol, FFA, plasma and TRL (d < 1.006g/ml) apoB and AI concentrations before and after ingestion of a fat load are shown in tables in the appendices (15 - 20).

Table 6.2: ß-Quantification of lipids and lipoproteins and apoE phenotypes.

Lipid levels (mmol/L)	Subjects								
	BC	MM	BJ	KE	HA	CN	JM	IC	AG
Plasma triglyceride	1.20	0.95	0.85	1.15	1.00	1.30	0.50	0.90	2.11
Plasma cholesterol	3.6	5.35	5.90	4.70	4.80	4.05	3.75	4.70	5.44
VLDL cholesterol	0.40	0.50	0.50	0.35	0.35	0.45	0.90	0.35	0.60
LDL cholesterol	2.35	3.35	4.10	2.65	2.95	2.40	2.10	2.70	3.60
HDL cholesterol	0.85	1.50	1.30	1.70	1.50	1.20	0.75	1.65	1.05
ApoE phenotype	4/3	3/3	3/3	3/2	3/3	4/3	3/3	4/3	3/2

Mean (±SD) plasma triglyceride concentrations before and after the ingestion of the fat load are shown in table 6.3 and figure 6.1. Mean plasma cholesterol concentrations did not change significantly during the course of the experiment. After the fat load, individual plasma triglyceride concentrations peaked between 3 - 7 hours postprandially, with triglyceride concentrations ranging from 1.75 - 6.04 mmol/L, and a mean peak of 3.45 ± 1.36 mmol/L (table 6.4). (Note table 6.3 shows mean triglyceride levels over the time course of the experiment while table 6.4 shows the mean maximal triglyceride value for subjects taken when their individual peaks occurred, usually at 4, 5, or 6 hours). When all individual plasma triglyceride response curves were combined, there was a peak at 5 hours postprandially with a mean triglyceride concentration of 2.84 ± 1.33 mmol/L (table 6.3). The mean plasma triglyceride concentration was significantly elevated ($p < 0.05$) above fasting concentration 1 - 8 hours postprandially (table 6.3, figure 6.1).

Table 6.3: ***Postprandial changes in mean triglyceride concentrations (mmol/L ± SD) in plasma and all TRL fractions in normal subjects after an oral fat load.*** *Significantly different from fasting concentration by paired t-test *p < 0.05, **p < 0.01, ***p < 0.005*

Time (hours)	Mean Plasma TG (mmol/L ± SD)	Mean TRL (d < 1.006 g/ml) TG (mmol/L ± SD)	Mean ApoB48 TRL TG (mmol/L ± SD)	Mean ApoB100 TRL TG (mmol/L ± SD)	Mean $VLDL_1$ TG (mmol/L ± SD)	Mean $VLDL_2$ TG (mmol/L ± SD)
0	1.11 ± 0.45	0.61 ± 0.36	0.21 ± 0.15	0.36 ± 0.21	0.22 ± 0.16	0.08 ± 0.03
1	1.26 ± 0.50**	0.75 ± 0.43*	0.32 ± 0.23*	0.38 ± 0.25	0.23 ± 0.17	0.07 ± 0.3
2	1.94 ± 0.84***	1.21 ± 0.57***	0.64 ± 0.32***	0.47 ± 0.23***	0.31 ± 0.20***	0.08 ± 0.03
3	2.55 ± 1.18***	1.63 ± 0.87***	0.98 ± 0.59***	0.52 ± 0.32***	0.35 ± 0.25**	0.08 ± 0.02
4	2.75 ± 1.34***	1.69 ± 0.82***	1.05 ± 0.58***	0.50 ± 0.30*	0.34 ± 0.21*	0.07 ± 0.02
5	2.84 ± 1.33***	1.88 ± 0.98***	1.29 ± 0.83***	0.56 ± 0.36*	0.37 ± 0.29	0.07 ± 0.03
6	2.78 ± 1.68**	1.84 ± 1.34*	1.20 ± 1.03*	0.49 ± 0.39	0.34 ± 0.32	0.07 ± 0.03
7	2.43 ± 1.31**	1.52 ± 1.01*	0.91 ± 0.65**	0.47 ± 0.38	0.31 ± 0.29	0.07 ± 0.03
8	1.94 ± 1.16*	1.21 ± 0.88	0.73 ± 0.58*	0.39 ± 0.31	0.23 ± 0.22	0.058 ± 0.04
9	1.78 ± 1.05	1.04 ± 0.81	0.63 ± 0.63	0.33 ± 0.26	0.21 ± 0.17	0.06 ± 0.04
10	1.39 ± 0.74	0.76 ± 0.58	0.43 ± 0.45	0.25 ± 0.17	0.14 ± 0.08	0.06 ± 0.04
11	1.05 ± 0.45	0.52 ± 0.34	0.26 ± 0.19	0.22 ± 0.13	0.11 ± 0.05	0.05 ± 0.03

TRL (d < 1.006g/ml) fractions were isolated from plasma by sequential flotation ultracentrifugation and were separated into apoB48 and apoB100 TRL species by immunoaffinity chromatography. The apoB100 TRL species were further separated into large and small VLDL subfractions ($VLDL_1$ and $VLDL_2$ respectively) by cumulative flotation.

In the fasting state (time = 0 hour) the mean plasma triglyceride concentration was 1.11 ± 0.45 mmol/L (table 6.4) and mean TRL (d < 1.006g/ml), apoB48, apoB100, $VLDL_1$ and $VLDL_2$ triglyceride concentrations were 0.61 ± 0.36 mmol/L, 0.21 ± 0.15 mmol/L, 0.36 ± 0.21 mmol/L, 0.22 ± 0.16 mmol/L and 0.08 ± 0.03 mmol/L respectively.

Table 6.4; Mean of maximal postprandial triglyceride levels for individual subjects after a fat load when mean peaks occurred, usually at 4, 5 or 6 hours.

Lipoprotein fraction	Mean fasting TG (mmol/L)	Mean individual peak TG (mmol/L)	Mean peak (hours)
Plasma	1.11 ± 0.45	3.45 ± 1.36	5
TRL (d < 1.006g/ml)	0.61 ± 0.36	1.88 ± 0.98	5
ApoB48	0.21 ± 0.15	1.56 ± 0.80	5
ApoB100	0.36 ± 0.21	0.63 ± 0.35	5
$VLDL_1$	0.22 ± 0.16	0.43 ± 0.29	5
$VLDL_2$	0.08 ± 0.03	0.08 ± 0.03	-

In the fed state at the time of maximal triglyceride increase (mean 5 hours postprandially for all lipoprotein fractions), there was a 68% increase in both mean plasma and mean TRL (d < 1.006g/ml) triglyceride concentrations. Mean apoB48 TRL triglyceride concentrations showed an 84% increase, with mean apoB100 TRL triglyceride levels increasing by 36%. Mean large $VLDL_1$ and small $VLDL_2$ triglyceride levels increased by 41% and 4% respectively.

Table 6.3 shows mean TRL (d < 1.006g/ml) triglyceride concentrations after the ingestion of a fat meal. Individual TRL (d < 1.006g/ml) triglyceride concentrations peaked between 3 - 7 hours postprandially, with triglyceride concentrations ranging from 1.09 - 4.35 mmol/L; and a mean peak of 1.88 ± 0.98 mmol/L (table 6.4). When all the individual TRL (d < 1.006g/ml) triglyceride response curves were combined, there was a mean peak at 5 hours postprandially with a mean triglyceride concentration of 1.88 ± 0.98 mmol/L (table 6.3). The mean TRL (d < 1.006g/ml) triglyceride

concentrations were significantly elevated ($p<0.05$) above fasting concentrations 1 - 7 hours postprandially, in accord with total plasma data (table 6.3).

The mean triglyceride concentrations of plasma, TRL (d<1.006g/ml), apoB48 and apoB100, $VLDL_1$ and $VLDL_2$ fractions before and after the fat load are shown in figure 6.2. (Note significant differences from fasting triglyceride concentrations in plasma and TRL (d<1.006g/ml) fractions before and after a fat load are shown in figure 6.2, while those for apoB48, apoB100, $VLDL_1$ and $VLDL_2$ fractions are shown in figure 6.3) After the fat load, individual apoB48 triglyceride concentrations peaked between 3 - 8 hours postprandially, with triglyceride concentrations ranging from 0.67 - 3.00 mmol/L; and a mean peak of 1.56 $\pm$ 0.80 mmol/L (table 6.4). When all individual apoB48 triglyceride response curves were combined there was a mean peak at 5 hours postprandially, with a mean triglyceride concentration of 1.29 $\pm$ 0.83 mmol/L (table 6.3). The mean apoB48 triglyceride concentration was significantly elevated above fasting concentrations 1 - 8 hours postprandially (table 6.3, figure 6.3). Again, the apoB48 results are in accord with plasma and TRL (d<1.006g/ml) data. The majority of the postprandial increase in plasma triglyceride concentrations seems to be due to the increase in apoB48 triglyceride concentrations. A smaller but significant increase was observed in mean apoB100 TRL triglyceride concentrations (table 6.3, figure 6.3). The individual apoB100 triglyceride concentrations increased between hours 1 - 5, peaking between 3 - 7 hours postprandially with triglyceride concentrations ranging from 0.3 - 1.44 mmol/L, and a mean peak of 0.63 $\pm$ 0.35 mmol/L (table 6.4). The mean apoB100 triglyceride concentrations were significantly elevated above fasting concentrations between 2 - 5 hours (figure 6.3, table 6.3). After peaking, the triglyceride concentration decreases rapidly after 8 hours, with the decrease becoming significant after 11 hours.

Large $VLDL_1$ triglyceride concentrations increased between 1 - 5 hours (figure 6.4), peaking individually between 3 - 6 hours postprandially, with triglyceride concentrations ranging from 0.18 - 1.13 mmol/L; and a mean peak with a triglyceride concentration of 0.43 $\pm$ 0.29 mmol/L (table 6.4). Individual $VLDL_1$ triglyceride response curves peaked then decreased rapidly after 8 hours. When all the individual large $VLDL_1$ triglyceride response curves were combined, there was a mean peak at 5 hours postprandially with a mean triglyceride concentration of 0.37 $\pm$ 0.29 mmol/L (table 6.3). Individual (appendix 14) and mean $VLDL_2$ triglyceride concentrations did not change significantly during the course of the experiment (figure 6.3, table 6.3), with triglyceride concentrations remaining relatively constant.

The contribution of apoB48 and apoB100 TRL fractions to the postprandial increase in TRL triglyceride and $VLDL_1$ and $VLDL_2$ fractions to the postprandial increase in total apoB100 TRL triglyceride (table 6.5) was quantitated by measuring the area under individual triglyceride response curves (fasting 0 hour concentration was taken as baseline). Mean areas under the curve were calculated for plasma, TRL ($d < 1.006$g/ml), apoB48, apoB100, $VLDL_1$ and $VLDL_2$ fractions (table 6.6). The increase in apoB48 TRL triglyceride was 5 times greater than the increase in apoB100 TRL triglyceride (6.13 $\pm$ 4.28 mmol.hour vs 1.25 $\pm$ 1.06 mmol.hour). Seventy nine percent ($\pm$ 8%) of the postprandial increase in TRL ($d < 1.006$g/ml) triglyceride was due to apoB48 TRL. The contribution of apoB100 TRL accounted for 15% ($\pm$ 8%) of the postprandial increase in TRL ($d < 1.006$g/ml) triglyceride. The increase in $VLDL_1$ triglyceride was 11 times greater than the increase in $VLDL_2$ triglyceride (0.94 $\pm$ 0.76 mmol.hour vs 0.086 $\pm$ 0.077 mmol.hour). Seventy five percent ($\pm$ 17%) of the postprandial increase in total apoB100 triglyceride was due to $VLDL_1$, with 10% ($\pm$ 10%) of the postprandial increase in total apoB100 accounted for by $VLDL_2$.

Table 6.5: ***Contribution of apoB48 and apoB100 TRL to postprandial increases in TRL ($d < 1.006$g/ml) triglyceride concentration, and of $VLDL_1$ and $VLDL_2$ to postprandial increases in the apoB100 TRL triglyceride concentration.*** *Quantitated by the measurement of area under the individual triglyceride response curves.*

Fraction	ApoB48	ApoB100	$VLDL_1$	$VLDL_2$
Triglyceride (mmol.hour)	6.31 $\pm$ 4.28 (79%)*	1.25 $\pm$ 1.06 (15%)*	0.94 $\pm$ 0.76 (75%)*	0.086 $\pm$ 0.077 (10%)*

****Values in parentheses represent increases expressed as percentages of the total.***

Mean cholesterol concentrations were also measured in the different TRL fractions and these results (mean cholesterol concentrations for each fraction) are shown in figure 6.5. A postprandial increase in total TRL ($d < 1.006$g/ml) cholesterol was observed in every subject, with mean TRL cholesterol concentrations increasing significantly 1 - 6 hours postprandially followed by a decrease in the mean TRL cholesterol to fasting concentrations. Mean apoB48 TRL cholesterol was significantly elevated 2 - 6 hours postprandially, returning to fasting concentrations 11 hours postprandially. There was no significant change in mean apoB100 TRL cholesterol. Mean $VLDL_1$ TRL cholesterol levels were significantly increased 2 - 4 hours

postprandially with no change in mean $VLDL_2$ TRL cholesterol concentrations observed.

Table 6.6: Individual and mean areas under the curve (mmol.hour) to assess the contribution of apoB48 and apoB100 TRL, $VLDL_1$ and $VLDL_2$ to postprandial increases in the plasma concentration of triglyceride.

Subject	Plasma (mmol.hr)	TRL (d < 1.006 g/ml) (mmol.hr)	ApoB48 (mmol.hr)	ApoB100 (mmol.hr)	$VLDL_1$ (mmol.hr)	$VLDL_2$ (mmol.hr)
BC	23.42	18.48	15.73	1.80	0.78	0.08
MM	8.10	6.33	5.08	0.72	0.58	0.03
BJ	13.20	6.13	5.37	0.45	0.88	0.13
KE	11.94	8.71	5.27	2.82	2.08	0.067
HA	2.87	1.98	1.67	0.24	0.14	0
CN	3.65	2.72	2.13	0.30	0.24	0
JM	10.24	8.28	6.37	1.32	1.15	0.12
IC	9.17	6.41	5.13	0.60	0.37	0.25
AG	18.40	12.63	10.02	2.97	2.56	0.09
Mean (± SD)	**11.20 ± 6.45**	**7.96 ± 5.06**	**6.31 ± 4.28**	**1.25 ± 1.06**	**0.94 ± 0.76**	**0.086 ± 0.077**

Mean plasma apoB, apoAI and TRL (d < 1.006g/ml) apoAI concentrations did not change significantly during the course of the experiment. Mean apoB TRL (d < 1.006g/ml) concentrations were significantly elevated above fasting concentrations 2 - 5 hours postprandially (figure 6.6).

Plasma FFA concentrations were measured and the results (mean plasma FFA concentrations) are shown in figure 6.7. There was a consistent initial decrease in plasma FFA concentrations 1 - 2 hours after ingestion of the fat meal in virtually all subjects (appendix 15) followed by a subsequent rise in plasma FFA concentrations and a fall back towards baseline. Mean plasma FFA concentrations were significantly elevated *($p < 0.05$)* above fasting plasma levels 3 - 11 hours postprandially, with a mean peak at 6 hours, reaching a plateau and then a decrease after 10 hours (figure 6.7).

Plasma insulin levels (table 6.7) were increased 2 hours after ingestion of the fat meal in virtually all subjects. Mean plasma insulin concentrations were significantly elevated *(p=0.039)* above fasting levels 2 hours postprandially.

Table 6.7: ***Postprandial changes in individual and mean plasma insulin concentrations (mU/ L).*** *Significantly different from fasting concentration by paired t-test: *p<0.05.*

Subjects	Time (hours) 0 hours	2 hours	Delta insulin
BC	7.00	14.10	7.10
MM	8.60	6.70	-1.90
BJ	6.20	8.40	2.20
KE	9.00	14.50	5.50
HA	8.40	10.00	1.60
CN	4.50	7.30	2.80
JM	2.50	11.70	9.20
IC	7.90	18.00	10.10
AG	10.60	38.90	28.30
Mean insulin (mU/L) ± SD	7.19 ± 2.48	14.40 ± 9.91*	7.21 ± 8.50

Correlations between variables.

To examine the effect of fasting triglyceride concentrations on the measured parameters of postprandial lipaemia, the data for normal subjects was analysed. There was no significant correlation between log fasting triglyceride and the area under the curve for plasma (r=0.29, *p=0.44*), apoB48 (r=0.248, *p=0.52*), apoB100 (r=0.47, *p=0.20*), $VLDL_1$ (r=0.43, *p=0.25*) triglyceride or between log fasting triglyceride and area under the curve for $VLDL_2$ triglyceride (r=-0.34, *p=0.37*).

To examine the effect of changes in insulin concentrations on the measured parameters of postprandial lipaemia, the data for normal subjects was analysed and revealed significant positive correlations between delta (*i.e.* a change in) insulin and the area under the curve for apoB100 (r=0.67, *p=0.045)* and $VLDL_1$ (r=0.72, *p=0.03*) triglyceride. In addition, there was also positive significant correlations between delta insulin and the delta apoB100 (r=0.70, *p=0.03*) and $VLDL_1$ (r=0.85, *p=0.004*) triglyceride concentrations (figure 6.8). A positive correlation, which failed to reach

significance was observed between delta insulin and area under the curve for plasma (r=0.52, *p=0.15*), apoB48 (r=0.45, *p=0.22*) and $VLDL_2$ (r=0.33, p=*0.39*) triglyceride and between delta insulin and delta apoB48 (r=0.65, *p=0.059*) and delta plasma (r=0.61, *p*=0.081).

No correlations between delta insulin and FFA area under the curve (r=-0.093, *p=0.81*) and delta insulin and delta FFA (r=-0.002, *p=0.99*), delta $VLDL_2$ (r=-0.18, *p=0.64*) were observed.

4 Discussion.

A specific monoclonal antibody for apoB100, LDL A4, as described previously in chapter III, was used to isolate the apoB48 and apoB100 containing TRL species from plasma obtained from normal subjects in the postprandial state. This allowed further examination of VLDL subfractions by cumulative flotation, from the apoB100 containing TRL fraction isolated by immunoaffinity chromatography. In order to obtain reliable quantitative data with the antibodies, single sample aliquots in individual eppendorf tubes containing the antibody bound to sepharose were used, rather than eluting fractions from an immunoaffinity chromatographic column, as described previously in chapter III.

The individual and mean plasma triglyceride concentrations increased in all patients, with the individual plasma triglyceride response curves showing a large variation between subjects which appeared to be independent of their fasting triglyceride levels. Mean plasma cholesterol concentrations remained unchanged. Mean TRL (d<1.006g/ml), apoB48, and $VLDL_1$ cholesterol concentrations were significantly elevated above fasting levels 1 - 6 hours in TRL (d<1.006g/ml) fraction, 2 - 6 hours for apoB48 and 2 - 4 hours postprandially for $VLDL_1$. Total apoB100 and $VLDL_2$ cholesterol concentrations remained relatively constant throughout the duration of the experiment.

A consistent initial decrease in plasma FFA concentrations was observed 1 - 2 hours postprandially in virtually all subjects followed by a subsequent rise in plasma FFA concentrations and a fall back to baseline. This phenomenon has previously been observed by others *(Lewis et al 1991, O'Meara et al 1992)* and is suggested to be related in timing to the early postprandial insulin rise. As seen in this study, plasma insulin concentrations increased significantly 2 hours postprandially. Plasma FFA concentrations were increased significantly above fasting levels 3 - 11 hours

postprandially, peaking after 6 hours, presumably as a result of the hydrolysis of the apoB48 (chylomicron) and apoB100 (VLDL) TRL associated triglyceride by LPL. It is likely that the capacity to incorporate FA into adipose tissue is exceeded.

Other investigators *(Cohn et al 1988 a and b)* have reported that the plasma concentration of apoB either does not change, or falls after a fat meal. In this study similar results were observed. Mean plasma apoB and AI and TRL ($d < 1.006$g/ml) apoAI concentrations did not alter during the postprandial period, while the TRL ($d < 1.006$g/ml) apoB concentrations were significantly elevated above fasting levels 2 - 5 hours postprandially and then decreased as noted by Cohn.

The increase in plasma triglyceride concentration after an oral fat load is predominantly due to triglyceride contained within the apoB48 containing lipoproteins in the TRL fraction of plasma, with apoB48 TRL and apoB100 TRL species accounting for approximately 79% and 15% of the postprandial increase in TRL triglyceride concentration, in agreement with earlier results, and $VLDL_1$ and $VLDL_2$ accounting for 75% and 10% of the postprandial increase in total apoB100 TRL triglyceride concentration. Although the increase in plasma triglyceride concentration in the fed state was predominantly due to an increase in apoB48 TRL triglyceride concentration, there was a significant increase in apoB100 TRL triglyceride concentration above fasting levels over hours 2 - 5 representing a 36% increase in apoB100 triglyceride concentration. It was also noted that triglyceride was present in the apoB48 (unbound) fraction at 0 hours. This may have been due to the presence of fasting chylomicrons or chylomicron remnants present in the plasma sample or to VLDL that failed to be retained by the antibody gel (see chapter III).

There may be several reasons for the increase in the concentration of apoB100 triglyceride in postprandial plasma. The first may be that the plasma clearance of apoB100 TRL is inhibited by the influx into plasma of postprandial chylomicrons. Second, the ingestion of a fat rich meal may stimulate the synthesis and secretion of apoB100 TRL from the intestine. Third, hepatic apoB100 TRL production may be enhanced postprandially in response to lipid of dietary origin reaching the liver via the portal vein or via chylomicron transport. The first possibility is supported by the studies of *Brunzell (1973)* who showed that chylomicrons and VLDL are catabolised by a common pathway. Triglycerides in both chylomicrons and VLDL are hydrolysed by LPL at the capillary endothelial surface and these lipoproteins can compete for enzyme mediated lipolysis. Reduced availability of lipolytic activity due to the postprandial presence of apoB48 containing chylomicrons could explain the observed increase in

apoB100 TRL triglyceride. An increase in the production of apoB100 TRL is another equally plausible explanation. *Cohn et al (1988 b)* previously found that in the fed state there is an increase in the plasma concentration of apoB100 in the TRL fraction, as well as an increase in the rate of production of apoB100 TRL *(Cohn et al 1990)*. They suggested that this represents a postprandial increase in synthesis and secretion of TRL by the liver, as it is normally assumed that apoB100 in plasma is of hepatic origin.

In the normal subjects, positive significant correlations (Pearson) were seen between delta insulin and the area under the curve for apoB100 and $VLDL_1$ triglyceride. In addition, there was also significant positive correlations between delta insulin and the delta apoB100 and delta $VLDL_1$ triglyceride concentrations. A possible explanation may be that as insulin activates LPL activity, there is competition between chylomicrons and VLDL for lipolysis, chylomicrons are the preferred substrate and as such are hydrolysed. As a result VLDL accumulates due to decreased catabolism and continued synthesis. A possible explanation of the preferential lipolysis of chylomicrons compared to VLDL might be differences in apolipoprotein contents between these two TRL species. A delayed clearance of VLDL might be due to alterations in the apolipoprotein and lipid composition of VLDL particles. *Bjorkegren et al (1997)* observed that large and small VLDL were enriched in cholesterol, apoE and CI, but depleted of apoCII, whereas the apoCIII, triglyceride and phospholipid contents were essentially unchanged in the postprandial state. ApoCII is necessary for the activation of LPL thus VLDL are poorly lipolysed possibly due to a lack of apoCII. *Bjorkegren et al (1997)* also noted an early transient increase in apoCIII contents of large $VLDL_1$ particles which may be implicated in the accumulation of this lipoprotein species during alimentary lipaemia *(Karpe et al 1993)*. Theoretically, the early apoCIII accumulation and the depletion of apoCII of large VLDL could explain part of the preferential lipolysis of chylomicrons and the ensuing postprandial accumulation of large VLDL observed both by *Bjorkegren et al (1996)* and the present study.

Positive significant correlations (Pearson) (see chapter VII) were seen between log fasting triglyceride concentrations and individual area under the curve for plasma, apoB48, apoB100 and $VLDL_1$ triglyceride response curves. This would indicate that fasting triglyceride concentrations have a bearing on postprandial triglyceride responses.

Other studies have suggested that the human intestine has the capacity to synthesise apoB100 *(Hoeg et al 1990)*, and it cannot be ruled out that in the fed state, apoB100 TRL is partly of intestinal origin. The postprandial state has been postulated to be potentially atherogenic on the basis that intestinal chylomicrons and their remnants

have the potential to cause lipid deposition in the cells of the artery wall *(Zilversmit 1979)*. Postprandial apoB100 may be of equal significance especially if it is the precursor of LDL which may be potentially atherogenic. The long term effects of repeated and prolonged alimentary lipaemia may be derangements in the metabolism of endogenous lipoproteins that are atherogenic.

