FACTORS INFLUENCING EXPRESSION OF LECITHIN: CHOLESTEROL ACYLTRANSFERASE

A thesis submitted to the University of London
in partial fulfilment of the requirements for
the degree of Doctor of Philosophy
in the Faculty of Science

by

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Acknowledgements

I would like to express my gratitude to Dr Jim Owen for his patience and support throughout the years and for his help with and critical reading of the thesis.

I would like to thank Professor Steve Humphries for allowing me to carry out some of the work in his laboratory and for his help and encouragement throughout my time there.

Many thanks to Dr David Harry and Dr David Brown for their kind advice, help and support throughout the years and to Dr Kaila Srai for his interest and encouragement.

I would like to thank my family and friends for their encouragement, understanding and support over the years.

Lastly, I wish to thank my husband for his unconditional support, patience, encouragement and great help throughout the writing of this thesis; without which it would probably have never been finished!
Dedication

I would like to dedicate this thesis to my son, Daniel Jack, who has brought so much joy into my life, and to my parents who have never stopped believing in me.
ABSTRACT

In this thesis, the regulation and secretion of lecithin: cholesterol acyltransferase (LCAT), the plasma enzyme which esterifies lipoprotein cholesterol, has been studied including LCAT mRNA tissue distribution and developmental expression in the guinea pig. The relationship between lipoprotein production and LCAT synthesis, as measured by Northern blotting, was examined in HepG2 (human hepatoblastoma) cells, after enhancing secretion of triglyceride-rich VLDL by oleic acid supplementation, and in the intestine of fat-fed guinea-pigs. In HepG2 cells, oleic acid diminished LCAT mRNA, while guinea pig intestinal LCAT mRNA was unchanged.

Apolipoprotein A1 (apo-A1) is the main LCAT co-factor and hence may influence LCAT expression. Their co-ordinate regulation was examined by altering production of apo-A1 in HepG2 cells and then measuring both LCAT and apo-A1 mRNA. Initially, HepG2 cells were pre-incubated with HDL to reduce their cholesterol content and suppress apo-A1 mRNA; when cholesterol levels were replenished, the rise in apo-A1 mRNA was accompanied by increased LCAT mRNA. Moreover, when apo-A1 expression was reduced by glucose deprivation, LCAT mRNA also fell even though the cells remained viable. However, when apo-A1 mRNA was increased by the hypolipidaemic drug, gemfibrozil, levels of LCAT mRNA fell. These findings suggest that, while hepatic LCAT mRNA is modulated by cellular lipid content and changes in lipoprotein production, there is no obvious co-ordinate regulation of LCAT and apo-A1 expression.

LCAT mRNA was reduced more than mRNA of other secreted proteins (apo-A1, albumin and transferrin), in 5 liver biopsy specimens from jaundiced patients compared to normal liver. Similarly, exposing HepG2 cells to increasing amounts of different hepatotoxic agents, generally reduced LCAT mRNA before apo-A1 mRNA fell. A broadly similar conclusion was drawn from experiments in vivo when mice were administered hepatotoxins (galactosamine, lipopolysaccharide and the purine analogue, 4-APP). These various studies support the emerging concept that plasma LCAT activity is a sensitive marker of liver synthetic function and a reliable diagnostic indicator of liver damage in humans.
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Abbreviations

The standard abbreviations used in the Biochemical Journal have been adopted throughout this thesis.

The following abbreviations have also been used:-

apo: apolipoprotein
BSA: bovine serum albumin
cDNA: complementary DNA
d: density
DNA: deoxyribonucleic acid
DMEM: Dulbecco's modified Eagle's medium
ER: endoplasmic reticulum
FCS: foetal calf serum
GlcNAc
HepG2: hepatoblastoma G2
Hep3B: hepatoblastoma 3B
IDL: intermediate density lipoprotein
kb: kilobases
kDa: kilodalton
EDTA: ethylenediamine tetra acetic acid
HDL: high density lipoprotein
LCAT: lecithin:cholesterol acyltransferase, EC 2.3.1.43
LDL: low density lipoprotein
mRNA: messenger RNA
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate buffered saline
RFHSM: Royal Free Hospital School of Medicine
RNA: ribonucleic acid
SDS: sodium dodecyl sulphate
SEM: standard error of the mean
TEMED: NNN' N- tetramethyl ethylenediamine
TLC: thin layer chromatography
Tris: Tris (hydroxymethyl) methylamine
UV: ultraviolet
VLDL: very low density lipoprotein
CHAPTER 1

INTRODUCTION
1. Introduction

1.1 Lipoproteins and apolipoproteins

The major lipids in plasma are cholesterol, cholesteryl ester, triglyceride and phospholipid. Because these lipids are insoluble in water, they are carried in large macromolecular complexes termed lipoproteins. The primary physiological function of lipoproteins is lipid transport. The lipoproteins of normal plasma are roughly spherical particles composed of a hydrophobic core of triglyceride and cholesteryl ester. They are surrounded by a single layer of phospholipid and cholesterol, which present their hydrophilic groups to the water of the surrounding plasma. The non-polar portions of these molecules project into the core of the particle. Specific polypeptides, or apolipoproteins, are associated with the lipoproteins. Apolipoproteins typically possess several helical regions that are amphipathic; one surface of the helix contains hydrophobic amino acid residues, which penetrate the non-polar core of the particle, while the other contains mainly polar amino acids which contact the aqueous environment. The lipoprotein structure is thus stabilised by non-covalent, mainly hydrophobic forces, but exchange and transfer of its constituent lipids and apolipoproteins occurs during metabolism.

The overall composition of lipoproteins reflects this general structure. The largest lipoproteins, chylomicrons and very low density lipoproteins (VLDL), contain mainly non-polar lipids and are triglyceride-rich. The smaller lipoproteins, low density lipoproteins (LDL) and high density lipoproteins (HDL), contain increased proportions of polar lipids and proteins. Their core region contains primarily cholesteryl esters. The polar lipids, and especially the proteins that compose the lipoprotein surface, are denser than the non polar lipids of the core. Thus, as the size of lipoprotein particles decreases and the ratio of protein to lipid increases, their density increases. This property provides the basis for the major method used to separate lipoprotein classes, ultracentrifugation. By adjusting the density of plasma with salt solutions,
specific classes of lipoproteins can be made to float or sink in an ultracentrifuge. **Chylomicrons** are very large particles that float on standing of plasma; on electrophoresis they remain at the origin. **VLDL** particles float at a density (d) of 1.006g/ml, the density of protein-free plasma and migrate to a pre-beta position on paper electrophoresis. **LDL** particles were originally defined as having a density range of 1.006 to 1.063g/ml. However, this fraction contains at least two types of particle; intermediate density lipoprotein (IDL, d 1.006-1.019 g/ml) and the major component ('true' LDL, d 1.019-1.063 g/ml). **HDL** float at a density of 1.063 to 1.21g/ml and migrate furthest on electrophoresis to the alpha position. HDL are further described later on in this section. The main cholesterol-carrying lipoproteins are LDL and HDL. In a normal individual, LDL contains approximately 70% and HDL 20% of the total plasma cholesterol.

**Apolipoproteins.** Lipoproteins cannot be synthesised and secreted from the liver and intestine without the corresponding structural apolipoprotein (Table 1). In addition to their structural role, apolipoproteins provide lipoproteins with recognition sites for cell-surface receptors (receptor ligands), and with cofactors for enzymes involved in their metabolism. Apolipoproteins are synthesised on the ribosomes of the rough endoplasmic reticulum and linked to lipid in the smooth endoplasmic reticulum where the enzymes for lipogenesis are found.

<table>
<thead>
<tr>
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<tr>
<td></td>
<td>µM</td>
<td>mol %</td>
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<tr>
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<td>41</td>
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<tr>
<td>apo E</td>
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<td>2</td>
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Table 1.1 The associations of apolipoproteins with the major non-transient lipoproteins (HDL, LDL, IDL and VLDL; modified from Havel et al, 1980)
1.2 Lipid Transport

There are three major routes of lipid transport in plasma: the exogenous, the endogenous and the reverse cholesterol transport pathways.

1.2.1 The exogenous pathway

The exogenous pathway is initiated by secretion of chylomicrons into intestinal lymph in response to dietary fat, mainly triglyceride with some phospholipid and cholesterol. After gastric digestion, the emulsified fats mix with bile salts in the duodenum and are acted on by pancreatic lipase to produce mono- and di-glycerides, fatty acids and lysophospholipids. These form mixed micelles with phospholipids and bile acids, thereby permitting intestinal uptake. In the enterocytes, long chain fatty acids and mono-glycerides are resynthesised into triglycerides and cholesterol is re-esterified. These are then complexed with phospholipids and apolipoproteins (apoB-48 and apo-A1) to produce chylomicrons and VLDL of intestinal origin. Further processing occurs in the Golgi complex, where the apoB-48 is glycosylated and the particles are then actively transported to the cell surface for secretion into lymph. On entering the circulation, via the thoracic duct, the chylomicrons acquire C and E apolipoproteins from HDL. Acquisition of apo C11 activates lipoprotein lipase, an enzyme present on the lumen of capillary endothelial cells, and this releases fatty acids (and glycerol) from core triglycerides for storage (in adipocytes) or energy purposes (by muscle and other tissues). Thus, circulating chylomicrons become progressively smaller as core triglycerides decrease and the redundant surface components (phospholipids, cholesterol and apolipoproteins C and A1) are released to contribute to the HDL pool. The remnant particles contain apoE and apoB-48, are relatively rich in cholesteryl esters, and are cleared in vivo by hepatocytes through an apoE-dependent, receptor-mediated process.

Although apoB-100 was the first ligand recognised for the LDL-receptor (apoB-48 lacks the necessary binding domain), apoE is also bound. It has been
proposed that 4-7 apoE molecules interact with one LDL or B,E receptor, and the receptor-recognition site has been identified by Weisgraber et al (1983). Nevertheless, it remains unclear whether apoE interaction with the LDL receptor is involved with the clearance of chylomicron remnants. Thus, several lines of evidence support the existence of a distinct hepatic receptor for apoE. Cooper (1977) demonstrated that chylomicron remnants, not intact chylomicrons, were recognised and metabolised by the liver. Since LDL did not compete with apoE for binding to the receptor, a distinct receptor for remnant particles was proposed. Further support came from studies in LDL-receptor-deficient rabbits (the Watanabe Heritable Hyperlipidaemic or WHHL rabbit; Yamamoto et al, 1986); although binding by LDL receptors is completely absent, binding of triglyceride rich particles through apoE remained significant (Ishii et al, 1990) and the rabbits catabolised chylomicron remnants normally (Kita et al, 1982). Furthermore, patients with defective LDL receptors who develop familial hypercholesterolaemia do not have elevated levels of chylomicron remnants (Rubenstein et al, 1990).

A candidate cDNA clone for the apoE receptor was sequenced in 1988. The predicted protein, termed 'low density lipoprotein receptor-related protein' (LRP) was 600 kDa and was proteolytically cleaved in the Golgi compartment to produce two fragments, of molecular masses 515 and 85 kDa. Several functional studies now implicate LRP as the apoE-receptor. It has calcium-binding properties (Herz et al, 1988), consistent with the known calcium-dependency of apoE binding by hepatic membranes, and cross-linking studies have demonstrated directly (Beisegel et al, 1989) that LRP binds apoE. Lipoprotein lipase has been suggested as a potential ligand for this receptor. Unlike apoB/E-receptors, the hepatic apoE receptor is not down regulated by entry of cholesterol into the liver (see below).
1.2.2 Endogenous lipid transport

In the endogenous pathway, triglyceride-rich VLDL are secreted from the liver into plasma in response to accumulation of fatty acids, which are not required for oxidative metabolism or for biliary lipid synthesis. Free fatty acids are derived from three sources: fat stored in adipose tissue; hydrolysis of lipoprotein triglycerides by lipoprotein lipase (LPL) on capillary endothelial cells; and from dietary carbohydrates which are converted to free fatty acids in the liver. Free fatty acids derived from the first two sources are transported in the blood bound to albumin. Triglycerides are synthesised in the liver from free fatty acids and the rate of triglyceride synthesis varies. In humans ingesting carbohydrate-rich diets, as much as 100g daily are secreted in VLDL (Wolfe & Ahuja, 1977), most of which is probably derived from hepatic lipogenesis. Thus, the liver has the capacity to secrete triglycerides at a rate approaching that of the intestine. VLDL particles contain apo C (C1, C11 and C111), apoE and apoB-100, which is synthesised in the liver. Each VLDL particle contains one molecule of apoB-100 and this is retained after secretion.

Once secreted, VLDL particles undergo partial degradation in the plasma (due to hydrolysis of core triglyceride by lipoprotein lipase) to produce VLDL remnants, or IDL; they are also acted upon by the enzyme hepatic lipase which hydrolysies much of the remaining triglyceride in the core of VLDL and LDL. Some IDL is also taken up by the liver via the apoE receptor. ApoE thus allows the LDL receptor to recognise VLDL remnants before they are catabolised through to LDL (see Fielding, 1992 for a review). In man, the rest of the IDL is converted to LDL through further hydrolysis of triglycerides. During the cascade through VLDL to LDL there is a loss of all the apolipoproteins except apoB-100 and the acquisition of cholesteryl ester from other lipoproteins, mainly HDL, through the action of cholesteryl ester transfer protein (CETP). The major function of CETP appears to be the transfer of cholesteryl ester from HDL to VLDL and thus to LDL, but it may also transport triglyceride from VLDL to the other lipoprotein fractions. Under most circumstances, nearly all LDL in
the circulation can be accounted for by the conversion of VLDL (Berman et al, 1978). However, in patients with homozygous familial hypercholesterolaemia and in some hypertriglyceridaemic subjects, the plasma LDL apolipoprotein B pool size appears to exceed the amount derived by flux from VLDL through the IDL pathway. Direct secretion of particles in the LDL density range has thus been proposed. LDL may also be secreted directly by the intestine; an LDL presumed to be of intestinal origin has been isolated from human chyle (Alaupovic et al, 1968).

The LDL particles thus formed are relatively enriched in cholesterol but are small enough to cross the vascular endothelium and enter the interstitial fluid, thereby coming into contact with virtually every cell in the body. Although the major site of LDL removal is the liver, peripheral cells also catabolise LDL; although most cells can synthesise cholesterol, the uptake and degradation of LDL would enable cells to conserve the energy required for de novo cholesterol synthesis. The cholesterol delivered by LDL is used by the cells for membrane and steroid hormone synthesis.

LDL can enter cells by one of two pathways; receptor-mediated or non-receptor-mediated.

**Receptor-mediated pathway.**

In 1973, Goldstein & Brown observed that fibroblasts which were exposed to human serum (which contains a mixture of cholesterol-carrying lipoproteins) could derive cholesterol only from LDL uptake (Brown et al, 1973; Brown & Goldstein, 1974). This suggested that there might be a specific mechanism for cells to interact with a particular lipoprotein class. Following these studies it was discovered that a specific cell surface receptor existed for LDL (Brown & Goldstein, 1974; Goldstein & Brown, 1974). Since then, the human LDL receptor cDNA has been purified, cloned and characterised (Yamamoto et al, 1984, Sudhof et al, 1985)
The sequence of reactions by which cells use this receptor is called the LDL receptor pathway (Brown & Goldstein, 1979). The LDL receptor recognises both apoB-100 and apoE, but both apolipoproteins must be associated with lipid to maintain the correct conformation. The recognition site for the receptor lies within the apolipoprotein structure (Yang et al, 1986). The LDL receptor is located in clusters in clathrin-coated pits and binding of LDL by the receptor is followed by internalisation within coated vesicles. These shed their coat to become endosomes and a drop in pH causes the receptor and ligand to uncouple. The receptor recycles back to the plasma membrane while the ligand is degraded in lysosomes (Brown et al, 1983); the apoB molecule is hydrolysed to its constituent amino acids and cholesteryl ester is hydrolysed to free cholesterol. The resulting free cholesterol crosses the lysosomal membrane and enters the cytoplasmic compartment, where it is responsible for several regulatory events occurring inside the cell.

Cholesterol inhibits further synthesis of LDL receptor thus preventing further influx of LDL-cholesterol (Dawson et al, 1988). Synthesis of the two key enzymes of cholesterol synthesis, hydroxy-methyl-glutaryl CoA reductase (HMG-CoA reductase) and hydroxy-methyl-glutaryl CoA synthase is also suppressed (Goldstein & Brown, 1990). This regulation occurs via a sterol regulatory element (SRE) which binds to a positive transcription factor (SP-1) in the promoter of the genes. This feedback mechanism is important in preventing overloading of the cells with cholesterol, however, this regulatory pathway potentially increases plasma LDL-cholesterol levels. As a result, high levels of intracellular cholesterol will cause hypercholesterolaemia. Another consequence of the release of intracellular free cholesterol by the LDL receptor pathway is the activation of the enzyme, acyl CoA: cholesterol acyl transferase (ACAT). This results in the re-esterification of cholesterol surplus to immediate requirements which can then be stored in the cytoplasm (Goldstein et al, 1974; Brown et al, 1975; Brown & Goldstein, 1979).
The LDL receptor does not remove all the LDL from plasma in normal subjects. The rest is removed by non-specific endocytosis (see below) and via the 'scavenger' pathway. The latter mechanism involves phagocytic cells of the reticulo-endothelial system and clears LDL which has been chemically modified, e.g. by oxidation, by methylation, or by complexing with other molecules, and which is no longer recognised by the LDL receptor.

**Non LDL-receptor-mediated pathway.**

This involves the binding of LDL to cell membranes and uptake at sites which do not possess apoB/E receptors. Unlike receptor mediated entry, in this pathway LDL uptake is not saturable, but continues to increase with increasing extracellular LDL concentration. Thus, when LDL levels are relatively high, the entry of cholesterol into cells by this route may be of greater quantitative significance than that via the apoB/E receptor which is both saturated and down-regulated (Goldstein et al, 1979). In adult humans, about one third of LDL is catabolised by receptors and two thirds by non-receptor-mediated pathways. In patients with homozygous type II hypercholesterolaemia where the LDL receptor mechanism is defective, the levels of LDL being catabolised through non-receptor mediated mechanisms rises further. If the LDL receptor is absent all plasma LDL is cleared by non-LDL-receptor mediated mechanisms.

A scavenger pathway for acetylated LDL was proposed (Brown & Goldstein, 1979) to account for the massive deposition of LDL-derived cholesteryl esters in macrophages of patients with LDL-receptor defects. In these patients, the LDL receptor pathway is blocked and each particle circulates in the plasma 2-3 times longer than it does in normal subjects, thus raising the lipoprotein concentration in the plasma to abnormally high levels (Packard et al, 1983). Eventually the LDL is degraded by another pathway. It was proposed that a binding site exists on macrophages and mediates the degradation *in vivo* of plasma LDL that has become denatured, or chemically modified. This pathway, via the 'scavenger' receptor, was suggested to serve as a 'backup' for receptor-
mediated degradation of normal LDL. Following various attempts to characterise this receptor, the structure of the 220kDa acetyl-LDL binding protein was finally elucidated by Freeman et al (1990) and was shown to exit as differentially spliced variants, types I and II.

1.2.3 Reverse cholesterol transport

Since peripheral tissues are unable to catabolise cholesterol, the only way of maintaining cholesterol homeostasis is by the removal of cholesterol from these tissues for elimination by the liver. This flux of cholesterol, from peripheral cells to the liver, was proposed to be mediated by HDL, in a process first termed 'Reverse cholesterol transport' by Glomset (1968). Evidence suggests that there may be several ways for removal of cellular cholesterol (Nichols et al, 1987) and it is likely that some pathways operate more efficiently in some tissues than in others.

High-density lipoproteins (HDL) are a heterogeneous group of particles with respect to their physical, chemical and functional properties (Eisenberg, 1984). The properties in common to all HDL subclasses are a high density (1.063 to 1.21g/ml), a relatively small size (diameters range between 7-12nm; Nichols et al, 1987) and the presence of apo-A1. Different subclasses of HDL have been defined by methods such as the following:-

**Ultracentrifugation** defines two major density classes, HDL\(_2\) (1.063-1.125g/ml) and HDL\(_3\) (1.125-1.21g/ml; Anderson et al, 1977). Analytical techniques define a minor sub-class HDL\(_1\), occurring within the HDL\(_2\) density range; it is a larger particle and richer in cholesteryl ester than HDL\(_2\), and appears to contain most of the apoE in peripheral venous HDL in normal humans.

**Immunoaffinity chromatography and gradient gel electrophoresis** have demonstrated the presence of two apolipoprotein-specific populations of plasma HDL, one containing both apo-A1 and apo-A11 (LpA1/A11) and another containing apo-A1 only (LpA1) (Cheung & Albers, 1984, Marz & Gros,
1988). A number of studies indicate that LpA-1 and LpA1/A11 are metabolically distinct (Mowri et al, 1994) and may perform different functions (Vadiveloo & Fidge, 1992; Ohta et al, 1992). Particles containing only apo-A1 may be the physiological acceptor of cellular cholesterol (Castro & Fielding, 1988) and HDL₂ containing both apo-A1 and apo-A11 constitute a better substrate for hepatic lipase than HDL₂ containing apo-A1 only. It is also suggested that plasma levels of LpA1 reflect the protective function of HDL better than plasma levels of LpA1/A11 and that apo-A11 antagonises the cholesterol-efflux mediating properties of HDL (Nowicka et al, 1990). HDL can also be classified by non-denaturing polyacrylamide gradient gel electrophoresis, which differentiates at least five subclasses (Blanche et al, 1981).

**Agarose gel electrophoresis** separates HDL particles into those fractions migrating with an α-mobility (the majority) and a smaller fraction (approximately 5% of total HDL) which have a pre-β mobility. The latter fraction can be further sub-divided upon 2-dimensional polyacrylamide gel electrophoresis to produce pre-β-1, pre-β-2 and pre-β-3-HDL (Francone et al, 1989). Pre-β-1 are the smallest of these particles; they are discoidal, and contain only apo-A1 (Castro & Fielding, 1988). Pre-β-2- and pre-β-3-HDL sequentially arise from pre-β-1 HDL. Unlike pre-β-HDL, α-HDL can contain both apo-A1 and apo-A11 (Cheung & Wolf, 1989).