The results from this study confirm the findings of both *Karpe et al (1993)* and *Bjorkegren et al (1996)*. A 3 fold increase in plasma triglyceride levels was accompanied by an increase of large $VLDL_1$ triglyceride concentrations (between 1 - 5 hours), in contrast $VLDL_2$ triglyceride levels remain unaltered. Bjorkegren concluded that the most likely explanation for the rise in *Sf 60 - 400* VLDL is delayed lipolysis of the particle due to competition for the sites of LPL action. Recently *Karpe and Hultin (1995)* have shown that endogenous TRL accumulate in rat plasma due to a failure to compete for a common lipolytic pathway with a chylomicron-like emulsion. It was observed in most subjects that as the apoB48 TRL triglyceride level (figure 6.3) dropped to below 1 mmol/L at 7 - 8 hours, apoB48, apoB100 and large $VLDL_1$ lipolysis levels became accelerated. Total apoB100 and large $VLDL_1$ triglyceride levels dropped to below almost half that of fasting triglyceride levels (figure 6.3). In contrast small $VLDL_2$ triglyceride levels remained relatively constant and unaffected by the levels of chylomicronaemia. It is suggested that this accelerated lipolysis is as a result of increased (excess) levels of LPL being present to hydrolyse the intestinal lipoproteins. As the levels of these decreases, LPL action over-compensates and lipolysis of the remaining lipoproteins becomes accelerated, with the result that the total apoB100 and large $VLDL_1$ triglyceride concentrations drop to below fasting levels.

The fat load given, contained a supra-normal amount of fat (137 grams), in that the normal fat intake for women is approximately 75 grams/day and for men 99 grams/day. This dose generated a clear response to the fat load, which was the objective of the study. In the literature alternative approaches have been taken for postprandial studies. These include a variety of either solid or liquid fat loads with either a constant dose of fat for all subjects or doses used as grams fat per kg body weight (ranging from 0.2 - 1.0 grams fat/ kg body weight) with meals containing from 15 - 75 grams of fat. None of these approaches appear to be superior to the other and for reasons of simplicity we chose to use a constant dose of 137 grams of fat contained in a liquid fat load, used successfully in previous investigations in this laboratory *(Warwick et al 1992)*.

Figure 6.1: ***Postprandial changes in the mean plasma triglyceride concentration.*** *Significantly different from fasting concentration by paired t-test: *p < 0.05, **p < 0.01, ***p < 0.005.*

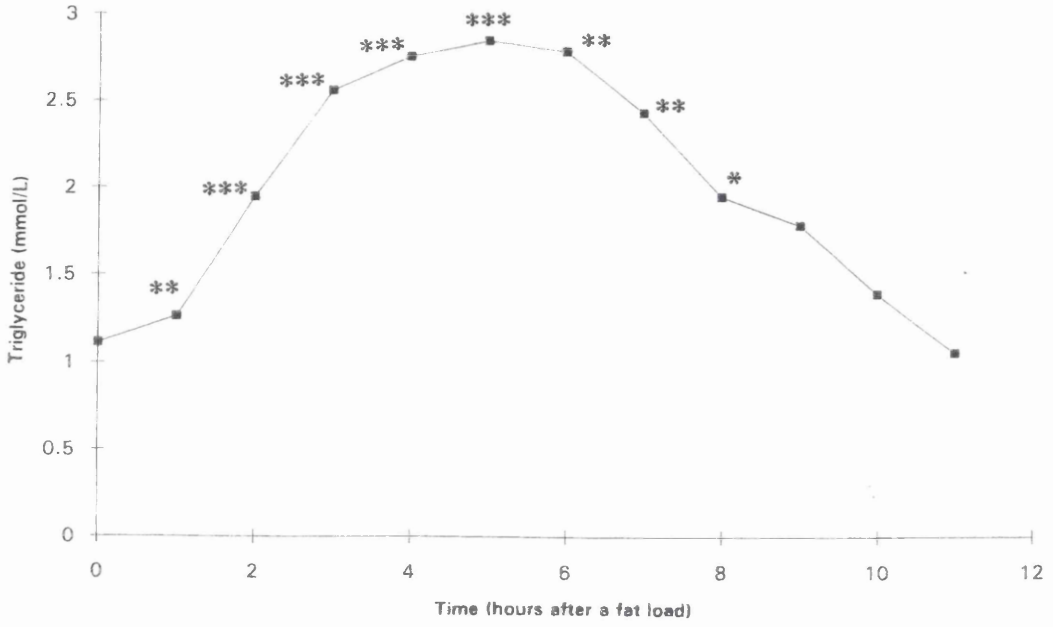


Figure 6.2: ***Postprandial changes in the mean triglyceride concentration of plasma and all TRL fractions.***

*Significantly different from fasting concentration by paired t-test: *p < 0.05, **p < 0.01, ***p < 0.005.*

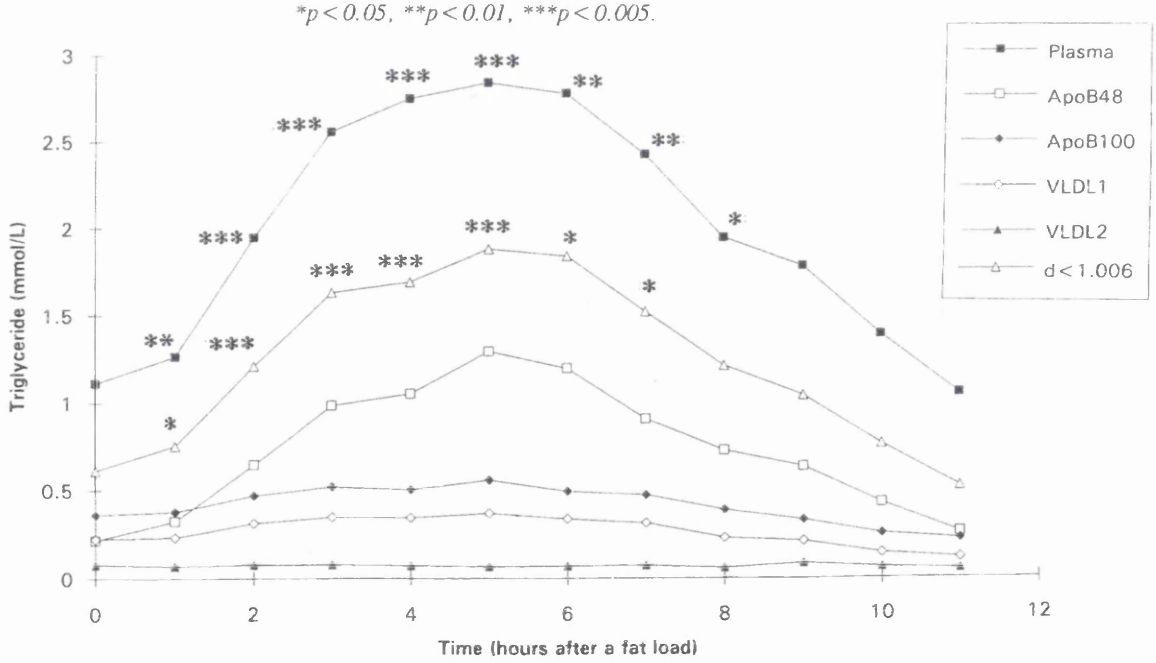


Figure 6.3: ***Postprandial changes in the mean triglyceride concentration of individual TRL fractions apoB48, apoB100, VLDL$_1$, VLDL$_2$.*** *Significantly different from fasting concentration by paired t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.*

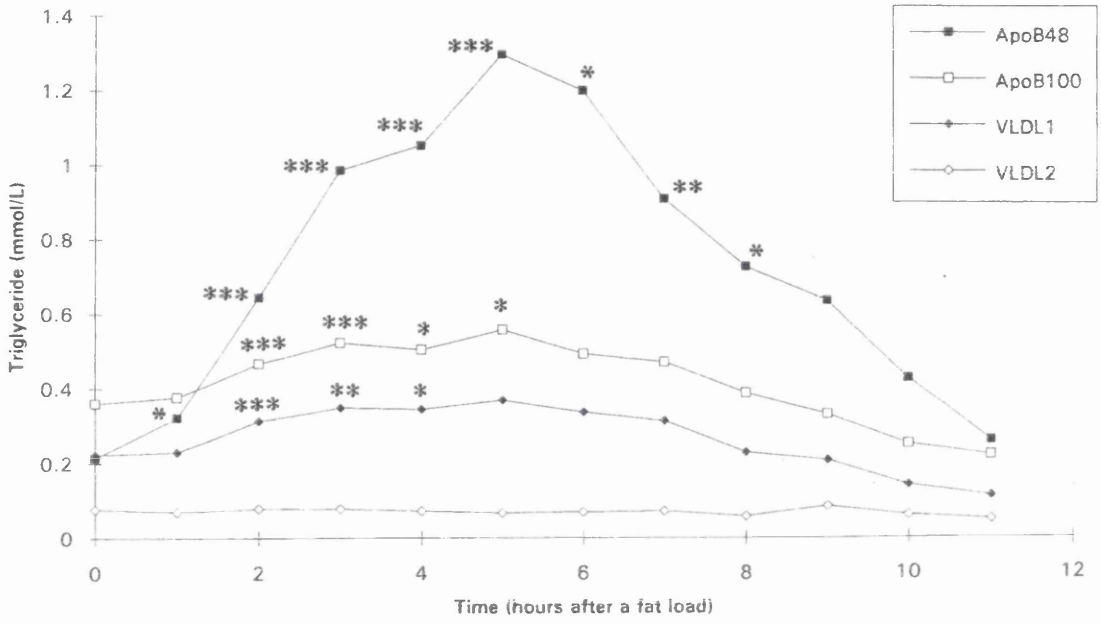


Figure 6.4: ***Increment in triglyceride concentrations in the individual VLDL$_1$ TRL fractions (baseline at zero time subtracted).***

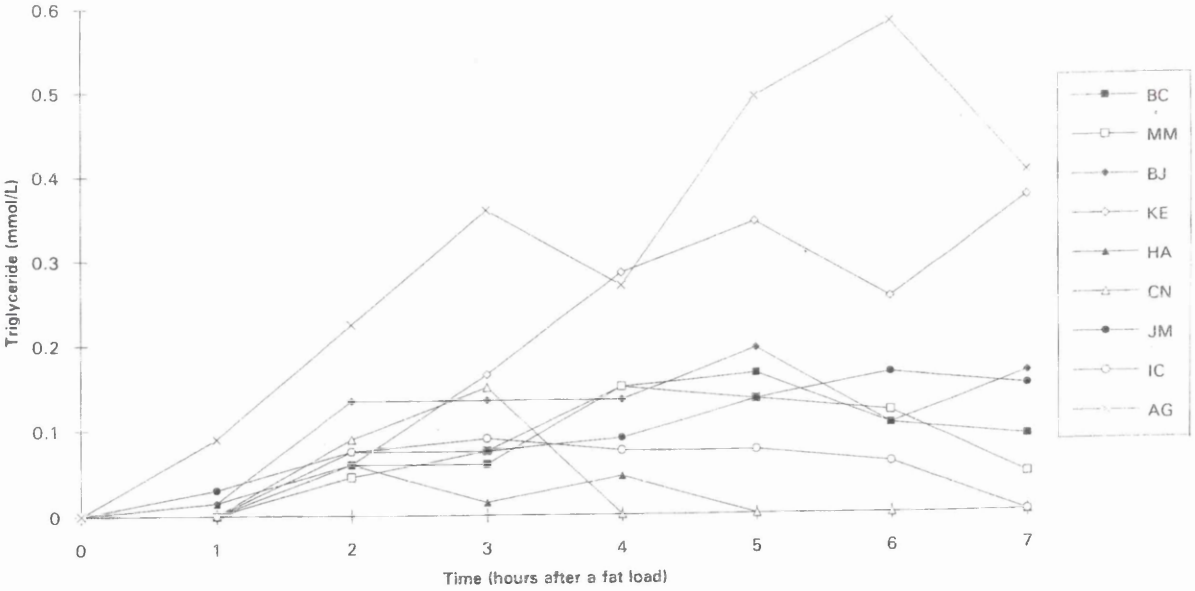


Figure 6.5: ***Postprandial changes in the mean cholesterol concentrations in all the TRL fractions.***
*Significantly different from fasting concentration by paired t-test: *p < 0.05, **p < 0.01, ***p < 0.005.*

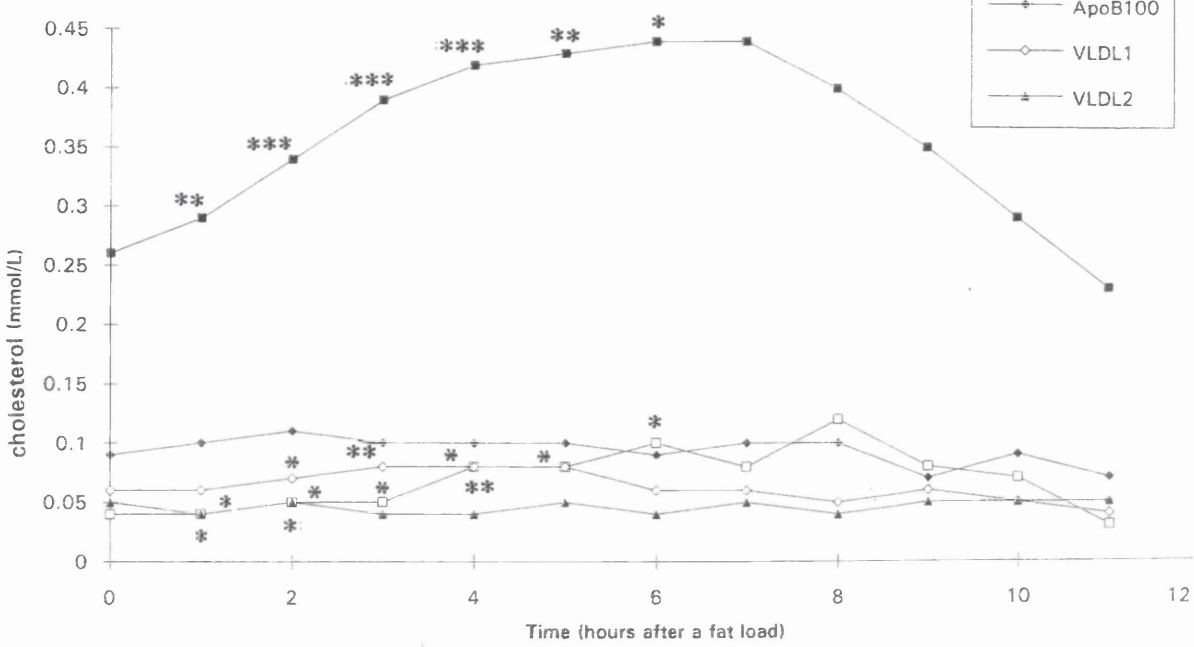


Figure 6.6: ***Postprandial changes in the mean plasma and mean TRL (d < 1.006g/ml) apoB and apoAI concentrations.*** *Significantly different from fasting concentration by paired t-test: *p < 0.05, **p < 0.01, ***p < 0.005.*

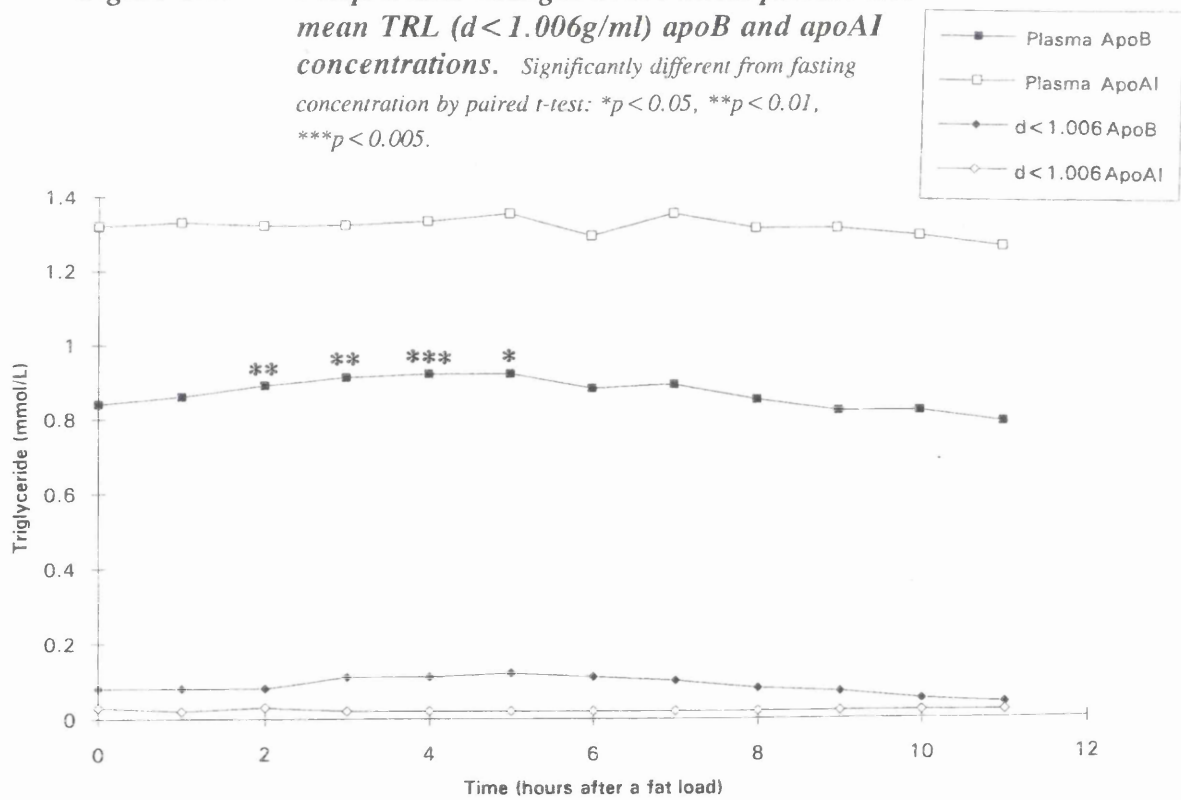


Figure 6.7: ***Postprandial changes in the mean plasma concentration of FFA.*** *Significantly different from fasting concentration by paired t-test: ***$p < 0.005$.*

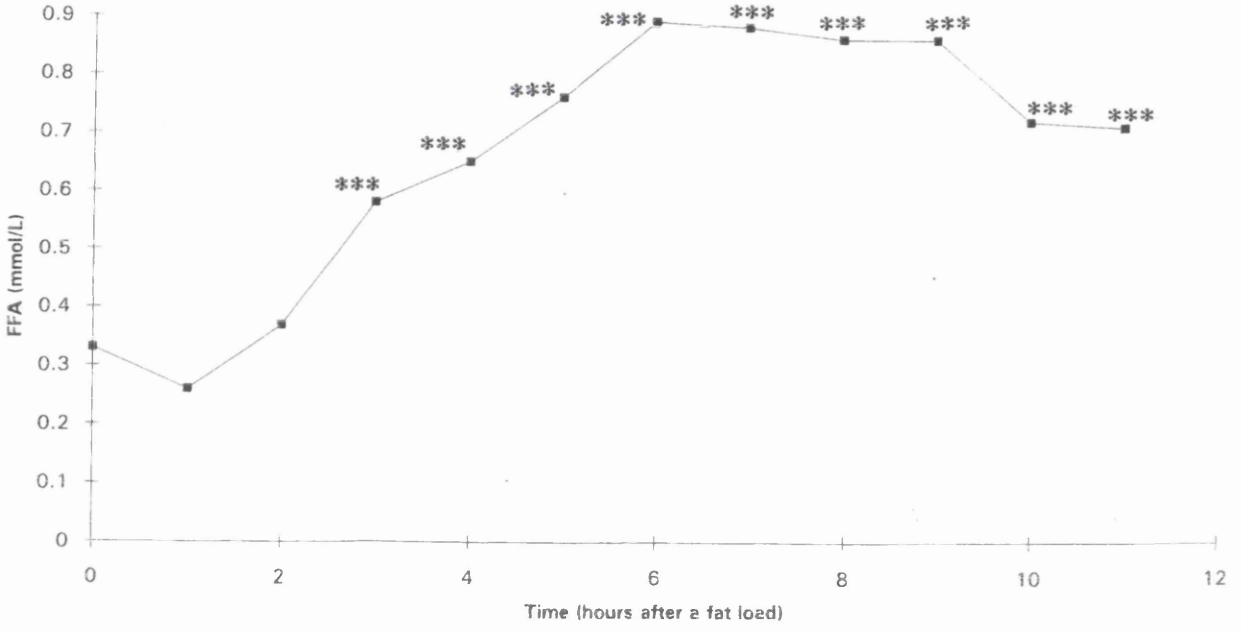
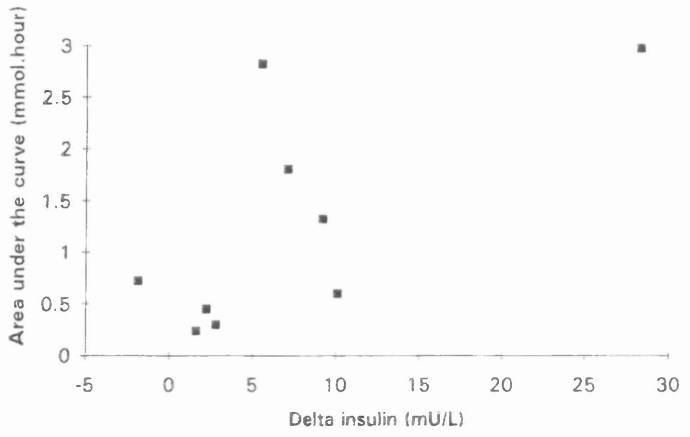
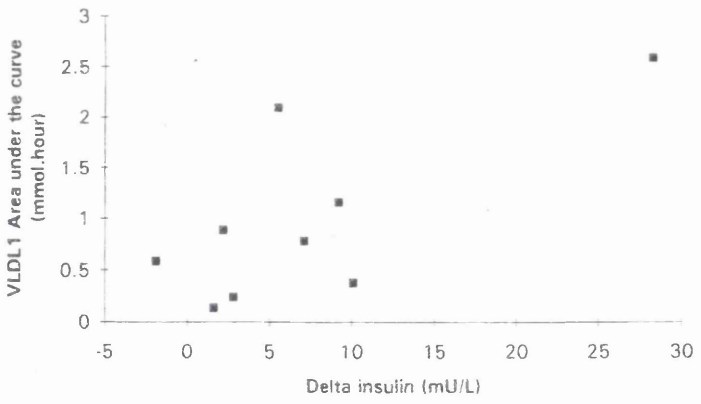


Figure 6.8: ***Pearson correlations.*** *Correlations between (a) delta insulin and individual areas under the curve (mmol.hour) for (i) apoB100 and (ii) VLDL$_1$ and between (b) delta insulin and the increase in (delta) (i) plasma, (ii) apoB100 and (iii) VLDL$_1$ triglyceride concentrations (mmol/L) for normal patients.*

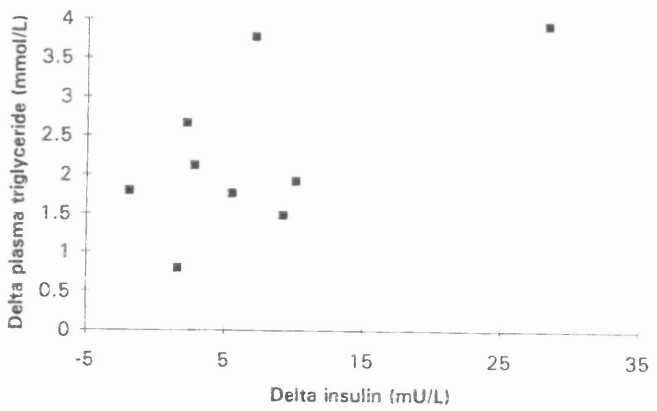
(a) (i)



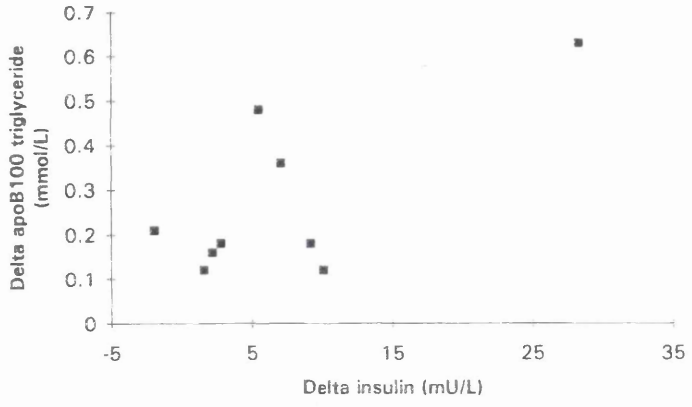
(a) (ii)



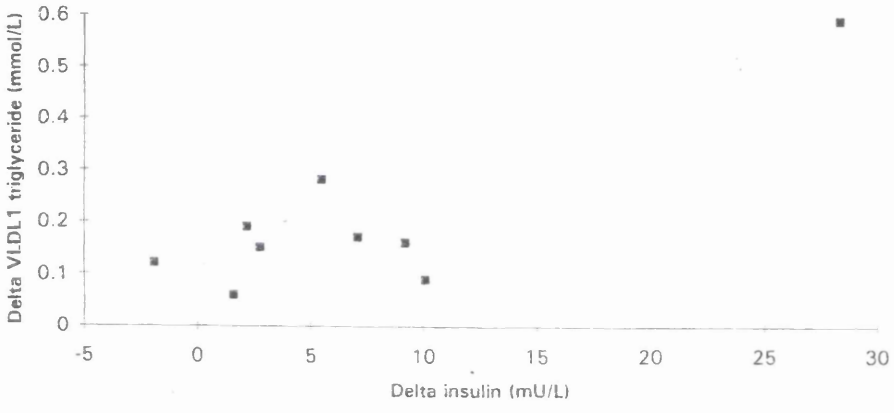
(b) (i)



(b) (ii)


Delta apoB100 triglyceride (mmol/L)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-5
5
15
25
35
Delta insulin (mU/L)

(b) (iii)


Delta VLDL1 triglyceride (mmol/L)
0.6
0.5
0.4
0.3
0.2
0.1
0
-5
0
5
10
15
20
25
30
Delta insulin (mU/L)

Chapter VII Metabolism of chylomicrons and VLDL in diabetic patients after a fat meal.

1 Introduction.

The current classification and diagnosis of diabetes was developed by the National Diabetes Data Group (NDDG) and published in 1979, who recognised 2 major forms of diabetes, which they termed insulin-dependent (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM), now termed type 1 and type 2 diabetes respectively. It is now considered to be important to move away from a system that appears to base its classification of the disease, in large part, on the type of pharmacological treatment used in its managemant toward a system based on the disease etiology where possible. The vast majority of cases of diabetes fall into 2 broad categories. In one category (type 1 diabetes), the cause is an absolute deficiency of insulin secretion resulting from a cellular-mediated autoimmune destruction of the ß-cells of the pancreas. In the other, (type 2 diabetes), the cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response (relative insulin deficiency). The current expert committee (1997) have now proposed changes and modifications to the NDDG diagnosis and classification scheme (Report Committee 1997). The revised criteria for the diagnosis of diabetes mellitus are a casual plasma glucose (PG) (casual is defined as any time of the day without regard to time from last meal) concentration $\geq$ 200 mg/dl (11.1 mmol/L, or a fasting (fasting defined as no calorie intake for 8 hours) PG $\geq$ 126 mg/dl (7.0 mmol/L) or a 2 hour PG $\geq$200mg/dl during an OGTT (oral glucose tolerance test). This new criteria would not alter the status of the diabetic patients recruited here (appendix 35).

1.1 Lipid and lipoprotein abnormalities in NIDDM.

As previously discussed in chapter I, the basic dyslipidaemia that accompanies diabetes was described several years ago *(Howard 1987)*. Recent work has focussed on abnormalities in lipoprotein alterations, composition and apolipoprotein content and the metabolic mechanisms that govern these changes.

The most common abnormality of lipid metabolism in patients with diabetes is hypertriglyceridaemia due to an elevation in VLDL *(Howard et al 1978, Mattock et al 1979, Briones et al 1984, Bierman 1992, Laws 1996)*. This is accompanied by a decrease in HDL cholesterol concentrations. Abnormalities in both production *(Abrams et al 1982, Kissebah et al 1982, Reaven 1987)* and clearance *(Abrams et al 1982, Kissebah et al 1982, Howard et al 1983, Taskinen et al 1986)* of VLDL triglyceride have been reported in NIDDM (Type 2 diabetics). *Kissebah et al (1982)* also found that hypertriglyceridaemic diabetics had elevated rates of VLDL apoB production, a finding compatible with data in non-diabetics. There appear to be changes in the composition of VLDL *(Schonfeld et al 1974 a, Taskinen et al 1986)*, which suggest that non-insulin dependent diabetics may have larger, triglyceride-rich VLDL as indicated by a higher triglyceride: apoB ratio. One of the general effects of NIDDM (Type 2 diabetics) on lipoprotein metabolism may be a relative decrease in LPL activity, consistent with the observed delayed clearance of VLDL.

The magnitude of postprandial lipaemia is known to be highly dependent on the fasting plasma triglyceride concentration *(Patsch 1987)*; since NIDDM (Type 2 diabetics) patients generally have a higher fasting plasma triglyceride, a disturbed postprandial triglyceride metabolism would be expected. However it is not yet known in diabetics if the exaggerated lipaemia, often present towards the latter hours after a fat feed, is due to the persistance of VLDL or due to failure to clear either or both chylomicrons and VLDL. The only way to answer this question is to separate apoB48 and apoB100 TRL.

1.2 Aim.

This study was undertaken to delineate the behaviour of VLDL subfractions, in both normal (see chapter VI) and diabetic patients during postprandial lipaemia. These lipoprotein particles were isolated by immunoaffinity chromatography and cumulative flotation. This is a novel study investigating mechanisms not previously examined in diabetics.

2 Methods.

2.1 Patient selection, clinical measurements and oral fat tolerance tests.

As in chapter VI methods section.

2.1.1 Diabetic subjects.

The diabetic subjects recruited were all attending the Diabetic clinic. All were well controlled Type 2 diabetics, and not on lipid treatment. All subjects were recruited by Dr Neil Bennett with the permission of Dr A. MacCuish. The patients were initially contacted by Dr Bennett during routine visits to the Diabetic clinic where he discussed the study with them and asked if they would consider taking part. If the patients agreed, an initial fasting blood sample was taken to check that lipid levels (table 7.1) were relatively normal (triglyceride approximately 2 mmol/L and cholesterol approximately 6 mmol/L). Plasma apoB, apoAI and FFA levels were also measured. The subjects were given an information sheet (appendix 21) about the study. Suitable subjects were contacted by telephone and then by letter. All subjects gave signed informed consent before the study was started (appendix 22). Initially 25 diabetic subjects were screened and from those, 5 diabetic subjects (3 males and 2 females) agreed to take part in the fat feeding study. Their mean plasma lipid levels are given in table 7.1.

Table 7.1: Diabetic subject characteristics.

Subjects (n=5)	**Mean (± SD)**
Age (years)	61.8 ± 9.0
BMI (kg/m^2)	31.2 ± 2.28
Triglyceride (mmol/L)	1.79 ± 1.15
Cholesterol (mmol/L)	5.04 ± 0.90
VLDL cholesterol (mmol/L)	0.68 ± 0.38
LDL cholesterol (mmol/L)	3.59 ± 0.65
HDL cholesterol (mmol/L)	0.70 ± 0.70

2.2 Statistical analysis.

Mean and standard deviation of means for plasma parameters were reported. Paired t-tests were used to determine the statistical significance between lipid, apoprotein and insulin measurements obtained from fasting (0 hour) and postprandial

samples. Unpaired t-tests were used to determine statistical significance between fasting plasma lipids and lipoproteins and the triglyceride response curves and area under the curves obtained from normal and diabetic subjects, where the baseline was the fasting 0 hour concentration. Area measurements were expressed as mmol.hour. The Minitab 10 statistical package was used to calculate regression coefficients and *p* values for correlations.

3 Results.

Plasma triglycerides, cholesterol, apoB, apoAI and FFA were studied in the 9 normal and 25 diabetic patients recruited. Plasma triglyceride, cholesterol and apoB concentrations were found to be significantly increased ($p<0.005$) in the diabetic population, with no difference observed in the plasma FFA and apoAI concentrations (table 7.2).

Individual ß-Quantification measurements for the initial fasted plasma samples for each of the diabetic patients are shown in table 7.3. We were unable to obtain HDL cholesterol results for technical reasons from two of the diabetic patients.

All triglyceride, cholesterol, apoB and apoAI concentrations in the various isolated lipoprotein fractions were adjusted back to plasma levels. The triglyceride concentration was used as the index of postprandial lipoprotein metabolism.

Table 7.2: Comparison of lipid parameters in normal and diabetic populations.

Parameter	**Normals (n=9) (Mean ± SD)**	**Diabetics (n=25) (Mean ± SD)**	**Significance (*p value*)**
Triglyceride (mmol/L)	1.22 ± 0.40	2.39 ± 1.12	**0.0001**
Cholesterol (mmol/L)	4.62 ± 0.72	5.32 ± 0.84	**0.028**
ApoB (g/L)	0.84 ± 0.18	1.16 ± 0.29	**0.0008**
ApoAI (g/L)	1.32 ± 0.27	1.26 ± 0.22	**0.59**
FFA (mmol/L)	0.32 ± 0.21	0.31 ± 0.22	**0.83**

Table 7.3: ß-Quantification of lipids and lipoproteins and apoE phenotypes in individual diabetic subjects involved in fat feeding studies.

Lipid levels (mmol/L)	Subjects				
	JC	SJ	PF	CE	MH
Plasma triglyceride	3.40	0.70	2.55	0.95	1.35
Plasma cholesterol	4.90	5.15	6.35	3.80	5.00
VLDL cholesterol	1.15	0.25	0.95	0.70	0.35
LDL cholesterol	2.85	3.20	4.50	3.95	3.45
HDL cholesterol	0.90	1.70	0.90	-	-
ApoE phenotype	4/3	3/3	3/3	3/2	4/3

Individual plasma, TRL (d < 1.006g/ml), apoB48, apoB100, $VLDL_1$ and $VLDL_2$ triglyceride concentrations before and after ingestion of a fat load are shown in tables in appendices 23 - 28. Individual plasma FFA, cholesterol, plasma and TRL (d < 1.006g/ml) apoB and apoAI concentrations before and after a fat load are shown in tables in appendices 29 - 34.

Mean (± SD) plasma triglyceride concentrations before and after the ingestion of the fat load are shown in table 7.4 and figure 7.1. Mean plasma cholesterol concentrations did not change significantly during the course of the experiment. After the fat load, individual plasma triglyceride concentrations peaked between 5 - 6 hours postprandially, (figure 7.2) at triglyceride concentrations ranging from 2.31 - 10.23 mmol/L, with a mean of 4.99 ± 3.07 mmol/L (table 7.5). Note table 7.4 shows mean triglyceride levels over the time course of the experiment, while table 7.5 shows the mean maximal triglyceride value for subjects taken when their individual peaks occurred, usually at 4, 5 or 6 hours. The mean plasma triglyceride concentration was significantly elevated ($p < 0.05$) above fasting concentrations from 2 to 5 hours postprandially (table 7.4, figure 7.1).

Table 7.4: ***Postprandial changes in mean triglyceride concentrations (mmol/L ± SD) in plasma and all TRL fractions in diabetic patients after an oral fat load.*** *Significantly different from fasting concentration by paired t-test: *p<0.05; **p<0.01.*

Time (hours)	Mean Plasma TG (mmol/L ± SD)	Mean TRL (d<1.006 g/ml) TG (mmol/L ± SD)	Mean ApoB48 TRL TG (mmol/L ± SD)	Mean ApoB100 TRL TG (mmol/L ± SD)	Mean $VLDL_1$ TG (mmol/L ± SD)	Mean $VLDL_2$ TG (mmol/L ± SD)
0	2.13 ± 1.12	1.32 ± 0.77	0.41 ± 0.26	0.71 ± 0.44	0.43 ± 0.27	0.11 ± 0.06
1	2.23 ± 1.08	1.44 ± 0.83	0.52 ± 0.30	0.75 ± 0.46	0.43 ± 0.29	0.11 ± 0.07
2	2.77 ± 1.18*	1.87 ± 0.91*	0.83 ± 0.42*	0.86 ± 0.49*	0.56 ± 0.30*	0.13 ± 0.07
3	3.31 ± 1.49**	2.18 ± 1.07*	1.09 ± 0.55*	0.92 ± 0.48**	0.59 ± 0.30*	0.14 ± 0.07
4	4.03 ± 2.11*	2.71 ± 1.44*	1.55 ± 0.93*	0.98 ± 0.54**	0.73 ± 0.43*	0.07 ± 0.04
5	4.65 ± 2.69*	3.17 ± 1.89*	1.85 ± 1.15*	1.09 ± 0.62*	0.76 ± 0.46*	0.08 ± 0.03
6	4.87 ± 3.16	3.35 ± 2.52	2.06 ± 1.55	1.07 ± 0.67	0.76 ± 0.53	0.10 ± 0.03
7	4.15 ± 3.45	3.26 ± 1.88	1.97 ± 1.30	1.06 ± 0.57	0.70 ± 0.43	0.12 ± 0.05
8	4.29 ± 3.10	3.34 ± 3.26	2.10 ± 2.4	0.95 ± 0.68	0.66 ± 0.53	0.10 ± 0.03
9	3.99 ± 2.61	3.31 ± 3.33	2.06 ± 2.4	1.03 ± 0.87	0.66 ± 0.62	0.11 ± 0.05
10	4.48 ± 3.73	3.34 ± 3.00	1.86 ± 2.29	0.98 ± 0.9	0.58 ± 0.64	0.09 ± 0.04
11	4.66 ± 5.43	2.76 ± 3.00	1.73 ± 2.4	0.83 ± 0.79	0.58 ± 0.62	0.09 ± 0.05

The plasma triglyceride concentrations in subjects JC, SJ, and PF began to increase again after the initial triglyceride peak at 10 hours postprandially but for the group as a whole this late increase was not significant compared to the baseline (0 hour) value (figure 7.2) (appendix 23).

In diabetic subjects, in the fasting state (time = 0 hour), the mean plasma triglyceride concentration was 2.13 ± 1.12 mmol/L (table 7.5) and mean TRL (d < 1.006g/ml), apoB48, apoB100, $VLDL_1$ and $VLDL_2$ triglyceride concentrations were 1.32 ± 0.77 mmol/L, 0.41 ± 0.26 mmol/L, 0.71 ± 0.44 mmol/L, 0.43 ± 0.27 mmol/L, and 0.11 ± 0.06 mmol/L respectively. These concentrations are significantly higher than those observed in normal subjects (with the exception of apoB48 and $VLDL_2$ fasting triglyceride concentrations), who in the fasting state (time = 0 hour) had a mean plasma triglyceride concentration of 1.11 ± 0.45 mmol/L (chapter VI, table 6.4) and mean TRL, apoB48, apoB100, $VLDL_1$ and $VLDL_2$ triglyceride concentrations of 0.61 ± 0.36 mmol/L, 0.21 ± 0.15 mmol/L, 0.36 ± 0.21 mmol/L, 0.22 ± 0.16 mmol/L and 0.08 ± 0.03 mmol/L respectively.

In the fed state at the time of maximal triglyceride increase (mean 6 hours postprandially for plasma, TRL (d < 1.006g/ml) and apoB48 TRL fractions, and 5 hours for the apoB100 TRL fractions: total apoB100, $VLDL_1$, and $VLDL_2$), there was a 57% increase in mean plasma triglyceride concentrations accompanied by a 64%, and an 81% increase in mean TRL (d < 1.006g/ml) and mean apoB48 TRL triglyceride concentrations, respectively. Mean apoB100 TRL, $VLDL_1$ and $VLDL_2$ triglyceride levels showed an increase of 37%, 38% and 57% respectively (table 7.5).

Table 7.5: Mean of maximal postprandial triglyceride levels for individual subjects after a fat load when mean peaks occurred, usually at 4, 5 or 6 hours.

Lipoprotein fraction	Mean fasting TG (mmol/L)	Mean of individual peak TG (mmol/L)	Mean peak (hours)
Plasma	2.13 ± 1.12	4.99 ± 3.07	6
TRL(d < 1.006g/ml)	1.32 ± 0.77	3.64 ± 2.34	6
ApoB48	0.41 ± 0.26	2.18 ± 1.46	6
ApoB100	0.71 ± 0.44	1.12 ± 0.66	5
$VLDL_1$	0.43 ± 0.27	0.69 ± 0.57	5
$VLDL_2$	0.11 ± 0.06	0.26 ± 0.23	5

Table 7.4 shows mean TRL (d < 1.006 g/ml) triglyceride concentrations after ingestion of a fat meal. Individual TRL triglyceride concentrations peaked between 5 - 7 hours, with triglyceride concentrations ranging from 1.85 - 7.56 mmol/L; and a mean peak of 3.64 ± 2.34 mmol/L (table 7.5). When all individual TRL (d < 1.006g/ml) triglyceride response curves were combined, there was a mean peak at 6 hours with a mean triglyceride concentration of 3.35 ± 2.52 mmol/L (table 7.4). The mean TRL (d < 1.006g/ml) triglyceride concentration was significantly elevated ($p < 0.05$) above fasting concentrations 2 - 5 hours postprandially, in accord with total plasma data.

The mean triglyceride concentrations of plasma, TRL (d < 1.006g/ml) and apoB48 are shown in figure 7.1 with the mean triglyceride concentrations of apoB100, $VLDL_1$ and $VLDL_2$ fractions, before and during the fat load are shown in figure 7.3. After the fat load, individual apoB48 triglyceride concentrations peaked between 5 - 7 hours, with triglyceride concentrations ranging from 1.17 - 4.75 mmol/L; and a mean peak of 2.18 ± 1.46 mmol/L (table 7.5). When all individual apoB48 triglyceride response curves were combined, there was a mean peak at 6 hours with a mean apoB48 triglyceride concentration at the peak of 2.06 ± 1.55 mmol/L, followed by a later increase then decrease in triglyceride concentrations around 8 hours in some subjects (figure 7.1, table 7.4). The mean apoB48 triglyceride concentration was significantly elevated above fasting concentrations from 2 to 5 hours postprandially. Again the apoB48 results are in accord with plasma and TRL (d < 1.006g/ml) data.

A smaller but significant increase was observed in both individual and mean apoB100 triglyceride concentrations (table 7.4, figure 7.3). The individual apoB100 triglyceride concentrations peaked between 3 - 6 hours postprandially at 0.42 - 2.1 mmol/L; with a mean peak value of 1.12 ± 0.66 mmol/L (table 7.5). When all individual apoB100 triglyceride response curves were combined, triglyceride concentrations increased over 1 - 5 hours, with a mean peak at 5 hours and a mean apoB100 triglyceride concentration at the peak of 1.09 ± 0.62 mmol/L (table 7.4). The mean apoB100 triglyceride concentrations were significantly elevated above fasting concentrations 2 - 5 hours postprandially (figure 7.3, table 7.4). After peaking the apoB100 triglyceride concentration remained elevated and appeared to reach a plateau.

Large $VLDL_1$ triglyceride concentrations increased between 1 - 5 hours postprandially (figure 7.4), peaking individually between 3 - 6 hours postprandially, with triglyceride concentrations ranging between 0.42 - 1.57 mmol/L (mean of 0.69 ± 0.57 mmol/L (table 7.5)). The mean $VLDL_1$ triglyceride concentration was significantly elevated ($p < 0.05$) above fasting concentrations 2 - 5 hours postprandially

(table 7.4, figures 7.3, 7.4), in accord with total apoB100 data. Some of the individual $VLDL_2$ triglyceride concentrations increased during the course of the experiment (appendix 28, table 7.4), but overall mean $VLDL_2$ triglyceride concentrations remained relatively constant during the course of the experiment (figure 7.3).

The contribution of apoB48 and apoB100 TRL fractions to the postprandial response in TRL (d < 1.006g/ml) triglyceride (table 7.6) was quantified by measuring the area under the individual triglyceride response curves (fasting 0 hour concentration was taken as baseline). $VLDL_1$ and $VLDL_2$ contributions to the postprandial increase in apoB100 TRL was also quantitated by measuring the area under individual response curves (fasting 0 hour was taken as baseline) (table 7.6). The increase in apoB48 TRL triglyceride was almost 5 times greater than the increase in apoB100 TRL triglyceride (13.3 ± 13.0 mmol.hour vs 2.9 ± 2.7 mmol.hour). Eighty three percent (± 9%) of the postprandial increase in TRL (d < 1.006 g/ml) triglyceride was due to apoB48 TRL (table 7.7). The contribution from apoB100 TRL accounted for 16% (± 6%) of the postprandial increase in TRL (d < 1.006g/ml) triglyceride. The increase in $VLDL_1$ triglyceride was 12 times greater than the increase in $VLDL_2$ triglyceride (2.41 ± 2.00 mmol.hour vs 0.20 ± 0.17 mmol.hour). Seventy three percent (±21%) of the postprandial increase in total apoB100 triglyceride was due to $VLDL_1$, with 13% (± 13%) of the postprandial increase in total apoB100 accounted for by $VLDL_2$ (table 7.7).

*Table 7.6: **Individual and mean areas under the curve (mmol.hour) to assess the contribution of apoB48 and apoB100 TRL, $VLDL_1$ and $VLDL_2$ to postprandial increases in the plasma concentration of triglyceride in diabetics.***

Subject	Plasma (mmol.hr)	TRL (d < 1.006 g/ml) (mmol.hr)	ApoB48 (mmol.hr)	ApoB100 (mmol.hr)	$VLDL_1$ (mmol.hr)	$VLDL_2$ (mmol.hr)
MH	12.1	7.9	7.1	1.2	0.81	0.2
CE	8.2	6.3	5.6	1.3	1.14	0.5
JC	55.4	48.1	37.9	7.5	7.57	0.07
SJ	11.0	9.1	6.2	1.6	0.72	0.2
PF	15.5	10.9	9.7	2.6	1.8	0.1
Mean (± SD)	**20.5 ± 19.7**	**16.5 ± 16.0**	**13.3 ± 13.0**	**2.9 ± 2.7**	**2.41 ± 2.0**	**0.2 ± 0.17**

Table 7.7: ***Contribution of apoB48 and apoB100 TRL to postprandial increases in the TRL (d<1.006g/ml) triglyceride concentration, and of $VLDL_1$ and $VLDL_2$ to postprandial increases in the total apoB100 triglyceride concentration.*** *Quantitated by the measurement of area under the individual triglyceride response curves.*

Fraction	ApoB48	ApoB100	$VLDL_1$	$VLDL_2$
Triglyceride (mmol.hour)	13.3 ± 13.0 (83%)*	2.9 ± 2.7 (16%)*	2.4 ± 2.0 (73%)*	0.20 ± 0.17 (13%)*

** Values in parentheses represent increases expressed as percentages of the total triglyceride in lipoprotein fractions.*

Mean cholesterol concentrations were also measured in the different TRL fractions. No significant increases in cholesterol concentrations were observed in the TRL (d<1.006g/ml), apoB100, $VLDL_1$ or $VLDL_2$ fractions. There was however a significant elevation in apoB48 cholesterol concentrations 3 - 5 hours postprandially.

Plasma and TRL (d<1.006g/ml) apoB and apoAI concentrations were also measured. Mean plasma apoB, apoAI and mean TRL (d<1.006g/ml) apoB and apoAI concentrations did not change significantly during the course of the experiment.

Plasma free fatty acid (FFA) concentrations are shown in figure 7.5. Mean plasma FFA concentrations were increased but not significantly by the fat meal (figure 7.5).

Plasma insulin levels (table 7.8) increased 2 hours after ingestion of the fat meal in all subjects. Mean plasma insulin concentrations were significantly elevated *($p=0.003$)* above fasting levels 2 hours postprandially.

Table 7.8: ***Postprandial changes in individual and mean plasma insulin concentrations (mU/L).*** *Significantly different from fasting concentration by paired t-test: *p< 0.005.*

Subjects	Time (hours) 0 hours	2 hours	Delta insulin
JC	19.2	32.8	13.6
SJ	6.9	15.0	8.1
PF	37.2	49.3	12.1
CE	7.5	24.7	17.5
MH	10.2	17.4	7.2
Mean insulin (mU/L) (± SD)	16.2 ± 12.7	27.8 ± 13.8*	11.7 ± 4.2

Correlations between variables.

To examine the effect of fasting triglyceride concentrations on the measured parameters of postprandial lipaemia, potentially important correlations were examined. Log fasting triglyceride was not significantly correlated with plasma (r=0.51, *p=0.379*), apoB48 (r=0.537, *p=0.351*), apoB100 (r=0.555, *p=0.332*), $VLDL_1$ (r=0.60, *p=0.285*) or $VLDL_2$ (r=-0.03, *p=0.962*) triglyceride areas under the curve. FFA area under the curve (r=0.709, *p=0.18*) and delta insulin (r=0.805, *p=0.10*) were positively correlated with log fasting triglyceride but these associations failed to reach significance.

A positive, significant correlation between areas under the curve for FFA and apoB48 (r=0.948, *p=0.014*) was observed (figure 7.6).

The effect of changes in insulin concentrations on the measured parameters of postprandial lipaemia was also examined by correlation analysis. This revealed no correlation between delta insulin and the area under the curve for plasma (r=0.196, *p=0.752*), apoB48 (r=0.232, *p=0.707*), apoB100 (r=0.261, *p=0.672*), $VLDL_1$ (r=0.316, *p=0.605*), $VLDL_2$ triglyceride (r=0.467, *p=0.428*) and FFA (r=0.494, *p=0.398*).

One of the diabetic patients (JC) produced exaggerated individual postprandial triglyceride responses for plasma, TRL (d < 1.006g/ml), apoB48, apoB100, $VLDL_1$ and $VLDL_2$ fractions (appendices 23 - 28). This exaggerated response was observed

particularly during the latter hours of lipaemia and was out of keeping with the rest of the diabetic patients. Triglyceride response curves for plasma, TRL ($d < 1.006$g/ml), apoB48, apoB100, $VLDL_1$ and $VLDL_2$ were re-plotted excluding JC. This resulted in a lowering of all triglyceride response curves, particularly in the latter stages of postprandial lipaemia. However there were no significant differences in plasma, TRL ($d < 1.006$g/ml), apoB48, apoB100, $VLDL_1$ and $VLDL_2$ results whether JC was included or not.