HDL originate from three sources: liver, intestine (Green et al, 1980) and lipolysis of chylomicrons and VLDL in the plasma (Tall et al, 1982; Eisenberg, 1984). Both intestinal and hepatic 'nascent' HDL consist of spherical and discoidal particles that are rich in phospholipids but poor in triglycerides and unesterified cholesterol. Newly synthesised hepatic HDL consist of apo-A1, apo-A11 and apoE, whereas intestinal nascent HDL contain only apo-A1 and apo-A1V. Another major source of HDL is in the surface components of the triglyceride-rich lipoproteins (Havel et al, 1973, Schaefer et al, 1978), remnants
rich in phospholipids, apoCs and apo-A1 which contribute to the plasma assembly of HDL. The apoE-rich core remnants which remain are then taken up by the liver via chylomicron remnant (i.e. apoE) receptors. The pathway of reverse cholesterol transport can be viewed as being composed of three stages which are: cholesterol movement from tissues to plasma, cholesterol movement between plasma lipoproteins and transfer of cholesterol from plasma lipoproteins to hepatocytes. These stages will be briefly reviewed.

**Cholesterol movement from tissues to plasma**

Due to their low cholesterol content, HDL precursors are excellent acceptors of excess cellular cholesterol. The preferential acceptors for cell membrane cholesterol are the pre-β-1 HDL (Castro & Fielding, 1988; Francone et al, 1989). However, another subfraction of HDL, γ-lipoprotein (Lp)E, also serves as an important initial acceptor of cellular cholesterol (Huang et al, 1994). This lipoprotein was detected by agarose gel electrophoresis of plasma briefly exposed to cells labelled with $^3$H-cholesterol and has electrophoretic γ mobility. γLpE contains apoE as its only apolipoprotein and is rich in sphingomyelin. The HDL particles involved in initial transport of cholesterol away from peripheral cells are not simply those present in plasma as the cells are bathed by interstitial fluid which is separated from the plasma compartment by the capillary endothelium. Interstitial HDL lipoproteins can be regarded as the precursors of mature plasma HDL. Interstitial HDL particles arise by filtration of plasma HDL across this barrier and by direct secretion of apolipoproteins by some peripheral cells (Sloop et al, 1987). Lymph HDL is relatively enriched in free cholesterol relative to cholesteryl ester and the HDL concentration in human peripheral lymph is about 10% of that of plasma.

Three general models have been proposed for the efflux of cellular cholesterol. Firstly, unesterified cholesterol from plasma membranes diffuses towards HDL through the aqueous phase between the plasma membrane and the acceptor
particles (passive diffusion model; Rothblat et al, 1982). Secondly, HDL binds to specific cell surface receptors (Oram et al, 1983), augmenting the translocation of free cholesterol to the plasma membrane before desorption into the extracellular fluid and adsorption by HDL particles that are free in solution. Apo-A1 has been described as the ligand for the binding of these HDL particles to macrophages and other cells. Thirdly, binding of HDL to its receptor is followed by internalisation and resecretion of cholesterol-enriched HDL particles (retroendocytosis; Schmitz et al, 1985a; 1985b). However the present evidence for an obligatory receptor-mediated pathway for efflux is not conclusive and the mechanism of cellular cholesterol efflux is still controversial.

**Cholesterol movement between plasma lipoproteins.**

Three processes probably contribute towards the intravascular phase: the LCAT reaction, exchange and transfer of lipids and apolipoproteins between different lipoproteins, and hydrolysis by lipoprotein lipase and hepatic endothelial lipase.

Subsequent to cellular release, cholesterol is transferred to the hydrophobic core of pre-β-1 HDL precursors, which become larger. Cholesterol is then transferred to pre-β-3 HDL (the site of esterification by LCAT), that contain (besides apo-A1) apoD, LCAT and CETP (Francone et al, 1989). The smaller, denser HDL\(_3\) particle is the preferred substrate for LCAT and cholesteryl ester production by this enzyme leads to the formation of larger HDL\(_2\) particles (Dieplinger, 1985; Zechner et al, 1987). Cholesteryl ester in HDL\(_2\) is exchanged by CETP with triglyceride in LDL or VLDL. The triglyceride exchanged into the HDL\(_2\) particle is attacked by hepatic lipase and HDL\(_2\) is reconverted to HDL\(_3\), while the cholesteryl ester is transferred to LDL and then to the liver. During this process, apo-A1 is thought to dissociate from the HDL particle, in a cyclical process where it may then complex with phospholipids to
form the pre-β migrating population of apo-A1-containing nascent HDL. These discoidal particles are then available to incorporate unesterified cholesterol from other lipoprotein fractions or from cell membranes. Subsequent esterification of this cholesterol by LCAT converts the discs into mature, spherical HDL, thus completing a cycle in which unesterified cholesterol in lipoproteins or cell membranes is used to expand the cholesteryl ester core of HDL particles. Hepatic lipase, CETP and VLDL therefore appear to act synergistically to reduce the core lipid content and the particle size of HDL but it is currently unknown whether cholesteryl esters are transferred to α-HDL via CETP or whether pre-β particles are converted to α-HDL (for example, by the uptake of apo-A11-containing particles).

**Cholesterol transport from plasma to liver**

Cholesteryl esters present in α-migrating HDL, especially HDL₂, are directed to the liver, where they can be eliminated via bile acid synthesis, by one of at least four mechanisms:

1. A subpopulation of HDL, which are apoE-rich and cholesteryl ester-rich, can be recognised and internalised by hepatic apoE receptors (Weisgraber & Mahley, 1980; Mahley, 1988).

2. HDL without apoE can be endocytosed by hepatocytes via a specific HDL receptor protein (Glass et al, 1985; Takata, 1988). The concept of an HDL receptor was first proposed by Kovanen and colleagues over 10 years ago (Kovanen et al, 1979) who described a separate uptake mechanism for HDL and LDL *in vivo*. Since then, the search for an HDL receptor led to the characterisation of such a structure between 1983 and 1987 (Graham and Oram, 1987) and the cloning of a plasma membrane HDL binding protein with several features predicted for an HDL receptor (McKnight et al, 1992). HDL
binding does not lead to a depletion of cellular cholesterol and apo-A1 is also reported to be the ligand for this receptor (Morrison et al, 1991, Allan et al, 1992). The functional domains involved in binding of HDL to the hepatic membrane were recently identified (Dalton & Swaney, 1993). More recently, a well defined cell surface HDL receptor for selective cholesterol uptake was discovered and characterised. This receptor (class B, type 1 scavenger receptor, SR B1) binds HDL with high affinity, is expressed primarily in the liver and steroidogenic tissues, and mediates selective cholesterol uptake from HDL to cells via a mechanism distinct from the LDL receptor (Acton et al, 1996).

3. Hydrolysis of the phosphatidylcholine components of HDL and of hepatic cell membranes by hepatic triglyceride lipase (HTGL) is proposed to result in the fusion of HDL with the outer leaflet of the plasma membrane (Van't Hoff et al, 1981). This results in the enhanced deposition of HDL cholesterol into hepatocytes.

4. Most of the cholesteryl ester generated by LCAT in HDL is transferred to apoB-containing lipoproteins, mainly VLDL, by CETP, thus allowing LCAT to esterify fresh free cholesterol entering the HDL particle along the concentration gradient (Tall, 1990). Since VLDL free cholesterol is a poor substrate for LCAT (Fielding, 1984), most VLDL cholesteryl ester must be acquired from other lipoproteins, namely HDL. As nascent VLDL contains very little cholesteryl ester (Akanuma & Glomset, 1968), the transfer must initially be predominantly from HDL to VLDL. This results in a continuous mass transfer of cholesteryl ester from HDL to VLDL via CETP, with 1 mole of cholesteryl ester from HDL being exchanged for 1 mole of triglyceride from VLDL, and eventually producing an increase in HDL core volume, since 1 mole of triglyceride occupies 1.5 X the volume of 1 mole cholesteryl ester. Thus, during triglyceride hydrolysis of VLDL, cholesteryl ester, made by LCAT, is transferred
from HDL to VLDL remnants via CEPT and hence to IDL and LDL where it is removed by the liver.

1.3. Lecithin : cholesterol acyltransferase : mechanism of action, molecular biology and structure

Lecithin : cholesterol acyltransferase (E.C.2.3.1.43; LCAT) plays a central role in the extracellular metabolism of plasma lipoproteins. LCAT catalyses the esterification of lipoprotein cholesterol by cleaving the \( sn-2 \) fatty acyl oxyester bond of phosphatidylcholine (lecithin) and transferring the resultant fatty acid onto the 3-\( \beta \)-hydroxyl group of cholesterol forming lysolecithin and cholesteryl ester respectively (Glomset, 1962). The majority of plasma cholesteryl ester in man is formed through the action of LCAT (Glomset, 1968) and this transesterification occurs preferentially on the surface of HDL (Fielding et al, 1972). The LCAT reaction regulates the distribution of unesterified cholesterol among lipoproteins and cell membranes by maintaining the ratio between free and esterified cholesterol (Glomset et al, 1983). This results in the modulation of plasma triglyceride-rich lipoprotein cholesterol content (Marcel & Vezina, 1973) thereby stabilising the normal shape and size of these lipoproteins. LCAT is also responsible for the transformation of excess surface cholesterol and phospholipid into spherical HDL during chylomicron and remnant hydrolysis by lipoprotein lipase. The action of LCAT converts its preferred substrate, nascent discoidal HDL, into mature, spherical HDL particles in the plasma; this is accompanied by the acquisition of cholesterol and phospholipids by HDL. The esterification of HDL cholesterol (particularly the pre-\( \beta \)-3 subfraction containing apo-A1; Francone et al, 1989) creates a concentration gradient between cell membrane cholesterol and HDL, since the newly esterified cholesterol cannot transfer back to the cell membrane. Instead, it is transferred to other HDL species and then to VLDL, IDL and LDL by CETP (Morton & Zilversmit, 1982). This transfer protects against cholesteryl ester accumulation within the HDL surface which would inhibit further LCAT
action, and thus serves as a second step in maintaining the concentration gradient between cell membranes or other lipoproteins and HDL (Dobiazova, 1983). LCAT thus facilitates the process of 'reverse cholesterol transport' from peripheral cells to the liver.

Figure 1.1  Action of lecithin: cholesterol acyltransferase. LCAT catalyses the transfer of a fatty acid from the 2-position of lecithin to the 3-β-hydroxyl group of unesterified cholesterol to form cholesteryl ester and lysolecithin.
Experiments with perfused rat liver (Simon & Boyer, 1971) first demonstrated that LCAT was released from the parenchyma to the perfusate, and direct evidence that liver parenchyma cells synthesise and secrete LCAT was provided by Nordby et al (1976). Until recently, LCAT was thought to be synthesised exclusively by the liver and present only within the plasma compartment; low levels of LCAT activity in intestinal lymph (Clark & Norum, 1977) and cerebrospinal fluid (Illingworth & Glover, 1970) were ascribed to filtration of the plasma activity. But it has since been demonstrated by Warden et al (1989) that LCAT mRNA, although most abundant in the liver, is also present in significant quantities in the brain and testes of rats. Both of these tissues are active in cholesterol metabolism and have blood-tissue barriers that do not allow the passage of plasma LCAT. Within the plasma compartment, LCAT is predominantly associated with high density lipoproteins, particularly with HDL₃ although smaller quantities have been found to be associated with pre-β₃ lipoprotein (Francone et al, 1989). LCAT has been described from the plasma of many animal species including rats (Pownall et al, 1985a), mice (Warden et al, 1989) monkeys (Parks et al, 1989), reptiles and amphibians (Gillett, 1978) and man (Sperry, 1935; Chen & Albers, 1983). LCAT has also been purified from pigs (Park & Lacko, 1986).

1.3.1. Substrates

LCAT is predominantly associated with HDL particles in the plasma. These apo-A1-containing lipoproteins are the preferred substrate for LCAT, particularly 'nascent' HDL. However, Cheung et al (1988) demonstrated that non-apo-A1-containing particles can also serve as physiological substrates for cholesterol esterification mediated by LCAT. Although much less effective than nascent discoidal HDL, 'reconstituted' HDL, LDL from humans and some animal species (pig) can act as a substrate for LCAT (Barter, 1983; Jonas et al, 1988; Knipping et al, 1987). Cheung et al (1988) demonstrated that LCAT mass remaining in apo-A1-lipoprotein-free plasma is distributed over the LDL and
VLDL size ranges. Furthermore, Chen & Albers (1985b) showed that cholesterol esterification in the plasma of apo-A1 or HDL-deficient patients proceeds at significant rates, while in patients with Fish-eye disease, LDL, not HDL, are the substrates for the LCAT reaction (Carlson et al, 1987); this may be due to a defect in LCAT that prevents it from binding to or being activated by HDL, rather than a defective catalytic action (Jonas, 1991).

1.3.2. Co-factors

LCAT requires a protein co-factor in order to express optimal catalytic activity. Apo-A1 is the major cofactor and most potent activator for the LCAT reaction under normolipidaemic conditions (Fielding et al, 1972). However, this may not be the case in dyslipoproteinaemias; in patients with Tangier disease, where only 2% of the normal apo-A1 concentration is present in plasma, LCAT activity is almost normal (Chen & Albers, 1985b) and patients with combined apo-A1/C111 deficiency have a normal total cholesterol:cholesteryl ester ratio in plasma LCAT (Schaefer et al, 1985). Indeed, since studies by various groups have demonstrated that other apolipoproteins activate LCAT (to a lesser extent) in vitro, it can be assumed that these apolipoproteins can also activate the LCAT reaction in vivo. These are: apo A1V (Chen & Albers, 1985b), E (Zorich et al, 1985; Soutar et al, 1975), and C1 and D (Steyrer & Kostner, 1988).

1.3.3. Inhibitors

The LCAT reaction can be inhibited by various chemicals (this is reviewed by Dobiasova, 1983), for example, sulphydryl inhibitors (e.g. DTNB), inhibitors of serine-histidine anti-esterase activity, heavy metal cations, and calcium (Glomset, 1968)

1.3.4. LCAT purification

A method for the purification of human plasma LCAT was first reported by Albers et al (1976) using ultracentrifugation, hydroxylapatite chromatography, and HDL and anti-apoD affinity chromatography. The enzyme preparation was
purified 12,000-fold and found to be homogeneous by the following criteria; it showed a single band on SDS-PAGE chromatography, with an apparent molecular weight of 68kDa and produced a single protein peak with a molecular weight of 70kDa on a Sephadex G-50 column. Several different methods have since been reported for LCAT isolation, with a purity of up to 50,000-fold. LCAT has also been purified from mammalian species other than human (Chen & Albers, 1985a).

1.3.5. Molecular Biology of LCAT

Full length human LCAT cDNA clones have been isolated and sequenced by McLean et al (1986), Tata et al (1987) and by Rogne et al (1987). Translation of the cDNA sequence predicted a mature protein of 416 amino acids with a calculated molecular weight of 47,090. The 5' end of LCAT cDNA contains an initiator codon (methionine) followed by a continuous open reading frame coding for a 440 amino acid polypeptide. The \( \text{NH}_2 \) terminus of the mature protein (beginning Phe-Trp-Leu) is preceded by a hydrophobic leader sequence of 24 amino acids which represents the signal peptide of the secreted protein. The protein sequence has six cysteine residues and four potential sites for post-translational glycosylation, which is consistent with the high oligosaccharide content of purified LCAT observed by Chung et al (1979).

Active site of LCAT.

The human LCAT amino acid sequence shows several linear stretches of hydrophobic residues which are analogous to the extended 'interfacial' active sites of other lipid-binding enzymes (Pujik et al, 1977; De Caro, 1981); these sequences surround a hexapeptide sequence (-Ile-Gly-His-Ser\(^{181}\)-Leu-Gly; residues 178-183), which by analogy to porcine pancreatic lipase (Guidoni et al, 1981), may represent the active site of LCAT, where ser-181 is the putative active site serine. Studies by Aron et al (1978) showed that a single serine residue was required for catalysis and ser-181 was later confirmed by Farooqi
and colleagues (1988) and by Francone & Fielding (1991) as the unique active site serine of LCAT.

The primary structure amino acid sequence of LCAT was confirmed by Yang and colleagues (1987) by direct sequence analysis using degradation and alignment of peptides obtained from tryptic and staphylococcal digestion and cyanogen bromide cleavage. They located two free sulphydryl groups (at Cys-31 and Cys-184), thought to be the essential acyl acceptors in the LCAT reaction, and two disulphide bridges were shown to be located between cysteine (Cys)-50 and Cys-74 and between Cys-313 and Cys-356. The N-terminus of LCAT was found to be phenylalanine while the C terminus was identified as glutamic acid. The N-glycosylated sites of the protein were assigned to asparagines at positions 20, 84, 272 and 384. The first glycosylation site to be confirmed was at residue 272 (McLean et al, 1986). By the use of predictive algorithms, they reported the secondary structure of LCAT to be composed of 21% α-helix, 24% β-pleated sheet and 55% other structures; these results were similar (24, 27 and 49% respectively) to those obtained by Chong et al (1983), based upon an analysis of the circular dichroic spectrum of LCAT. Yang et al (1987) also predicted the presence of several amphiphilic regions in the LCAT protein flanked by proline residues. This, by analogy with similarly endowed proteins (Boguski et al, 1986), suggested that the peptide backbone is able to change direction. Furthermore, the suggestion that Cys-31 and Cys-184 are adjacent to the active site serine and within 3.50 - 3.62 Å of each other (Jauhiainen & Dolphin, 1988) places a constraint on the way in which the polypeptide chain is folded. Although the tertiary structure of LCAT is unknown, a molecular model for human LCAT has recently been proposed (Peelman et al, 1999), based upon the structural homology between LCAT and lipases. This model proposes that LCAT belongs to the α/β hydrolase fold family and that its central domain consists of a mixed seven stranded β-pleated sheet with four α-helices and loops linking the β strands. The catalytic triad of
LCAT was identified, by site-directed mutagenesis, as Asp-345 and His-377, as well as the previously defined ser-181 residue (Farooqi et al, 1988).

The cDNA probe detected LCAT mRNA sequences about 1550 bases long in human adult liver (McLean et al, 1986) foetal liver and rat liver RNA, but not in RNA from human colon or upper small intestine (Tata et al, 1987). This is consistent with an open reading frame of 1320 bases, a 5'-untranslated region of about 28 bases, a 3'-untranslated region of 23 bases and a poly(A) tail estimated to contain about 200 bases. A 1600 nucleotide-long LCAT mRNA has also been detected in the brain and testes of the rat (Warden et al, 1989).

The LCAT gene has been mapped to chromosome 16 in humans (Azoulay et al, 1987), in the location 16q22, and to chromosome 8 in mice (Warden et al, 1989). The LCAT gene has been sequenced to completion by McLean et al (1986). The gene is divided into six exons spanning about 4,200 base pairs, of which 1400 base pairs represent the coding sequences. The first exon encodes the 24 amino acid-long signal prepeptide and the first 27 amino acids of the mature protein. Exon five contains two regions of homology with other lipid-interacting proteins. One of these regions, by homology with porcine pancreatic lipase, codes for the active site of the enzyme. The other region shares a 66% DNA sequence homology with the 3’ terminal coding region of the apoE gene and may encode an amphipathic α-helix, a structure implicated in lipid binding. Exon six, which codes for nearly half of the protein, has a very short (23 nucleotides long) 3’ untranslated region. Thus the polyadenylation signal AATAAA which precedes the poly A site in eukaryotes by 20-30 nucleotides (Proudfoot & Brownlee, 1981), is partially contained in the codon for the COOH-terminal glutamine (GAA) and in the translation stop codon TAA. All intron-exon borders conform to the GT-AT rule and to reasonable variants of the surrounding splice site consensus sequences. The largest intron in the LCAT gene lies between exons 5 and 6 and contains three 'Alu' repeat
sequence elements. The 5' mRNA start site was assigned to nucleotide 809+/-2 by primer extension and RNAsese mapping. Southern blot analysis suggests there is only one LCAT gene in humans, although more distantly related genes may exist.

**The LCAT gene promoter**

A putative TATA box consensus sequence (GATAA) was identified 23 base pairs upstream from the proposed start site (McLean et al, 1986), and a second sequence was found (GGCAATCT) at 100 base pairs 5' to the start site which resembles the consensus CAAT box sequence GGP_yCAATCT. Both these sequences are recognisable elements of many eukaryotic gene promoters. More recently, Meroni et al (1991) used the cloramphenicol acetyl transferase (CAT) gene to identify the promoter sequences responsible for LCAT transcription. These workers isolated 2.9kb of the 5' flanking sequences of the LCAT gene incorporating the LCAT promoter and used them to generate a series of deletion fragments with different 5' ends and a common 3' end at position +14 relative to the transcription start site. These fragments were then fused upstream to the CAT gene and transfected into both Hep3B (hepatic) and HeLa (non-hepatic) cells. Their results showed that approximately 300 base pairs of DNA upstream of the transcriptional start site of the LCAT gene was sufficient for its expression in both Hep3B and HeLa cells. The 71 bp construct dropped to 50% of the full CAT activity, while further deletion to 42 bp reduced CAT expression to background values comparable to those obtained with the promoterless expression vector. The region between sequences -71 and -42 was found to contain two consensus sequences for the Sp1 transcription factor and one potential recognition site for the liver specific transcription factor LF-AI, required for the expression of several genes in the hepatocyte. The presence of a TATA box-like sequence was confirmed at position -30 upstream from the transcriptional start (CAP) site. Site directed mutagenesis on the LF-AI site ruled out the importance of this sequence for LCAT gene transcription, while mutagenesis at both distal and proximal Sp1 sites demonstrated a reduction in
CAT activity to 70% and 35%, respectively. Mutation of both Sp1 sites resulted in a decrease in CAT activity to almost background levels. The two Sp1 binding sites therefore appear essential for promoter activity.

A recent study (Hoppe & Francone, 1998) has now defined the cis-elements and identified the transcription factors for the proximal LCAT gene promoter. In this study deletion mutants were used to identify GC-rich regions (-29 to -47) and (-49 to -65) that stimulate transcription in HepG2 and HeLa cells. They also identified Sp1 and Sp3 as the transcription factors interacting at these sites. They suggested that Sp1 activates the human LCAT promoter, while Sp3 functions as a dose-dependent repressor of Sp1-mediated activation and that Sp3 levels or the nuclear Sp1/Sp3 ratio may determine the transcriptional activity of the LCAT promoter in vivo.

**DNA polymorphism**

To date only one polymorphism has been identified for LCAT, in baboons (Hixon et al, 1990). Digestion of the pUCLCAT.10 plasmid containing human LCAT cDNA (McLean et al, 1986) by the enzyme Pvu11 (CAG/CTG) produced two bands corresponding to a two allele polymorphism, at either 2.9 (Pv1) or 2.7kb (Pv2). The Pvu11 site has been mapped to intron 5 (Rainwater et al, 1992). These authors found that baboons heterozygous for the Pv1/Pv2 alleles had greater LCAT activity than homozygous baboons (Pv1/Pv1). The heterozygotes also exhibited higher plasma HDL-cholesterol and HDL-apo-A1 levels. Homozygotes for the rarer allele (Pv2/Pv2) were also described by Kammerer et al (1993) who observed that animals homozygous for this allele had HDL cholesterol levels that averaged 18-19% lower than animals homozygous for the more common (Pv1) allele.

### 1.3.6 Catalytic mechanism of LCAT

The LCAT reaction has been studied using a variety of substrates including vesicles (Nichols & Gong, 1971), native lipoproteins (Dobiasova, 1983) and
reconstituted HDL (rHDL), (Matz & Jonas, 1982; Bolin & Jonas, 1994). The natural acyl donor for the LCAT reaction, due to its relative abundance in HDL (74% of phospholipids) is lecithin (Glomset, 1968), although phosphatidylethanolamine may be a superior substrate (Pownall et al, 1985b). LCAT favours the C-2 fatty acid acyl group of lecithin for transacylation (Glomset, 1962), preferably longer chain, more unsaturated fatty acids (Goutas, 1972), although the enzyme exhibits incomplete position specificity, i.e. fatty acids in the C-1 position can also be used for cholesteryl ester formation (Aron et al, 1978; Assmann et al, 1978). A phosphate group appears important for substrate recognition, while the sterol acyl acceptor (e.g. cholesterol) requires a 3-β-hydroxyl group and a trans configuration of the A/B rings (Piran & Nishida, 1979). LCAT will also transfer the C-2 fatty acid of lecithin to water, diglyceride and lysolecithin (Czarnecka & Yokoyama, 1993).

**General mechanism of LCAT action**

The first step in the LCAT reaction is a phospholipase A₂ activity (Aron et al, 1978; Yokoyama et al, 1980) to produce lysolecithin and an acylated enzyme and is followed by the transfer of the acyl group (Jahiainen & Dolphin, 1986) to cholesterol (or other acceptors). Chemical modifications have been used to identify regions of the LCAT molecule essential for catalysis. Modifying serine residues of pure LCAT (Jauhiainen & Dolphin, 1986, Jauhiainen et al, 1987) inhibited phospholipase A₂ activity, the first portion of the LCAT reaction, while similarly, blocking the two free sulphydryl groups of cysteine selectively reduced cholesteryl ester formation without significantly affecting phospholipase activity. They concluded that transfer of the acyl group from the sn-2 position of lecithin involved serine residues but not sulphydryl groups, while the last part of the reaction, fatty acid transfer to cholesterol, required free sulphydryl groups. Titrating the sulphydryl modifications indicated that both cysteines were required for full transacylase activity, suggesting that these two LCAT sulphydryl groups were equally involved in catalysis. Later work supported this suggestion since both cysteine thiol groups were located close
to the serine hydroxyl group (Jauhiainen et al, 1987). Accordingly, they proposed a general reaction mechanism for the catalytic action of LCAT. Cleavage of the sn-2 ester bond of lecithin by LCAT involves transfer of the fatty acyl group to Ser-181 to form a serine oxyester acyl enzyme intermediate and lysolecithin. The fatty acyl group is then transacylated to the nearby sulphur atom of either Cys-31 or Cys-184 with which it forms a thioester intermediate. The cysteine thioester subsequently donates its fatty acyl group to cholesterol, forming cholesteryl ester and regenerating the non-acylated enzyme. Acylated LCAT may also cleave a second lecithin molecule forming another serine oxyester which donates its fatty acyl to the unused cysteine; the dithioesterified enzyme then sequentially transfers both fatty acyl groups to cholesterol acceptors.

Histidine also seemed to be required for full LCAT activity. Thus, diethylpyrocarbonate (DEPC) which modifies histidine, inhibited both phospholipase A\textsubscript{2} and transacylase activities, suggesting that histidine participates in initial hydrolysis of lecithin and formation of the serine-fatty acyl intermediate, and possibly in the subsequent transacylation reactions. Later work using boronic acids (Jauhiainen et al, 1987) demonstrated that a tetrahedral adduct might be formed between serine and lecithin, the enzyme-substrate adduct decaying, with C-O bond cleavage to form a fatty acyl serine oxyester. This mechanism would be similar to that employed by pancreatic lipase and lipoprotein lipase and suggests that serine, histidine and aspartic acid may form a 'proton relay' or 'catalytic triad' to produce a fatty acyl-serine acyl-enzyme with final donation of the fatty acid to the 3-OH group of cholesterol (Jauhianin et al, 1987).

Lysolecithin endogenously generated by deacylation of lecithin in the first step of the LCAT reaction (the phospholipase A\textsubscript{2} step) is also a good fatty acyl acceptor in what is essentially a reversal of this step, showing that the LCAT reaction is always partly 'idling' (Czarnecka & Yokoyama, 1993). In this
lysolecithin acyltransferase or 'LAT' reaction, the preferred substrate is LDL and the reaction may require apoB (Subbaiah & Bagdade, 1979; Subbaiah et al, 1980), but there is no net formation of lecithin or lysolecithin. The reacylation of lysolecithin is probably the major portion of the lipid esterification by LCAT on LDL, but the rate of the LCAT reaction is overall much lower compared to that in HDL (Czarnecka & Yokoyama, 1993). Therefore, a significant part of acyl chains cleaved from glycerophospholipids by LCAT appears to be 'wasted', i.e. used for reacylation of lysolecithin on plasma lipoproteins. This evidence, along with the observations by Sorci-Thomas et al (1990) that LCAT is able to react with cholesteryl ester to produce free cholesterol, suggests that the entire LCAT reaction may be reversible. However, no evidence was found for the formation of free fatty acids or phospholipids and only a small percentage (5%) of the total reaction products were in the form of free cholesterol. It was thus proposed that the cholesteryl ester substrate produces free cholesterol and an ester acyl enzyme intermediate, which can only produce another cholesteryl ester product, but not the oxyester acyl enzyme intermediate. The LCAT reaction thus appears only partially reversible.

Site-directed mutagenesis has been used by several groups to confirm or further elucidate the role of specific residues in the catalytic mechanism of LCAT. Francone & Fielding (1991) demonstrated that replacement of the free cysteine residues with glycine did not result in a reduction of expressed LCAT specific activity, as predicted from the chemical modification studies in cultured Chinese hampster ovary cells stably transfected with the human mutant gene. More recently, Qu et al (1993a) found that replacing the free cysteines at residues 31 or 184 resulted in a 50% reduction of LCAT activity compared to the wild type. These data suggest that the free cysteines at residues 31 or 184 may enhance, but are not essential, for LCAT activity, since they are not directly involved in catalysis and that the acyl-cysteine intermediate proposed
by Jauhiainen & Dolphin (1986) is not required for the acyltransferase step. Qu and colleagues (1993a) also found that disruption of the disulphide bonds, by replacing either of the cysteines in the disulphide link, resulted in a reduction (Cys 50 -Cys 74 link) or complete inhibition (Cys 313-Cys 356 link) in LCAT secretion from the cells. They concluded that the disulphide bond between Cys 313 and Cys 356 is required for secretion and activity of the enzyme.

Francone & Fielding (1991) found that substitution of serine residues in the human LCAT gene at position 186 or 216 by residues which do not form acylated intermediates in catalysis (glycine, alanine or threonine) provided strong evidence that Ser-181 of LCAT does form part of the active site of LCAT (none of the three mutants at this position had any detectable residual activity), while the modification of Ser-216, although resulting in a mutant enzyme that still retained LCAT activity, nevertheless provided evidence of an indirect role in the LCAT catalytic mechanism. Substitution of the serine at this position by alanine increased the LCAT catalytic rate, while substitution by glycine resulted in a 90% drop in enzymic activity. This region is therefore extremely sensitive to modification and may be comparable to a similar sequence in pancreatic lipase representing the beginning of a helical 'flap' which covers the active site in the absence of substrate. The modification of Ser-216 may reduce the energy required to transform LCAT from a 'closed' to an 'open' structure involving the adjacent helical loop if this structure is also present in LCAT. Therefore, Ser-216 may have an important role in orienting substrates for effective LCAT action. This data, together with the mutagenesis data of Francone and others (Francone & Fielding, 1991; Qu et al, 1993a) provides evidence against a unique role of free cysteines in the catalytic mechanism of LCAT. This also suggests that the structure of LCAT is closely related with and has a similar catalytic mechanism to that of other lipases in which serine, histidine and aspartic acid are the components of an active site triad forming a
fatty acyl-serine acyl-enzyme where the fatty acid is then donated to the 3-OH group of cholesterol.

**1.3.7 LCAT as a glycoprotein**

LCAT is a glycoprotein with a carbohydrate content of 25% of the molecular mass (approximately 65-69 kDa) of which 13% is hexose (mannose and galactose), 6% glucosamine and 5% sialic acid (Chung et al, 1979). LCAT has four potential N-glycosylation sites - the asparagine (Asn) residue acceptor is located in the tripeptide sequence N....Asn-aa-Ser/Thr....C, where aa represents any of the 20 common amino acids. Removal of the terminal sialic residues has been reported to result in a 1.5-fold increase in enzyme activity (Doi & Nishida 1983). Protein-bound oligosaccharides are classified according to the covalent linkage between amino acid and carbohydrate, the most common classes of glycoproteins being those which contain N-glycosidically linked oligosaccharide chains attached to Asn residues, and O- glycosidically linked oligosaccharide chains linked to Ser or Thr residues in the polypeptide (Lennarz, 1983). Asn-linked glycoproteins can be divided into two main categories: high mannose, comprised of 8 mannose residues attached to the polypeptide and complex oligosaccharide-containing proteins containing 3 mannose residues attached to at least two sialic acids, galactose and N-acetyl-glycosamine (GlcNAc) moieties. A third type is a hybrid mixture of the two. The initial steps in the formation of the glycoprotein are carried out in the endoplasmic reticulum (ER) and involve the transfer of dolichol-linked, glucose-capped high-mannose structures to the asparagine residues of the polypeptide. Within the ER, the high-mannose chains are uncapped by sequential glucosidase activities. These structures are further modified in both the ER and golgi compartment by the action of specific glycosidases, followed by the addition of hexose, hexosamine and sialic acid residues in the golgi compartment to produce the oligosaccharide sequences of the mature protein. Collett & Fielding (1991) demonstrated that digestion of purified plasma LCAT
by N-glycanase to remove N-linked oligosaccharide chains produced a protein with an apparent molecular weight of about 46 kDa, which was comparable to the molecular weight (46.917 kDa) of the mature protein moiety. This suggested that LCAT contains a large N-linked carbohydrate moiety and no, or very little, O-linked sugar. Treatment with endo F, an enzyme that cleaves the bonds of many high-mannose N-linked sugars also reduced the molecular weight of LCAT, indicating the presence of high-mannose chains. Similarly, the use of tunicamycin in transfected cells, which inhibits the synthesis of the dolichol-linked sugars initially attached to asparagine sites (Tkacz & Lampen, 1975) thereby preventing the attachment of N-linked carbohydrate chains, confirmed the predominance of N-linked sugars in this enzyme. Thus, newly secreted LCAT, like the circulating plasma protein, contains a substantial carbohydrate moiety. These inhibitors of N-linked sugar processing had no effect on LCAT protein secretion or rate of accumulation of LCAT protein in the culture medium of CHO cells transfected with the human LCAT gene, however they were very effective in suppressing activity of the enzyme. The data therefore suggest an unusual role for high-mannose carbohydrates for the catalytic mechanism of LCAT, in that LCAT activity, but not the syntheses or secretion of LCAT protein, is dependent upon the addition of N-linked carbohydrate to the nascent LCAT polypeptide of at least one uncapped high-mannose chain.

Site-directed mutagenesis was carried out to eliminate each of the four potential sites for N-linked glycosylation (Asn-20, Asn-84, Asn-272 and Asn-384) by the substitution of asparagine by glycine and the mutant proteins expressed in COS cells (O et al, 1993a). The amount of secreted LCAT protein although similar to that of the wild-type enzyme, had a 3-4 kDa reduction in molecular mass but the rate of secretion appeared to be unaffected. Loss of carbohydrate at positions 20, 84 or 272 produced a decrease in LCAT activity of 18%, 82% and 16%, respectively, whereas loss of glycosylation at position
384 resulted in a two-fold increase in enzyme activity. This suggests that the carbohydrate group at position 384 may have an inhibitory effect on LCAT activity, possibly by sterically hindering access of the substrate to the active site. The elimination of all four potential glycosylation sites in a quadruple mutant reduced both the secretion and catalytic activity of LCAT by \( \approx 90\% \). Thus, while complete N-linked glycosylation does not appear to be required for LCAT secretion, it does appear to be necessary for generation of the full-sized LCAT protein. Site-directed mutagenesis studies by Qu et al (1993b) have confirmed all four potential sites to be N-glycosylated. Asn-272 was implicated as essential for the intracellular processing of LCAT while glycosylation at residues 20 and 384 was not essential for intracellular processing, secretion or activity. The amounts of LCAT found within cells transfected with wild-type or mutant LCAT cDNA corresponding to the mutations Asn-20-> Thr, Asn-84->Thr, or Asn-384 ->Thr, were similar and there was little difference in the amount of LCAT in the media, suggesting that glycosylation at these three sites is not required for effective secretion of LCAT. By contrast, site-directed mutagenesis at residue 272 resulted in the absence of LCAT, either intracellularly, or secreted into the cell culture medium. This indicates that the carbohydrate linkage at Asn-272 is necessary for secretion-competent LCAT. Although the amounts of LCAT found in the media of secretion-competent transfectants of LCAT were normal, their specific activities differed. Kinetic data from these experiments showed that carbohydrates at residues 20 and 384 have little effect on the activity and specificity of LCAT, whereas the specific activity of LCAT mutant Asn-84-> Thr was significantly reduced compared to the wild-type. Qu and colleagues inferred that the major effect of this residue is on the catalytic step and suggested that although none of the glycosylation sites of LCAT are apparently near the active site of the primary structure, the mechanism may involve a steric effect if Asn-84 is close to the active site in the three-dimensional structure. Residue 84 therefore appears to be essential for full activity of the enzyme, but not for intracellular processing.
and secretion. A greater substrate affinity was demonstrated by the LCAT mutant Asn-384. This could be attributed to either the loss of carbohydrate causing a resultant decrease in free energy of transfer of the phosphatidylcholine (lecithin) molecules into the active site region, or the removal of hydrophilic carbohydrate, thus increasing the hydrophobic association of an interfacial binding site to the substrate surface.

1.4 Physiological role of LCAT

1.4.1 LCAT deficiency

LCAT deficiency is characterised by a combination of clinical, tissue and plasma lipoprotein abnormalities that result from the failure of LCAT to esterify cholesterol in the plasma. Primary or familial LCAT deficiency is inherited as an autosomal recessive trait and was first described by Norum & Gjone (1967) in a Norwegian family. Subsequently, more than 50 patients have been described, from over 30 families in different geographical areas world-wide, and the physiological and pathological manifestations associated with familial LCAT deficiency have been extensively reviewed, by Glomset (1983) and others. Studies with antibodies have demonstrated that some individuals with LCAT deficiency have no detectable serum LCAT protein, while low, but variable, levels of non-functional LCAT protein have been detected in others. This implies that different mutations in the LCAT gene have arisen independently to produce the phenotypic heterogeneity observed in familial LCAT deficiency (Humphries et al, 1988).

The primary effect of the absence of LCAT is the inability to convert free cholesterol to its esterified form in plasma. This results in the accumulation of abnormally high levels of free cholesterol and phospholipid in all lipoprotein fractions. Affected individuals thus show abnormal serum lipids and lipoproteins, although the total cholesterol concentration may be normal. VLDL
contain low amounts of protein, especially apo C-11 and C-111 and are high in free cholesterol. LDL are depleted in apoB and after agarose gel electrophoresis usually yield three well-defined fractions, compared to normal LDL which is composed of one fraction only. One of the subfractions is disc-shaped and is enriched in free cholesterol, lecithin and apoC, thereby resembling lipoprotein-X, which otherwise is found only in cholestasis (Hamilton et al, 1971). HDL from LCAT-deficient patients consists of two types of unusually small particles enriched in lecithin and free cholesterol. One type is spherical and contains only apo-A1, the other type is discoidal and contains either apoE or apo-A1 and apo-A11. The discoidal apoE particles resemble the 'nascent' HDL particles found in rat and monkey perfusates or those secreted by HepG2 cells (McCall et al, 1988; Norum et al, 1989). In the presence of exogenously supplied LCAT, both 'nascent' HDL-like and discoidal apoE particles from LCAT-deficient patients are converted into plasma-like HDL, with a similar size, shape and cholesteryl ester content. Similarly, the small apo-A1-containing particles isolated from either LCAT-deficient patients or from the medium of Hep G2 cells are an even better substrate for LCAT than apoE discs. These particles thus represent newly secreted lipoproteins, either synthesised by the liver or intestine, or derived from the redundant surface coat of triglyceride-rich lipoproteins during lipolysis. The failure to transform these particles to mature HDL₂ and HDL₃ appears to be a direct metabolic consequence of LCAT deficiency.

Clinical abnormalities caused by the widespread disturbance of lipid metabolism lead to the accumulation of lipid in many organs, particularly in the glomeruli of the kidneys causing renal failure and death. Other symptoms include corneal opacities caused by lipid deposition, anaemia and frequently (but not always) proteinurea. The tissue abnormalities include foam cells in the bone marrow and spleen, as well as the presence of 'sea-blue histiocytes' in which the granules are composed of a lamellar arrangement of membranes
which are thought to be made up of cholesterol and lecithin (McIntyre, 1988). Target-shaped erythrocytes contain abnormally high amounts of unesterified cholesterol and lecithin and have a reduced life-span. Some patients present with early atherosclerosis due to lipid accumulation in the vessel walls of the aorta and large arteries, and foam cells are also present; however, only 35% of the cholesterol is in the form of cholesteryl ester, compared to about 75% in normal atheroma.

1.4.2 Genetic basis of familial LCAT deficiency

LCAT deficiency has been shown to be associated with point mutations in the LCAT gene and does not appear to be the result of large deletions or rearrangements (Humphries et al, 1988). A number of mutations in the LCAT gene have been reported, leading to familial LCAT deficiency. These include missense mutations in codons 147 (exon 4; Taramelli et al, 1990), 228 (Gotoda et al, 1991), 293 (in exon 6; Maeda et al, 1991), 156 (Klein et al, 1993a), a single nucleotide insertion in exon 1 (Bujo et al, 1991), an in frame GGC (Arg) insertion between codons 140 and 141 (Gotoda et al, 1991), and a substitution at codon 83 converting Tyr to a stop codon (Klein et al, 1993a). Thus, so far no common defect of the gene has been identified; each case family is caused by different and independent defects of the LCAT structural gene, leading to differential phenotypic expression of familial LCAT deficiency. This suggests that the normal functioning of LCAT requires the structural integrity of several different domains. Most patients with familial LCAT deficiency carry two identical defective alleles, although there are exceptions where a clinically affected patient has been identified as a compound heterozygote for two different mutations (Funke et al, 1993; Taramelli et al, 1990). Clinical abnormalities in heterozygotes are not seen, although these types exhibit marked reductions in HDL cholesterol and apo A-1 concentrations compared to unaffected individuals (Funke et al, 1993). In most cases the loss of LCAT
activity is associated with the absence of LCAT mass from the plasma compartment. Exceptions are the mutations in codons 147, 228 and 293, where no or very little LCAT activity but 40-60% of normal mass is found, and in 'Fish-eye disease' where the ability of LCAT to act on small substrate particles is lost, despite the presence of half normal LCAT mass. Fish-eye disease (Carlson & Holmquist, 1985) has been described as a second form of LCAT deficiency, phenotypically distinct from 'classic' LCAT deficiency and is so named because the first patients described had corneal opacities that caused their eyes to resemble those of boiled fish. Fish-eye disease (FED) patients also present with partial LCAT deficiency and levels of HDL protein and lipid about 10% that of normal and are characterised by a marked loss of LCAT activity towards HDL substrates. This activity has been termed α-LCAT in contrast with β-LCAT which describes cholesterol esterification in apoB-containing lipoproteins, i.e. LDL and VLDL. The β-LCAT activity is fully maintained in patients with FED but is absent in familial LCAT deficiency (Carlson & Holmquist, 1985; Holmquist & Carlson, 1987). Both LCAT activities (α and βLCAT) reside on the same protein (O et al, 1993b). Thus, FED patients retain the ability to esterify cholesterol, resulting in a near normal ratio of plasma unesterified cholesterol / total cholesterol. Absence of α-LCAT in the presence of β-LCAT results in net cholesterol esterification rates and unesterified cholesterol / total cholesterol ratios that are near normal in plasma but decreased in HDL. Furthermore, in the plasma of FED patients, the percentage decrease in LCAT mass is less marked than LCAT activity so that specific LCAT activity (activity per mass) is severely reduced. Renal disease is not a feature of FED, in contrast to patients with classical LCAT deficiency, presumably because cholesterol esterification in plasma does proceed through alternative lipoprotein substrates. Most FED cases for which a defect has been defined have a C-T substitution at codon 123 (threonine for isoleucine), away from the proposed active site (O et al, 1993b; Funke et al, 1991). This is consistent with changes in the structural organisation of the enzyme affecting
substrate access to the active site and hence the preferred lipoprotein substrates for the LCAT reaction switch from HDL to other lipoproteins in plasma (Assmann et al, 1991). However, recently, Klein et al (1993b) reported a unique defect in the LCAT gene of a patient presenting with FED, indicating that α-LCAT deficiency is not a prerequisite for FED. DNA sequence analysis identified a mutation in codon 300 resulting in the deletion of leucine-300. Expression of both the mutant and normal enzyme in human embryonic kidney-293 cells confirmed that the specific α-LCAT activity of the mutant LCAT enzyme was similar to that of normal LCAT; however, only a small fraction of mass and activity of the LCAT-deletion mutant was detected extracellularly, suggesting either a defect in LCAT secretion or enhanced degradation. Thus, deletion of leucine-300 results in the synthesis of an enzyme with normal specific activity but with decreased plasma levels. The distribution of the mutant enzyme on α and β- lipoproteins was found to be similar to that of normal plasma LCAT and significant levels of LCAT activity were detected in the patient's HDL fraction. On the basis of this study, the authors therefore proposed that the phenotypic heterogeneity of primary LCAT deficiencies is primarily dependent on the residual levels of activity of the mutant LCAT enzyme, and not on its location on the lipoprotein particles, and that classic LCAT deficiency and FED represent different clinical manifestations of the same genetic disorder.