3.1 Comparison of normal and diabetic patients.

Normal subjects were compared with diabetics. Since JC had an aberrant postprandial response statistical analysis was performed with and without this subject. Several variables reached significance when he was excluded from the diabetic group, but inclusion or exclusion of JC did not affect overall interpretation of data.

Mean fasting plasma cholesterol concentrations were comparable in normals and diabetics (4.84 $\pm$ 0.72 mmol/L vs 5.48 $\pm$ 0.6 mmol/L respectively). No significant changes occurred between normal and diabetic groups in mean plasma cholesterol concentrations during the course of the experiment.

Mean plasma insulin concentrations were significantly different *($p < 0.05$)* between the normal and diabetic groups (fasting 7.19 $\pm$ 2.48 mU/L vs 16.2 $\pm$ 12.7 mU/L and at 2 hours 14.40 $\pm$ 9.91 mU/L vs 27.8 $\pm$ 13.8 mU/L respectively).

Mean fasting FFA concentrations were significantly different between the groups, with the FFA concentration in the normal subjects being lower than that in diabetic subjects (0.32 $\pm$ 0.21 mmol/L for normals, 0.62 $\pm$0.19 mmol/L for diabetics *($p = 0.02$)* (Note this value differs from the data in table 7.2 probably because of patient selection). FFA concentrations in the normal subject group were also significantly decreased compared to diabetic groups at 1 hour post meal but thereafter the difference was lost. After the meal, there was a consistent initial decrease in FFA concentrations in both groups (0 - 2 hours in normals, 0 - 3 hours in the diabetic group). FFA concentrations subsequently rose to reach peak levels at 6 hours postprandially for normals and 7 - 10 hours postprandially for the diabetic group (figure 7.7). Thereafter, FFA levels started to fall in all groups. There was no significant difference in the mean area under the curve between the normal and diabetic subject groups (4.15 $\pm$ 1.67 mmol.hour vs 2.91 $\pm$ 3.12 mmol.hour respectively).

Mean fasting plasma apoAI and mean TRL (d < 1.006g/ml) apoB and apoAI concentrations were not significantly different between the normal and diabetic groups. During the course of the experiment there were no significant changes in the mean plasma apoAI and mean TRL (d < 1.006g/ml) apoB concentrations and response curves between the normal and diabetic groups. The mean TRL (d < 1.006g/ml) apoAI response curves were not significantly different between the normal and diabetic group, however exclusion of JC from the diabetic group allowed the differences at 2 - 5 and 7 - 9 hours postprandially to reach significance, compared to the normal subject group. The mean fasting plasma apoB concentrations were significantly different between the normal and both diabetic groups (0.84 $\pm$ 0.18 g/L for normals vs 1.146 $\pm$ 0.24 g/L for diabetics ($p=0.006$)). The mean plasma apoB response curve concentrations in the diabetic groups remained significantly elevated above that of the normal subject group during the course of the experiment (0 - 11 hours postprandially), although levels did not change during the course of the experiment (figure 7.8).

Mean plasma triglycerides peaked at 5 hours after ingestion of the fat meal in the normal group, whereas the peak of mean plasma triglycerides seemed to occur 1 - 2 hours later in the diabetic subject group (figure 7.9). Mean plasma triglyceride concentrations returned to fasting levels or even lower by 11 hours in the normal group, but remained elevated above the fasting levels even after 11 hours in the diabetic group. Seven to ten hours after the meal, mean plasma triglyceride response curves and concentrations in the normal subject group decreased significantly ($p<0.05$) compared to the diabetic group (figure 7.9). The mean areas under the plasma triglyceride response curves were not significantly different (11.2 $\pm$ 6.5 mmol.hour for normals; 20.5 $\pm$ 19.7 mmol.hour for diabetics) between the groups.

Mean fasting and postprandial apoB48 triglyceride concentrations in the subject groups are shown in figure 7.10. Mean apoB48 triglycerides peaked at 5 hours postprandially in the normal group, whereas the peak for mean apoB48 triglycerides seemed to occur 1 - 2 hours later in the diabetic group (6 hours postprandially). Mean apoB48 triglyceride response curves and concentrations returned to fasting levels at 11 hours postprandially in the normal subject group but remained elevated above fasting concentrations in the diabetic subject group, suggesting that there may be a problem with clearance of dietary triglyceride or chylomicrons. The mean apoB48 fasting triglyceride concentration was decreased in the normal subject group compared to the diabetic subject group, but did not reach significance (0.21 $\pm$ 0.15 mmol/L for normals compared to 0.41 $\pm$ 0.26 mmol/L for diabetics). Mean apoB48 triglyceride concentrations were significantly lower in the normal subject group compared to the

diabetic subject group, when JC was excluded, at 7 ($p<0.001$), 10 and 11 ($p<0.05$) hours postprandially. This suggests that the diabetics may have delayed clearance of chylomicron triglyceride. The mean area under the apoB48 triglyceride curves was higher in diabetics, but not significantly (6.31 $\pm$ 4.28 mmol.hour for normals; 13.3 $\pm$ 13.0 mmol.hour for diabetics).

Mean fasting and postprandial apoB100 concentrations in the subject groups are shown in figure 7.11. Mean apoB100 triglyceride concentrations peaked at 5 hours postprandially in both patient groups. Mean apoB100 triglyceride response curves and concentrations returned to below fasting levels 11 hours postprandially in the normal group while remaining slightly elevated in the diabetic subject group. The mean apoB100 triglyceride response curve concentrations in the diabetic group remained significantly elevated ($p<0.05$) above that of the normal subject group throughout the course of the experiment (0 - 11 hours postprandially) (figure 7.11). The mean apoB100 triglyceride response curve concentrations in the diabetic group, if JC was excluded, were significantly elevated ($p<0.05$) above that of the normal subject group 6, 7, 10, 11 hours postprandially. The mean area under the apoB100 triglyceride curves was higher in diabetics, but not significantly (1.3 $\pm$ 1.1 mmol.hour for normals; 2.9 $\pm$ 2.7 mmol/hour for diabetics).

Fasting and postprandial triglyceride concentrations of $VLDL_1$ and $VLDL_2$ subfractions in the subject groups are shown in figure 7.12. Mean $VLDL_1$ triglyceride concentrations peaked at 5 hours postprandially in all subject groups, in accord with apoB100 data. Mean $VLDL_1$ triglyceride response curves and concentrations returned to below fasting levels 11 hours postprandially in the normal while remaining slightly elevated in the diabetic group. The mean $VLDL_1$ triglyceride response curve and triglyceride concentrations in the diabetic group remained significantly elevated ($p<0.05$) above that of the normal subject group during the course of the experiment (0 - 11 hours postprandially) (figures 7.12). Exclusion of JC meant that this significance was lost. The mean areas under the $VLDL_1$ triglyceride curves were again higher in diabetics but showed no significant differences (0.94 $\pm$ 0.76 mmol.hour for normals; 2.41 $\pm$ 2.00 mmol.hour for diabetics). Mean $VLDL_2$ triglyceride response concentrations remained relatively constant in normal subjects during the course of the experiment (figure 7.12). However, in the diabetic subject group, mean $VLDL_2$ triglyceride concentrations appeared to have a double peak postprandially and decreased to approximately baseline concentrations. The mean $VLDL_2$ triglyceride response curve concentrations in the diabetic group were significantly elevated above that of the normal subject group 3 and 6 - 9 hours postprandially ($p<0.05$). As a result, the mean areas

under the curves were comparable between the normal and diabetic group (0.086 ± 0.07 mmol.hour for normals compared to 0.20 ± 0.17 mmol.hour for diabetics) but the diabetic group was significantly different from the normal group when JC was excluded (0.086 ± 0.07 mmol.hour for normals compared to 0.24 ± 0.18 mmol.hour (*p=0.02*)).

Recovery of initial TRL added to the immunoaffinity gel was 88 ± 6%, and comparable with the recoveries of 89 ± 10% obtained from normal patients, as estimated by measuring the recovered triglyceride in the apoB100 (bound) and apoB48 (unbound) fractions.

The distribution of apoE phenotypes was similar in normal and diabetic patient groups.

3.2 Correlations between variables.

To examine the influence of variation in fasting triglyceride on postprandial lipoprotein metabolism, the combined data for all subjects was analysed. This revealed significant positive correlations between the log fasting plasma triglyceride concentration and the area under the curve for plasma *(r=0.534, p=0.049)*, apoB48 *(r=0.549, p=0.042)*, apoB100 *(r=0.623, p=0.017)*, $VLDL_1$ *(r=0.618, p=0.019)* triglyceride (figure 7.13), and with delta insulin (r=0.56, *p=0.037*) (figure 7.14). Log fasting triglyceride was not significantly correlated with $VLDL_2$ *(r=0.131, p=0.654)* or FFA area under the curve *(r=0.325, p=0.257)*.

A positive significant correlation between area under the curve for FFA and area under the curve for apoB48 (r=0.541, *p=0.046*) was observed (figure 7.15).

To examine the influence of changes in insulin concentrations on the measured parameters of postprandial lipaemia, the data for all subjects was analysed. No significant correlation between delta insulin and area under the curve was seen for plasma (r=0.342, p=0.231), apoB48 (r=0.324, *p=0.259*), apoB100 (r=0.461, *p=0.097*), $VLDL_1$ (r=0.42, *p=0.135*), or $VLDL_2$ (r=0.385, *p=0.174*) triglyceride. Delta insulin was significantly correlated with delta $VLDL_1$ (r=0.610, *p=0.021*). No correlations were observed between area under the curve for delta insulin and FFA (r=-0.013, *p=0.965*) or between delta insulin and delta $VLDL_2$ (r=-0.204, *p=0.485*).

4 Discussion.

In this study an analysis of both chylomicron and VLDL subfraction metabolism in the postprandial state was undertaken in patients with NIDDM (Type 2 diabetes).

As in the previous chapter, a specific monoclonal antibody for apoB100 was used to separate the apoB48 and apoB100 containing TRL species from plasma obtained from diabetic subjects in a postprandial state. This allowed further isolation of VLDL subfractions, by cumulative flotation from the apoB100 fraction isolated by immunoaffinity chromatography. Again, single sample aliquots in individual eppendorf tubes containing antibody bound to sepharose were used, rather than eluting fractions from an immunoaffinity column, to obtain reliable quantitative data. Adequacy of separation was controlled by monitoring the apolipoprotein composition of isolated fractions by SDS gel electrophoresis (chapter III).

This study demonstrates that the lipid and lipoprotein responses to a fat meal in normal and NIDDM (Type 2 diabetic) subjects depend on the fasting triglyceride level and not on the underlying diabetic state, as observed by others *(Lewis et al 1991)*, although the fasting triglyceride concentration can reflect diabetic control. Significant positive correlations between fasting triglyceride (log) and lipid and lipoprotein responses to the fat meal were seen when the combined data for all subjects were analysed. These correlations lost significance when the data from the diabetic patients was analysed separately. NIDDM (Type 2 diabetic) patients with normal fasting triglyceride levels have postprandial responses that are similar to those of non-diabetic controls. By contrast, the NIDDM (Type 2 diabetic) patient (JC) with an increased fasting triglyceride level demonstrated several differences after ingestion of a fat meal; including greater and prolonged elevation of total plasma, TRL ($d < 1.006$g/ml), apoB48, apoB100, $VLDL_1$ and or $VLDL_2$ triglyceride concentrations and plasma FFA concentrations. The triglyceride response curves were elevated and prolonged particularly in the latter stages of postprandial lipaemia, suggesting that there may be a problem with clearance of triglyceride (chylomicrons and their remnants) by this NIDDM (Type 2 diabetic) subject (JC), yet plasma cholesterol, apoB and apoAI concentrations remained normal and comparable to the normal subjects. Other investigators have demonstrated the association between fasting triglyceride concentration and postprandial triglyceride elevation in non-diabetic subjects *(Cohn et al 1988)*. The differences observed between normal and diabetic subjects in apoB48, apoB100, $VLDL_1$ and $VLDL_2$ triglyceride concentrations occur as stated previously in the latter hours of lipaemia. These differences would not have been seen without the

separation of these lipoprotein fractions by immunoaffinity chromatography and then cumulative flotation.

There were no significant differences between the normal and diabetic groups as regards mean plasma cholesterol, FFA and plasma apoAI concentrations and response curves. The mean areas under the triglyceride response curves were calculated for plasma, TRL ($d < 1.006$g/ml), apoB48 , apoB100, $VLDL_1$ and $VLDL_2$ fractions. No significant differences in the areas under the curves were found between the both groups, suggesting that the overall production and clearance of each of these lipoprotein species occurs at the same rate in normal and NIDDM (Type 2 diabetic) patients. The exception was $VLDL_2$ where the diabetics had a significantly greater area under the triglyceride response curve. This suggests that there may be a difference in the rate of production or clearance of $VLDL_2$. However significant differences were noted in the plasma, TRL ($d < 1.006$g/ml) and apoB48 triglyceride response curves between the normal and diabetic (NIDDM) groups in the later hours of postprandial lipaemia (7 - 10 hours postprandially). This indicates that although the differences in area under the curves did not reach significance, the differences seen in the triglyceride response curves may indicate a delayed rate of triglyceride clearance in NIDDM (Type 2 diabetic) subjects, but in order to confirm this a larger NIDDM (Type 2 diabetic) patient population would be required.

Total apoB100 and $VLDL_1$ mean triglyceride response curves were significantly elevated between 0 - 11 hours postprandially in the diabetic group compared to the normal subject group, and 6, 7, 10, and 11 hours postprandially when JC was excluded from the diabetic group compared to the normal subject group. This suggests increased $VLDL_1$ triglyceride concentrations and the possibility of a delayed clearance rate for total apoB100 and $VLDL_1$ triglyceride. $VLDL_2$ triglyceride response curves are relatively unchanged throughout the course of the experiment, although there are significant differences 3 and 6 - 9 hours postprandially in the diabetic subjects compared to normals. The differences in the response of $VLDL_1$ and $VLDL_2$ subfractions to the fat meal may suggest independent regulation and separate synthesis pathways for the VLDL subfractions. This was a finding of a recent study by *Malmstrom et al (1997 a)*. Possible explanations for elevated levels of $VLDL_1$ in normal subjects have been discussed in the previous chapter, however in NIDDM (Type 2 diabetic) patients the situation may be exacerbated because NIDDM (Type 2 diabetic) subjects are insulin resistant. In NIDDM (Type 2 diabetic) subjects the antilipolytic effects of insulin may be diminished and as a result, excess circulating fatty acids are released from adipose tissue resulting in increased hepatic triglyceride rich VLDL synthesis with reduced LPL

activity impeding the removal of triglyceride from the circulation. This theory is compatible with the results of this study which show that there may be delayed clearance of chylomicrons and VLDL according to the triglyceride response curves although the differences in mean area under the curve data did not reach significance.

Plasma FFA concentrations increased but did not change significantly during the course of the experiment and were not significantly different among the groups. A consistent initial decrease in plasma FFA concentrations 1 - 3 hours after ingestion of the fat meal was observed in virtually all subjects in each group followed by a subsequent rise in plasma FFA concentrations. This phenomenon has previously been observed by others *(Lewis et al 1991, O'Meara et al 1992)* and is suggested to be related in timing to the early postprandial insulin rise as also seen in this study. The source of the plasma FFA in normal and diabetic subjects still requires to be clarified; in normals most of the FFA will be present in plasma as a result of the hydrolysis of chylomicron and VLDL triglyceride by LPL. In diabetics (NIDDM), the situation is slightly different, the FFA may arrive in plasma from different sources; *i.e.* NIDDM (Type 2 diabetics) who are insulin resistant may have FFA present in plasma as a result of the lack of inhibition of HSL in adipose tissue. HSL is normally suppressed by the action of insulin and LPL activated by it, therefore in subjects who are insulin resistant the HSL could be activated resulting in production of excess FFA from stored triglycerides from adipose tissue resulting in increased hepatic VLDL synthesis, LPL will not be properly activated and therefore there could be decreased hydrolysis of chylomicrons and VLDL triglyceride by LPL and removal of triglyceride from plasma.

Positive, significant correlations (Pearson) were seen between fasting triglyceride concentrations and individual areas under the curve for plasma, apoB48, apoB100 and $VLDL_1$ triglyceride response curves, suggesting that the lipoprotein responses to a fat meal in normal and diabetic patients depends on the fasting triglyceride concentration and not necessarily the underlying diabetic state. In normal subjects, positive, significant correlations (Pearson) were seen between delta insulin and area under the curve for apoB100 and $VLDL_1$ as well as between delta insulin and delta apoB100 and delta $VLDL_1$ but these were not observed in the diabetic subject population. In the diabetic population and the subject group as a whole a positive, significant correlation (Pearson) was seen between area under the curve for FFA and area under the curve for apoB48 indicating that these two variables may be closely linked *e.g.* higher FFA may cause LPL loss from endothelial sites and impair apoB48 particle removal. The magnitude of postprandial lipaemia is known to be highly dependent on the fasting plasma triglyceride concentration *(Patsch 1987)*, since NIDDM (Type 2 diabetic)

patients generally have a higher fasting plasma triglyceride a disturbed postprandial triglyceride metabolism would be expected. However this study demonstrated NIDDM (Type 2 diabetic) patients with normal fasting plasma triglyceride concentrations had postprandial responses similar to those of non-diabetic controls.

The fact that there were differences in age and BMI between the diabetic and control subjects should be kept in mind when interpreting data from the postprandial studies. The type 2 diabetics recruited here were older and had a higher BMI compared to controls and therefore were probably less fit than controls. It has been shown that chylomicron removal decreases with age and that fitness may lead to an increase in LPL levels, enhancing chylomicron removal. Weight loss, increasing exercise and dietetic changes may all reduce postprandial hyperlipidaemia in the diabetic subjects The normal subjects recruited here may have been fitter and would also have had a different diet compared to the diabetic group. These differences should be considered before patient recruitment for future postprandial studies.

Figure 7.1: ***Postprandial changes in the mean triglyceride concentration of plasma, TRL (d<1.006g/ml) and apoB48 fractions in diabetic subjects.***

*Significantly different from fasting concentration by paired t-test: *p<0.05, **p<0.01.*

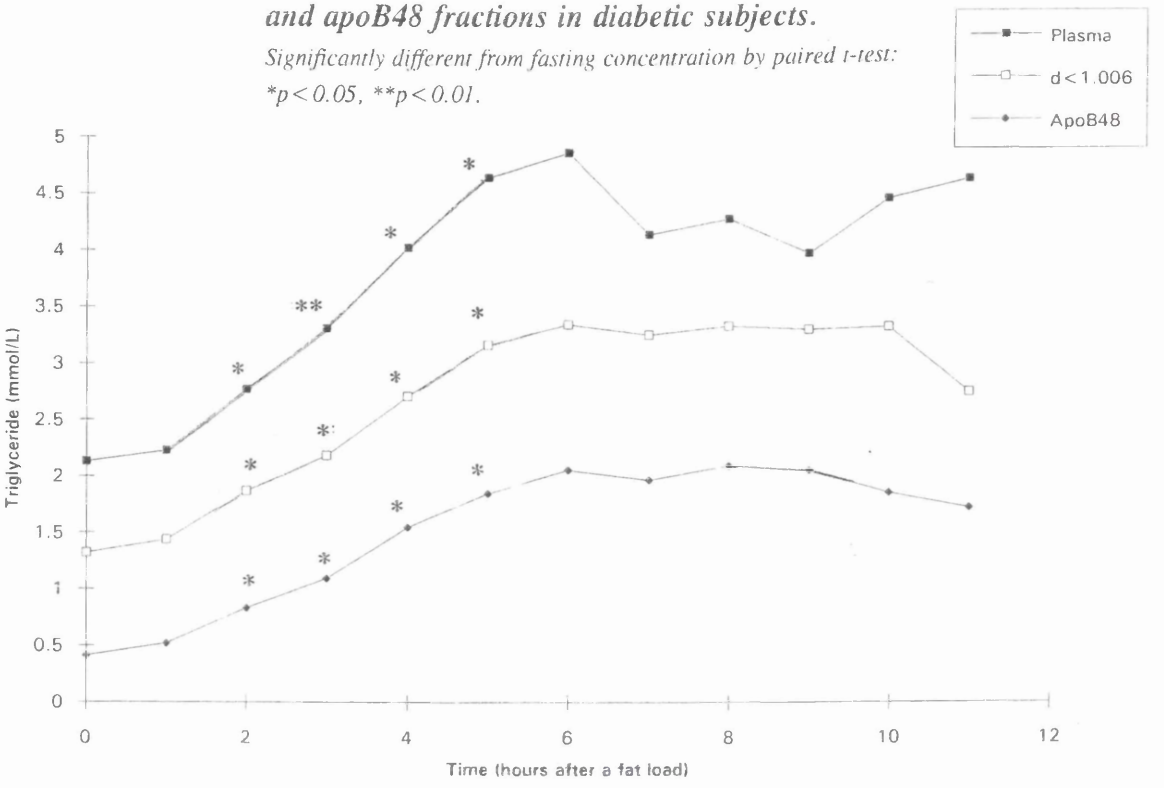


Figure 7.2: ***Postprandial changes in the individual plasma concentration of triglycerides in diabetic subjects.***

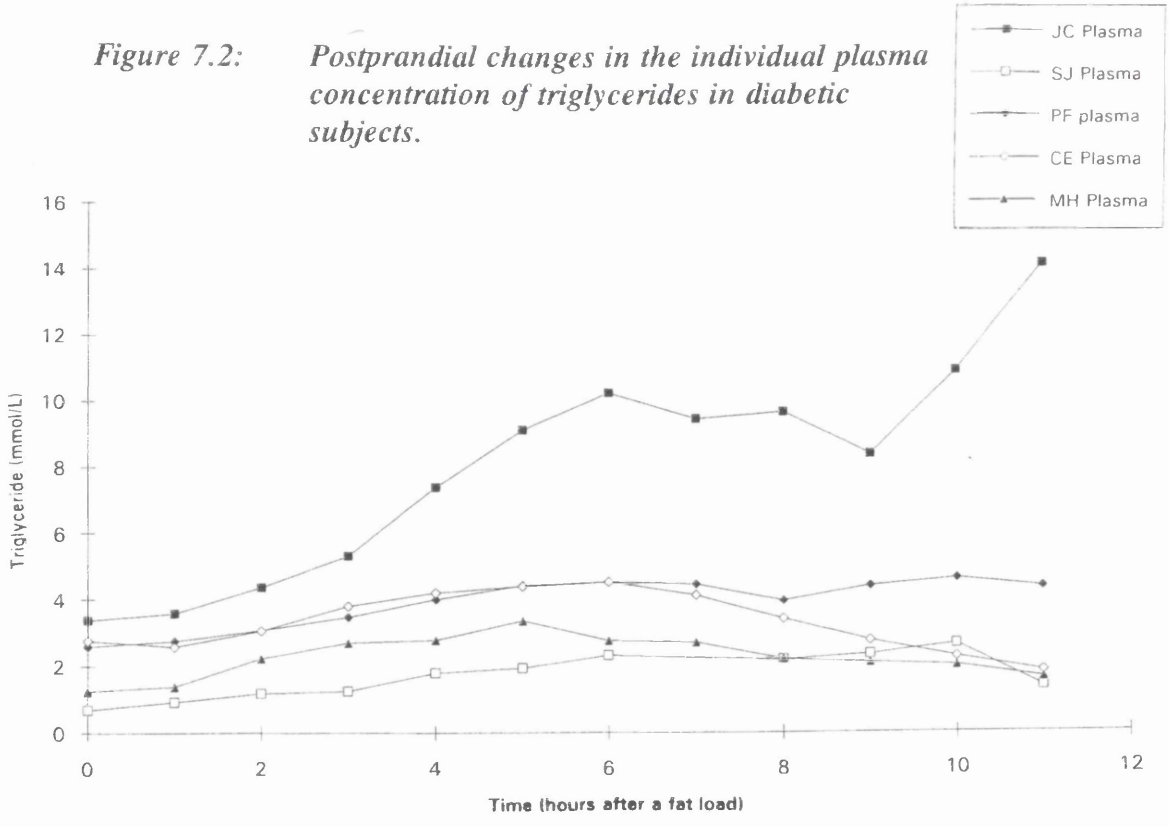


Figure 7.3: ***Postprandial changes in the mean triglyceride concentration of apoB100, $VLDL_1$ and $VLDL_2$ fractions in diabetic subjects.*** *Significantly different from fasting concentration by paired t-test: *$p < 0.05$, **$p < 0.01$.*

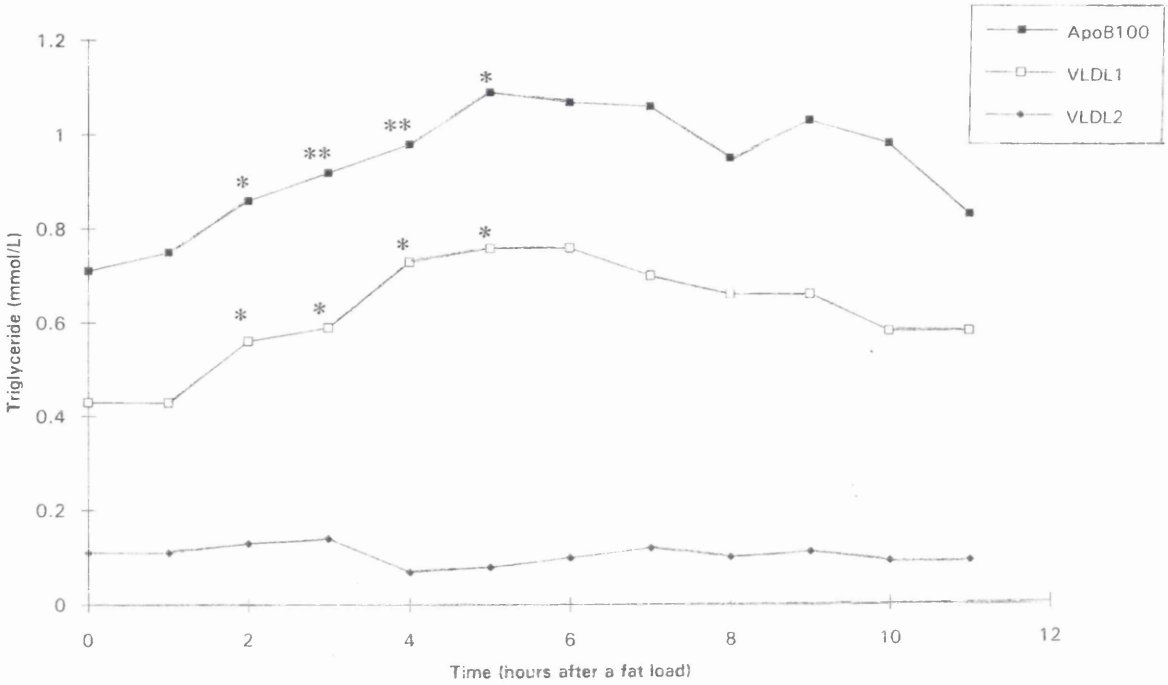


Figure 7.4: Postprandial changes in triglyceride concentrations in the individual VLDL$_1$ TRL fractions in diabetic subjects.

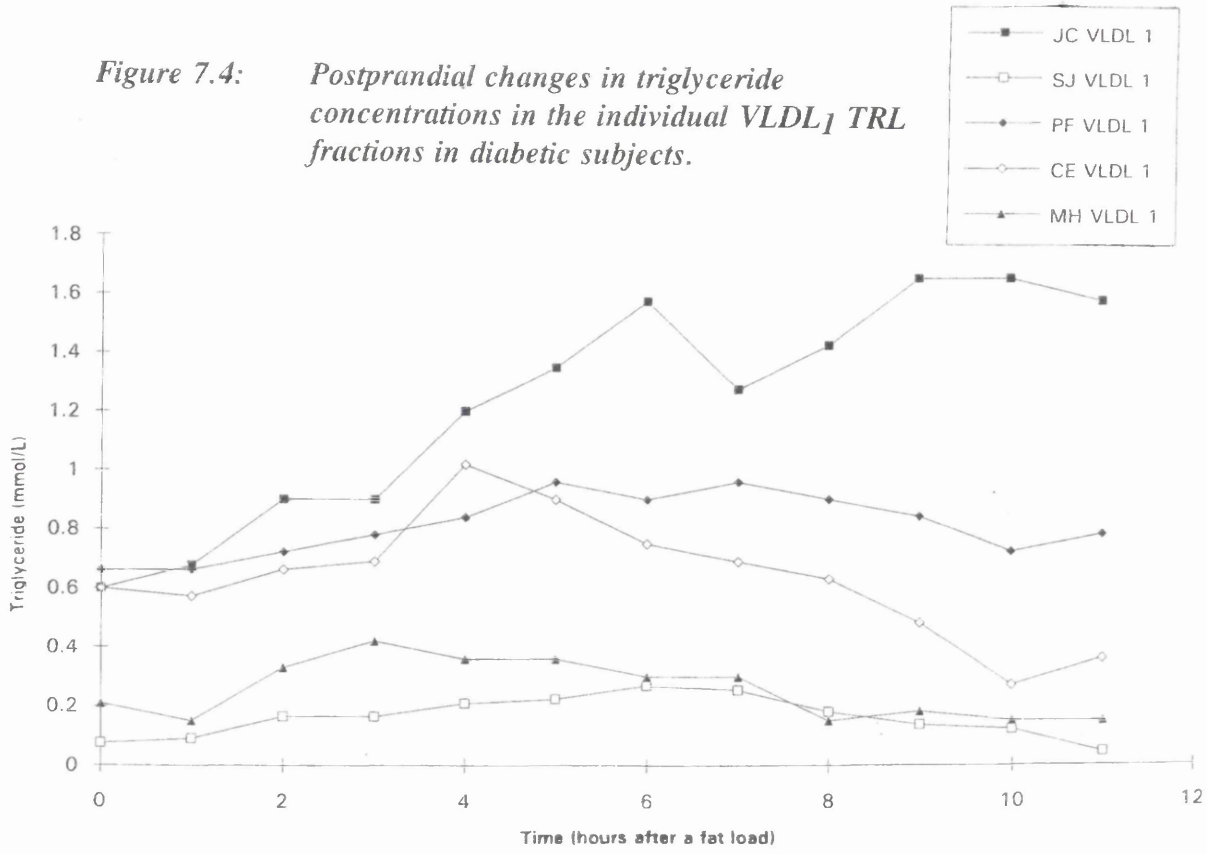


Figure 7.5: Postprandial changes in the mean plasma concentration of FFA in diabetic subjects.

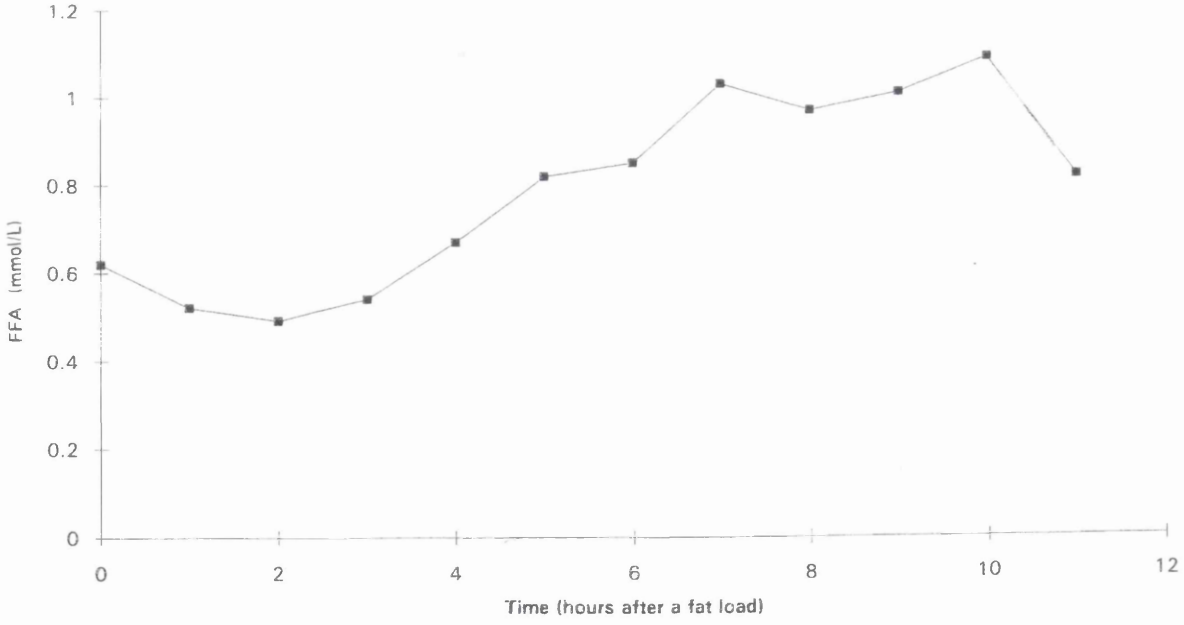


Figure 7.6 : ***Pearson correlation between FFA area under the curve and apoB48 area under the curve in diabetic subjects.***

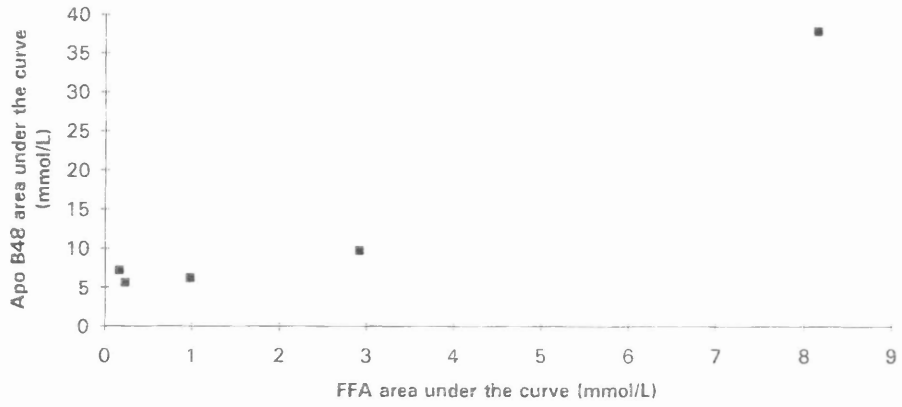


Figure 7.7: ***Postprandial changes in the mean plasma FFA concentration for normal and diabetic subjects, with and without JC.*** *Significantly different from fasting concentration by paired t-test: *p < 0.05.*

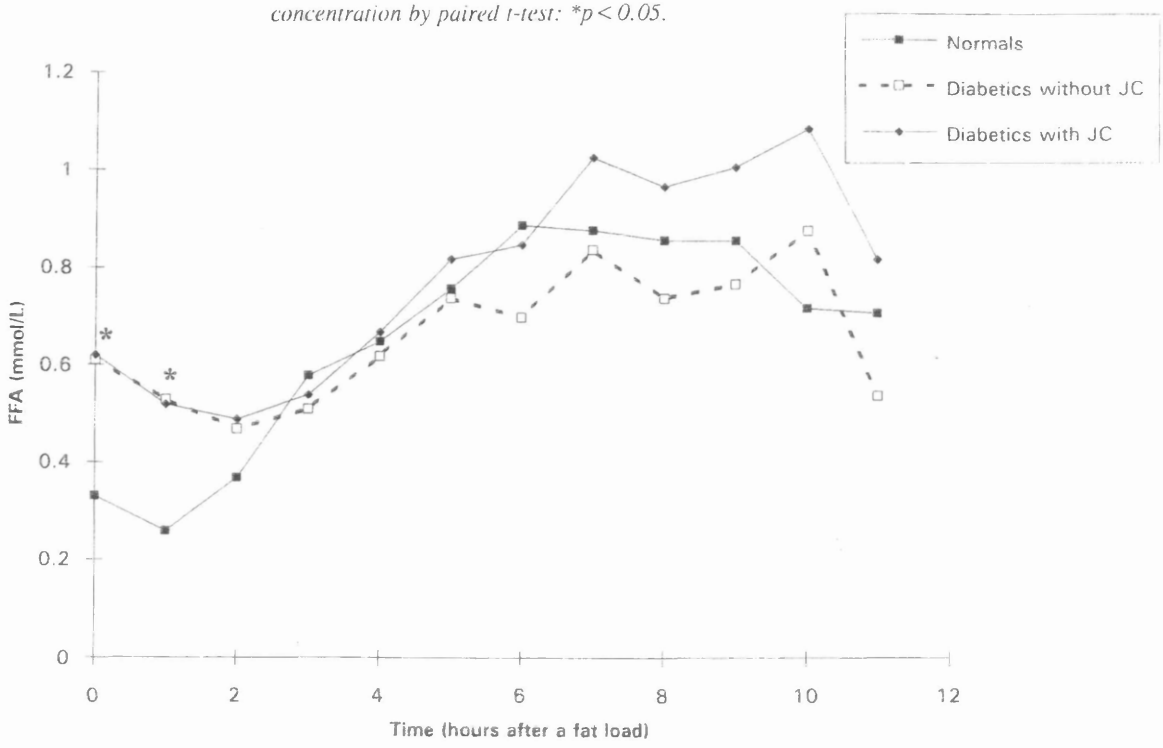


Figure 7.8: ***Postprandial changes in the mean plasma apoB concentration for normal and diabetic subjects, with and without JC.*** *Significantly different from fasting concentration by paired t-test: *p < 0.05.*

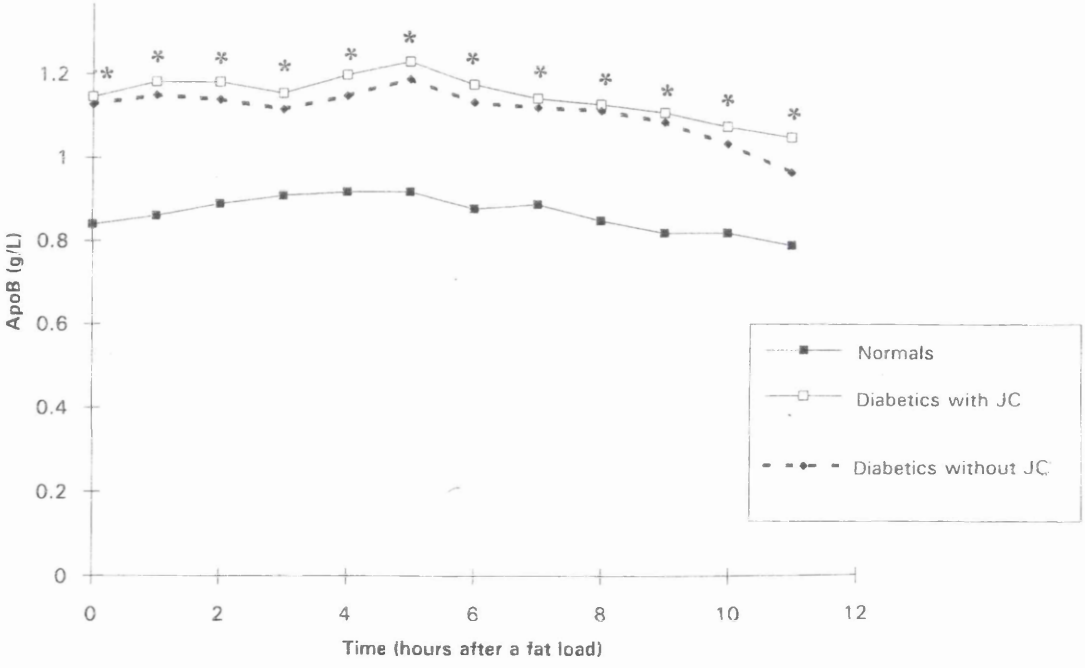


Figure 7.9: ***Postprandial changes in the mean plasma triglyceride concentration for normal and diabetic subjects, with and without JC.*** *Significantly different from fasting concentration by paired t-test: *p < 0.05.*

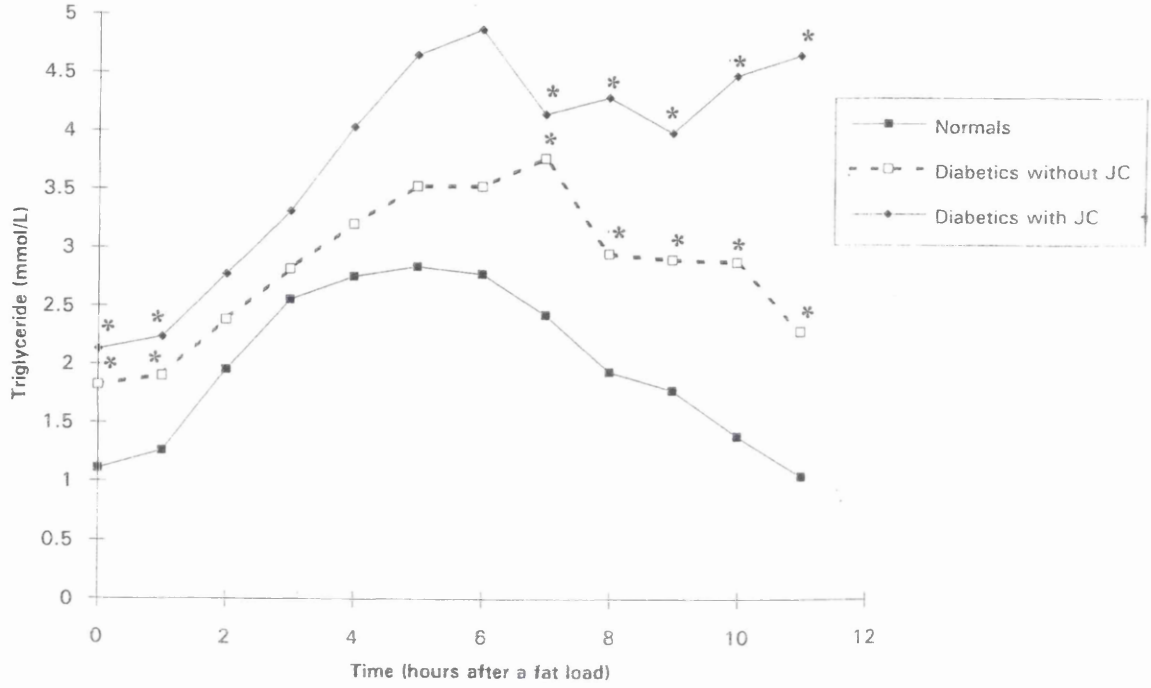


Figure 7.10: ***Postprandial changes in the mean apoB48 triglyceride concentrations for normal and diabetic subjects, with and without JC.*** *Significantly different from fasting concentration by paired t-test: *p < 0.05; ***p < 0.005.*

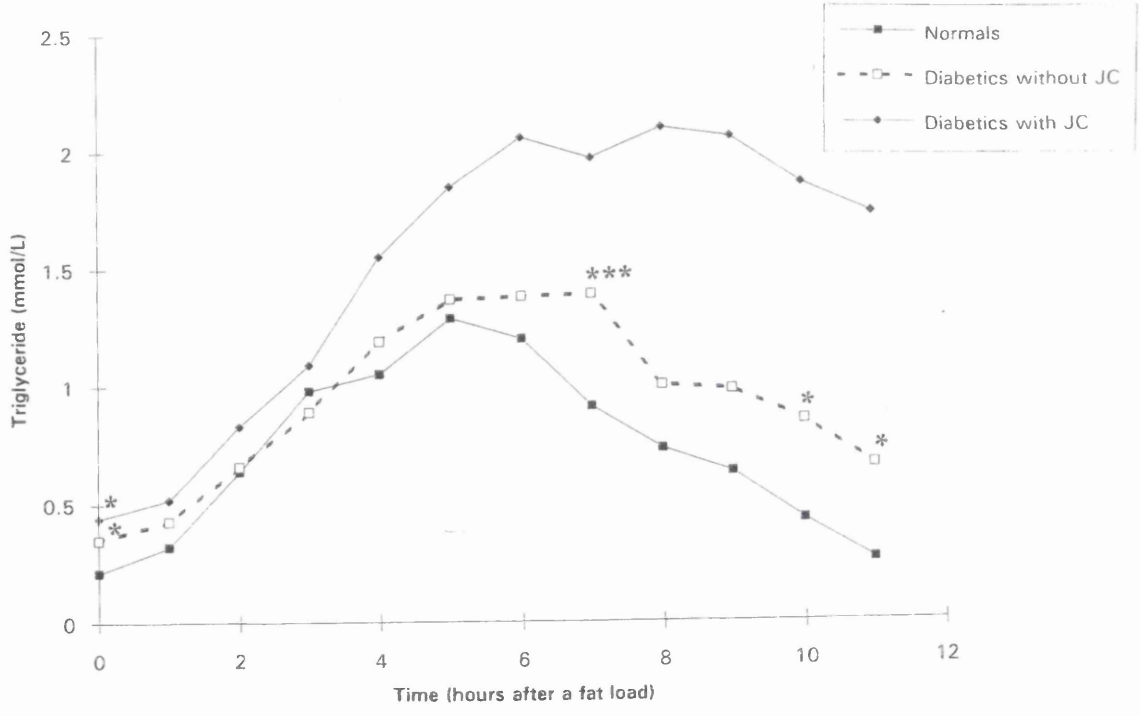


Figure 7.11: ***Postprandial changes in the mean apoB100 triglyceride concentration for normal and diabetic subjects, with and without JC.*** *Significantly different from fasting concentration by paired t-test: *p < 0.05.*

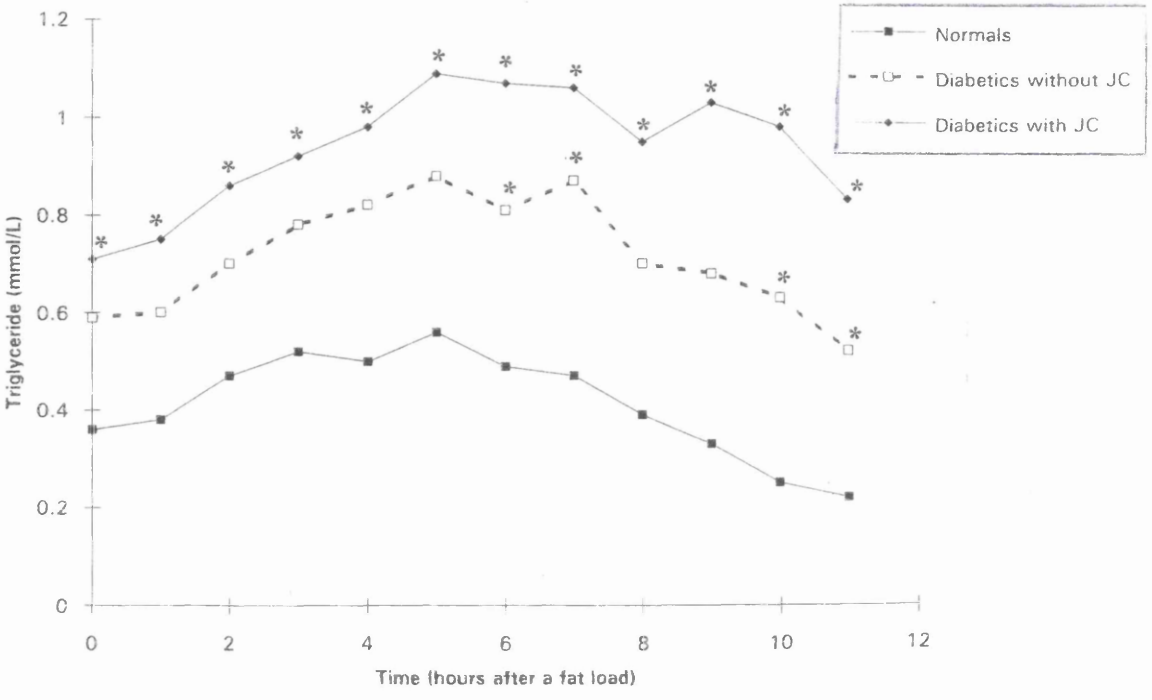


Figure 7.12: ***Postprandial changes in the mean $VLDL_1$ and $VLDL_2$ triglyceride concentration for normal and diabetic subjects, with and without JC.***

*Significantly different from fasting concentration by paired t-test: *p < 0.05.*