1.4.3 Secondary LCAT-deficiency syndromes

LCAT activity is also reduced in some conditions of severe HDL deficiency which are not genetically related to mutations in the LCAT locus. These include Tangier disease, apo-A1/apo-C111 deficiency and apo-A1 milano. In these three conditions, the plasma levels of apo-A1 compared with normal controls vary from about 50% (in apo-A1 milano; Franceschini et al, 1990), to 0% (in the apo-A1/apo-C111 deficiency; Jonas, 1991). The LCAT mass and activity are
reduced to between 60-20% of normal, but the specific activity of the enzyme is essentially normal (Jonas, 1991), indicating that a fully functional enzyme is present in these conditions but that its level is reduced. The decreased LCAT mass may be associated with the increased catabolism of certain HDL subfractions containing bound LCAT (Holmquist & Carlson, 1987). The most common cause of secondary LCAT deficiency is liver disease.

1.4.4 Liver disease and LCAT deficiency

In severe liver disease, plasma LCAT activity is often reduced. This reduction has been shown to correlate with the amount of plasma cholesterol present as ester and appears to be due to reduced LCAT mass (Floren et al., 1987) and not to inhibitors, absence of activators or lack of suitable substrate lipoproteins (Simon & Schieg, 1970; Calandra et al., 1971). It was therefore suggested that the low LCAT activity is due to defective synthesis or impaired release of the enzyme from the damaged liver. The effects of hepatic and biliary disease can be separated in terms of the influences of cholestasis (a functional disorder of bile flow) and parenchymal damage (Miller, 1990). Intrahepatic cholestasis may occur in primary biliary cirrhosis and in some cases of alcoholic and viral hepatitis, while extrahepatic cholestasis is the result of obstruction of major bile ducts (commonly caused by gallstones or by carcinoma of the pancreas or bile ducts). Cholestatic liver disease is widely known to be associated with secondary hyperlipidaemia (Durrington, 1989) and is characterised by a disproportionate increase in plasma free cholesterol, accompanied by an equimolar increase of phospholipid, predominantly lecithin (Phillips, 1960), and cholesteryl ester and lysolecithin concentrations may also be reduced (Miller, 1990). Low LCAT activity may be partly responsible for the hyperlipidaemia of cholestasis, in view of the hyperlipidaemia seen in patients with familial LCAT deficiency and the similarities between their lipoproteins and those of patients with hepatic disease (Glomset et al., 1983) but this can only be a partial
explanation since the hyperlipidaemia of familial LCAT deficiency is frequently mild compared to that of patients with cholestasis, who may have normal LCAT activity, for example in the early stages of primary biliary cirrhosis (Miller, 1990).

The hyperlipidaemia of cholestasis is also associated with the presence of two abnormal LDL particles, LP-X and LP-Y; normal LDL is present although in reduced concentrations. LP-Y is an LDL-like particle which is triglyceride-rich and represents an accumulation of remnants (Durrington, 1989). LP-X comprises 25% cholesterol and more than 60% phospholipid (Durrington, 1989), and its origin is still the subject of speculation. Since LP-X largely consists of LCAT substrates and is also found in familial LCAT deficiency and in cholestatic patients with low LCAT activity, it has been suggested that reduced LCAT activity is a prerequisite for its formation. Furthermore, these particles are not seen in cholestatic patients with well-preserved levels of LCAT activity. However, they are not usually seen in cirrhotic patients, who may also have low LCAT activity (Day et al, 1979). Thus LP-X may appear only when the delivery of free cholesterol and lecithin into plasma exceeds the ability of the LCAT enzyme to cope with it. A lipoprotein particle similar to LP-Y is also seen in familial LCAT deficiency and in cholestasis when LCAT activity is low (Agorastos et al, 1978). HDL is often present in reduced amounts (Clifton et al, 1988) and the particles may resemble the disc-shaped, 'nascent' HDL secreted by the liver and intestine (Hamilton et al, 1976). Under the electron microscope this takes on the appearance of stacked discs. Concentrations of apo-A1 and apo-A11 may also be reduced, while that of apoE is increased (Danielson et al, 1975; Clifton et al, 1988). The observed increase in apoE is also seen in familial LCAT deficiency (Glomset et al, 1983) and therefore may partly be due to reduced LCAT activity. However, HDL concentrations are not always reduced in cholestasis; in patients in the early stages of primary biliary cirrhosis, HDL levels (especially HDL$_2$) may be increased. This is in contrast to
the reduced HDL found in more advanced disease and in patients with acute biliary obstruction due to other causes (Miller, 1990). Serum triglyceride concentrations may also be elevated in liver disease and in rats this has been shown to be partly explained by a defect in clearance of triglyceride (Komai, 1987). Hepatic secretion of triglyceride-rich particles may also be reduced. It has been argued that many of the lipoprotein changes observed in liver disease result from reduced LCAT activity, since they are not found in cholestatic patients with well preserved levels of the enzyme. Work by Floren et al (1987) suggests that variability in cholesterol esterification and apparent LCAT activity are indeed due to changes in circulating LCAT mass. In primary biliary cirrhosis, where there is a gradual progression of cholestasis, the reduction in LCAT activity may result partly from deteriorating hepatocyte function, but other mechanisms are probably also involved (Miller, 1990); Clifton et al (1988) observed that LCAT activity may fall to about 8% of normal in 2 weeks when hepatocyte function is still well preserved. Erythrocytes may appear as "target" cells in cholestasis due to an increase in their membrane cholesterol content as a result of the circulating free cholesterol-rich lipoproteins. Altered cell membrane lipid composition induced by abnormal lipoproteins affects the ability of membrane proteins to function, most commonly because the fluidity of the lipid bilayer is decreased (Owen, 1990).

The distinction between cholestatic and parenchymal liver disease is not clear-cut and in practice the two may co-exist in the same patient. Hepatocellular liver disease is often accompanied by hypertriglyceridaemia and hypercholesterolaemia. In some cases, an increased flux of fatty acids leading to increased hepatic synthesis of triglycerides may be set against an inability to secrete these adequately into the circulation; the fatty liver of alcoholic liver disease may be the result of such a mechanism (Durrington, 1989). VLDL levels are decreased and LDL is rich in triglycerides (McIntyre, 1988), but relatively depleted in cholesteryl ester. HDL, present at a stage closer to that of
nascent HDL, is rich in apoE and levels of apo-A1 and apo-A11 may be very low (Chang et al, 1986). In acute alcoholic hepatitis at least, the reduced concentration of apo-A1 is due to the increase in the fractional catabolic rate (Nestel et al, 1980). In alcoholic liver disease, HDL cholesterol and apo-A1 concentrations were found to decrease progressively with increasing severity of liver disease (Miller, 1990). Patients with chronic hepatocellular disease have a wide range of LCAT activities (Day et al, 1979). Patients with high LCAT activities had normal total serum cholesterol concentrations and HDL of normal composition and concentrations. Patients with low LCAT activities had reduced total cholesterol and phospholipids but normal total triglyceride levels. HDL was reduced and rich in free cholesterol, phospholipid and triglyceride and depleted in cholesteryl esters and protein. The low LCAT activity in parenchymal liver disease appears to reflect LCAT mass and is probably the result of impaired LCAT synthesis (Floren et al, 1987). Many of the effects seen in parenchymal liver disease may be explained in terms of the often profound LCAT deficiency which accompanies hepatocellular failure (McIntyre, 1988). Failure to esterify HDL cholesterol by LCAT prevents the development of the HDL core and the resultant diminished supply of cholesteryl ester from HDL to VLDL and LDL limits the reciprocal outward flow of triglycerides. In addition, a deficiency of hepatic triglyceride lipase may affect the removal of remnant-like particles and the abnormal LDL from the circulation.
1.5 Aims of the thesis

Since LCAT plays a central role in normal lipoprotein metabolism and because of the clinical complications associated with LCAT deficiency, it is important to understand the factors which affect LCAT activity as well as its synthesis and secretion. Studies of the LCAT gene may lead to a better understanding of the regulation of LCAT activity and its metabolism and will ultimately lead to the further identification of defects responsible for other LCAT deficiency states. Much research has to date centred around the characterisation of the properties of LCAT and while some workers have provided detailed information on the gene structure of LCAT, there is relatively little data available on the genetic level of regulation of this enzyme. This thesis attempts to study the relationship between LCAT synthesis and secretion and lipoprotein production in order to elucidate whether these two processes are linked. Factors that may be important in influencing LCAT regulation were explored by asking three basic questions:

1. Is LCAT synthesis and secretion linked to an increased production of triglyceride-rich lipoproteins?

Since LCAT is required for removal of excess surface components from hepatic VLDL, hepatic LCAT production may be linked to that of VLDL secretion. This question was examined using oleic acid to stimulate hepatic triglyceride and VLDL production in the human hepatoblastoma cell line, HepG2, and then measuring the changes in LCAT mRNA levels.

The presence of functional LCAT activity has been demonstrated in mesenteric lymph, suggesting that the small intestine may also produce LCAT (Clark and Norum, 1977). Although LCAT mRNA has not been detected in the colon (Tata et al, 1987) or the terminal ileum (McLean et al, 1986), the upper small intestine (duodenum), which is the major site of chylomicron secretion, has not previously been examined. As with hepatic VLDL, LCAT is involved in the clearance of the redundant surface components from chylomicrons following
lipolysis and expression of LCAT may therefore be linked to this process. Thus, RNA isolated from the duodenal enterocytes of the rat and guinea-pig was examined for LCAT expression. The question of whether LCAT mRNA or protein increases in response to oral fat (and hence chylomicron production) was also addressed, by measuring the LCAT protein and mRNA produced from the duodenal enterocytes of fat-fed guinea-pigs and rats.

2. **Is there a co-ordinate regulation between LCAT mRNA and the mRNA of apo-A1?**

Apo-A1 was chosen as it is the main co-factor for the LCAT reaction and thus may be similarly influenced by factors that affect LCAT expression. HDL or apo-A1 secretion were altered by use of the hypolipidaemic drug, gemfibrozil, by glucose deprivation in HepG2 cells, or by cholesterol-loading; any changes in either apo-A1 or LCAT mRNA levels were monitored by northern and slot-blotting.

3. **Is expression of the LCAT gene altered in liver disease?**

The reduced LCAT activity observed in patients with liver disease may be due to a defect(s) in LCAT gene expression. This hypothesis was tested by setting up various models for liver disease and examining LCAT mRNA levels, both in vitro, using HepG2 cells exposed to paracetamol, galactosamine and hydrogen peroxide, and in vivo, by injecting mice with galactosamine and other agents. Normal and diseased human liver samples were also obtained and LCAT mRNA levels similarly analysed.
CHAPTER 2

METHODS AND MATERIALS
2. Methods & Materials

2.1. Cell Culture Experiments

2.1.1 Cell lines

Several cell lines (listed below) were used in the study of LCAT expression.

**HepG2**: The hepatoma cell line HepG2 was established from a liver tumour biopsy (hepatoblastoma) of a caucasian male, aged 15, from Argentina; its morphological characteristics and epithelial cell shape are compatible with those of parenchymal cells. Histology of the liver biopsy revealed well differentiated hepatocellular carcinoma (Aden et al, 1979). This cell line was provided by Dr. A. Magee, National Institute of Medical Research (N.I.M.R.), Mill Hill, UK.

**Hep3B**: The human hepatocellular carcinoma derived cell line Hep3B was established from a liver tumour biopsy obtained during extended lobectomies of an 8 year old black male from the USA. by Aden et al, (1979). This cell line was obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, USA.

**Mahlavu**: The hepatocellular carcinoma derived cell line, Mahlavu, was established from a liver biopsy of a male from South Africa by Prozesky et al, (1973). It was obtained from Dr Jenny Walters, Department of Medicine, St. Mary’s Hospital, London.

**SK55**: This cell line has an apparent doubling time of 48 h and the epithelial cell shape is compatible with those of liver parenchymal cells. This cell line was a gift from Dr J. Monjardino, Department of Medicine, St. Mary’s Hospital, London, UK.

**PLC/PRF/5**: The human hepatoma cell line PLC/PRF/5 (often termed Alexander cells), was derived from a primary hepatocellular carcinoma of a 24 year old Shangaan male from Mozambique (Alexander et al, 1976). The cells
are epitheloid, resemble hepatocytes and have an apparent doubling time of 35 to 40 h. The cells were obtained from Dr Maria Barros, Department of Medicine, St. Mary's Hospital, London, UK.

**Skin Fibroblasts:** Primary cultures of fibroblasts were established in this department by Dr Bingle (RFHSM) from a skin biopsy taken from a patient with Wilson's disease.

Stocks of the human hepatoma cell lines, HepG2, Hep3B, Mahlavu, PLC/PRF/5, SK55 and human skin fibroblasts were removed from liquid nitrogen and quickly thawed at 37°C in a water bath. The cells were diluted into 10 ml of DMEM (Gibco) supplemented with 10 % (v/v) heat-inactivated foetal-calf serum (FCS), glutamine (2mM) and penicillin/streptomycin (100IU/ml and 100µg/ml respectively) and incubated at 37°C [in a plastic culture flask (75cm²)] in a humid atmosphere of 95% air and 5% CO₂. The medium was changed after approximately 24h. Growing cells were supplied with fresh growth medium every 48h until confluent and passaged weekly, when 1.5 x10⁶ cells were seeded into 75cm² (T-75) flasks. Passaging of cells was carried out as follows: monolayer cultures were washed three times in PBS (5 ml; Gibco) and released by incubation with 0.25% (w/v) trypsin-EDTA (Gibco) for 5-20 minutes at 37°C. The enzyme reaction was stopped by addition of growth medium (10 ml) and the cell suspension was centrifuged at 150 g for 5 min. The supernatant was discarded and the cells resuspended in growth media (1ml) as described above. The cells were then subcultured at split ratios of between 1:3 and 1:8 into plastic culture T-75 flasks for cell experiments or for continuing the cell line. For cells used in experiments, nearly confluent cells were washed with serum-free medium and then appropriate medium with various experimental supplements was added. Cell viability during experiments was assayed by use of the Trypan blue exclusion method.
2.1.2 Oleic acid treatment in HepG2 and Hep3B cells

In cell culture experiments with oleic acid, cells were grown to sub-confluency in T-75 flasks. Full medium was removed and replaced with 15ml medium, without foetal calf serum but containing [³H]-glycerol (100μCi per flask) and increasing concentrations of oleic acid (0.2-0.8mM), complexed to bovine serum albumin (BSA) (see 2.6). Control cells received medium containing BSA alone. The cells were incubated for 20h to allow incorporation of glycerol into lipids and the radioactivity was measured (see 2.2).

2.1.3 Preparation of gemfibrozil

Gemfibrozil (Parke-Davis Research Laboratories, UK) was dissolved in ethanol (1M stock solution) and was added to the culture media to give a final concentration of 100μM. This dose was chosen as it was similar to plasma levels (16-23 mg/l or 75-105μM) observed in men treated with 600mg gemfibrozil twice daily (product information, Parke-Davis Laboratories, UK, 1986). As with the oleic acid experiments, cells were grown in the presence of [³H]-glycerol in order to radioactively label newly synthesised triglycerides. Medium for control cells was supplemented with ethanol.

2.1.4 Protocol for lipoprotein-deficient serum experiments

The HepG2 cell line was grown to near confluence in T-75 flasks and the medium then changed to one containing 10% lipoprotein deficient serum (LPDS; Monge et al., 1989). After 24h in DMEM plus LPDS the cells were changed to fresh DMEM plus LPDS (control) or DMEM plus LPDS containing one of the following: LDL (200μg protein/ml), HDL (100μg protein/ml), or cholesterol (100μg/ml, added from a 5mg ml⁻¹ stock solution in absolute ethanol). After 24h the cells were harvested for RNA analysis.

In further experiments, cells were pre-incubated in a medium containing LPDS plus HDL for 24h. The cells were then divided into 3 groups according to their
subsequent treatment; continued incubation in LPDS plus HDL, incubation with LPDS plus LDL or incubation in LPDS plus free cholesterol.

2.1.5 Protocol for reduced glucose concentration

HepG2 cells were grown to near confluence in full medium (4500mg/l glucose) and then incubated for 2, 4, 6, 8 and 10h in glucose-free DMEM containing 5% foetal calf serum (dialysed against PBS to remove traces of glucose). Control cells were those grown throughout in medium containing glucose. The cells were lysed with guanidium thiocyanate and total RNA isolated (see 2.3.3). In other experiments, HepG2 cells were incubated for 16h in medium containing glucose at concentrations of 0, 500, 1000, 2000, 3000 and 4500 mg/l.

In order to determine whether the changes in apo-A1 and LCAT expression noted were due to lack of availability of glucose, rather than cell death, an assay was carried out to measure incorporation of $^{35}$S-methionine into cellular protein. This was achieved by growing HepG2 cells under the experimental conditions stated, but with $^{35}$S-methionine added to the cell culture medium. At the end of the experiment the amount of newly synthesised protein incorporated into the glucose-deprived cells was measured and compared to that in control cells. Measurement of protein incorporation was achieved by counting $^{35}$S-radioactivity in a beta-counter. A protein estimation was also carried out according to the method of Lowry (1951).

2.2 Measurement of Radiolabelled Triglycerides in cell cultures

Medium was removed from experimental flasks, centrifuged to remove any cell debris and 2ml taken for analysis of total triglyceride secretion by thin layer chromatography (TLC, see below). For measurement of intracellular triglyceride secretion, the cells were washed with PBS, then lysed using methanol. A lipid extraction was carried out before separation and analysis by TLC. The
remainder was dialysed against PBS to remove unincorporated glycerol for separation of the different lipoprotein classes. These were separated from medium by sequential ultracentrifugation. Fresh normal plasma (0.5ml) was added to the dialysed medium (as a carrier to increase the total mass of lipoprotein) and the density of the medium was adjusted to <1.019g/ml to allow the triglyceride-rich lipoproteins (VLDL and IDL) to float to the top of the tube after centrifugation. After centrifugation for 20h, at 16°C, 105,000g, the VLDL/IDL fraction was collected and further sequential centrifugations carried out at increased density (with addition of NaCl/NaBr) to isolate the LDL (d1.019-1.063g/ml) and HDL (d1.063-1.21g/ml) classes.

The Bligh and Dyer (1959) method of lipid extraction was used to remove proteins and other impurities and the lipid components separated by thin layer chromatography (TLC) on silica gel G plastic plates. An aliquot (100μl) of [14C]-cholesterol in isopropanol was added to each sample as an internal standard prior to application to the silica plate; this allowed calculation of recovery of each sample. Following chromatography, the [14C]-cholesterol and the [3H]-triglyceride bands were cut out, placed in scintillation fluid and the radioactivity quantified in a liquid scintillation counter.

### 2.3 Northern blot analysis

#### 2.3.1 Large scale preparation of plasmid DNA for cDNA probes

The following procedures are taken from Sambrook, Fritsch and Maniatis, (1989):

**Growth of Bacteria**

Plasmids were produced as follows: glycerol stocks of E.Coli bacteria containing the relevant plasmid were streaked onto agar plates with the appropriate antibiotic. A single bacterial colony was removed from the plate and grown overnight at 37°C in 10ml L-B broth medium (per litre: 10g Tryptone;
5g yeast extract; 10g NaCl) containing the relevant antibiotic at a concentration of 2μ/ml. The fresh overnight culture (2ml) was then added to 500ml of sterile L-B broth, with 2μ/ml of the appropriate antibiotic, in a 2L flask and grown overnight at 37°C in a shaking incubator.

**Amplification of plasmids**

This procedure was carried out on plasmids with a low copy number (e.g. PBR322) and was achieved by adding 100mg/ml of chloramphenicol to the L-B broth. This stops the growth of E.Coli but allows the plasmids within the bacteria to continue replicating.

**Plasmid DNA Extraction**

L-B broth containing the transformed E.Coli were centrifuged at 5000rpm at 4°C for 15 min. The supernatant was discarded and the bacterial pellet resuspended in 4.2ml of 15% sucrose, 50mM Tris-HCl pH 8.0 and 1ml of lysozyme (10mg/ml in water). This was left on ice for 15min with intermittent swirling before adding 2.3ml of 0.25M EDTA for a further 10min. Lysis mix (4.8ml) was then added (20mg/ml triton; 62.5mM EDTA; 50mM Tris-Cl, pH 8, in H2O) and the mixture swirled on ice. The lysed cell mixture comprising precipitated protein and debris was centrifuged at 30,000rpm in an angle rotor for 30min and the pellet containing E. Coli chromosomal DNA removed. The supernatant was adjusted to 12.5ml with Tris-EDTA buffer (T.E. buffer; 10mM Tris, 1mM EDTA) and 12.5g of caesium chloride added before centrifuging at 30,000rpm for 30min at 25°C. The floating pellet was discarded and 1.25ml of 10mg/ml ethidium bromide was added. The tubes were rebalanced and centrifuged at 37,000rpm for 48h at 20°C. The plasmid band (identified by ethidium bromide under UV light) was removed and the ethidium bromide removed with 3 or more extractions with isopropanol saturated with CsCl solution. The CsCl was removed by dialysing against T.E. overnight. The T.E solution containing the plasmid was extracted with an equal volume of phenol and ether, then ethanol precipitated at -70°C overnight before centrifuging.
(microfuge) for 30 min and resuspending in 200-300\(\mu\)l TE. The DNA was checked by digesting a small aliquot for analysis on an agarose gel.

**Assessment of DNA concentration**

The DNA was diluted 1:50 in TE and scanned by spectrophotometry at wavelengths 220-300nm. DNA produces a peak at about 260nm and in its pure form gives a ratio of 2.0 when divided by the optical density (OD) reading at 280nm (protein). However, if there is any protein contamination the ratio between the OD at these two wavelengths is decreased. The DNA concentration was calculated as follows:

Double strand DNA, 1 OD unit \(\text{260nm} = 50\mu\text{g/ml}\)

DNA concentration = \(\frac{\text{OD}_{260nm}}{50}\) (for dilution) \(\times 50\mu\text{g/ml}\)

Average yields obtained were 500-1000\(\mu\)g, which was then dissolved in 200-300\(\mu\)l T.E.