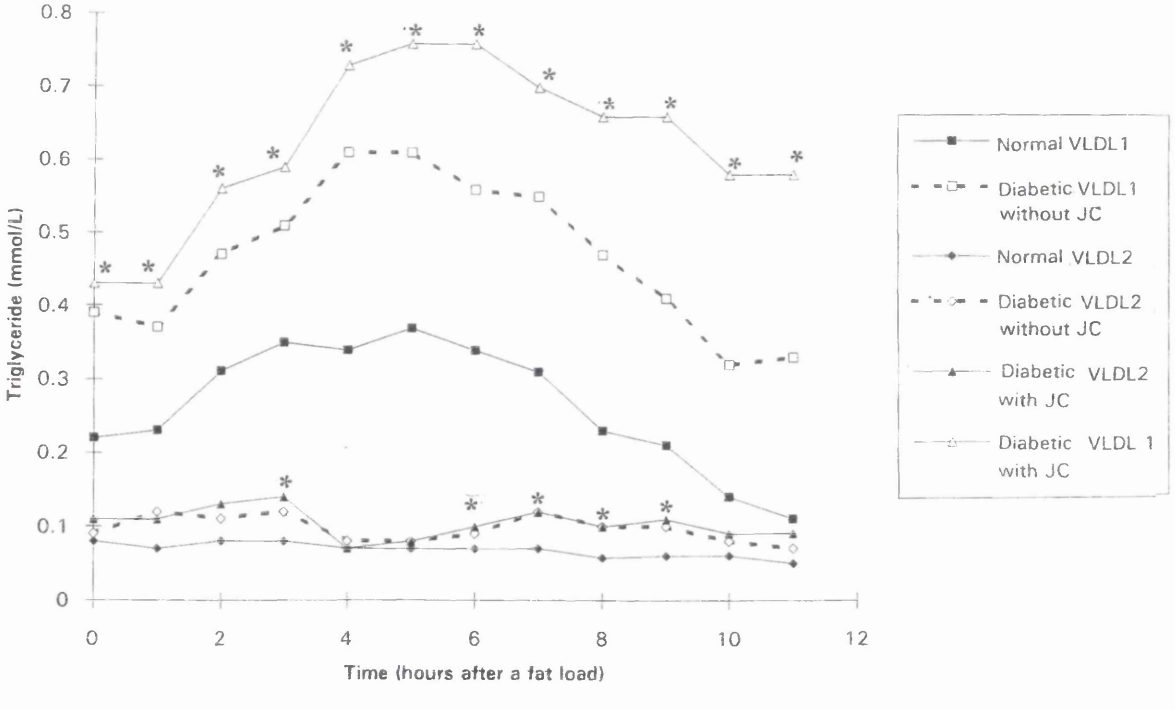
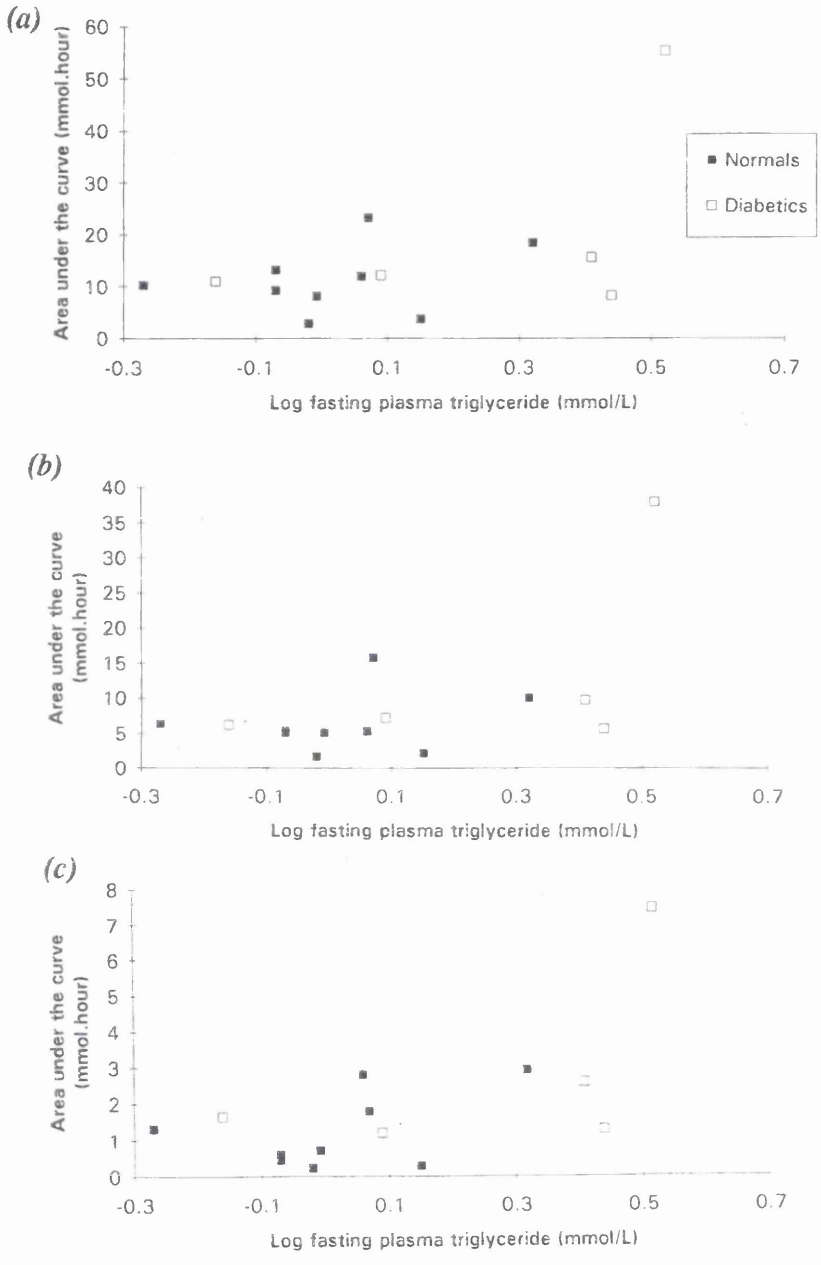
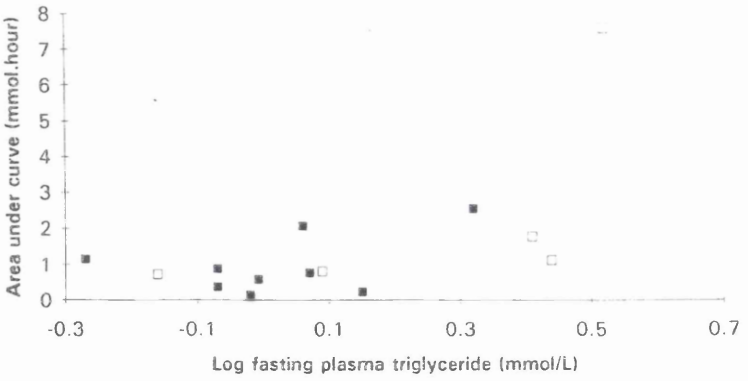


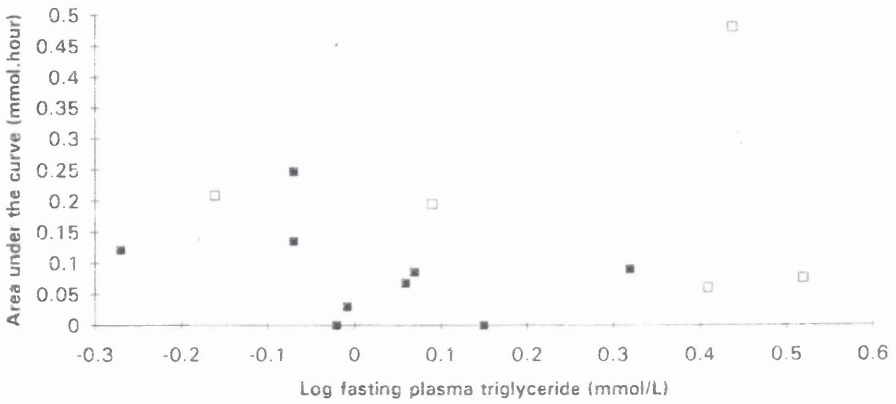
Figure 7.13: ***Pearson correlations.*** *Correlations between log fasting triglyceride concentration (mmol/L) and individual areas under the curve (mmol.hour) for (a) plasma, (b) apoB48, (c) apoB100, (d) $VLDL_1$, (e) $VLDL_2$ triglyceride and (f) FFA response curves, including all normal and diabetic subjects.*



(d)


Area under curve (mmol.hour)
8
7
6
5
4
3
2
1
0
-0.3
-0.1
0.1
0.3
0.5
0.7
Log fasting plasma triglyceride (mmol/L)

(e)


Area under the curve (mmol.hour)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
Log fasting plasma triglyceride (mmol/L)

(f)

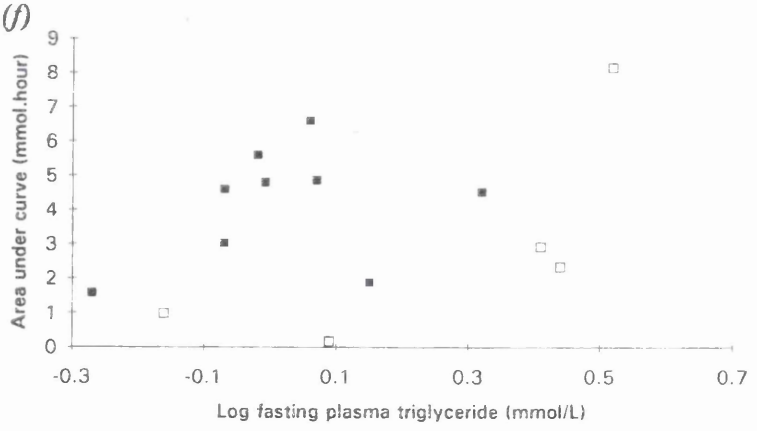

Area under curve (mmol.hour)
9
8
7
6
5
4
3
2
1
0
-0.3
-0.1
0.1
0.3
0.5
0.7
Log fasting plasma triglyceride (mmol/L)

Figure 7.14 *Pearson correlation, including all subjects between log fasting plasma triglyceride and delta insulin.*

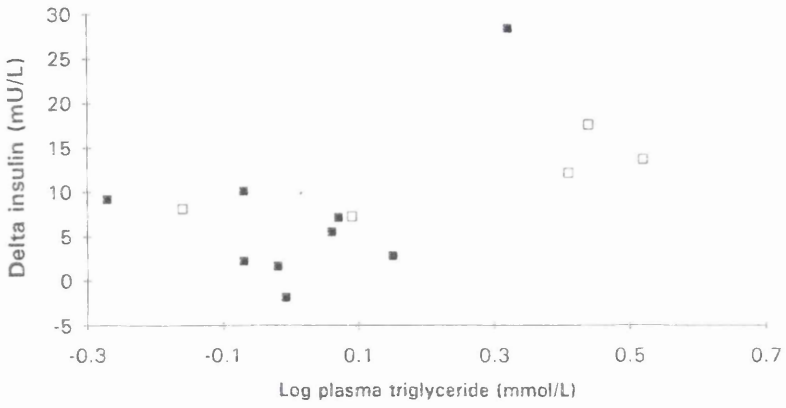
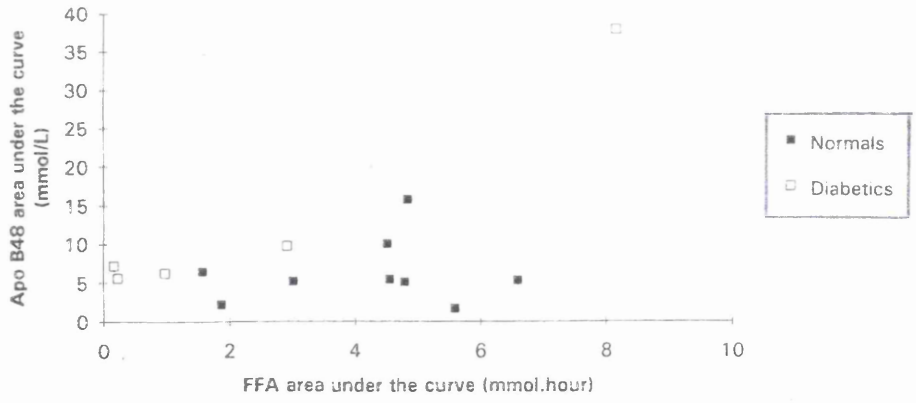


Figure 7.15: *Pearson correlation between FFA area under the curve and apoB48 area under the curve for all subjects.*



Chapter VIII Conclusions and future directions.

The aim of this thesis was to understand better the regulation of triglyceride metabolism in both man and rat models, in which it is aberrant. This objective has largely been met, and further insight has been gained into the regulation of triglyceride metabolism in two inter-related areas. First, in postprandial lipid and lipoprotein metabolism and second in the regulation of HSL activity and adipocyte lipolysis. A schematic representation of triglyceride metabolism, which includes information derived from the present studies is illustrated in figure 8.1. What follows is a short discussion integrating the main findings relating to the regulation of triglyceride metabolism.

1 Postprandial studies.

Postprandial triglyceridaemia describes the presence, following a meal, in plasma of triglyceride-rich lipoprotein particles, a heterogeneous population of lipoprotein particles comprising chylomicrons and VLDL. In order to delineate the behaviour of VLDL and its subfractions during alimentary lipaemia (in normal subjects, chapter VI and in diabetic subjects, chapter VII) it is essential to separate chylomicrons (apoB48 TRL) and VLDL (apoB100 TRL) fractions using a reliable method. Immunoaffinity chromatography is the current method of choice and has been successfully used by other groups. Simple measurement of plasma triglyceride concentrations alone may mask a defect in diabetic chylomicron metabolism, as both chylomicron and VLDL accumulate postprandially (chapter VII). The abnormalities in postprandial lipaemia *i.e.* in diabetics which were attributed to chylomicrons (apoB48 TRL) could conceivably have been due to VLDL (apoB100 TRL) accumulation, although it is true that the majority of triglyceride is present in chylomicrons. The exaggerated lipaemia, often present particularly towards the latter hours after a fat feed in diabetic subjects, could be due to the persistence of VLDL or failure to clear efficiently either or both chylomicrons and VLDL. Without the immunoaffinity chromatographic separation (using a Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes) of chylomicron and VLDL lipoprotein particles it would be impossible to study changes in the behaviour of $VLDL_1$.

The development of the methodology to separate chylomicrons and VLDL (chapter III), was based on that already published in the literature *(Schneeman et al 1993, Campos et al 1992, Milne et al 1984 and Cohn et al 1988, Bjorkegren et al 1997)*, however we used a different monoclonal antibody and made some changes to the methodology from that used by *Cohn et al (1993)*. Development and testing of the immunoaffinity chromatography methodology was, like the postprandial studies, labour intensive and time consuming. Acceptable recovery rates (85 - 95%) quoted by Cohn were reproduced by this methodology (89 $\pm$ 10%). Adequate separation of apoB48 (chylomicrons) and apoB100 (VLDL) TRL containing fractions for the purposes of the present study was achieved. Quality of separation was monitored by gel electrophoresis of the isolated fractions. Some contamination of apoB48 fractions was noted, leading to a small underestimation of apoB100 TRL lipid concentrations. Additional chromatography was not carried out to remove contaminating apoB100 material as this would adversely affect recovery. Thus in the interpretation of results it should be noted that while the retained fraction (apoB100 bound) was demonstrated free of chylomicrons, the non-retained fraction (apoB48 unbound) had some apoB100 containing lipoproteins present. Minor contamination of the unbound fraction was also noted by *Cohn et al 1993* who also observed faint apoB100 bands often present in the apoB48 fraction. There may be two reasons for this contamination, either the immunoaffinity gel was saturated or there is apoB100 that fails to bind to the antibody. The latter is more likely to be the case, since part of the validation process for the immunoaffinity chromatography method involved preparation of a saturation curve. The small underestimation of apoB100 TRL lipid concentrations, if taken into account, would only increase the contribution of apoB100 TRL and enhance the significance of the present results. However to what extent does the presence of apoB100 TRL confound the apoB48 TRL data. Since apoB48 TRL triglyceride accounts for 79% and 83%, in normals and diabetics respectively, and apoB100 for 15% and 16%, in normals and diabetics respectively, of the increase in TRL ($d < 1.006$g/ml) triglyceride concentrations after a fat meal, and with the vast majority of apoB100 TRL retained in the immunoaffinity resin, the amount of triglyceride in apoB100 particles contaminating the 'apoB48' unbound fraction by apoB100 TRL is small. Therefore we cautiously label the unretained fraction 'apoB48 unbound' (chapters VI, VII). At present there is no method that we or others are aware of that can separate clearly apoB100 containing lipoproteins from apoB48 containing lipoproteins. The use of immunoaffinity chromatography to isolate VLDL allowed subsequent separation of VLDL subfractions, into large $VLDL_1$ and small $VLDL_2$ by cumulative flotation. This allowed a clearer insight into what was happening to VLDL metabolism postprandially. In both normal and diabetic subjects, $VLDL_1$ accounted for the behaviour of the total

VLDL TRL fraction with $VLDL_2$ remaining essentially unchanged during alimentary lipaemia. $VLDL_1$ can be viewed as the liver's chylomicron delivering FFAs as an energy source in the fasting state. Postprandially, the normal response to the increased availability of lipoprotein triglyceride in the form of chylomicrons is to reduce $VLDL_1$ release from the liver *(Malmstrom et al 1997 a, b)* and to suspend $VLDL_1$ lipolysis (chapter VI) *(Karpe et al 1993, Bjorkegren et al 1996)* thereby relieving postprandial competition between chylomicrons and VLDL via the same lipolytic pathway. Karpe demonstrated that the plasma concentration of large $VLDL_1$ *(Sf 60 - 400)* apoB100 increases after an oral fat load both in healthy subjects and in normo- and hypertriglyceridaemic patients with CHD *(Karpe et al 1993)*, and $VLDL_2$ levels remain unchanged. Bjorkegren also concluded from a recent study that chylomicrons (and their remnants) impede the normal lipolytic degradation of VLDL and it may be that the most likely explanation for the rise in $VLDL_1$ *(Sf 60 - 400)* is delayed lipolysis of the particle due to competition for the same lipolytic pathway involving LPL.

A number of observations can be made that add to current concepts of the regulation of triglyceride metabolism.

(1) The postprandial response of apoB48 lipoproteins in diabetics has not previously been studied, but it is clear from chapter VII that the triglyceride response curves were elevated and prolonged particularly in the latter stages of postprandial lipaemia indicating late clearance of chylomicrons and their remnants from the plasma. This also contributes to differences between normal and CHD patients *(Simpson et al 1990)* who develop exaggerated lipaemia when given a standard fat load test meal. Chylomicron remnants are removed from the circulation, by the liver via receptor-mediated endocytosis which is believed to involve both the LDL receptor (LDL-R) and the LDL receptor-related protein (LRP) (figure 8.1). Insulin resistance may impair this removal suggesting that insulin may have some control over the LRP.

(2) Also $VLDL_1$ lipolysis is virtually halted during the early part of alimentary lipaemia as seen in chapters VI and VII. $VLDL_1$ increases 2 fold in normal (and 1.6 fold in diabetic) subjects over the first 5 hours after ingestion of the fat load, at a time when insulin suppresses the release of $VLDL_1$ *(Malmstrom et al 1997 a)* in normal subjects. It is possible that the relationship between $VLDL_1$ area under the curve and the increase in insulin are both reflections of insulin resistance. A rise in insulin postprandially is a reflection principally of insulin resistance and an rise in $VLDL_1$ maybe due to a failure of insulin to act to suppress the release of lipoproteins from the liver explaining the correlation observed in normal subjects between rise in insulin and

$VLDL_1$ area under the curve. The lack of such a correlation in diabetics is a reflection that the group are inherently resistant to insulin action.

A delayed clearance of VLDL may be due to an altered apolipoprotein and lipid composition of VLDL particles (figure 8.1) thereby increasing their potential atherogenicity. *Bjorkegren et al (1997)* observed that large and small VLDL were enriched in cholesterol, apoE and CI, but depleted of apoCII. The apoCIII, triglyceride and phospholipid contents were essentially unchanged in the postprandial state. *Bjorkegren et al (1997)* also noted an early transient increase in the apoCIII content of large $VLDL_1$ particles which may be implicated in the accumulation of this lipoprotein species during alimentary lipaemia *(Karpe et al 1993)*. ApoCII is necessary for the activation of LPL thus VLDL are poorly lipolysed due to a lack of apoCII. Theoretically, the early apoCIII accumulation and the depletion of apoCII of large VLDL could explain part of the preferential lipolysis of chylomicrons and the ensuing postprandial accumulation of large VLDL observed both by *Bjorkegren et al (1996)* and the present study.

$VLDL_1$ metabolism gives rise to remnants in the $VLDL_2$ and IDL ranges *(Packard and Shepherd 1997)* and small dense LDL (figure 8.1). Prolonged chylomicronaemia causes an increased residence time of $VLDL_1$ in the circulation. CETP mediates the exchange of neutral lipid between lipoproteins with the result that when circulating levels of TRL (chylomicrons and VLDL) are high, a proportion of their core triglyceride is transferred to LDL (and HDL) and replaced with cholesteryl ester from the denser lipoprotein species (figure 8.1). Triglyceride-enriched LDL is then a substrate for endothelial-bound lipases and their action leads to the formation of smaller, lipid depleted particles. Inefficient clearance of chylomicrons may therefore lead to atherogenesis in an indirect way. That is if VLDL fails to be cleared efficiently from the circulation, it may once lipolysis restarts form a cascade of atherogenic lipoproteins, including not only small LDL but also long-lived remnants of VLDL and IDL, which are believed to contribute to increased risk of CHD. The results from *Rapp et al 1994* support the view that VLDL and VLDL remnants are potentially atherogenic and can indeed cross the endothelium and enter both human atherosclerotic plaques and the arterial wall.

FFA, although products of lipolysis, were not previously thought to have an important role in regulating lipolysis but now increased levels of FFA (released by the enzymatic hydrolysis of triglyceride) have been shown to release LPL from the endothelial sites (figure 8.1). This may lead to inefficient clearance of lipoproteins

(Saxena, Witte and Goldberg 1989, Olivecrona et al 1993, Coniglio 1994). As such it remains difficult to interpret the relationship between FFA and the clearance of lipoproteins. However, in diabetics there was a correlation between FFA area under the curve and apoB48 area under the curve, which was not observed in normal subjects. Therefore one reason for the accumulation of FFA in diabetics could be that diabetics are more sensitive to FFA accumulation as they possibly have less lipase activity as a result of their underlying insulin resistance.

2 Hormone-sensitive lipase.

Hormone-sensitive lipase is believed to be a key enzyme in hypertriglyceridaemia, diabetes, and coronary heart disease (CHD). It is a principal factor in insulin resistance, and in some subjects, the underlying defect may be an inability to shut down HSL activity. This causes an increase in FFA fasting levels which results in impaired glucose disposal and insulin resistance. The uncontrolled flow of FFA from adipose tissue to the liver is a major promoter of $VLDL_1$ production (figure 8.1). It was felt that when the relationship between chylomicron and $VLDL_1$ metabolism (liver-adipose axis) had been elucidated in human studies, that control of HSL would be a key element in altering triglyceride regulation to produce clinical benefit.

The general aim was to understand how control of HSL activity by drugs could affect the flux of triglyceride postprandially and change the liver-adipose tissue relationship in fatty acid metabolism.

Abnormally high rates of adipocyte lipolysis are thought not only to result in elevated plasma lipid levels linked to an increased risk of atherosclerosis and the development of CHD, but are closely linked to the metabolic disturbances characterised by insulin resistance. Thus the selective inhibition of HSL (figure 8.1) may contribute towards the therapeutic control of excessive adipose tissue lipolysis in various disease states. The antilipolytic mechanism of action of a series of compounds was investigated in isolated rat adipocytes *in vitro* (chapter VI), with *in vivo* dose response experiments using nicotinic acid carried out in Zucker fatty rats after initial experiments in Alderley Park (Wistar-derived) rats (chapter V). *In vitro*, in the isolated rat adipocytes, insulin was the most potent antilipolytic agent, nicotinic acid (and analogues) were found to be G_i receptor agonists (depending on adenosine removal to exert their antilipolytic effect), and compound 304205 was a direct inhibitor of HSL (figure 8.1). TZD, fibrates and a $ß_3$ agonist had no inhibitory effects on HSL activity in the isolated rat adipocyte.

In vivo, the antilipolytic effect of nicotinic acid on HSL mediated adipocyte lipolysis in the Zucker and Alderley Park rats suggests the decrease in plasma FFA levels observed is a result of nicotinic acid administration. It appears to be an immediate effect, with the Zucker rats appearing to be more sensitive to the effects of nicotinic acid than the Alderley Park rats.

These preliminary studies with nicotinic acid in the Zucker rat were carried out with the long term aim of defining the dose of nicotinic acid which would normalise insulin levels in the Zucker rat. This would be of great use, as Zucker rats have increased plasma FFA levels, are hyperinsulinaemic and develop marked hyperlipoproteinaemia probably as a result of over-production of VLDL. It was hoped that by defining a dose of nicotinic acid which would suppress plasma FFA levels *e.g.* for 24 hours or longer, that this would normalise plasma insulin levels and maybe even start to correct some of the defects associated with the hyperlipoproteinaemia observed in the Zucker rats. Administration *in vivo* of nicotinic acid to the Zucker rat inhibited HSL activity, and would modify the FFA flux to the liver.

3 Directions for future research.

A number of questions have arisen from the studies in this thesis, which could be the subject of future research.

Now that the methodology for separation of chylomicrons and VLDL subfractions from postprandial blood samples is established, it would be of interest to further investigate a larger population of NIDDM subjects; including both normo- and hypertriglyceridaemic diabetics. The study of more normotriglyceridaemic NIDDM subjects would hopefully confirm the findings of this thesis. The diabetic patient included in this thesis who had a fasting triglyceride greater than the normal reference range, thus classified as hypertriglyceridaemic, showed several differences in response to ingestion of a fat meal compared to the other diabetic patients; including a greater and prolonged elevation of triglyceride response curves particularly in the later hours of lipaemia suggesting a defect in triglyceride clearance.

It would be of interest to perform oral fat load tests in other patient groups who also suffer from disease states that display insulin resistance for example CHD and obese subjects to observe if their postprandial responses were similar to those of the NIDDM subjects.

postprandial studies in NIDDM subjects could be undertaken to allow apoB metabolism to be followed. The use of FFA tracers during a fat load may help clarify the sources of the FFA in normal and NIDDM subjects.

Further investigations are required in the Zucker fatty rat to examine the *in vivo* effects of other antilipolytic agents upon plasma FFA levels and their consequent influence on VLDL levels. This may lead to the identification of a drug with an identical mode of action as nicotinic acid but without the side effects.

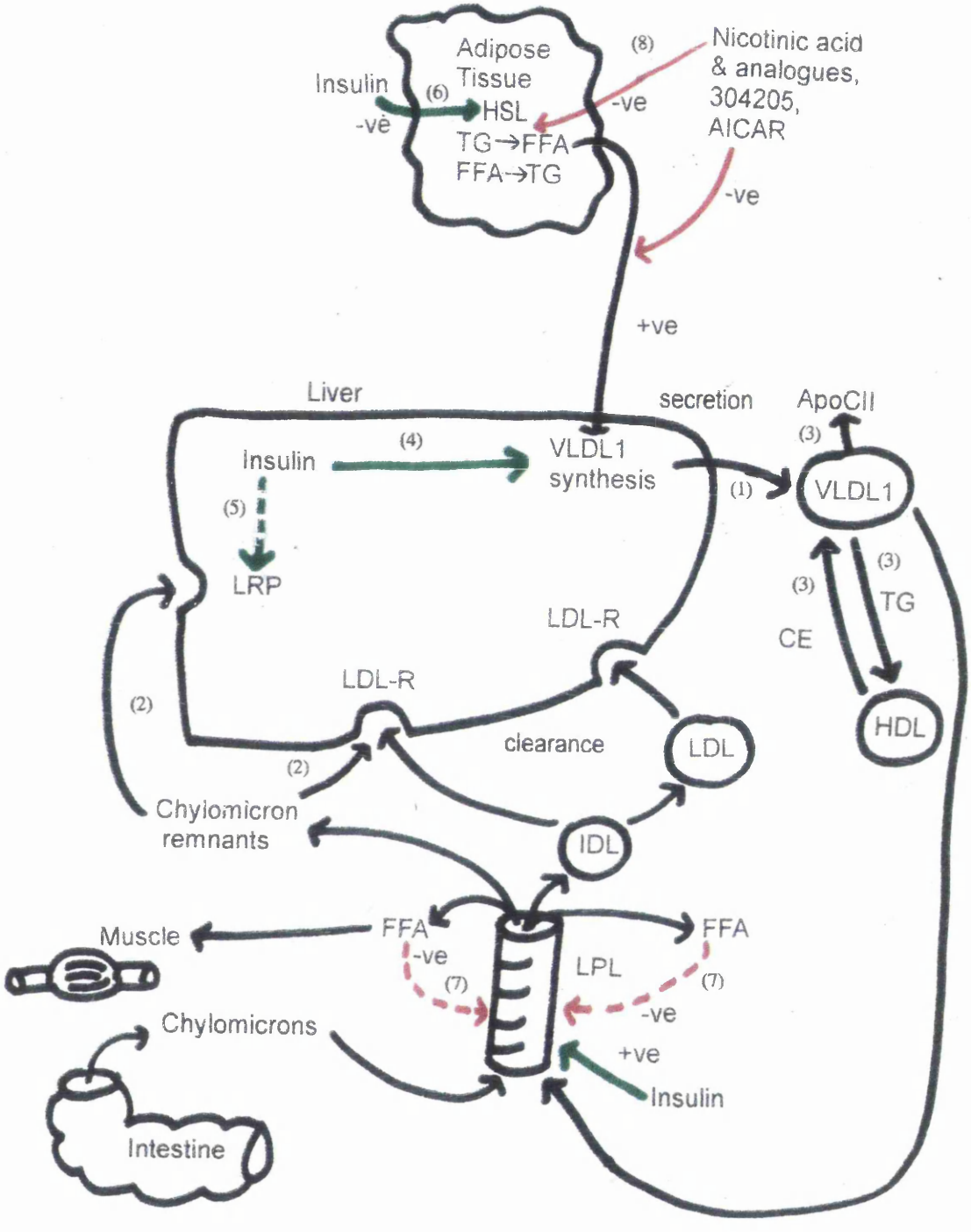
Further investigations in isolated primary rat adipocytes using radiolabelled triglyceride would help to determine if FFA esterification is occurring within the adipocytes.

4 Characterisation of Triglyceride-rich lipoproteins (TRLs)

Triglyceride-rich lipoproteins can be characterised by either their apolipoprotein content or their physiochemical properties; size, lipid content and charge. These properties are responsible for a variety of processes, determining rapidity of lipolysis, clearance by the liver, rate of delivery of FA and ability to attract cholesteryl ester.

Apolipoproteins have a variety of roles within lipoprotein metabolism (chapter 1). They are required for secretion of lipoproteins, stabilisation of the surface coat and hence maintain the structure of the whole lipoprotein transport system, lipoproteins cannot be synthesised and secreted from the liver or intestine without the correspnding structural apolipoprotein (apoB100, B48). The presence of apoB100 or apoB48 (in man) determines whether a lipoprotein particle was derived from the gut or intestine. Chylomicrons and VLDL have different sites of synthesis (intestine and liver respectively) and clearance. Chylomicrons are cleared by the LRP, through apoE, while VLDL is cleared by the both the LDL receptor, which recognises as ligands both apoB100 and apoE, and the VLDL receptor. Chylomicrons and VLDL have different metabolic pathways, but can overlap in size and other physiochemical properties. Postprandially there is a delayed clearance of VLDL by LPL and *Bjorkegren et al 1997* obsevered enrichment of apoE, CI and depletion of CII in VLDL, apoCII is necessary for activation of LPL resulting in accumulation of VLDL. Categorising TRLs by apolipoprotein content rather than physiochemical properties is therefore a better method to isolate metabolically derived TRL particles with the aim of studying their distinct roles in triglyceride metabolism.

Figure 8.1: ***Schematic representation of triglyceride metabolism.*** *AICAR = 5-amino-4-imidazole carboxamide, CE = cholesterol ester, FFA = free fatty acids, HSL = hormone-sensitive lipase, LPL = lipoprotein lipase, LRP = LDL receptor-related protein, LDL-R = LDL receptor, TG = triglyceride, 304205 = p-nitrophenylethylhexyl phosphonate. Inefficient clearance of lipoprotein and abnormally high rates of adipocyte lipolysis are thought to result in elevated plasma lipid levels, linked to an increased risk of atherosclerosis and CHD; these in turn are closely linked to metabolic disturbances characterised by insulin resistance. Postprandially the normal response to an increased availability of lipoprotein triglyceride in the form of chylomicrons is to reduce $VLDL_1$ release from the liver (1) and to suspend its lipolysis thereby relieving postprandial competition between chylomicrons and VLDL via the same lipolytic pathway. Chylomicron remnants are removed from the circulation by both LRP (2) and LDL-R (2). A delayed clearance of $VLDL_1$ may be due to an altered apolipoprotein and lipid composition of VLDL, in particular depletion of apoCII (3), and enrichment of cholesterol (3). Failure to clear VLDL efficiently from the circulation may lead to formation of a cascade of atherogenic lipoproteins. Insulin has a number of effects upon triglyceride metabolism, on (4) $VLDL_1$ synthesis, (5) LRP and (6) HSL action, these effects may not be seen in those subjects who are insulin resistant. FFA, products of lipolysis have been shown to release LPL from its endothelial binding sites (7). Increased flow of FFA from adipose tissue to the liver is a major promoter of $VLDL_1$ production. Selective inhibition of HSL (8) may contribute towards the therapeutic control of excessive adipose tissue lipolysis in various disease states.*


Adipose
Tissue
HSL
TG→FFA
FFA→TG
Insulin
(6)
-ve
(8)
Nicotinic acid
& analogues,
304205,
AICAR
-ve
-ve
+ve
Liver
secretion
ApoCII
(3)
Insulin
(4)
VLDL1
synthesis
(1)
VLDL1
(5)
LRP
(3)
TG
(3)
CE
LDL-R
LDL-R
(2)
clearance
LDL
HDL
(2)
Chylomicron
remnants
IDL
Muscle
FFA
-ve
(7)
LPL
FFA
(7)
-ve
Chylomicrons
+ve
Insulin
Intestine

Appendix 1.

Manufacturers and suppliers of reagents, hardware and software.

Amicon Inc.
Beverley, MA 01915,
USA.

Baker Instruments Ltd,
Rusham Park,
Whitehall Lane,
Egham,
Surrey,
TW20 9NW,
UK.

Beckman Instruments (UK) Ltd,
Analytical Sales and Service Operation,
Progress Road,
Sands Industrial Estate,
High Wycombe,
Bucks, HP12 4JL,
UK.

Becton Dickinson Labware,
2 Bridgewater Lane,
Lincoln Park,
New Jersey 07035,
(800) 235-5953
USA.

BDH Laboratory Supplies,
McQuilkin and Co,
21 Polmadie Avenue,
Glasgow,
G5 OBB,
UK.

Bio-Rad Laboratories,
2000 Alfred Nobel Drive,
Hercules,
CA 94547,
USA.

Boehringer Mannheim UK (diagnostics and biochemicals) Ltd,
Bell Lane,
Lewes,
East Sussex,
BN7 1LG,
UK.

Chromacol Ltd,
3 Little Mundells,
Welwyn Garden City,
Herts,
AL7 1EW,
UK.

Denley Tech Ltd,
Sussex,
England.

Dynatech,
Guernsey,
Great Britain.

Fisons Instruments,
Crewe Road,
Wythenshaw,
Manchester,
M23 9BE,
UK.

Hoefer Scientific Instruments,
654 Minnesota Street,
San Fransisco 94107,
USA.

Innogenetics NV,
Canadastraat 21 Haven 1009,
b-2070 Zwijndrecht,
Belgium.

Lennart Krabish,
Servicon AB,
Thomanders v.20
224 65 Lund,
Sweden.

Microcal Software Inc,
22 Industrial Drive,
East Northampton,
MA 01060,
USA.

Microsoft Corporation,
1 Microsoft Way,
Redmond, WA,
USA.

Minitab Inc.,
3081 Enterprise Drive,
State College,
PA 16801-3008.

Orion Diagnostica,
Espoo,
Finland.

Paar Scientific Ltd,
594 Kingston Road,
Raynes Park,
London,
SW20 8DN.

Pharmacia Biotech Ltd,
23 Grosvenor Road,
St Albans,
Herts,
AL1 3AW.

Scottish Antibody Production Unit (SAPU),
Law Hospital,
Carluke,
Lanarkshire,
ML8 5ES,
UK.

Sigma Chemical Company,
Fancy Road,
Poole,
Dorset,
BH17 7TG,
UK.

Techne,
Scotlab,
Kirkshaw Road,
Coatbridge,
Lanarkshire,
ML5 8AD.

Treff Lab.,
Treff AG,
CH 9113,
Switzerland.

Wako Chemicals GmbH,
Nissanstr. 2,
D-41468
Neuss,
Germany.

Whatmann International Ltd,
Whatmann Labsales Ltd,
St. Leonards Road, 20/20
Maidstone,
Kent,
ME16 OLS.

Worthington Biochemical Corporation,
UK.

Appendix 2.

TO MAKE DRUG SOLUTIONS IN DMSO

Weight accurately. approx. 3-5mg

eg. 5/mol. wt (in mg) = x mmoles of compound weighed.

Std solutions; Top std = 5mM
X mmoles/5 = volume of DMSO in mls to be added to give a 5mM solution.

tube nos/std	vol std(μl)	vol DMSO(μl)	CONC.(mM)	FINAL CONC.(M)
1	100 (5mM)	0	5	1×10^{-4}
2	100 of 1 (5mM)	200	1.66	3×10^{-5}
3	100 of 1 (5mM)	900	0.5	1×10^{-5}
4	100 of 2 (1.66mM)	900	0.166	3×10^{-6}
5	100 of 3 (0.5mM)	900	0.05	1×10^{-6}
6	100 of 4 (0.166mM)	900	0.0166	3×10^{-7}
7	100 of 5 (0.05mM)	900	0.005	1×10^{-7}
8	100 of 6 (0.0166mM)	900	0.0016	3×10^{-8}
9	100 of 7 0.005mM)	900	0.0005	1×10^{-8}
10	100 of 8 (0.00166nM)	900	0.000166	3×10^{-9}
11	100 of 9 (0.0005mM)	900	0.00005	1×10^{-9}
12	100 of 10 (0.000166mM)	900	0.000016	3×10^{-10}
13	100 of 11 (0.00005mM)	900	0.000005	1×10^{-10}
14	100 of 12 (0.0000166mM)	900	0.00000166	3×10^{-11}
15	100 of 13 (0.000005mM)	900	0.0000005	1×10^{-11}
16	100 of 14 (0.00000166mM)	900	0.000000166	3×10^{-12}

Appendix 3.

Standard nicotinic acid solutions for Zucker rat dose response experiments.

Nicotinic acid solutions:

Stock 200 mg/kg = 40 mg/ ml/ 200 gram rat.

Want 35 ml = 35 x 40 = **1400 mg/ 35 ml.**

100 mg/kg = 1 in 2 = 10 ml stock + 10 ml excipient.
50 mg/kg = 1 in 4 = 4 ml stock + 12 ml excipient.
25 mg/kg = 1 in 8 = 2 ml stock + 14 ml excipient.
10 mg/kg = 1 in 10 = 2 ml (100 mg/kg solution)+ 18 ml excipient.
5 mg/kg = 1 in 10 = 2 ml (50 mg/kg solution)+ 18 ml excipient.
2.5 mg/kg = 1 in 10 = 2 ml (25 mg/kg solution)+ 18 ml excipient.
1.25 mg/kg = 1 in 8 = 2 ml (10 mg/kg solution)+ 14 ml excipient.

Appendix 4.

Body weights (in grams) of Zucker rats used for nicotinic acid dose response experiment 1.

Control	10 mg/kg	25 mg/kg	50 mg/kg	100 mg/kg	200 mg/kg
624	634	558	600	652	625
606	658	626	626	616	656
626	565	592	720	565	540
565	600	574	580	552	560
620	610	527	565	506	634
650	628	582	608	498	570

Appendix 5.

Body weights (in grams) of Zucker rats used for nicotinic acid dose response experiment 2.

Control	1.25 mg/kg	2.5 mg/kg	5 mg/kg	10 mg/kg	25 mg/kg
568	448	484	525	600	546
578	486	580	586	590	562
528	462	538	546	620	532
510	498	530	574	608	558
565	484	480	552	610	560
628	527	508	554	570	540

Appendix 6.

Body weights (in grams) of Zucker rats used in nicotinic acid multiple dosing experiment.

Controls	**Nicotinic acid (140 mg/kg) 12 post dose**	**Nicotinic acid (140 mg/kg) 2 post dose**
670	700	550
630	670	580
580	560	555
630	570	630
660	540	640
650	560	640

Appendix 7.

Reference ranges for fasting plasma lipid, lipoprotein and insulin concentrations.

Parameter	Reference Range
Plasma triglyceride	< 2.3 mmol/L
Plasma total cholesterol	<6.5 mmol/L
LDL cholesterol	<4.5 mmol/L
HDL cholesterol	>1.0 mmol/L in males >1.2 mmol/L in females
Plasma insulin	<13 mU/L

Appendix 8.

FAT FEEDING PROTOCOL

Patient should fast for 10 hours before start of experiment. Patient fed FAT MEAL after blood sample(0 hour) is removed. Patient not allowed to eat during the duration of the experiment but can drink low calorie drinks. After fat meal patient must fast for a further twelve hours, the duration of the experiment.

BLEEDING SCHEDULE

TIME (hours)	BLOOD VOLUME (mls)	ANALYSES	PLASMA VOLUME (mls)
0	4.5ml Lithium Heparin	INSULIN	
	30ml Na_2EDTA	B Quant	5ml
		Apo E phenotype	0.5ml
		Apo B, Apo AI	0.5ml
		FFA	0.5ml
		TG/CHOL	0.5ml
		sequential preparation of lipoprotein	4ml
1	4.5ml Lithium Heparin	INSULIN	
	20ml Na_2EDTA	FFA	0.5ml
		TG/CHOL	0.5ml
		Apo B,AI	0.5ml
		sequential preparation of lipoproteins	4ml
2	4.5ml Lithium Heparin	INSULIN	
	20ml Na_2EDTA	FFA	0.5ml
		TG/CHOL	0.5ml
		Apo B,AI	0.5ml
		sequential preparation of lipoproteins	4ml
3	20ml Na_2EDTA	FFA	0.5ml
		TG/CHOL	0.5ml
		Apo B,AI	0.5ml
		sequential preparation of lipoproteins	4ml

4	20ml Na_2EDTA	FFA	0.5ml
		TG/CHOL	0.5ml
		ApoB,AI	0.5ml
		sequential preparation of lipoproteins	4ml
5	20ml Na_2EDTA	FFA	0.5ml
		TG/CHOL	0.5ml
		PROTEIN	
		Apo B,AI	0.5ml
		sequential preparation of lipoproteins	4ml
6	20ml Na_2EDTA	FFA	0.5ml
		TG/CHOL	0.5ml
		Apo B,AI	0.5ml
		sequential preparation of lipoproteins	4ml
7	20ml Na_2EDTA	FFA	0.5ml
		TG/CHOL	0.5ml
		Apo B,AI	0.5ml
		sequential preparation of lipoproteins	4ml
8	20 ml Na_2EDTA	FFA	0.5ml
		TG/CHOL	0.5ml
		Apo B,AI	0.5ml
		sequential preparation of lipoproteins	4ml
9	20ml Na_2EDTA	FFA	0.5ml
		TG/CHOL	0.5ml
		Apo B,AI	0.5ml
		sequential preparation of lipoproteins	4ml
10	20ml Na_2EDTA	FFA	0.5ml
		TG/CHOL	0.5ml
		Apo B,AI	0.5ml
		sequential preparation of lipoproteins	4ml
11	20ml Na_2EDTA	FFA	0.5ml
		TG/CHOL	0.5ml
		ApoB,AI	0.5ml
		sequential preparation of lipoproteins	4ml

Appendix 9.

Postprandial changes in individual plasma triglyceride concentrations (mmol/L) in normal subjects.

Time (hours)	BC	MM	BJ	KE	HA	CN	JM	IC	AG
0	1.18	0.98	0.85	1.15	0.95	1.42	0.53	0.85	2.11
1	1.37	1.07	1.13	1.34	1.05	1.32	0.70	0.92	2.47
2	1.82	1.38	2.14	1.53	1.50	2.71	0.99	1.68	3.77
3	3.28	2.16	3.08	2.09	1.14	3.54	0.97	2.11	4.62
4	4.22	2.44	3.51	2.46	1.75	1.66	1.54	1.78	5.37
5	4.42	2.77	2.94	2.91	1.58	1.11	2.01	2.50	5.33
6	4.95	2.32	2.55	2.37	1.36	0.76	1.88	2.77	6.04
7	4.20	2.18	2.56	3.41	1.14	0.67	1.61	1.72	4.34
8	3.65	1.42	1.45	3.01	0.89	0.59	1.91	1.24	3.34
9	3.98	1.18	1.05	2.78	0.85	0.67	1.79	1.73	2.00
10	3.18	0.86	1.20	1.49	0.81	0.72	1.47	1.19	1.57
11	2.11	0.75	0.81	1.20	0.63	0.71	1.20	0.88	1.17

Appendix 10.

Postprandial changes in individual triglyceride concentrations (mmol/L) in the TRL (d < 1.006g/ml) fraction isolated by sequential flotation ultracentrifugation in normal subjects.

Time (hours)	BC	MM	BJ	KE	HA	CN	JM	IC	AG
0	0.68	0.53	0.45	0.6	0.46	0.89	0.17	0.30	1.37
1	0.85	0.58	0.62	0.75	0.55	0.84	0.37	0.35	1.79
2	1.23	0.89	1.15	0.89	0.92	1.84	0.62	0.92	2.43
3	2.36	1.50	1.65	1.28	0.60	2.46	0.57	1.07	3.13
4	0.27	1.70	1.88	1.61	1.09	1.09	0.83	1.02	3.24
5	3.19	1.86	1.70	1.83	0.82	0.63	1.42	1.83	3.64
6	3.77	1.56	1.31	1.36	0.72	0.33	1.42	1.76	4.35
7	2.97	1.46	1.47	2.29	0.54	0.22	1.01	0.79	2.92
8	2.695	0.93	0.77	2.00	0.38	0.18	1.39	0.45	2.07
9	2.75	0.67	0.50	1.79	0.31	0.26	0.19	0.74	1.19
10	2.14	0.38	0.54	0.81	0.30	0.33	0.95	0.42	0.93
11	1.27	0.31	0.35	0.61	0.19	0.31	0.74	0.28	0.64

Appendix 11.

Postprandial changes in individual triglyceride concentrations (mmol/L) in the apoB48 TRL fraction isolated by immunoaffinity chromatography, using a Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes, from the TRL ($d < 1.006$g/ml) fraction in normal subjects.

Time (hours)	BC	MM	BJ	KE	HA	CN	JM	IC	AG
0	0.27	0.23	0.05	0.22	0.17	0.30	0.03	0.13	0.52
1	0.35	0.27	0.22	0.37	0.25	0.20	0.17	0.15	0.9
2	0.70	0.47	0.45	0.45	0.50	0.95	0.35	0.57	1.35
3	1.60	1.00	0.90	0.65	0.25	1.50	0.28	0.72	1.95
4	2.03	1.15	1.10	0.85	0.67	0.57	0.50	0.62	1.95
5	2.73	1.35	0.95	0.95	0.45	0.27	1.05	1.42	2.47
6	2.85	1.00	0.60	0.60	0.42	0.15	0.90	1.25	3.00
7	2.20	0.87	0.85	1.10	0.32	0.07	0.60	0.55	1.57
8	1.93	0.55	0.42	1.15	0.2	0.10	0.95	0.27	0.97
9	2.15	0.40	0.20	0.95	0.18	0.10	0.78	0.5	0.45
10	1.55	0.20	0.15	0.37	0.15	0.15	0.62	0.27	0.35
11	0.66	0.20	0.07	0.30	0.10	0.13	0.45	0.15	0.25

Appendix 12.

Postprandial changes in individual triglyceride concentrations (mmol/L) in the apoB100 TRL fraction isolated by immunoaffinity chromatography, using a Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes, from the TRL ($d < 1.006$g/ml) fraction in normal subjects.

Time (hours)	BC	MM	BJ	KE	HA	CN	JM	IC	AG
0	0.33	0.36	0.24	0.36	0.27	0.54	0.12	0.21	0.81
1	0.30	0.36	0.27	0.36	0.27	0.48	0.18	0.18	0.99
2	0.45	0.45	0.30	0.42	0.39	0.66	0.24	0.30	0.99
3	0.48	0.45	0.30	0.60	0.30	0.72	0.24	0.33	1.26
4	0.54	0.57	0.24	0.72	0.33	0.39	0.24	0.33	1.17
5	0.69	0.57	0.30	0.84	0.30	0.33	0.30	0.33	1.35
6	0.60	0.45	0.30	0.66	0.21	0.15	0.30	0.33	1.44
7	0.51	0.39	0.42	0.90	0.15	0.09	0.27	0.24	1.26
8	0.48	0.36	0.24	0.78	0.12	0.09	0.24	0.18	0.99
9	0.45	0.27	0.12	0.78	0.09	0.12	0.24	0.18	0.72
10	0.48	0.15	0.15	0.36	0.09	0.15	0.21	0.12	0.54
11	0.42	0.18	0.12	0.30	0.09	0.15	0.18	0.12	0.42

Appendix 13.

Postprandial changes in individual triglyceride concentrations (mmol/L) in the large $VLDL_1$ subfraction after isolation by immunoaffinity chromatography, using a Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes, followed by cumulative flotation in normal subjects.

Time (hours)	BC	MM	BJ	KE	HA	CN	JM	IC	AG
0	0.22	0.24	0.20	0.16	0.12	0.39	0.02	0.10	0.54
1	0.19	0.22	0.21	0.16	0.13	0.36	0.04	0.10	0.63
2	0.28	0.28	0.33	0.22	0.18	0.48	0.09	0.18	0.76
3	0.28	0.31	0.33	0.33	0.13	0.54	0.09	0.19	0.90
4	0.37	0.39	0.33	0.45	0.16	0.28	0.10	0.18	0.81
5	0.39	0.37	0.39	0.51	0.12	0.16	0.15	0.18	1.03
6	0.33	0.36	0.30	0.42	0.07	0.07	0.18	0.16	1.13
7	0.31	0.28	0.36	0.54	0.07	0.06	0.16	0.07	0.94
8	0.30	0.25	-	0.48	0.06	0.06	0.16	0.06	0.67
9	0.30	0.18	0.12	0.45	0.04	0.07	0.12	0.09	0.49
10	0.25	0.10	0.14	0.16	0.04	0.12	0.12	0.04	0.27
11	0.15	0.15	0.12	0.12	0.04	0.10	0.09	0.04	0.18

Appendix 14.

Postprandial changes in individual triglyceride concentrations (mmol/L) in the small $VLDL_2$ subfraction after isolation by immunoaffinity chromatography, using a Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes, followed by cumulative flotation in normal subjects.

Time (hours)	BC	MM	BJ	KE	HA	CN	JM	IC	AG
0	0.037	0.105	0.083	0.09	0.09	0.105	0.045	0.0375	0.09
1	0.037	0.112	0.038	0.09	0.075	0.09	0.045	0.0375	0.09
2	0.045	0.112	0.105	0.097	0.0675	0.105	0.0675	0.0375	0.0675
3	0.045	0.12	0.09	0.09	0.0825	0.09	0.0675	0.045	0.0675
4	0.045	0.105	0.09	0.09	0.0675	0.0825	0.06	0.03	0.0675
5	0.03	0.105	0.105	0.09	0.0525	0.0675	0.06	0.0375	0.045
6	0.037	0.097	0.105	0.105	0.06	0.0525	0.045	0.045	0.0675
7	0.053	0.105	0.135	0.09	0.0375	0.03	0.06	0.045	0.09
8	0.053	0.105	-	0.105	0.03	0.0225	0.0525	0.0375	0.1125
9	0.045	0.105	0.067	0.12	0.0225	0.015	0.0525	0.0225	0.1125
10	0.06	0.09	0.06	0.09	0.015	0.03	0.06	0.015	0.12
11	0.06	0.045	0.052	0.083	0.015	0.03	0.045	0.0075	0.105

Appendix 15.