### 2.3.2 Probe preparation and radioactive labelling by random oligonucleotide primer extension

The following procedures were based on the method described by Feinberg and Vogelstein (1983)

**Preparation of probe**

The plasmid containing the cloned DNA to be used as a probe was digested with appropriate restriction enzymes to cut out the insert. Sufficient plasmid was used to give 2-5\(\mu\)g of insert DNA. After checking for complete digestion, the remaining sample was loaded onto a 1% low-melting point agarose gel and subjected to electrophoresis alongside a size marker until the insert band was clearly separated from the vector band. The insert band was then cut out of the gel, with minimal accompanying agarose. It was weighed and at least 3x the weight of water was added to give a final concentration of insert of approximately 1.5-3ng/\(\mu\)l, which was divided into 32\(\mu\)l aliquots (~ 50-100ng) and stored at -20°C.
Description of Probes:

The DNA probes used were as follows:

<table>
<thead>
<tr>
<th>GENE</th>
<th>VECTOR</th>
<th>SOURCE</th>
<th>LENGTH</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo-A1</td>
<td>pUC8</td>
<td>genomic</td>
<td>2.2Kb</td>
<td>Karanthasis et al, 1983</td>
</tr>
<tr>
<td>Actin</td>
<td>pAM91/PBR322</td>
<td>mouse cDNA</td>
<td>1.1Kb</td>
<td>Humphries et al, 1981</td>
</tr>
<tr>
<td>LCAT</td>
<td>pUC19</td>
<td>cDNA</td>
<td>1.43Kb</td>
<td>McLean et al, 1986</td>
</tr>
</tbody>
</table>

Labelling Probes

An aliquot of probe was boiled for 3min immediately prior to use. The labelling reaction consisted of 10\mu l oligolabelling buffer (see below), 2\mu l BSA (10mg/ml), 32\mu l DNA insert/agarose solution, 5\mu l \(\alpha^32P\) dCTP (10 mCi/ml) and 1\mu l (1U/\mu l) Klenow polymerase fragment in a total volume of 50\mu l. The mixture was incubated at room temperature for 16-20h before column fractionation.

Composition of oligolabelling buffer

To make the oligolabelling buffer, solutions A,B and C were mixed in the ratio of 1:2.5:1.5 (by volume). The buffer was divided into aliquots and stored at -20°C.

Solution O: 1.25 M Tris-HCl, 0.125 M MgCl₂, pH 8
Solution A: 1 ml solution O
18\mu l 2- mercaptoethanol
5\mu l dATP (0.1M in 3mM Tris-HCl, 0.2mM EDTA, pH7 [=TE])
5\mu l dTTP (TE, pH7)
5\mu l dGTP (TE, pH7)
Solution B: 2M HEPES, titrated to pH6.6 with 4M NaOH.
Solution C: Hexadeoxyribonucleotides in TE at 90 OD units ml⁻¹.

Separation of the labelled probe from nucleotides

A column of Sephadex-G50 beads, saturated with 3 x SSC (pH7) (1 x SSC is 150mM NaCl, 15mM sodium citrate, adjusted to pH7 with 1M HCl), was poured into a small Pasteur pipette plugged with polyallomer wool. The oligolabelling
reaction mix was mixed with 50μl dextran-blue dye solution, loaded onto the column and fractions collected. The dextran blue fractions, containing the labelled DNA, were pooled. 50μl was counted in a β-scintillation counter and the number of counts per minute were obtained.

**Restriction enzyme digestion**

Restriction enzyme digestion of plasmid DNA was usually carried out in a total volume of 40μl. The reaction mixture was as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>5μg</td>
</tr>
<tr>
<td>Salt buffer</td>
<td>4μl</td>
</tr>
<tr>
<td>BSA (1mg/ml)</td>
<td>4μl</td>
</tr>
<tr>
<td>restriction enzyme</td>
<td>5-20 U</td>
</tr>
<tr>
<td>H2O to 40μl</td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>40μl</td>
</tr>
</tbody>
</table>

Restriction enzyme buffers with varying salt concentrations were used according to the manufacturers' recommendations. The mixture was incubated at 37°C in a water bath for 12-16 h. The completion of digestion was checked by running a sample on an agarose gel (see below). If digestion was not complete, more enzyme was added to the sample, which was incubated further, prior to re-checking.

**Salt Buffer**

<table>
<thead>
<tr>
<th>Salt Buffer</th>
<th>NaCl</th>
<th>Tris (pH 7.5)</th>
<th>MgCl2</th>
<th>DTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>1M</td>
<td>500mM</td>
<td>100mM</td>
<td>10mM</td>
</tr>
<tr>
<td>Medium</td>
<td>500mM</td>
<td>100mM</td>
<td>100mM</td>
<td>10mM</td>
</tr>
<tr>
<td>Low</td>
<td>0</td>
<td>100mM</td>
<td>100mM</td>
<td>10mM</td>
</tr>
</tbody>
</table>

**Agarose gel electrophoresis for DNA separation**

For agarose gel production, the required volume of 1 x TAE buffer plus 1-1.2% (W/v) agarose suspension was prepared and heated in a microwave oven until dissolved. The gel was poured in a gel former, allowed to set and submerged in an elecrophoresis tank with 1 x TAE buffer (with or without
ethidium bromide). In the case of a low melting point agarose gel, the preparation was usually carried out at 4°C.

**Checking gels**
To determine whether digestion of plasmid DNA had taken place, one tenth of each sample was mixed with 10 x loading buffer and subjected to electrophoresis at 100 volts (V) in a 1% agarose gel. It was then visualised in the presence of ethidium bromide with a UV transilluminater. Gels were run with at least one well containing a size marker.

**Size markers**
Lambda-Hind III (Sigma) 8 bands of sizes between 23.1 and 0.125kb.
1 kb ladder (Sigma) 18 bands of sizes between 12.2 and 0.2kb.

To separate any rejoined cohesive ends, the size markers were heated to 65°C for 5 min prior to loading on gels. The gel was photographed, with a ruler alongside the size marker for sizing of bands, using a mounted polaroid camera and UV transillumination.

**Buffers used were as follows:**

<table>
<thead>
<tr>
<th>10x Loading buffer</th>
<th>Electrophoresis buffer (TAE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25% bromophenol blue (BPB) (w/v)</td>
<td>0.04M Tris</td>
</tr>
<tr>
<td>25% Ficoll 400 (w/v)</td>
<td>0.001M EDTA</td>
</tr>
<tr>
<td>0.1M EDTA</td>
<td>titrated to pH 8 with glacial acetic acid</td>
</tr>
<tr>
<td></td>
<td>0.5μg/ml ethidium bromide</td>
</tr>
</tbody>
</table>

**2.3.3 Isolation of steady-state mRNA**
The following method is described in Current Protocols in Molecular Biology (1987-1991). The cell monolayer (20x10⁶ cells per flask) was washed with PBS and lysed by the addition of 5ml guanidium thiocyanate (GT; 4M). The lysate was taken three times through a 21G needle to shear chromosomal DNA in
order to minimise its co-precipitation with RNA. Tissues snap-frozen in liquid nitrogen were ground under liquid nitrogen with a pestle and mortar (on ice), with approximately 1ml of GT and were then taken sequentially through 19G, 21G and 23G needles. The lysate was then transferred to an eppendorf tube and microcentrifuged at high speed (14000g) for 5 minutes, to remove cell debris. The supernatant containing the RNA was then removed.

Total RNA was isolated by caesium chloride ultracentrifugation essentially as described by Glison et al, (1974). The GT cell or tissue lysate was layered onto 3.5ml cushion of caesium chloride (5.7M CsCl) to create a step gradient and centrifuged in a swing out rotor at 33,000rpm for 20h. After aspiration of the supernatant (containing the DNA), the RNA pellet was washed in 70% ethanol and briefly air-dried. The pellet was dissolved in water and reprecipitated on dry ice plus ethanol for 30min or overnight at -20°C with 1/10 vol. of 3M sodium acetate (pH 5.2) and 2 vol. of ethanol. The pellet was washed in 70% ethanol, dried briefly and dissolved in sterile distilled water. The optical density was read at 260nm and 280nm and the total amount of RNA calculated using the formula:-

\[
1 \text{ absorbance unit at 260 nm} = 40 \mu g \text{ ml}^{-1} \text{ of RNA}
\]

2.3.4 Electrophoresis of steady-state RNA for analysis (Northern Blotting)

For Northern blot analysis, total RNA was separated by electrophoresis in formaldehyde-containing agarose gels (Sambrook, Fritsch and Maniatis, 1989; book 1). Comparable loading in each lane was verified by ethidium bromide staining and visualisation of ribosomal bands (28s: 4.85 Kb and 18s: 1.74 Kb). An RNA ladder (0.24- 9.5 Kb; BRL) was also run on the same gel and later stained with ethidium bromide. The distance run down the gel by the different fragments was then plotted against fragment size in order to be able to identify any signals picked up on the autoradiograph (see later). Between 10 and 20μg
of RNA was denatured at 55°C for 15min in the presence of formaldehyde. The samples were loaded on a 1.2% agarose / 2.2M formaldehyde gel and electrophoresis carried out for 4-6h. After blotting overnight, the RNA was fixed to the membrane by UV irradiation for 3min, and prehybridised overnight at 42°C in 50% formamide, 5 x Denhardt’s solution, 4 x SSC, 0.5% SDS and 0.02mg/ml of denatured salmon sperm DNA. The solution was replaced with fresh prehybridisation solution and the denatured (or single stranded) radioactive DNA probe was added to yield a final activity of 1 x 10^6 cpm ml^{-1}. The hybridisation was continued for at least 48h after which the filters were washed as follows: twice at room temperature in 2 x SSC + 0.1% SDS; at 55°C in 2 x SSC + 0.1% SDS; at 55°C in 1 x SSC + 0.1% SDS, and finally at 55°C in 0.1 x SSC + 0.1% SDS until background levels only could be picked up by a Geiger-counter. Filters were wrapped in cling-film and autoradiographed. The signal intensity on the autoradiograph was measured using a densitometer (Joyce-Loebl Chromoscan). Autoradiographs were photographed by the Medical Illustration Department of the RFHSM.

For slot blot analysis, five doubling dilutions of each sample starting from 10µg of total RNA (i.e. 5, 2.5, 1.25, 0.62 and 0.31µg) were denatured and applied to the wells of the minifold apparatus (Schleicher and Schuell) for blotting onto nylon filters. RNA from THP-1 cells was used as a negative control for all probes in slot blots (Tsuchiya et al, 1980).

2.4 Detection of secreted [^{35}S]-labelled LCAT protein from guinea-pig duodenum

2.4.1. Enterocyte isolation

Villus cells were isolated from duodenal and ileal segments as follows. Briefly, a 15cm length of intestine beginning 5cm from the pyloric sphincter or ending 5cm from the ileal-caecal junction was removed and washed through with cold NaCl (154mM) containing dithiothreitol (DTT, 0.5mM). One end was ligated and
the segment filled with buffer (96mM NaCl, 1.5mM KCl, 8mM KH$_2$PO$_4$, 5.6mM Na$_2$HPO$_4$, 27mM sodium citrate, 0.5mM β-hydroxybutyric acid and BSA, 1mg/ml), pH 7.3. The open end was also ligated and the segment incubated for 15 min at 37°C. The luminal solution was discarded and replaced with a second buffer (137mM NaCl, 2.2mM KCl, 11.5mM KH$_2$PO$_4$, 8mM Na$_2$HPO$_4$, 0.5mM β-hydroxybutyric acid, 1.5mM EDTA, 0.5mM DTT and BSA, 1mg/ml). The segment was incubated for a further 30 min and villus enterocytes were collected by manually dislodging the cells and washing with ice-cold bicarbonate saline containing BSA (1mg/ml). Cells were kept on ice and used within 60 min of isolation.

2.4.2 $^{35}$S methionine-labelling of enterocytes and protein isolation

$^{35}$S methionine (200µCi) in methionine-free medium was added to the guinea pig isolated duodenal enterocytes in PBS. The cells were incubated for 45 min in a waterbath at 37°C to allow incorporation of the radioactive methionine into newly synthesised cellular proteins and subsequent secretion of these proteins into the medium. The cells were then centrifuged at 200g for 5 min at 4°C and the supernatant removed to perform an immunoassay in order to detect whether any LCAT protein had been secreted from the enterocytes. Detection of LCAT protein was accomplished by the use of a polyclonal antibody to human LCAT and subsequent separation of proteins by polyacrylamide gel electrophoresis, followed by silver staining and fluography to visualise bands (see Lima, 1989). Detection of radiolabelled bands corresponding to the antigen-antibody complex was achieved by exposure to autoradiographic film at -70°C for 48h. HepG2 cells were used as a positive control for the procedure. The time course of protein secretion from enterocytes incubated in radioactive methionine was also followed. This was achieved by removing 1ml of the incubation medium after 10, 20, 30, 45 and 60 min time intervals followed by centrifugation to obtain the cell supernatant.
20μl of pre-immune serum and 10μl protease inhibitor (Trasylol 20,000 units/ml) were added to 1ml of medium from cultures of duodenal enterocytes. This solution was mixed for two hours on a rotating wheel at 4°C (to clear excess non-specific protein) before adding 20μl of protein-A sepharose beads. The solution was gently mixed for 14 hours, then centrifuged to pellet the beads associated with antibodies and any proteins bound non-specifically. The supernatant (containing radioactive LCAT protein) was removed, added to anti-LCAT antibody (20μl) and more protein A sepharose beads and allowed to mix for 5h to allow LCAT protein to bind to the antibody. The protein-A sepharose/medium suspension was centrifuged for 2min at 13,000g at 4°C. The resulting pellet was then washed three times in a phosphate buffer, and frozen at -20°C until required.

2.4.3 SDS-Polyacrylamide gel electrophoresis (PAGE)

**Gel preparation and casting**

10% SDS-PAGE gels were prepared using gel-casting glass plates (Genetic Research Instrumentation) arranged to give a thickness of 1.5mm. The gel-casting solution was prepared using 15.7ml water, 15ml 1M Tris-HCl (pH 8.8) and 8ml of 50% polyacrylamide; this was polymerised using 400μl of 10% SDS, 99μl of 10% ammonium persulphate and 30μl of NNN'N tetramethylethylenediamine (TEMED). The gel was allowed to set for approximately 30 min. The 5% stacking gel was made similarly (but with 5% SDS) and allowed to set on top of the resolving gel. A twelve well comb was inserted into the top of the stacking gel before the gel set. The glass plate containing the gel was placed in an electrophoresis chamber (Genetic Research Instrumentation). The chamber was filled as appropriate with gel running Buffer.
The samples were thawed and dissolved by boiling for 5 min in 30μl of PBS (with bromophenol blue). After cooling and centrifuging (Microcentaur centrifuge) for 2 min at 6500g, the samples were then ready for loading onto the SDS-PAGE gel. The twelve-well comb was removed and the pre-prepared samples were loaded into the well lanes using a Hamilton syringe. Electrophoresis was performed for 1h (or the time taken for the Bromophenol blue to reach the bottom of the gel) at 40mA per gel.

After the gel was run a silver stain kit (Silver stain plus; Bio Rad) was used to visualise proteins (solutions were freshly made as required). The gel was gently agitated in Fixative Enhancer Solution for 30min, washed three times (10 min per wash) in distilled water, then soaked in 0.2% AgNO₃ in 1mM formaldehyde for 30min. The gel was rinsed (30-60s) with water then placed in 20ml of the Silver Stain Developer (2 x 8 min). The reaction was stopped with 3.5% acetic acid (5-10min incubation). The gel was wrapped in cling film and dried under vacuum overnight before ³⁵S autoradiography.

**Buffer and Solutions**

<table>
<thead>
<tr>
<th>Gel running buffer, pH 8.3</th>
<th>Dye solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05M Tris</td>
<td>0.0625 M Tris-HCl, pH 6.8</td>
</tr>
<tr>
<td>0.384M Glycine</td>
<td>0.4% (w/v) SDS</td>
</tr>
<tr>
<td>0.1% (w/v) SDS</td>
<td>20% (v/v) Glycerol</td>
</tr>
<tr>
<td></td>
<td>10% (v/v) 2-mercaptoethanol</td>
</tr>
</tbody>
</table>

**Acrylamide/bis stock solution**

30% (w/v) acrylamide  
0.8% (w/v) NN'-Methylenebisacrylamide

**2.4.4 Detection of ³⁵S-radiolabelled proteins from HepG2 cells**

Confluent HepG2 cells were incubated with the experimental medium in six-well plates, and then washed in PBS, before adding 1ml cell lysis buffer (see
below). TCA was added to the lysed cell mixture to a final concentration of 10% and the suspension left overnight at 4°C to precipitate the protein pellet. This was washed in 10% TCA to remove any unincorporated radioactivity, and the radiolabelled protein pellet redissolved in 1ml 0.1M NaOH. A Lowry assay was carried out on each sample. Another 100μl was taken to measure 35S-radioactivity.

**Cell Lysis Buffer**

- 20mM Tris-HCl, pH 7.4
- 1mM phenylmethyl-sulphonyl fluoride
- 150mM NaCl
- 0.5% (w/v) sodium deoxycholate
- 0.5% (v/v) Nonidet P-40
- 1mM EDTA
- 100 units/ml aprotinin (Trasylol)
- 0.1% (w/v) SDS

### 2.5 In Vivo experiments

#### 2.5.1 Animals

The animals used for the experiments, Sprague-Dawley rats, Dunkin Hartley guinea-pigs and white T/O mice were maintained in the Comparative Biology Unit (CBU) of the Royal Free Hospital (RFH). Guinea-pigs were fed according to the original Frant Diet formula (Special Diets services, U.K. -see Appendix) and were allowed free access to water and hay until required for the fat feeding experiments.
2.5.2 Assay of plasma LCAT activity

This assay is based on the proteoliposome method for measurement of enzyme activity (Gillet and Owen, 1992). This assay is based on the use of a highly efficient substrate for LCAT (apo-A1: lecithin: labelled unesterified cholesterol, in the molar ratio 0.8: 250: 12.5), so that endogenous HDL is bypassed; this method is thus considered to reflect LCAT mass in the plasma sample. An aliquot of 100 μl serum taken from each mouse was thawed on ice. 0.23 ml of [14C]-cholesterol radiolabelled LCAT proteoliposome substrate (containing apo-A1) was also thawed. Each sample was measured in duplicate and normal plasma and saline were used as controls. Test serum (7.5 μl) was added to the substrate, vortexed and the samples then incubated for 1h at 37°C. A Folch extraction was then carried out in order to isolate the lipids. The samples were transferred to tubes containing 5 ml chloroform: methanol (2:1) and vortexed. Water (1 ml) was added to form 2 layers and the samples were mixed and centrifuged for 5 minutes. The top aqueous layer and the interface containing cell proteins were removed and the bottom layer containing the lipids dried under nitrogen. The residue was dissolved in 100 μl chloroform: methanol (2:1) and redried. Each sample was then dissolved in 30 μl of chloroform and applied to a silica-coated TLC plate. The developing solvent used to separate the lipid components (cholesterol, triglyceride and cholesteryl ester) was hexane/diethylether/glacial acetic acid (90:20:1). The bands corresponding to free cholesterol and cholesteryl ester were cut out and 14C counted in a liquid scintillation counter. The overall reaction for the proteoliposome method for calculation of LCAT activity is as follows:

\[
\text{Free Cholesterol}^* + \text{Lecithin} \rightarrow \text{Cholesteryl ester}^* + \text{Lysolecithin} \\
^* \text{radioactive label}
\]

Plasma LCAT activity was calculated as nmol of free cholesterol converted to cholesteryl ester per ml of test plasma per hour at 37°C (nmol/ml/h).
2.5.3 Preparation of hepatotoxic drugs

4-aminopyrazolopyrimidine (4-APP) was dissolved in 150mM NaCl and 20mM sodium phosphate (pH 2.5) at a stock concentration of 2mg ml\(^{-1}\). Mice were injected i.p. with 1mg 4-APP (i.e. \(~50\text{mg kg}^{-1}\)) daily or vehicle and were killed after 5 days. The livers were removed and snap-frozen in liquid nitrogen. Galactosamine and paracetamol were dissolved in saline to give stock solutions of 100mM and 25mM, respectively. Lipopolysaccharide stock solution was prepared in saline at 1.2mg ml\(^{-1}\).

2.5.4 Protocol for fat-feeding experiments

In the initial experiment, a male adult guinea-pig and male adult rat were both fasted overnight. The following morning (9.30 a.m.) the animals were given an oral fat load comprising a dispersion of olive oil, egg white (albumin) and a saline carrier in the ratio 2 : 1 : 1. Controls were fed saline and egg-white. The animals were allowed three hours to digest the fatty meal (in humans, plasma triglyceride levels peak approximately 4h after a fatty meal, however metabolism in the rodent is likely to be more rapid). The animals were killed by exposure to \(CO_2\) and the duodenum was removed and placed into physiological buffer for isolation of the enterocytes. Isolation of the duodenal enterocytes was carried out according to Weiser (1972). The duodenum was briefly incubated in sodium citrate buffer (pH 7.3) to dissociate the cells, followed by further treatment with 1.5mM EDTA in phosphate-buffered saline. This method isolates only epithelial cells and excludes serosal and interstitial cells.

In a further experiment, larger numbers of animals were used to confirm the preliminary results obtained. This experiment was performed only in guinea-pigs. The fat-feeding was carried out as described above using 3 treated animals and 3 controls. Following the oral fat-load, the liver was removed and snap-frozen in liquid nitrogen for isolation of total RNA. The duodenal
enterocytes were isolated as described (see 2.4.1); some were snap-frozen in PBS for RNA extraction and the remainder were incubated with $^{35}$S-methionine to determine whether the cells were secreting $^{35}$S-LCAT protein.

2.6 Materials

Unless otherwise stated chemicals were obtained from Sigma Chemical Co, BDH or BCL; enzymes and biochemicals were obtained from Sigma, BCL, BRL or Anglian Biotechnology. Radioisotopes were obtained from Amersham, and standards for SDS and PAGE were obtained from Sigma. Cell culture reagents were obtained from Gibco and Flow laboratories.

LCAT antibodies

LCAT antibodies were prepared by Dr Lima in our laboratory (RFHSM). Briefly, LCAT was purified 30,000-fold from 1l of human plasma and used to raise antibodies in New Zealand White Rabbits by injecting 30μg of LCAT in Freund's adjuvant over a period of several weeks. The resulting anti-sera completely inhibited LCAT activity in human serum. Preimmune serum was also collected from rabbits.

Preparation of Oleic acid/BSA

Oleic acid complexed to albumin was prepared using the method described by Ellsworth et al (1986). Oleic acid (40mg) was dissolved in 5ml 95% ethanol containing approx. 1mg ml$^{-1}$ phenolphthalein as a pH indicator. Whilst vortexing 1M NaOH was added dropwise until the colourless solution just turned pink. The solution was evaporated to dryness under nitrogen gas. Solvent traces were removed by drying in a vacuum desiccator for 1-2h. The sodium oleate was dissolved by adding 4ml warm Dulbecco's PBS. Defatted bovine serum albumin (1g BSA) was dissolved in 5ml PBS and after neutralisation with 60μl 1M NaOH was chilled on ice. Whilst vortexing, oleate solution (warmed to 55°C) was added dropwise to the ice-cold BSA. This oleate/BSA complex was stirred magnetically overnight at room temperature.
The following day the solution was centrifuged at 13000g for 10min, the supernatant removed and passed through a 0.2μm filter. An aliquot of 100μl was removed and the oleate concentration estimated using a commercial kit for free fatty acids. The value was typically about 16mM, but this was usually diluted to 10mM with BSA solution since this proved more stable at 4°C than the original preparation. A control solution was made by adding 4ml PBS to 5ml of BSA solution and this was also passed through a 0.2μm filter.

**Human Tissue**

Human liver tissue was obtained from in-patients at the RFH undergoing liver transplantation. The samples were snap-frozen in liquid nitrogen before extraction of RNA (see 2.3.3).
CHAPTER 3

Manipulation of Lipoprotein Levels
3 Manipulation of Lipoprotein Levels

3.1 Screening of human hepatoma cell lines for LCAT and apo-AI mRNA

3.1.1 Introduction

The aim of this initial set of experiments was to identify a cell type that modelled the secretion of LCAT by the liver. Secretion of LCAT protein by cultured HepG2 cells has been reported (Erickson & Fielding, 1986; Lima et al, 1987; McCall et al, 1989) but there is little information available for other human hepatoma cell lines. However, several hepatoma-derived cell lines, including HepG2, Hep3B, Alexander [PLC/PRF/5], Mahlavu and SK55 have previously been screened in this laboratory for their ability to secrete LCAT protein (Table 3.1). The present study was carried out to determine whether LCAT gene expression in these cells reflects this pattern of protein secretion. The cell type selected in these experiments was then used to investigate the expression of LCAT mRNA under different experimental conditions; for example, treatment with oleic acid, gemfibrozil or cholesterol, and exposure to reduced glucose concentrations.

3.1.2 Results

LCAT mRNA was present in all five human hepatoma cell lines tested: HepG2, Hep3B, Alexander [PLC/PRF/5], Mahlavu and SK55 (Table 3.1; Figure 3.1). LCAT mRNA levels were 3-4 times greater in HepG2 cells compared to Hep3B, Alexander, SK55 and Mahlavu cell types. Apo-AI mRNA was observed in HepG2, Hep3B and Alexander; again expression was greatest in Hep G2 cells.

3.1.3 Discussion

The catalytic activity of LCAT (as previously described by Lima, 1989) appeared to correlate with LCAT mRNA levels observed in the present study.
LCAT mRNA levels, protein secretion and LCAT activity were greatest in HepG2 cells. Furthermore, the co-factor for LCAT, apo-A1, was expressed and

Figure 3.1 Northern blot of different cell lines, hybridised to (a) LCAT and (b) apo-A1 probes; (c) actin probe demonstrates presence and loading of total RNA in each lane.
secreted by this cell type. Forte et al (1989) have previously demonstrated that HepG2, Hep3B and Alexander cell lines secrete lipoproteins into the cell culture medium. They found that Hep3B and Alexander secreted discoidal HDL-like particles into the cell culture medium, as previously demonstrated for HepG2 cells (Cheung et al, 1989). Furthermore, it was suggested that the morphology of this HDL was similar to that previously reported for plasma HDL from LCAT-deficient patients i.e. that LCAT was absent or non-functional in all three cell lines. In the case of HepG2 cells, where LCAT protein was secreted into the culture medium, LCAT was suggested to be non-functional, whereas in Alexander cells, where no LCAT protein was detected in the medium, a true LCAT deficiency was suggested. However, in our laboratory both LCAT mRNA and LCAT protein were detected in Alexander cells and the cell culture medium respectively, (Figure3.1, Table 3.1), although the catalytic activity of the protein was found to be only 1/10 that of LCAT secreted by HepG2 cells. The reasons for these differences observed compared to previous published work is unknown. Similarly, for Hep3B cells, LCAT mRNA was approximately 1/3 that of HepG2 cells and this correlates with the pattern of catalytic activity shown in these cells (Table 3.1).

<table>
<thead>
<tr>
<th>human hepatoma cell line</th>
<th>LCAT protein secretion</th>
<th>LCAT catalytic activity (%)</th>
<th>LCAT mRNA</th>
<th>Apo-AI protein secretion</th>
<th>Apo-AI mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>+</td>
<td>100</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hep3B</td>
<td>+</td>
<td>30</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alexander</td>
<td>+</td>
<td>10</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mahlavu</td>
<td>-</td>
<td>0</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SK55</td>
<td>-</td>
<td>0</td>
<td>+</td>
<td>-</td>
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</tr>
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</table>

Table 3.1 LCAT and apo-AI protein secretion and mRNA in different human hepatoma cell lines. LCAT catalytic activity was also measured and expressed as a percentage of the activity found in HepG2 cells. + and - represent the presence or absence of a feature, respectively. LCAT protein secretion, LCAT catalytic activity and apo-A1 protein secretion were taken from Lima, 1989.
HepG2 cells therefore appeared to be the better model for further investigations since they appear to have good LCAT activity and mRNA. It should also be noted that the two cell lines that did not express apo-A1 mRNA, Mahlavu and SK55, did not secrete LCAT protein although LCAT mRNA was expressed.

Thus, both transcriptional and post-transcriptional regulation of LCAT and the presence of apo-A1 may be important for production of a functional LCAT protein in these cell lines.

3.2 Oleic acid treatment of HepG2 and Hep3B cells

3.2.1 Introduction

A number of studies in rat perfused liver (Marsh, 1976; Johnson et al, 1985), liver slices (Radding et al, 1958) or cultured liver cells (Davis et al, 1979; Bell-Quint & Forte, 1981) have shown that long chain fatty acids have a stimulatory effect on the synthesis of triglyceride secreted in the form of VLDL. However, similar studies on the regulation of human hepatic lipoprotein synthesis and secretion have been hampered by the lack of an adequate experimental model. Although the use of primary cultures of human hepatocytes has been very informative in understanding lipoprotein metabolism (Brown & Goldstein; 1986, Edge et al; 1986, Havekes et al, 1986), the major disadvantage of such cultures is variability between preparations and limited life span.

Since HepG2 cells were first isolated and reported (Aden et al, 1979), this cell line has been used in several hundred metabolic studies (reviewed in Javitt 1990). The production of lipoproteins and associated proteins by this cell line has been well documented (Thrift et al, 1986; Craig & Cooper, 1988; Havinga et al, 1987; Dashti et al, 1987; McCall et al, 1988; Faust & Albers, 1987), and also compared with other hepatoma-derived transformed cell lines (Forte et al,
HepG2 cells express all hepatic components essential for triglyceride and cholesterol metabolism (Zannis et al, 1981) and are therefore considered to be a good model for studying the role of the liver in lipid metabolism. For example, studies evaluating the effect of lipoproteins on apo-A1 expression (Craig & Cooper, 1988; Monge et al, 1989) and free fatty acids on apolipoprotein levels (Dashti & Wolfbauer, 1987; Wong et al, 1989) have utilised this cell line. Furthermore, the regulation of apolipoprotein biosynthesis has been studied to some extent. For example, the stimulatory effect of hormones, like oestrogen, on secretion and mRNA levels of apo-AI and apo-CII have been investigated in HepG2 cells (Tam et al, 1986, Archer et al, 1986).

The HepG2 cell line is thus considered a suitable model for studying factors that regulate human hepatic lipoprotein production. Another human hepatoma cell line, Hep3B has also been examined for its suitability as a model. This particular cell line has been more commonly used in the study of fibrinogen expression during the acute phase response (Darlington et al, 1986; Oliviero et al, 1987).

The main physiological roles of LCAT are to help clear the redundant surface coat of triglyceride-rich lipoproteins (hepatic VLDL and intestinal chylomicrons) following lipolysis and to regulate the interconversion of HDL particles (see Chapter 1). The purpose of this study was to test the hypothesis that LCAT synthesis and secretion, as its physiological role might suggest, is linked to VLDL production. This was achieved by measuring LCAT mRNA levels during increased triglyceride synthesis induced by oleic-acid in HepG2 and Hep3B cells. The expression of apo-AI (a major protein constituent of HDL and co-factor for LCAT) under these conditions was also studied by measuring apo-AI mRNA levels.
3.2.2 Results

Triglyceride and mRNA analysis in HepG2 cells

HepG2 cells treated with increasing concentrations of oleic acid (Figure 3.2), responded with a corresponding increase in secreted triglyceride from the cells up to a concentration of 0.4 mM oleic acid. At higher concentrations of oleic acid, triglyceride secretion fell and a value equivalent to that obtained from untreated cells was recorded at 0.8 mM. Intracellular triglyceride levels were also examined in HepG2 cells (Figure 3.3); there was a concomitant rise in intracellular triglyceride levels with increasing concentration of oleic acid and triglyceride levels were still elevated with the highest dose of oleic acid used, 0.8 mM.

In later experiments, the distribution of triglyceride within the lipoprotein classes was measured. In HepG2 cells the major triglyceride fraction was LDL in both treated and untreated cells (Figure 3.4). When the cells were incubated with increasing concentrations of oleic acid, there was a continuous shift in distribution of $^3$H-triglyceride from LDL to VLDL, while the HDL fraction steadily decreased (Figure 3.4).

In HepG2 cells, LCAT expression was reduced with oleic acid concentrations equal to or greater than 0.2 mM (Figures 3.5A, 3.8). Further increases in oleic acid concentration were accompanied by a further decline in LCAT mRNA levels. In HepG2 cells incubated with 0.6 mM oleic acid, LCAT mRNA levels had dropped to 30% of the control value. There was a reduction in apo-AI expression with oleic acid treatment; apo-A1 levels were reduced with 0.05 mM oleic acid and this was maintained at higher concentrations of oleic acid (Figure 3.5B).
Figure 3.2 Effect of 20h incubation with oleic acid (0.1-0.8mM) on $^3$H-labelled triglyceride ($^3$H-TG) secretion from HepG2 (■) or Hep3B (▼) cells into the culture medium. HepG2 or Hep3B cells were incubated with medium containing [$^3$H]-glycerol and increasing concentrations of oleic acid. Incorporation of radioactive glycerol into triglyceride was measured following separation of phospholipid and triglyceride by thin layer chromatography. Counts were expressed as DPM/ml/medium. (n=1)

Figure 3.3 Effect of 20h incubation with oleic acid (0.1-0.8mM) on intracellular $^3$H-labelled triglyceride ($^3$H-TG) synthesis in HepG2 cells. (n=1)
Figure 3.4 Effect of 20h incubation with oleic acid (0.05-0.6mM) on secretion of specific fractions of $^3$H-labelled triglyceride ($^3$H-TG) from HepG2 cells. A Graph showing the effect of oleic acid on the secretion of ● very low density lipoprotein (VLDL); ▼ low density lipoprotein (LDL) and ■ high density lipoprotein (HDL). B Histogram showing the proportion of (cross-hatched) VLDL, (non-hatched) LDL and (hatched) HDL expressed as a percentage of the total triglyceride secreted. (n=1)
Figure 3.5 Effect of 20h incubation with oleic acid (0.05-0.6mM) on A LCAT and B Apo-AI mRNA levels in HepG2 cells. Control cells (0mM) were grown in the absence of oleic acid. Total RNA from HepG2 cells was blotted in triplicate in the range from 2.5 to 10µg and hybridised with either the LCAT probe or the apo-AI probe. The amount of mRNA was quantified by scanning densitometry of autoradiographs and the absorbance values were normalised to those for actin. The changes in mRNA were expressed as a percentage of control absorbance values. C Effect of oleic acid on $^3$H-labelled triglyceride ($^3$H-TG) secretion in HepG2 cells. The results shown are the average from two separate experiments.

**Triglyceride and mRNA Analysis in Hep3B cells**

Hep3B cells showed a dose-related increase in triglyceride secretion when incubated with oleic acid (Figures 3.2 and 3.7C). Unlike HepG2, the major triglyceride-containing fraction in Hep3B cells was initially HDL and this was reduced by oleic acid treatment at the higher concentrations of oleic acid (Figure 3.6). Although there was a steady increase in VLDL-triglyceride...
secretion with the addition of oleic acid (Figure 3.6A), surprisingly and unlike the response observed in HepG2 cells, there was no shift in the distribution of triglyceride towards the VLDL fraction until the highest concentration of oleic acid (0.6 mM) was used (Figure 3.6B). LDL triglyceride secretion rose at concentrations greater than 0.1 mM oleic acid, (3.6A), while percentage LDL steadily increased between 0.1 and 0.6 mM oleic acid (3.6B).

![Graph showing the effect of oleic acid on the secretion of very low density lipoprotein (VLDL); low density lipoprotein (LDL) and high density lipoprotein (HDL).](image)

**Figure 3.6** Effect of 20h incubation with oleic acid (0.05-0.6 mM) on secretion of specific fractions of 3H-labelled triglyceride (3H-TG) from Hep3B cells. A Graph showing the effect of oleic acid on the secretion of very low density lipoprotein (VLDL); ▼ low density lipoprotein (LDL) and ■ high density lipoprotein (HDL). B Histogram showing the proportion of (cross-hatched) VLDL, (non-hatched) LDL and (hatched) HDL expressed as a percentage of the total triglyceride secreted. (n=1)

Incubation of Hep3B cells with oleic acid resulted in a clear stimulation of LCAT expression (Figure 3.7A), with LCAT mRNA levels in the treated cells reaching a value 7.5 times greater than control at 0.6 mM. However, oleic acid treatment
did not have the same effect on apo-AI mRNA. There was an apparent marked drop in expression with 0.05 mM oleic acid in apo-A1 mRNA which was reduced to 5% of the control value at 0.6 mM oleic acid (Figure 3.7B).

**Figure 3.7** Effect of 20h incubation with oleic acid (0.05-0.6mM) on A LCAT and B Apo-AI mRNA levels in Hep3B cells. Control cells (0mM) were grown in the absence of oleic acid. Total RNA from Hep3B cells was blotted in triplicate in the range from 2.5 to 10µg and hybridised with either the LCAT probe or the Apo-AI probe. The amount of mRNA was quantified by scanning densitometry of autoradiographs and the absorbance values were normalised to those for actin. The changes in mRNA were expressed as a percentage of control absorbance values. C Effect of oleic acid on ³H-labelled triglyceride (³H-TG) secretion in Hep3B cells. The results shown are the average from two separate experiments.
3.2.3. Discussion

An increased triglyceride secretion from the liver may lead to a stimulation of LCAT secretion; in humans hypertriglyceridaemia is often associated with increased LCAT activity while a positive correlation between plasma triglyceride and LCAT activity has been demonstrated (Wallentin, 1975). Furthermore, studies on isolated rat hepatocytes (Nordby, 1978) have shown that addition of oleic acid to the incubation medium results in increased triglyceride secretion from the hepatocytes which is paralleled by an increase in LCAT secretion.

Figure 3.8 Northern blot using total RNA from HepG2 cells treated with different concentrations of oleic acid, hybridised to the LCAT probe
In the present study, oleic acid stimulated the production of triglyceride in HepG2 cells; this has also been demonstrated by Ellsworth et al (1986), who similarly reported preferential increases in the VLDL and LDL fractions with oleic acid treatment. This enhanced triglyceride secretion was accompanied by a reduction rather than the anticipated increase in LCAT expression. It is possible however, that LCAT activity may not reflect this reduction in expression; indeed, one report that LCAT activity is unaffected by linoleate in the culture medium of HepG2 cells (Erickson & Fielding, 1986) suggests a refractory response to free fatty acid supplementation. One explanation for the reduction of LCAT mRNA is that HepG2 cells are sensitive to high doses of oleic acid, since triglyceride production by the cells drops at values greater than 0.4 mM oleic acid (see Figures 3.2 and 3.4). By contrast, intracellular HepG2 triglyceride levels show that synthesis of triglyceride steadily increases with oleic acid up to a concentration of 0.8 mM (Figure 3.3); this suggests that secretion of triglyceride is impaired at doses of oleic acid greater than 0.4 mM in this cell line, possibly due to a non-specific toxic effect.

The Hep3B cell line was also examined for the response of LCAT to oleic acid. When the response of HepG2 and Hep3B cells to oleic acid treatment were compared (Figure 3.2), basal triglyceride secretion from Hep3B cells was low (10 fold less than that of HepG2). Thus, HepG2 appeared to be the more suitable cell line of the two. However, unlike HepG2 cells, LCAT expression is stimulated by oleic acid (Figure 3.7A) in the Hep3B cell line. It is also noteworthy that, unlike HepG2 cells, the Hep3B cell line maintains an almost linear increase of triglyceride secretion in response to increasing concentrations of oleic acid (Figures 3.2 and 3.7C).

Apolipoprotein production by Hep3B cells, after an initial lag, has also been shown (Forte et al, 1989) to be essentially linear between 3 and 24h incubation. Forte et al (1989) further demonstrated that a distinguishing
difference between Hep3B cells and HepG2 cells is the ability of the former to accumulate small quantities of 'VLDL' particles in serum-free medium, whereas HepG2 cells do not and suggested a 'precursor-product' relationship between 'VLDL' and 'LDL' in these cells. However in the oleic acid experiments there were found to be measurable, albeit small, quantities of VLDL in the culture medium (Figure 3.4) of untreated HepG2 cells. Oleic acid treatment appears to remodel the lipoprotein profile in HepG2 cells (Figure 3.4), causing a reduction of LDL and HDL, paralleled by an increase in VLDL. Similarly, in Hep3B cells, oleic acid supplementation causes the triglycerides secreted to redistribute from mainly LDL to similar proportions of HDL, LDL and VLDL. The reduction of LCAT mRNA in HepG2 cells may be linked to the decline in expression of its co-factor apo-AI (Figure 3.5B), although this is not the case in the Hep3B cell line, where LCAT mRNA is stimulated, but apo-AI mRNA is reduced, in the presence of oleic acid.

Ellsworth et al (1986) demonstrated that oleic acid failed to cause an increase in apolipoprotein synthesis. Thus, free fatty acid availability and increased triglyceride synthesis alone may not be adequate stimuli for apolipoprotein synthesis in HepG2 cells. A study by Miller and Nanjee (1991) showing that reverse cholesterol transport is stimulated by lipolysis of triglyceride-rich lipoproteins may partly explain this. Rabbits universally pre-labelled with [3H]-cholesterol, were injected with heparin to release lipoprotein lipase and changes in plasma lipids monitored over time. Under these conditions there was a rapid decrease in plasma triglyceride and an increase in HDL cholesterol. Thus, lipolysis of triglycerides may be a more important factor in HDL production (and LCAT synthesis) in HepG2 cells. However, the cell culture conditions used for our experiments were more suited for synthesis of triglycerides, rather than facilitating their lipolysis.
In conclusion, although oleic acid was able to stimulate triglyceride secretion in both HepG2 and Hep3B cells, the changes in mRNA levels of LCAT and apo-AI were quite different and no obvious factor influencing LCAT expression could be identified.

3.3. Gemfibrozil treatment of HepG2 cells

3.3.1. Introduction
The fibric acid derivative, gemfibrozil, is a lipid regulating drug which exerts its major effects by decreasing plasma triglyceride and increasing HDL cholesterol (Kaukola et al, 1986). Gemfibrozil is thought to increase plasma HDL levels by stimulating HDL synthesis and to decrease plasma triglycerides both by decreasing production of VLDL (Kesaniemi & Grundy, 1984) and by increasing the activity of lipoprotein lipase, (Saku et al, 1983). This increase in lipoprotein lipase activity is associated with the presence of smaller VLDL particles with an increased apoCII/CIII ratio. These changes are suggested to be accomplished through changes in the hepatic synthesis of specific proteins. For example, an increase in apo-AI synthesis has been reported during gemfibrozil treatment (Kashyap & Saki, 1991). This stimulation was associated with the presence in plasma of smaller and denser HDL particles. An increase in expression of apo-AI mRNA in HepG2 cells has also been demonstrated by Tam (1991); exposure of HepG2 cells to gemfibrozil resulted in a two-fold induction of apo-AI mRNA.

The purpose of the present study was to utilise HepG2 cells to investigate whether treatment with gemfibrozil alters hepatic LCAT gene expression and whether this is accompanied by a change in apo-AI expression.
3.3.2. Results

Effect of gemfibrozil on triglyceride secretion

Incubation of HepG2 cells for 6 h with 100μM gemfibrozil resulted in a small reduction in total triglyceride secretion from HepG2 cells (Figure 3.9). This was reflected by a small decrease in the triglyceride content in all three of the lipoprotein fractions measured (VLDL, LDL and HDL).

![Figure 3.9](image)

Figure 3.9 Effect of a 6h incubation with 100μM gemfibrozil (hatched) on total ^3^H-labelled triglyceride (^3^H-TG) secretion from HepG2 cells. The effect of gemfibrozil on the specific lipoprotein fractions (VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein) is also shown. Control HepG2 cells (non-hatched) were grown in the absence of gemfibrozil. (n=1)

Effect of gemfibrozil on LCAT mRNA and apo-AI mRNA

A 6h Incubation of HepG2 cells with gemfibrozil resulted in a 41% reduction in LCAT mRNA compared to control cells (0h). In contrast, exposure of HepG2 cells to gemfibrozil resulted in a two-fold induction in apo-AI mRNA after 6h (data not shown).

3.3.3. Discussion

In studies in humans, gemfibrozil has been shown to lower the free fatty acid concentration in the liver, thereby reducing hepatic triglyceride production (Kissebah et al, 1976). In the present study, a reduction in LCAT mRNA was
observed in response to gemfibrozil treatment, accompanied by a reduction in triglyceride secretion. Thus a reduced output of VLDL in the liver may contribute to the observed drop in LCAT mRNA suggesting a direct correlation between hepatic LCAT mRNA and secreted lipoprotein levels. However, the reduction in secretion of VLDL triglyceride levels appeared to be slight. One explanation for the poor effect of gemfibrozil on lowering triglyceride levels in HepG2 cells is that the level of triglyceride synthesis by the cells is low. Studies in rats have shown that although gemfibrozil reduces high levels of plasma triglycerides, it does not significantly reduce normal levels (Krause & Newton, 1986). The observed increase in apo-A1 mRNA is consistent with existing data showing an increase in HDL cholesterol during fibrate therapy, suggested to be partly due to increased apo-A1 synthesis (Ginsberg, 1987). Another mechanism contributing to elevation of HDL cholesterol is the increase of surface components from the degradation of VLDL by lipoprotein lipase, which can be used for HDL synthesis (Nikkila, 1984). In addition, gemfibrozil stimulates rat hepatocytes to synthesise sterols at a faster rate, secreting them predominantly into the HDL fraction (Newton, 1985).