Postprandial changes in individual plasma FFA concentrations (mmol/L) in normal subjects.

Time (hours)	BC	MM	BJ	KE	HA	CN	JM	IC	AG
0	0.183	0.25	0.16	0.41	0.27	0.75	0.36	0.49	0.04
1	0.174	0.31	0.13	0.44	0.39	0.37	0.15	0.34	0.06
2	0.155	0.33	0.35	0.53	0.39	0.57	0.26	0.59	0.15
3	0.466	0.61	0.64	0.72	0.62	0.92	0.28	0.63	0.31
4	0.64	0.72	0.59	0.68	0.68	0.72	0.39	0.92	0.47
5	0.615	0.81	0.77	0.98	0.94	0.82	0.52	0.73	0.66
6	0.83	0.90	0.95	0.77	0.93	0.74	0.72	1.34	0.83
7	0.908	1.09	0.73	1.42	0.77	0.92	0.57	0.91	0.62
8	0.828	0.88	0.53	1.45	1.00	1.05	0.71	0.71	0.61
9	0.892	0.68	0.53	1.77	0.85	1.15	0.61	0.91	0.33
10	0.807	0.56	0.55	1.11	1.10	0.98	0.53	0.63	0.24
11	0.509	0.67	0.47	1.29	0.96	1.27	0.43	0.61	0.20

Appendix 16.

Postprandial changes in individual plasma apoB concentrations (g/L) in normal subjects.

Time (hours)	BC	MM	BJ	KE	HA	CN	JM	IC	AG
0	0.65	0.77	1.03	0.74	0.76	0.89	0.72	0.78	1.21
1	0.65	0.8	1.11	0.77	0.79	0.9	0.73	0.76	1.21
2	0.73	0.83	1.03	0.84	0.84	0.95	0.75	0.75	1.25
3	0.82	0.79	1.08	0.83	0.89	0.96	0.73	0.76	1.3
4	0.78	0.82	1.08	0.87	0.88	0.91	0.77	0.81	1.37
5	0.79	0.88	1.27	0.86	0.88	0.8	0.75	0.83	1.26
6	0.78	0.85	0.99	0.89	0.78	0.84	0.72	0.75	1.32
7	0.86	0.87	1.07	0.92	0.78	0.83	0.71	0.68	1.31
8	0.81	0.79	0.98	0.9	0.76	0.81	0.72	0.69	1.18
9	0.84	0.75	0.93	0.88	0.81	0.7	0.67	0.73	1.08
10	0.85	0.78	0.94	0.75	0.75	0.85	0.69	0.65	1.11
11	0.82	0.75	0.94	0.73	0.78	0.8	0.71	0.65	0.95

Appendix 17.

Postprandial changes in individual plasma apoAI concentrations (g/L) in normal subjects.

Time (hours)	BC	MM	BJ	KE	HA	CN	JM	IC	AG
0	0.83	1.29	1.39	1.65	1.25	1.52	1.02	1.62	1.29
1	0.86	1.25	1.45	1.73	1.34	1.38	1.08	1.49	1.35
2	0.88	1.19	1.31	1.77	1.36	1.52	1.1	1.47	1.31
3	0.93	1.18	1.3	1.79	1.41	1.45	1.12	1.42	1.31
4	0.92	1.3	1.24	1.77	1.39	1.34	1.21	1.41	1.37
5	0.96	1.25	1.56	1.81	1.48	1.3	1.16	1.36	1.29
6	0.9	1.24	1.28	1.83	1.29	1.31	1.14	1.35	1.31
7	1.01	1.32	1.36	1.88	1.32	1.38	1.18	1.32	1.35
8	1.02	1.33	1.34	1.76	1.22	1.32	1.15	1.34	1.28
9	1.02	1.29	1.31	1.69	1.32	1.33	1.16	1.43	1.25
10	0.99	1.24	1.36	1.59	1.22	1.39	1.12	1.43	1.29
11	0.95	1.17	1.37	1.49	1.26	1.44	1.07	1.41	1.2

Appendix 18.

Postprandial changes in individual TRL (d < 1.006 g/ml) apoB concentrations (g/L) in normal subjects.

Time (hours)	BC	MM	BJ	KE	HA	CN	JM	IC	AG
0	0.25	0.045	0.025	0.05	0.075	0.06	0.045	0.04	0.125
1	0.225	0.055	0.025	0.06	0.075	0.05	0.045	0.05	0.165
2	0.045	0.055	0.045	0.075	0.08	0.16	0.035	0.05	0.185
3	0.09	0.01	0.095	0.06	0.06	0.20	0.035	0.065	0.26
4	0.105	0.11	0.12	0.15	0.07	0.07	0.05	0.05	0.255
5	0.12	0.185	0.11	0.16	0.055	0.035	0.065	0.055	0.29
6	0.18	0.105	0.065	0.145	0.05	0.01	0.055	0.055	0.35
7	0.13	0.13	0.08	0.215	0.04	0.02	0.055	0.045	0.175
8	0.13	0.075	0.03	0.17	0.03	0.005	0.05	0.05	0.2
9	0.13	0.05	0.015	0.175	0.03	0.01	0.055	0.045	0.13
10	0.11	0.025	0.005	0.1	0.03	0.015	0.05	0.06	0.06
11	0.068	0.02	0.01	0.055	0.03	0.005	0.045	0.055	0.085

Appendix 19.

Postprandial changes in individual TRL (d < 1.006g/ml) apoAI concentrations (g/L) in normal subjects.

Time (hours)	BC	MM	BJ	KE	HA	CN	JM	IC	AG
0	0.1	0.02	0.01	0.02	0.02	0.05	0.035	0.01	0
1	0.05	0.02	0	0.015	0.02	0.025	0.03	0.01	0
2	0.1	0.025	0.015	0.015	0.02	0.02	0.03	0.005	0
3	0.025	0.025	0.015	0.015	0.025	0.02	0.025	0.01	0
4	0.03	0.025	0.025	0.015	0.025	0.01	0.045	0.005	0
5	0.04	0.03	0.015	0.02	0.015	0.05	0.045	0.005	0
6	0.06	0.005	0.015	0.015	0.02	0.005	0.045	0.025	0
7	0.04	0.025	0.015	0.015	0.025	0.005	0.04	0.005	0
8	0.035	0.025	0.02	0.025	0.02	0.035	0.05	0.002	0
9	0.035	0.025	0.02	0.015	0.015	0.03	0.045	0.002	0
10	0.025	0	0.015	0.005	0.02	0.03	0.04	0.002	0
11	0.015	0.02	0.01	0.005	0.02	0.025	0.05	0.002	0

Appendix 20.

Postprandial changes in individual plasma cholesterol concentrations (mmol/L) in normal subjects.

Time (hours)	BC	MM	BJ	KE	HA	CN	JM	IC	AG
0	3.7	5.55	5.89	4.75	4.88	4.71	3.89	4.77	5.44
1	3.79	5.69	6.1	5.15	4.93	4.6	4.11	4.95	5.67
2	3.83	5.32	5.66	5.11	5.22	4.63	4.11	4.85	5.56
3	3.94	5.26	5.57	5.01	5.21	4.91	4.18	4.86	5.67
4	3.91	5.47	5.41	5.06	5.38	4.82	4.32	4.81	5.84
5	4.41	5.38	5.66	5.04	5.38	4.73	4.14	4.8	5.51
6	4.08	5.26	5.26	5.1	5.37	4.64	4.16	4.65	5.91
7	4.18	5.61	5.61	5.14	5.36	4.77	4.17	4.64	5.93
8	4.25	5.25	5.52	4.95	5.03	4.7	4.18	4.77	5.8
9	4.29	5.43	5.23	4.81	5.48	4.55	4.15	4.68	5.5
10	4.37	5.36	5.47	4.78	5.12	4.7	4.24	4.64	5.43
11	4.17	5.45	5.51	4.51	5.32	4.7	4.19	4.58	5.35

Appendix 21.

Dear Patient,

We are looking for someone to help us with a study to see how people with Diabetes handle fat from their diet.

As you know, Diabetics have trouble controlling blood sugar levels. It is also true that they may have difficulties in clearing from the bloodstream, the fat that they eat. We are wanting to find out if people with Diabetes are able to keep their blood fat levels under control when given a standard fat load test.

We are inviting people who attend the Diabetic Centre to volunteer to receive a creamy drink in the morning, after fasting overnight. After giving you the drink, we can follow the appearance of fat in the blood by taking regular samples over a period of eleven hours. Special techniques that we use in the laboratory enable us to separate the fat that comes from the creamy drink from other fats in your bloodstream that the liver makes. We are testing the possibility that people with Diabetes have difficulties in keeping the levels of both kinds of fat (from the diet and liver) in good control.

It means a day in hospital in our quiet, comfortable clinical suite. If you agree to be involved you can bring your favourite videos and books, or read some of ours. We would be delighted to talk through study details with you either today or at a future date.

The people mentioned below are involved in this effort and can be contacted at the telephone numbers given below.

Dr Neil Bennit
Lynne Crawford 0141 211 4589

Appendix 22.

Patient Information and Consent Form

Title: THE REGULATION OF INTESTINAL AND HEPATIC LIPOPROTEIN SYNTHESIS IN NORMAL, CORONARY HEART DISEASE AND DIABETIC PATIENTS AFTER A FAT MEAL.

I understand that Professor Shepherd and his team are conducting studies to discover how blood fat levels are controlled. These studies involve fasting for about 12 hours before being given a test breakfast made up mainly of cream with flavourings. After the breakfast I understand that I will not be able to eat until about 4.00 p.m. in the afternoon when I will receive a light fat-free meal.

Blood samples will be taken over the day of the study with a total of about 200mls of blood being donated.

The object of the study is to investigate how my body handles fat in the diet and to measure the rate of appearance and disappearance of fat in the blood. The fat test meal is able to show differences between people who have normal blood fat levels and those who have coronary heart disease or who have diabetes. The period of fasting is necessary so that the liver does not produce fat and confuse the results from the test breakfast.

In some cases I will be given a small amount of substances that are used by the body to make lipoproteins (the particles that carry fat in the blood) these substances will either be included in the test meal or be given by injection just prior to eating the breakfast. I appreciate that some mild discomfort may be associated with the injection and the taking of blood samples.

Taking part in the study will be of little or no direct benefit to myself but will help other patients in the future. If I wish to stop taking part I may do so at any time and this will not affect my present or future medical treatment. My General Practitioner will be told of my participation and will be given details of my results.

CONSENT:

I,(Name)..... of (Address) .....

.....

agree to take part in the Research Project/Study Programme described above.

Dr/Mr ..... has explained to me what I have to do, how it might affect me and the purpose of the Research Project/Study Programme.

Signed ..... Date .....

Witness ..... Date .....

Appendix 23.

Postprandial changes in individual plasma triglyceride concentrations (mmol/L) in diabetic subjects.

Time (hours)	JC	SJ	PF	CE	MH
0	3.37	0.69	2.59	2.76	1.24
1	3.57	0.91	2.74	2.57	1.37
2	4.36	1.17	3.07	3.05	2.22
3	5.31	1.25	3.47	3.81	2.69
4	7.38	1.79	4.01	4.21	2.78
5	9.11	1.94	4.42	4.40	3.36
6	10.23	2.31	4.52	4.52	2.75
7	9.45	-	4.46	4.14	2.71
8	9.66	2.20	3.96	3.41	2.21
9	8.37	2.33	4.41	2.76	2.09
10	10.89	2.64	4.62	2.25	1.99
11	14.13	1.36	4.35	1.82	1.62

Appendix 24.

Postprandial changes in individual triglyceride concentrations (mmol/L) in the TRL ($d < 1.006$g/ml) fraction isolated from plasma by sequential flotation ultracentrifugation in diabetic subjects.

Time	JC	SJ	PF	CE	MH
0	2.01	0.31	1.825	1.78	0.67
1	2.33	0.37	2.075	1.58	0.83
2	3.155	0.73	2.07	2.03	1.345
3	3.63	0.74	2.46	2.40	1.665
4	4.92	1.11	2.825	2.89	1.82
5	6.22	1.24	3.13	3.185	2.082
6	7.56	1.66	3.405	2.94	1.176
7	6.45	1.855	3.07	3.09	1.85
8	9.06	1.47	2.745	2.24	1.185
9	9.06	1.23	3.35	1.65	1.26
10	9.525	1.36	3.31	1.335	1.145
11	8.26	0.66	2.955	0.95	0.95

Appendix 25.

Postprandial changes in individual triglyceride concentrations (mmol/L) in the apoB48 TRL fraction isolated by immunoaffinity chromatography, using a Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes, from the TRL ($d < 1.006$g/ml) fraction in diabetic subjects.

Time (hours)	JC	SJ	PF	CE	MH
0	0.625	0.10	0.5	0.65	0.15
1	0.875	0.125	0.7	0.6	0.3
2	1.5	0.375	0.7	0.9	0.65
3	1.875	0.35	1.1	1.2	0.9
4	3.00	0.55	1.4	1.8	1.00
5	3.75	0.675	1.6	1.85	1.35
6	4.75	1.025	1.8	1.65	1.05
7	4.25	1.175	1.6	1.7	1.1
8	6.5	0.9	1.4	1.05	0.65
9	6.375	0.775	1.8	0.65	0.7
10	5.875	0.75	1.6	0.45	0.6
11	6.00	0.40	1.5	0.25	0.5

Appendix 26.

Postprandial changes in individual triglyceride concentrations (mmol/L) in the apoB100 TRL fraction isolated by immunoaffinity chromatography, using a Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes, from the TRL ($d < 1.006$g/ml) fraction in diabetic subjects.

Time (hours)	JC	SJ	PF	CE	MH
0	1.2	0.15	0.96	0.90	0.36
1	1.35	0.18	0.96	0.84	0.42
2	1.5	0.27	1.08	0.96	0.48
3	1.5	0.30	1.2	1.02	0.6
4	1.65	0.33	1.2	1.2	0.54
5	1.95	0.39	1.32	1.2	0.6
6	2.1	0.42	1.2	1.08	0.54
7	1.8	0.42	1.32	1.2	0.54
8	1.95	0.33	1.2	0.96	0.30
9	2.4	0.27	1.32	0.78	0.36
10	2.4	0.24	1.32	0.66	0.30
11	2.1	0.15	1.08	0.54	0.30

Appendix 27.

Postprandial changes in individual triglyceride concentrations (mmol/L) in the large $VLDL_1$ subfraction isolated by immunoaffinity chromatography, using a Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes, followed by cumulative flotation in diabetic subjects.

Time (hours)	JC	SJ	PF	CE	MH
0	0.6	0.075	0.66	0.60	0.21
1	0.675	0.09	0.66	0.57	0.15
2	0.9	0.165	0.72	0.66	0.33
3	0.9	0.165	0.78	0.69	0.42
4	1.2	0.21	0.84	1.02	0.36
5	1.35	0.225	0.96	0.90	0.36
6	1.575	0.27	0.90	0.75	0.3
7	1.275	0.255	0.96	0.69	0.3
8	1.425	0.18	0.90	0.63	0.15
9	1.65	0.135	0.84	0.48	0.18
10	1.65	0.12	0.72	0.27	0.15
11	1.575	0.045	0.78	0.36	0.15

Appendix 28.

Postprandial changes in individual triglyceride concentrations (mmol/L) in the small $VLDL_2$ subfraction isolated by immunoaffinity chromatography, using a Sepharose bound LDL A4 monoclonal antibody in Eppendorf tubes, followed by cumulative flotation in diabetic subjects.

Time (hours)	JC	SJ	PF	CE	MH
0	0.18	0.03	0.12	0.09	0.12
1	0.037	0.03	0.15	0.12	0.195
2	0.23	0.04	0.09	0.14	0.18
3	0.23	0.05	0.12	0.12	0.18
4	0.038	-	0.03	0.12	0.075
5	0.038	0.053	0.09	0.12	0.075
6	0.15	0.068	0.09	0.12	0.09
7	0.11	0.068	0.12	0.21	0.09
8	0.11	0.060	0.12	0.12	0.09
9	0.15	0.053	0.15	0.15	0.06
10	0.11	0.053	0.09	0.14	0.05
11	0.15	0.037	0.09	0.12	0.03

Appendix 29.

Postprandial changes in individual plasma FFA concentrations (mmol/L) in diabetic subjects.

Time (hours)	JC	SJ	PF	CE	MH
0	0.622	0.907	0.423	0.477	0.65
1	0.49	0.41	0.534	0.512	0.678
2	0.557	0.258	0.492	0.589	0.55
3	0.667	0.279	0.496	0.677	0.574
4	0.903	0.55	0.664	0.803	0.444
5	1.167	0.709	0.740	0.912	0.581
6	1.464	0.857	0.542	0.757	0.633
7	1.809	0.955	0.741	0.872	0.79
8	1.858	1.071	0.622	0.654	0.624
9	1.96	1.132	0.793	0.724	0.420
10	1.961	1.447	1.08	0.609	0.367
11	1.962	0.869	0.865	0.244	0.163

Appendix 30.

Postprandial changes in individual plasma cholesterol concentrations (mmol/L) in diabetic subjects after an oral fat load.

Time (hours)	JC	SJ	PF	CE	MH
0	5.01	5.25	6.52	5.37	5.25
1	5.07	6.56	5.35	5.16	5.2
2	5.01	5.12	6.69	4.91	5.13
3	4.91	5.19	6.17	4.94	5.13
4	5.15	5.19	6.61	5.08	5.02
5	5.29	5.26	6.64	5.12	4.98
6	5.41	5.30	6.4	5.11	5.00
7	5.35	2.7	6.49	5.16	5.26
8	5.51	5.19	6.54	5.4	5.19
9	5.53	5.38	6.67	5.37	5.34
10	5.5	5.18	6.65	4.89	4.93
11	5.5	5.1	6.53	4.83	4.81

Appendix 31.

Postprandial changes in individual plasma apoB concentrations (g/L) in diabetic subjects.

Time (hours)	JC	SJ	PF	CE	MH
0	1.22	0.8	1.34	1.38	0.99
1	1.31	0.8	1.5	1.32	0.98
2	1.35	0.81	1.46	1.31	0.98
3	1.31	0.83	1.32	1.31	1.01
4	1.4	0.84	1.49	1.33	0.94
5	1.4	0.83	1.54	1.34	1.05
6	1.35	0.81	1.5	1.31	0.92
7	1.23	0.78	1.57	1.26	0.88
8	1.19	0.8	1.46	1.31	0.89
9	1.2	0.81	1.36	1.21	0.97
10	1.24	0.75	1.39	1.12	0.88
11	1.39	0.76	1.17	1.06	0.87

Appendix 32.

Postprandial changes in individual plasma apoAI concentrations (g/L) in diabetic subjects.

Time (hours)	JC	SJ	PF	CE	MH
0	1.1	1.41	1.24	1.01	1.41
1	1.25	1.43	1.29	0.9	1.36
2	1.14	1.39	1.29	0.88	1.37
3	1.18	1.38	1.14	0.92	1.35
4	1.31	1.4	1.22	0.92	1.29
5	1.27	1.34	1.23	0.97	1.4
6	1.29	1.21	1.21	0.93	1.27
7	1.2	1.25	1.22	0.99	1.32
8	1.16	1.29	1.27	0.95	1.31
9	1.19	1.27	1.29	0.91	1.46
10	1.15	1.22	1.27	0.83	1.33
11	1.12	1.24	1.3	0.83	1.25

Appendix 33.

Postprandial changes in individual TRL (d < 1.006g/ml) apoB concentrations (g/L) in diabetic subjects.

Time (hours)	JC	SJ	PF	CE	MH
0	0.25	0.02	0.19	0.09	0.055
1	0.29	0.02	0.21	0.22	0.045
2	0.355	0.02	0.195	0.26	0.09
3	0.345	0.025	0.205	0.25	0.1
4	0.41	0.03	0.205	0.315	0.115
5	0.505	0.03	0.225	0.355	0.13
6	0.64	0.08	0.24	0.28	0.115
7	0.395	0.035	0.22	0.34	0.12
8	0.41	0.02	0.2	0.265	0.075
9	0.625	0.01	0.23	0.205	0.075
10	0.595	0.005	0.215	0.155	0.055
11	0.655	0.005	0.2	0.125	0.055

Appendix 34.

Postprandial changes in individual TRL (d < 1.006g/ml) apoAI concentration (g/L) in diabetic subjects.

Time (hours)	JC	SJ	PF	CE	MH
0	0.02	0.05	0.005	0	0
1	0.005	0.05	0.02	0	0
2	0.02	0	0.01	0	0
3	0.02	0.01	0.01	0	0
4	0.04	0.01	0.015	0	0
5	0.055	0	0.025	0	0
6	0.09	0.05	0.015	0	0
7	0.07	0.01	0.02	0	0
8	0.11	0.01	0.02	0	0
9	0.165	0.01	0.025	0	0
10	0.165	0.02	0.025	0	0
11	0.175	0	0.015	0	0

Appendix 35

Individual clinical status of diabetic subjects.

Parmeter	JC	SJ	PF	CE	MH
Age (years)	74	69	63	46	65
BMI (kg/m^2)	29	31	35	30	31
BP (mmHg)	180/100	150/80	150/80	134/92	154/76
Therapy	Diet only	Diet only	Metformin	Metformin	Metformin
Neuropathy	Present	None	Present	None	None
Retinopathy	None	PDR*	None	None	BDR**
HbA_1 (%)	9.5	-	7.7	10.1	10.4
HbA_{1c} (%)	7.1	-	5.6	7.4	13.8
Duration of diabetes (years)	12	6	5	3	4

*PDR- Proliferative diabetic retinopathy

**BDR- Background diabetic retinopathy

Normal range HbA_1 4.9-7.6%
Normal range HbA_{1c} 3.4-5.2%

Glossary.

ACC	acetyl CoA-carboxylase
ADA	adenosine deaminase
AICAR	5-amino-4-imidazole carboximide
ANOVA	analysis of variance
Apo	apolipoprotein
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAD	coronary artery disease
CE	cholesterol ester
CETP	cholesterol ester transfer protein
CHD	coronary heart disease
cm	centimetre
CNBr	cyanogen bromide
CoA	co-enzyme A
cpm	counts per minute
d	density
DAG	diacylglycerol
dl	decilitre
DMSO	dimethyl sulphoxide
dpm	disintegrations per minute
EDTA	ethylenediaminetetra-acetic acid disodium salt
ELISA	enzyme linked immunosorbent assay
FA	fatty acids
FC	free cholesterol
FCS	foetal calf serum
FFA	free fatty acids
g	grams
g's	gravitational force
G_i	inhibitory G protein
G_s	stimulatory G protein
HAT	hypoxanthine aminopterin thymidine solution
HDL	high density lipoprotein
HL	hepatic lipase
HMG CoA-reductase	3-hydroxy-3-methyl glutaryl co-enzyme A-reductase
HMW	high molecular weight marker

HRP	horse radish peroxidase
HSL	hormone-sensitive lipase
H_2O	water
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	inhibitor concentration at 50% inhibition
IDDM	insulin-dependent diabetes mellitus
IDL	intermediate density lipoprotein
IgG	immunoglobulin
kcal	kilocalorie
kg	kilogram
L	litre
LCAT	lecithin cholesterol acyl-transferase
LDL	low density lipoprotein
LMW	low molecular weight markers
Lp(a)	Lipoprotein(a)
LPDP	lipoprotein deficient plasma
LPL	lipoprotein lipase
LRP	LDL receptor-related protein
M	molar solution
mA	milliamps
MAG	monoacylglycerol
mEq	milliequivalents
mg	milligram
mins	minutes
ml	millilitre
mM	millimolar
mmol	millimoles
MOME	monoacylmonoalkylglycerol
mRNA	messenger ribonucleic acid
mU	milliunits
3M5P	3-methyl-5-pyrazolecarboxylic acid
NaOH	sodium hydroxide
NaSCN	sodium thiocyanate
NEFA	non-esterified fatty acids
ng	nanogram
NIDDM	non-insulin dependent diabetes mellitus
nM	nanomolar
nmol	nanomole

3PAA	3-pyridyl acetic acid hydrochloride
PEG	polyethylene glycol
PIA	phenyl isopropyl adenosine
PL	phospholipids
PP	protein phosphatases
PPi	pyrophosphate
304205	p-nitrophenylethylhexyl phosphonate
RE	retinyl ester
RER	rough endoplasmic reticulum
rpm	revolutions per minute
RPMI 1640	Roswell Park Memorial Institute Medium 1640
SDS	sodium dodecyl sulphate
SDS PAGE	sodium dodecyl sulphate polyacrylamide slab gel electrophoresis
SER	smooth endoplasmic reticulum
TAG	triacylglycerol
TEMED	tetramethylethylenediamine
TG	triglyceride
TRL	triglyceride-rich lipoproteins
TZD	thiazolidinediones
μg	microgram
μl	microlitre
μM	micromolar
μmol	micromole
v	volume
VLDL	very low density lipoprotein
w	weight
ZMP	5-amino-4-imidazolecarboxamide
ß-VLDL	VLDL remnant, refers to migration pattern on agarose gels

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