Among the major lipid-lowering agents, fibric acids belong to a series of abnormal fatty acids called peroxisomal proliferators that are known to interact with a liver nuclear receptor. This causes several changes in fatty acid metabolism, including changes in gene expression (for a review see Sirtori & Colli, 1993). Until recently, gemfibrozil was thought to act predominantly on VLDL clearance, rather than synthesis, this mechanism contributing to the lower plasma triglyceride levels observed on gemfibrozil therapy (Kesaniemi, 1984). An alternative explanation for the triglyceride-lowering potential of gemfibrozil has been suggested by Lamb et al (1993), who demonstrated that addition of gemfibrozil to primary cultures of adult rat hepatocytes significantly decreased the incorporation of radiolabelled glycerol into cellular triglyceride and the secretion of labelled VLDL-triglyceride. Gemfibrozil was also shown to
increase the incorporation of labelled glycerol into cellular phosphatidylcholine and the secretion of labelled HDL-phosphatidylcholine. These workers suggested that the triglyceride-lowering effect of gemfibrozil may be partly due to its ability to shunt liver cell diglycerides into phosphatidylcholine rather than triglyceride via CDP choline. Studies in rats have also shown that gemfibrozil inhibits the in vivo incorporation of long chain fatty acids into newly synthesised triglycerides (Rodney, 1976). Thus, gemfibrozil appears to lower triglyceride levels by acting on both synthesis and catabolism of triglyceride. Gemfibrozil has also been shown to change the composition of VLDL by lowering its free cholesterol, cholesteryl ester and raising the protein content (Tsai et al, 1992).

In conclusion, the results from these studies demonstrate that LCAT is down-regulated by gemfibrozil in HepG2 cells and the observed reduction in triglyceride secretion indicates a possible link between hepatic LCAT mRNA concentration and secreted triglyceride-rich lipoproteins. Gemfibrozil, like other fibrates, may exert its effect on LCAT by reducing the transcription rate of the LCAT gene (Staels et al, 1992); however an alternative explanation that the reduction in LCAT mRNA may involve post transcriptional regulation cannot be excluded.

3.4. Effect of glucose deprivation on LCAT and apo-AI mRNA levels

3.4.1. Introduction
A high carbohydrate diet is known to induce lipoprotein synthesis and the secretion of VLDL triglyceride by the liver (Tepperman et al, 1958; Boogaerts et al, 1984). Furthermore, triglyceride synthesis has been shown to increase in primary rat hepatocytes following the addition of glucose (Durrington et al, 1982). Moreover, Dashti et al (1989) and Cianflone et al (1990) have reported
that addition of glucose to the medium of HepG2 cells stimulated triglyceride synthesis and secretion, as well as increasing apo-A1 secretion.

Stimulation of apo-A1 secretion may indicate an increase in apo-A1 mRNA; this was investigated by studying the effect of varying concentrations of glucose on apo-A1 mRNA levels in cultured HepG2 cells. Since LCAT is required for the interconversion of HDL particles (for which apo-A1 is the major protein constituent) and furthermore clears the redundant surface coat of triglyceride-rich lipoproteins (hepatic VLDL and intestinal chylomicrons) following lipolysis (see Introduction 1.3), LCAT mRNA was also measured to determine whether it could be altered under these conditions.

3.4.2. Results

Apo-AI mRNA dropped sharply after three hours of incubation in glucose-free medium and thereafter declined steadily, reaching only 2% of the control value after 9h incubation (Figures 3.10 and 3.11). LCAT mRNA levels steadily dropped when grown in glucose-free medium (Figures 3.10 and 3.11), reaching 16% of the control by 9h. Both apo-AI and LCAT mRNA levels were significantly reduced by growing the cells for 9h in media with decreasing glucose concentrations (Figure 3.13). When HepG2 cells were incubated in DMEM with low glucose (1.25mM), there was a significant reduction in apo-AI expression (Figures 3.12 and 3.13B) compared to cells grown in normal glucose (5.5mM) and high glucose (25mM). Figure 3.12 shows a separate experiment carried out prior to results shown on Figure 3.13, illustrating the effect of different concentrations of glucose on LCAT and apo-A1 mRNA.
Figure 3.10  Northern blot using total RNA from HepG2 cells incubated in glucose-free medium and harvested at varying time points. Control cells (0h) were grown in medium containing 25mM glucose. The blot was hybridised to LCAT, apo-A1 and actin probes (details in Figure 3.11).
Figure 3.11 Time course of the effects of a 9h incubation of HepG2 cells in glucose-free medium on A LCAT and B Apo-AI mRNA levels. Control cells (0h) were grown in medium containing the high glucose concentration (25mM). Total RNA from HepG2 cells was blotted in triplicate in the range from 2.5 to 10μg and hybridised with either the LCAT probe or the apo-AI probe. The amount of mRNA was quantified by scanning densitometry of autoradiographs and the absorbance values were normalised to those for actin. The changes in mRNA were expressed as a percentage of control absorbance values. Each time point is the mean from 4 replicate flasks and the vertical lines show the SEM. Significant differences (determined using Student's t-test for unpaired data) between control and treated groups are shown as * P < 0.05 and ** P < 0.01.
Figure 3.12 Northern blot using total RNA from HepG2 cells grown in medium containing different concentrations of glucose, hybridised to LCAT, apo-AI and actin probes. This was a preliminary experiment, using one flask per concentration point.
LCAT mRNA (Figure 3.13A). also showed a stepwise reduction in expression with decreasing amounts of glucose in the medium. In order to determine whether the changes in expression noted were due to lack of availability of glucose, rather than to loss of cell viability or death, an assay to measure the secretion of radio-labelled total cellular protein by the HepG2 cells was also carried out (see Methods, Chapter 2.15). Figure 3.13C shows that lowering the glucose concentration of the medium did not significantly reduce $^{35}$S...
protein secretion, indicating that the cells were still actively producing and secreting cellular proteins at levels comparable to control cells.

3.4.3. Discussion

These experiments demonstrate that partial or total glucose deprivation significantly reduces LCAT and apo-A1 mRNA levels in HepG2 cells. Furthermore, since protein secretion was not reduced by reducing glucose concentration, it is unlikely that the effects observed were due to cell death. The observation that glucose deprivation reduces apo-AI mRNA may have a basis in glucoregulatory physiology. Apo-AI production by cultured rat hepatocytes has been observed to be stimulated by treatment with insulin and depressed by glucagon treatment (Elshourbagy et al, 1985). Although glucose may exert its stimulatory effects by generating more glycolytic products, this is unlikely as it has now been shown that lactate added to the culture medium of HepG2 cells has no effect on apo-A1 secretion (personal communication, T. Hughes, Sandoz Research Institute, East Hanover). Since glucose has been shown to stimulate VLDL triglyceride synthesis in rat hepatocytes (Davis et al, 1979, Yamamoto et al, 1987), a more obvious explanation is that glucose may directly (or after conversion into glucose-6-phosphate) generate intracellular fatty acids and triglycerides by inducing the expression, or mRNA stability, of the lipogenic enzymes acetyl CoA carboxylase or fatty acid synthetase. This may also explain the effect of glucose on LCAT mRNA; an increased output of hepatic VLDL may lead to an increased requirement for LCAT protein to clear excess surface cholesterol from VLDL remnants. Conceivably, the observed reduction in LCAT mRNA when HepG2 cells are grown in glucose-deficient medium could reflect a decreased output of VLDL with a corresponding decline in the requirement for LCAT. However, an important role for fatty acid/triglyceride synthesis seems unlikely as exogenous oleic acid, in contrast to glucose, was unable to stimulate apo-A1 or LCAT mRNA and indeed appeared to produce an inhibitory response (see 3.2.1).
A direct effect of glucose-6-phosphate on apo-A1 and LCAT gene transcription may be an alternative explanation. Evidence is now mounting to suggest that dietary factors (such as glucose) are specific modifiers of gene expression (for a review, see Clarke & Abraham, 1992). For example, dietary glucose has been shown to be a key determinant of hepatic transcription rate for several genes, such as apo-E (Strobl et al, 1989). The apo-AI gene has several features thought to be characteristic of "housekeeping" genes (including GC-rich sequences in the apo-AI promoter region, multiple potential Sp1 binding sites (Higuchi et al, 1988) and the lack of a typical TATA element (Araki et al, 1987). The production of apo-AI by HepG2 cells may thus be regulated by "housekeeping" influences subject to control by basic metabolic processes. The fall in apo-AI mRNA levels in response to acute glucose deprivation in HepG2 cells has been shown to be blocked by prior treatment with cycloheximide (personal communication, T. Hughes). This indicates that a protein may be produced by HepG2 cells in response to glucose deprivation that either decreases apo-AI transcriptional activity, impairs processing of the apo-AI mRNA, or facilitates degradation of the apo-AI gene transcript. Thus, in HepG2 cells apo-A1 secretion is strongly dependent on ongoing protein synthesis. Hepatic LCAT mRNA synthesis may also be regulated by glucose sensitive "control" elements similar to those found in the pyruvate kinase promoter region (Thomson & Towle, 1991) i.e. the LCAT gene may have specific carbohydrate-responsive nucleotide sequences. Glucose may affect LCAT at the translational level (Liang et al, 1990); reduced glucose availability could interfere with the processing of carbohydrate and secretion of the mature glycoprotein. This would have two possible effects, firstly it may lead to a build-up of LCAT within the ER of the hepatocytes, with a reduced output of the protein or secondly, it may lead to a feedback signal to the hepatocytes to discontinue synthesis at the transcriptional level.
3.5. Treatment of HepG2 cells with lipoproteins and free cholesterol

3.5.1. Introduction

The membrane level of unesterified (free) cholesterol is tightly regulated, as changes in membrane cholesterol composition have major effects, both direct and indirect, on a wide array of cellular functions. Since free cholesterol exchanges freely between lipid surfaces, its level in the plasma membrane depends on the steady state equilibrium of cholesterol between the cell surface and plasma or interstitial lipoproteins in the extracellular medium (Fielding, 1984). Cholesterol is also delivered to the cell indirectly, as LDL-cholesterol; uptake occurs via the LDL receptor and after degradation of the lipoprotein particle the cholesterol released is available for cell metabolism and to suppress endogenous cholesterol synthesis. In this way, the LDL receptor facilitates delivery of circulating cholesterol to individual peripheral tissues. Cellular cholesterol homeostasis is maintained by an additional lipoprotein-dependent pathway which permits efflux of cell cholesterol into the circulation. This is thought to involve newly synthesised apo-AI-containing pre-beta HDL particles which are efficient acceptors of excess membrane cholesterol. Incubation of HepG2 cells with lipoproteins has previously been shown to alter apo-AI expression (Monge et al., 1989) leading to the hypothesis that intracellular cholesterol and the HDL and LDL receptor pathways may be linked to apo-AI expression. The aim of the following studies was to decrease or increase the level of apo-AI mRNA by incubating HepG2 cells with HDL or LDL (or free cholesterol), respectively and to observe the effects of these treatments on LCAT gene expression.

3.5.2. Results

Exposure of HepG2 cells to both HDL and LDL for 24 h led to a decline in apo-AI mRNA levels whereas free cholesterol treatment caused a modest stimulation in apo-AI expression (Figure 3.14B). LCAT mRNA levels were
reduced on incubation of HepG2 cells with both LDL and free cholesterol but were essentially unaffected by HDL treatment (Figure 3.14A). Both the magnitude of changes and the response to treatment were altered by pre-incubating the cells for 24h in DMEM plus lipoprotein-deficient serum containing HDL at a concentration of 100μg of protein ml⁻¹ medium. In cells preincubated with HDL, further incubation with LDL or free cholesterol increased LCAT mRNA (Figure 3.14C). LDL but not free cholesterol increased apo-AI expression (Figure 3.14D).

3.5.3. Discussion

When HepG2 cells are incubated with either free cholesterol or LDL-cholesterol, the subsequent accumulation of cholesterol suggests the need for an alternative mechanism by which excess cholesterol can be removed from the cells. Because HDL provides such a route, it is possible that HepG2 cells might respond to treatment with LDL or free cholesterol by increasing apo-AI mRNA levels and secretion of HDL. However, in the first experiment, stimulation of apo-AI mRNA by free cholesterol appeared to be minimal (confirming the data of Monge et al., 1989) and was actually depressed by LDL cholesterol. Although incubation of HepG2 cells with HDL-cholesterol lowered apo-A1 mRNA levels, there was no effect on LCAT mRNA (Figures 3.14 A&B).

A second experiment was therefore carried out in order to maximise any changes in mRNA; This was achieved by first pre-incubating HepG2 cells in medium containing HDL (this has been shown to deplete the cells of cholesterol - Monge et al, 1989) and then re-exposing the cells to a cholesterol supply (Figures 3.14 C & D).
Figure 3.14 The effect of 24h incubation of HepG2 cells with high density lipoprotein (HDL; 100μg ml⁻¹ protein), low density lipoprotein (LDL; 100μg ml⁻¹ protein) and free cholesterol (FC; 50μg ml⁻¹) on A LCAT and B Apo-AI mRNA. Control (CON) HepG2 cells were grown for 24h in the absence of lipoproteins or cholesterol. In separate experiments, HepG2 cells were preincubated with HDL (100μg ml⁻¹ protein) 24h prior to further incubation (24h) with either HDL (100μg ml⁻¹ protein), LDL (100μg ml⁻¹ protein) and FC (50μg ml⁻¹), to determine the effect on C LCAT and D Apo-AI mRNA. Total RNA from HepG2 cells was blotted in triplicate in the range from 2.5 to 10μg and hybridised with either the LCAT probe or the apo-AI probe. The amount of mRNA was quantified by scanning densitometry of autoradiographs and the absorbance values were normalised to those for actin. Data are expressed as a percentage of the absorbance values obtained from cells grown throughout in control (A, B) or HDL-enriched medium (C, D). Each bar represents pooled mRNA from three replicate flasks.

The finding that incubation with LDL cholesterol stimulates apo-AI mRNA (Figure 3.14D) suggests that an apparent increased requirement for HDL in the medium surrounding the cells may be linked to apo-AI mRNA levels. In contrast, incubation of HepG2 cells with HDL in the medium would result in the efficient removal of any excess cellular cholesterol, with the eventual need to switch off this mechanism in order to maintain cellular cholesterol homeostasis.
The depression of levels of apo-AI mRNA in the presence of HDL are consistent with this suggestion as the need for HDL production would be minimal. Direct interaction between LDL and LCAT has also been demonstrated (Barter, 1983). This worker proposed a model whereby cholesterol was directly incorporated into LDL as a result of the direct interaction of LCAT on the free cholesterol associated with LDL. He concluded that while most of the LCAT may interact with particles in the HDL fraction, a small proportion of the enzyme interacts directly with LDL. This direct esterification of the free cholesterol in VLDL-LDL may be important; for example, it may account for the esterified cholesterol of LCAT origin which is found in the VLDL and LDL of patients with Tangier disease (Chen & Albers, 1985b). In this disorder, characterised by a severe HDL deficiency secondary to a very rapid catabolism of HDL, the residence time of apo-A1 is less than 5% of normal and both the plasma LCAT levels and LCAT activity are dramatically reduced. Furthermore, in the A1 Milano condition (Franceschini et al, 1990), apo-A1M monomers and normal apo-A1 are also rapidly catabolised and LCAT mass is again decreased. These findings suggest that a relationship may exist between the catabolism of HDL (or apo-A1) and LCAT level in the individual. Thus, the presence of HDL under normal physiological conditions and the modification of these particles by LCAT may be important determinants of the receptor-mediated catabolism of LDL by the liver and hence the development of atherosclerosis.

Since the cellular free cholesterol transferred to HDL is largely sequestered through esterification by LCAT, and then delivered to the liver via the 'reverse cholesterol transport' process, there may be an increased requirement for LCAT when cells are exposed to LDL or free cholesterol. This explanation is supported by the observation that LCAT mRNA was clearly stimulated by LDL and free cholesterol in cells preincubated with HDL (Figure 3.14C). The suppression of LCAT mRNA by HDL (Figure 3.14C) suggests that the removal
of cellular cholesterol via LCAT and the reverse cholesterol transport pathway was not required and that the reduction of LCAT expression was necessary in order to temporarily shut down this pathway and regain cholesterol homeostasis. However, in an individual, the cells are continually exposed to HDL, therefore an alternative explanation is that incubation with either LDL or free cholesterol may remove some inhibitory element that suppresses LCAT expression or reduces the stability of LCAT mRNA. Another explanation is that treatment with the other lipoproteins (LDL and free cholesterol) merely returns the HDL-induced decrease in apo-A1 expression to normal levels.
CHAPTER 4

LCAT IN HUMAN DISEASED LIVER
4. LCAT in Human Diseased Liver

4.1. Introduction

Moderate and severe liver disease is characterised by partial plasma LCAT deficiency with lipoprotein abnormalities which may exacerbate several clinical problems, including anaemia, renal dysfunction and lipid deposition, as discussed earlier (Introduction pp 32-40) due to the accumulation of unesterified cholesterol and lecithin in cell plasma membranes. The reduced esterification rate of plasma cholesterol in parenchymal liver diseases has been shown to be attributable to defective LCAT production by damaged liver cells (Simon, 1979) and it is possible that reduced transcription of the LCAT gene contributes to the decreased mass of LCAT protein in liver disease. Plasma LCAT assays give a good indication of liver function (Horton & Owen 1990, Higashi et al 1990), however LCAT mRNA levels have not previously been measured in the liver of patients with hepatic disease.

This study was carried out to identify whether the low concentrations of plasma LCAT observed in liver disease are due to defects at the transcriptional level, rather than failure to secrete normal amounts of the protein, by comparing the amounts of liver mRNA of LCAT and three other secretory proteins, apo-A1, albumin and transferrin, from patients with various liver diseases. Diseased liver samples were obtained from patients undergoing liver transplantation: two with fulminant hepatic failure (FHF) due to paracetamol overdose; one with primary liver cell carcinoma (PLCC) and one with primary biliary cirrhosis (PBC). The grafted liver of the PBC patient was rejected 26 weeks after transplantation and a specimen of this was also obtained (PBC-r). 'Normal' liver was obtained from the adjacent non-tumourous tissue of the patient with primary liver cell carcinoma and from a patient with oxalosis. These tissues were immediately frozen in liquid nitrogen prior to extraction of total RNA for northern blot analysis (see Chapter 2.3.3). The mRNA levels for LCAT,
albumin, transferrin and apo-A1 were estimated and normalised to 28S ribosomal RNA.

4.2 Results

For all four secreted proteins (LCAT, apo-A1, albumin and transferrin - Figures 4.1 and 4.2), the greatest reduction in mRNA hepatic expression was found in the patients studied with fulminant hepatic failure, with LCAT mRNA showing the largest decrease in expression compared to the control. Apo-A1 was reduced to 90% of the control value in the rejected liver of the patient with primary biliary cirrhosis (PBC) but showed no reduction, compared to the controls, in the livers of patients with primary liver cell carcinoma (PLCC) or PBC. On the other hand, transferrin mRNA expression in the livers from the patients with PLCC and PBC-rejected liver was reduced to 67% and 87% of the control values respectively.

Surprisingly, both transferrin and apoA1 mRNA levels appeared to be elevated in the PBC liver, compared to the control values. By contrast, both LCAT and albumin mRNA were substantially reduced in the livers of the patients with PLCC (to 4% and 57% control respectively); PBC (to 50% and 51% respectively) and in the rejected liver of the PBC patient (to 19% and 38% respectively).
Figure 4.1 mRNA levels of the LCAT, apo-A1, albumin and transferrin genes in liver samples from patients with primary liver cell carcinoma (PLCC), primary biliary cirrhosis (PBC) and the rejected liver from the PBC patient as well as two patients with fulminant hepatic failure (FHF). Data (expressed in arbitrary densitometric units) was normalised to 28s ribosomal RNA and to the average of the two control livers (mRNA = 100 for each secretory protein).

Figure 4.2 Northern blot of liver tissue from different patients with liver disease
4.3. Discussion

Plasma LCAT activity has previously been shown to be a sensitive marker in the assessment of liver function and has even been used as a predictor of allograft viability in liver transplantation (Higashi et al, 1990). The value of plasma LCAT activity in the accurate prognosis of liver disease is supported by the observations made in this study at the gene level. Thus, although mRNA levels of all four proteins were markedly reduced in the livers from patients with fulminant hepatic failure, both LCAT and albumin expression also appeared to be sensitive indicators in the patients with PLCC and PBC. Moreover, while both LCAT and albumin mRNA levels were reduced similarly by 50% in the PBC liver, LCAT expression was clearly lower than that of albumin in the liver which had been rejected in this patient. This substantial reduction of LCAT mRNA in the 5 diseased liver samples suggests that the plasma LCAT deficiency observed in liver disease results from a defect in expression of the LCAT gene and not to a defect in protein secretion. Plasma LCAT activity measured in patients undergoing orthotopic liver transplantation in our department (Karatapanis et al; 1994) was shown to be significantly lower in 12 patients before transplantation compared to 10 normals (0.98% CE formed/h versus 6.5%, p<0.001), as was the percentage of total plasma cholesterol as cholesteryl ester (40% versus 72%). In this study, plasma LCAT activity showed a gradual increase following liver transplantation. Hepatic LCAT mRNA levels thus appear to reflect plasma LCAT activity in such patients. Further research would also be of value to determine whether the reduced mRNA levels in liver disease are a result of gross gene rearrangements, or whether they are due to a defect in the transcription of the LCAT gene, or to reduced stability of the newly transcribed RNA.
CHAPTER 5

Response of LCAT to Hepatotoxic Agents
5. Response of LCAT to Hepatotoxic Agents

5.1. Introduction

The study on human diseased liver (see Chapter 4) indicated that LCAT is more sensitive than other liver secreted proteins to different types of hepatocellular damage. However, there is little information on the response pattern of the LCAT gene and protein in such cases, or possible mechanisms for the response. As human patients were not available for such studies, various models for hepatocyte injury were used in order to assess whether the pattern of response of LCAT mRNA found in the previous study was reproducible in culture and in vivo. This was achieved by exposing liver cells (HepG2 cells) and whole animals to various hepatotoxins. LCAT mRNA was measured and, for the experiments in vivo, correlated with catalytic activity of the enzyme in order to obtain a complete picture of any resulting changes. Apo-A1 mRNA was also measured to see whether any changes in gene expression under these conditions were specific to LCAT. Furthermore, in order to show that any reduction in LCAT mRNA levels in cultured cells were real and not due to cell death, the secretion of $^{35}$S-labelled proteins from the HepG2 cells were measured throughout these experiments.

Many toxic chemicals and carcinogens undergo oxidative metabolism to form highly electrophilic reactive intermediates which may then become covalently bound to enzymes and DNA, or generate free radicals which may similarly damage intracellular constituents. Thus, the addition of $\text{H}_2\text{O}_2$ to Chinese hamster ovary (CHO) cells has been shown to induce oxidative stress through the production of hydroxyl radicals (OH; Gelvan et al, 1995), which are cytotoxic. Similarly, the hepatic necrosis seen in patients with paracetamol overdose is thought to be caused by a reactive metabolite rather than the parent compound itself. Galactosamine has been also been shown to induce hepatotoxicity in rats (Sabesin et al, 1975). Thus, the addition of paracetamol, galactosamine and $\text{H}_2\text{O}_2$ to HepG2 cells may provide a convenient model for
chronic liver cell injury allowing the investigation of LCAT regulation under these conditions.

5.2. The effects of hepatotoxic agents in vitro

5.2.1. Addition of paracetamol to HepG2 cells

Paracetamol (5–40mM) was added to confluent HepG2 cells for 20h. The cells were then removed into GT and total RNA extracted as described earlier (see Chapter 2).

Incubation of HepG2 cells with paracetamol caused a dose-related decrease in mRNA expression for both LCAT and apo-A1, compared to actin (Figure 5.1). At the highest concentration of paracetamol used (40mM), LCAT expression was reduced by 90% compared to untreated cells. Although LCAT expression was more sensitive to high concentrations of paracetamol than apo-A1, at 5mM of paracetamol in the incubation medium, apo-A1 mRNA dropped to 50% of the control value whereas LCAT was not significantly reduced. A time course was also carried out to determine the effect of paracetamol at a defined concentration (Figures 5.2 and 5.3). HepG2 cells were incubated with 30mM paracetamol and both LCAT and apo-A1 expression determined at fixed time intervals over 9h.

Figure 5.1C shows the mean results taken from three experiments looking at total cellular protein secretion after 20h of incubation with varying paracetamol concentrations. Interestingly, the pattern of protein secretion did not parallel LCAT mRNA expression; at 5mM paracetamol concentration, cellular protein production had increased nearly four-fold, compared to untreated cells and was still higher from cells treated with 20mM paracetamol than control cells.

LCAT expression in paracetamol treated HepG2 cells showed a steady drop with time, falling to 23% of the control value after 9h (Figures 5.2 and 5.3). Apo-A1 mRNA showed an initial increase after three hours of incubation followed by a steady reduction over the next six hours of incubation.
Figure 5.1 The effect of a 20h incubation of HepG2 cells with paracetamol (5-40mM) on A LCAT, B apo-A1 mRNA levels and C secreted cellular protein levels. Control cells were grown in standard medium in the absence of paracetamol (0mM). Total RNA from HepG2 cells was blotted in triplicate in the range from 2.5 to 10μg and hybridised with either the LCAT probe or the apo-A1 probe. The amount of mRNA was quantified by scanning densitometry of autoradiographs and the absorbance values were normalised to those for actin. The changes in mRNA were expressed as a percentage of control absorbance values. Data shown is from cells pooled from 3 separate flasks. C: the vertical lines show the SEM. Significant differences (determined using Student's t-test for unpaired data) between control and treated groups are shown as ** P < 0.01.
Figure 5.2 Time course of the effect of incubation of HepG2 cells with 30mM paracetamol on A LCAT and B apo-A1 mRNA levels. Control cells were grown in standard medium in the absence of paracetamol (0h). Total RNA from HepG2 cells was blotted in triplicate in the range from 2.5 to 10µg and hybridised with either the LCAT probe or the apo-A1 probe. The amount of mRNA was quantified by scanning densitometry of autoradiographs and the absorbance values were normalised to those for actin. The changes in mRNA were expressed as a percentage of control absorbance values. Data shown is from cells pooled from 3 replicate flasks.
5.2.2. Galactosamine treatment of HepG2 cells

Figure 5.4 shows the effect of galactosamine (5-50mM) on LCAT and apo-A1 expression in HepG2 cells. There was a sharp reduction in LCAT mRNA from 5 to 20 mM galactosamine, following which suppression of LCAT mRNA was maintained at a steady level of about 20% of the control value through to 50mM galactosamine. In contrast, apo-A1 appeared resistant to galactosamine treatment at the lower concentrations (5 and 10mM), but there was a sharp drop in apo-A1 mRNA between 10mM and 30mM galactosamine to 20% of the control value and this was further reduced (to 10%) at 40mM and 50mM.

Figure 5.5 shows the mean results taken from three experiments looking at LCAT expression (Figure 5.5A) and total cellular protein secretion (Figure 5.5B) in HepG2 cells treated for 20h with increasing concentrations of galactosamine. Protein synthesis levels have been proposed as good markers
of cellular metabolic competence, a reduction indicating cytotoxicity (Gwynn et al, 1979). Protein secretion from HepG2 at 30mM galactosamine was much higher (more than three-fold) compared to untreated cells and the cells were still actively secreting protein at a rate higher than control cells at 50mM galactosamine (Figure 5.5B). LCAT mRNA steadily declined from the control value with increasing concentrations of galactosamine in the HepG2 cell medium, and reached its lowest value at the highest concentration of galactosamine (50mM) (Figure 5.5A).

Figure 5.4 The effect of a 20h incubation of HepG2 cells with galactosamine (5-50mM) on A LCAT and B apo-A1 mRNA levels. Control cells were grown in standard medium in the absence of galactosamine (0mM). Total RNA from HepG2 cells was blotted in triplicate in the range from 2.5 to 10µg and hybridised with either the LCAT probe or the apo-A1 probe. The amount of mRNA was quantified by scanning densitometry of autoradiographs and the absorbance values were normalised to those for actin. The changes in mRNA were expressed as a percentage of control absorbance values. Data shown are from a single preliminary experiment using one flask per concentration point.
Figure 5.5 The effect of 20h incubation of HepG2 cells with galactosamine (5-50mM) on A LCAT mRNA and B secreted cellular protein levels. Control cells (0mM) were grown in standard medium in the absence of galactosamine. Total RNA from HepG2 cells was blotted in triplicate in the range from 2.5 to 10µg and hybridised with either the LCAT probe or the apo-A1 probe. The amount of mRNA was quantified by scanning densitometry of autoradiographs and the absorbance values were normalised to those for actin. The changes in mRNA were expressed as a percentage of control absorbance values. Each point is the mean from 3 replicate flasks and the vertical lines show the SEM. Significant differences (determined using Student’s t-test for unpaired data) between control and treated groups are shown as * P < 0.05

5.2.3. Incubation of HepG2 cells with hydrogen peroxide (H₂O₂)

Both LCAT and apo-A1 mRNA were reduced by H₂O₂ treatment (Figure 5.6). H₂O₂ caused a significant concentration-dependent reduction in LCAT mRNA. Maximum inhibition of LCAT mRNA was observed at 1mM. Protein secretion was similar to control values for up to 1mM H₂O₂. However, 2mM H₂O₂ was associated with a marked reduction in protein secretion, presumably due to H₂O₂ induced cell death (Figure 5.6C). Apo-A1 expression, after an initial apparent rise, was also reduced by H₂O₂. This appeared to parallel the reduction in LCAT, reaching 10% of the control mRNA value at 2mM H₂O₂. At a concentration of 2mM, mRNA levels of both secretory proteins were reduced to 10% or less of control value.
Figure 5.6 The effect of a 20h incubation of HepG2 cells with hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}; 0.1-2mM) on A LCAT and B apo-A1 mRNA levels. Control cells were grown in standard medium in the absence of H\textsubscript{2}O\textsubscript{2} (0mM). Total RNA from HepG2 cells was blotted in triplicate in the range from 2.5 to 10\textmu g and hybridised with either the LCAT probe or the apo-A1 probe. The amount of mRNA was quantified by scanning densitometry of autoradiographs and the absorbance values were normalised to those for actin. The changes in mRNA were expressed as a percentage of control absorbance values. A LCAT mRNA: each point is the mean from 3 replicate flasks and the vertical lines show the SEM. Significant differences (determined using Student's t-test for unpaired data) between control and treated groups are shown as ** P < 0.01. B apo-A1 mRNA: Data shown is from a single experiment.

5.2.4 Discussion

The above studies demonstrate that LCAT expression was reduced by exposure of HepG2 cells to various hepatotoxic drugs. This reduction in expression appeared to act selectively at the mRNA level without significantly affecting normal cellular protein synthesis or secretion at low concentrations. Although apo-A1 mRNA was also reduced by exposure to these drugs, LCAT mRNA levels appeared to be more sensitive to hepatotoxic drugs at the lower
concentrations. Furthermore, apo-A1 expression appeared to be initially stimulated by exposure to all three drugs (Figures 5.2-5.5), thus apparently exhibiting an acute-phase response to injury.

Tissue injury and infection lead to a large array of metabolic changes collectively known as the acute phase response, among which are changes in the hepatic synthesis of a number of plasma proteins referred to as acute-phase proteins (Fey & Fuller, 1987). Several studies have reported variations in the lipid and apolipoprotein composition during conditions that could promote the acute-phase response, such as microbial infections (Alvarez & Ramos, 1986), myocardial infarction (Heldenberg et al, 1980), and other tissue destructive processes. Studies in primary hepatocyte cultures and hepatoma cell lines have shown that hepatic synthesis of human acute-phase proteins can be influenced by several cytokines including interleukin-6 (IL-6), tumour necrosis factor α (TNFα). Furthermore, transforming growth factor β1 (TGF-β1) has also been shown to induce hepatic synthesis and secretion of a subset of acute-phase proteins, both directly and by modulating the effect of interleukin-6 (Mackiewicz et al, 1990). During the acute-phase response, VLDL-triglyceride and VLDL-cholesterol increase while HDL-triglyceride and HDL-cholesterol decrease (Cabana et al, 1989). In these experiments, LCAT mRNA levels were reduced when HepG2 cells were treated with various hepatotoxins. Furthermore, work recently done by Skretting et al (1995) has shown that treating HepG2 cells with TGF-β reduced both LCAT activity in the medium and LCAT mRNA levels in these cells. Reduced LCAT activity may also explain reported decreases in HDL-cholesterol during the acute-phase response (Cabana et al, 1989). Thus, LCAT appears to be involved in the acute-phase response, by acting as a negative acute-phase protein.

Hepatotoxins are usually classified as non-genotoxic (those that do not form DNA adducts or produce positive results in mutagenicity assays; Cohen et al,
1990), but cause ongoing liver cell necrosis, inflammation and regenerative proliferation, or they interact with a specific receptor that mediates effects on gene expression (for a review, see Ockner et al, 1993). LCAT gene expression was suppressed by hydrogen peroxide treatment in HepG2 cells, although cellular protein synthesis appeared to be normal (see Figure 5.6C). Hydrogen peroxide generation has been shown to increase oxidative stress, which in turn leads to alterations in gene expression (see Ockner et al, 1993). Thus, an alternative mechanism for the observed reduction in LCAT expression due to \( \text{H}_2\text{O}_2 \) might be the diminution of Sp1 binding to the LCAT gene promoter by the resulting hydroxyl radicals; Kwak et al (1995) detected endogenous intracellular glutathionyl radicals in a neuroblastoma cell line under \( \text{H}_2\text{O}_2 \) oxidative stress. This resulted in glutathione depletion. Furthermore, Ammendola et al (1995) reported that \( \text{H}_2\text{O}_2 \) added to nuclear extracts decreases Sp1 binding activity. The Sp1 box (GGGCGG) allows Sp1 factors to bind to binding sites within specific gene promoters resulting in their constitutive activation and hence transcription, provided that other binding sites which may also be needed for induction of gene expression are available. Thus, \( \text{H}_2\text{O}_2 \) may be mimicking the natural suppression and regulation of specific genes by hydroxyl radicals within cells.

Intraperitoneal administration of galactosamine to rats results in a dose-dependent depletion of uridine phosphates by the formation of UDP-sugar derivatives (De Oliveira et al, 1992). The resulting deficiencies in uridine phosphates and UDP-hexoses leads to plasma membrane injury resulting from the inhibition of RNA and protein synthesis (including plasma membrane proteins). Furthermore, both paracetamol and galactosamine-induced hepatocyte injury are associated with an increase in intracellular calcium concentration (Matsumoto et al, 1992), perhaps leading to membrane damage by the activation of phospholipases. It is thus possible that the use of
paracetamol and galactosamine results in non-infectious cell injury (Cabana et al, 1989) which elicits an acute-phase response in HepG2 cells.

Emergency transplantation for acute liver failure has a significantly inferior outcome than transplantations performed for elective indications (Devlin et al, 1995). Thus, the ability to predict the onset of liver disease would provide a valuable tool for successful treatment. Our experiments suggest that LCAT mRNA may be a sensitive and reliable indicator of liver dysfunction in these cases.

5.3. The effect of hepatotoxic agents in vivo

5.3.1. Galactosamine-induced hepatitis in mice

Intraperitoneal administration of galactosamine is known to cause acute hepatitis in rats, a consequence of which is a marked deficiency of LCAT activity in the plasma compartment (Matsuura & Swaney, 1991). In the present study galactosamine was used to induce experimental hepatitis in mice. The mice (weight 20g) were fasted overnight. The following day groups of 6 were injected with 15mg galactosamine in 0.5 ml of saline/mouse for varying times (8h, 16h, 24h or 48h) after which the mice were killed. The blood was clotted on ice for 4h, then the sera frozen at -70 °C to assay LCAT activity later. The livers were removed and snap-frozen in liquid nitrogen for RNA extraction in order to measure hepatic LCAT expression. A group of control mice was injected with saline and killed after 48h.

Results

Figure 5.7A shows the effect of galactosamine on plasma LCAT activity in mice. Galactosamine caused a significant fall in plasma LCAT activity (P<0.05; Students' unpaired t-test); eight hours after galactosamine injection LCAT activity had fallen to 54% of control value. However, between eight and sixteen hours following galactosamine treatment, plasma LCAT activity started to
recover, reaching 76% of control by 24 hours and remaining at this level for the next 24 hours. The percentage of esterified cholesterol in plasma was also monitored over this time period (Figure 5.7B), as was hepatic LCAT expression (Figure 5.8A). Sixteen hours after galactosamine injection, there was a significant (three-fold) increase in LCAT expression. From 16h to 24h, hepatic LCAT expression fell but was still greater than in the control animals; LCAT expression then continued to fall until at 48h post-injection of galactosamine it was lower than control levels. Percentage total cholesteryl ester formation followed a similar course to that of plasma LCAT activity (Figure 5.7B); but a drop in percentage total cholesteryl ester was noted at 16h (8h after the observed fall in plasma LCAT activity).

Similarly, apo-A1 expression dropped between 16h and 24h after galactosamine injection and continued to fall to well below the control values at 48h post-injection of galactosamine (Figure 5.8B).

5.3.2. Intraperitoneal administration with 4-amino-pyrazolo-{3,4-d} pyrimidine (APP) to mice

The purine analogue 4-aminopyrazolopyrimidine (4-APP) is known to block lipoprotein secretion from the liver (Andersen & Dietschy, 1976), causing a fall in the plasma concentration of cholesterol and triglyceride (Senior & Gregoriadis, 1983). Thus, the hypothesis that administration of 4-APP to mice, resulting in reduced lipoprotein secretion, may involve or influence the LCAT gene and protein was investigated. Briefly, mice were injected intraperitoneally (i.p) with cumulative doses of 4-APP and the livers removed and analysed for LCAT mRNA content. Plasma LCAT activity was also measured (see Methods and Materials, Chapter 2.5.2).
Figure 5.7  A Time course of change in plasma LCAT activity in mice injected with galactosamine. Mice were given an i.p. injection of 750mg kg⁻¹ galactosamine. Control animals were injected with saline and killed after 48h. Plasma LCAT activity was expressed as the amount of LCAT esterified in 1ml of plasma in 1h (nmol ml⁻¹ h⁻¹). Each time point is the mean of plasma LCAT activity from 6 animals and the vertical lines show the SEM. Significant differences (determined using Student's t-test for unpaired data) between control and treated groups are shown as * P < 0.05. B Time course of plasma cholesteryl ester measured in the same mice.
Figure 5.8 Time course of the effect of galactosamine treatment on A hepatic LCAT and B hepatic apo-A1 mRNA levels in mice. The animals were given an i.p. injection of 750mg kg$^{-1}$ galactosamine. Control animals were injected with saline and killed after 48h. Total RNA from the liver of control and treated animals was blotted in triplicate in the range from 2.5 to 10μg and hybridised with either the LCAT probe or the apo-A1 probe. The amount of mRNA was quantified by scanning densitometry of autoradiographs and the absorbance values were normalised to those for actin. The changes in mRNA were expressed as a percentage of control absorbance values. Each time point is the mean from 6 animals and the vertical lines show the SEM. Significant differences (determined using Student's t-test for unpaired data) between control and treated groups are shown as * $P < 0.05$ and ** $P < 0.01$. 
Results

Treatment of mice with 50mg kg\(^{-1}\) APP caused a dramatic fall in both LCAT and apo-A1 mRNA at 24h (Figures 5.10 and 5.9). LCAT expression was undetectable and this loss was maintained at higher cumulative doses of APP. Similarly, apo-A1 was dramatically reduced by 50mg kg\(^{-1}\) APP, and this loss in apo-A1 expression was maintained at the highest cumulative dose used (200mg kg\(^{-1}\)). Plasma LCAT activity initially appeared to be increased by APP at a dose of 50mg kg\(^{-1}\), although this was not significant, but was reduced to 83% of the control value at 100mg kg\(^{-1}\) APP. A cumulative dose of 150mg kg\(^{-1}\) APP caused a 42% reduction in plasma LCAT activity, while the highest cumulative dose of 200 mg kg\(^{-1}\) APP resulted in almost complete loss (0.02% of control).

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Figure 5.9  Northern blot of mouse liver exposed to increasing concentrations of 4-APP. Three lanes (mice) per group.
Figure 5.10 Effect of 4-amino-pyrazolo-[3,4-d]pyrimidine (APP) treatment in mice on A LCAT, B apo-A1 hepatic mRNA and C plasma LCAT activity. Animals were injected i.p. daily, over four days with APP (50mg kg\(^{-1}\), 4, 3, 2 or 1 injections; see methods). Control mice were given four injections (1 per day) of saline. All five groups of mice were killed on the fifth day. Total RNA from the liver of control and treated animals was blotted in triplicate in the range from 2.5 to 10μg and hybridised with either the LCAT probe or the apo-A1 probe. The amount of mRNA was quantified by scanning densitometry of autoradiographs and the absorbance values were normalised to those for actin. The changes in mRNA were expressed as a percentage of control absorbance values. Plasma LCAT activity was expressed as the amount of LCAT esterified in 1ml of plasma in 1h (nmol ml\(^{-1}\) h\(^{-1}\)). Each point is the mean mRNA level or plasma LCAT activity from 3 animals and the vertical lines show the SEM. Significant differences (determined using Student's t-test for unpaired data) between control and treated groups are shown as * P< 0.05 and ** P< 0.01.
5.3.3. Lipopolysaccharide treatment of mice

Lipopolysaccharide, or endotoxin, is a complex macromolecule containing lipid and polysaccharides, which is present in the cell wall of gram-negative bacteria (Morrison & Ulevitch, 1978). When lipopolysaccharide is injected into experimental animals in sub-lethal doses, it triggers increased or decreased synthesis and secretion into plasma of the acute-phase reactant proteins. This process is known to be mediated by cytokines, which are thought to be responsible for many of the host’s metabolic responses that occur during infection (Tracey et al, 1988; Grunfeld & Feingold, 1991), which is associated with changes in plasma lipids, resulting in hyperlipidemia (Heldenberg et al, 1980). From my in vitro studies (see Chapter 5.2.4), it was postulated that LCAT may be an acute-phase protein. Thus, in these experiments, endotoxin administration to rats was followed by monitoring any changes in plasma LCAT activity and in LCAT mRNA levels over time.

**Results**

Intraperitoneal injection of mice with 20mg/kg of lipopolysaccharide (LPS) resulted in a tendency towards a reduction of LCAT activity (Figure 5.11A). However, due to variation within the group, this did not attain significance. Plasma cholesteryl ester also showed a small decline in LPS-injected mice compared to control (Figure 5.11B). By contrast, LCAT mRNA tended to increase (by 964 ± 355%) 24h after LPS treatment, however this did not attain statistical significance compared to control mice injected with saline (100 ± 23%; p< 0.06).

5.3.4 Discussion

Infection, inflammation and trauma frequently induce changes in lipid metabolism and lead to immediate local responses and metabolic alterations collectively known as the acute phase response, among which are changes in
the hepatic synthesis of a number of plasma proteins referred to as acute phase proteins or reactants (for a review, see Fey & Fuller, 1987).

Figure 5.11 Effect of lipopolysaccharide (LPS) on A plasma LCAT activity, B plasma cholesteryl ester (%) and C LCAT mRNA in mice. Mice were injected with LPS (solid bar; 20mg kg\(^{-1}\), i.p.). Control mice were injected with saline (open bar; 0.5ml, i.p.). Plasma LCAT activity was expressed as the amount of LCAT esterified in 1ml of plasma in 1h (nmol ml\(^{-1}\) h\(^{-1}\)). Total RNA from control and treated animals was blotted in triplicate in the range from 2.5 to 10\(\mu\)g and hybridised with the LCAT probe. The amount of mRNA was quantified by scanning densitometry of autoradiographs and the absorbance values were normalised to those for actin. The changes in mRNA were expressed as a percentage of control absorbance values. Each bar represents the mean from 6 animals and the vertical lines show the SEM. There were no significant differences (determined using Student’s t-test for unpaired data) between control and treated groups although there was a tendency for LPS to reduce cholesteryl ester and to increase LCAT mRNA expression.
The synthesis of positive acute phase proteins increase while the synthesis of negative acute phase proteins decrease during the acute phase response. This host response to injury is mediated by cytokines such as tumour necrosis factor α (TNFα), the interleukins (IL) and the interferons (IFN) (see Fey & Fuller, 1987).

In the present study, maximum reduction of plasma LCAT activity was observed eight hours after galactosamine injection. However, LCAT mRNA was not reduced as expected. This suggests that galactosamine may directly affect secretion of LCAT protein, leading to a loss in catalytic activity. In rats, galactosamine induces an LCAT deficiency by trapping the liver’s uridine pool as UDP-galactosamine derivatives, thus inhibiting UDP-dependent synthesis of glycoproteins, glycolipids, glycogen, nucleic acids and other macromolecules (Decker & Keppler, 1972). LCAT secretion, which requires the protein to be glycosylated, may thus be impaired; this may offer a mechanism for the effect of galactosamine on LCAT at the translational level. By contrast, LCAT mRNA rose and could be seen as a compensatory mechanism to maintain homeostasis and may explain the partial recovery in LCAT activity 8-16h after galactosamine administration (Figure 5.7).

Galactosamine induced hepatitis in rats results in plasma lipoproteins with increased levels of free cholesterol, phospholipids and triglycerides and reduced levels of cholesteryl ester (Cartwright et al, 1982). Cholesterol shifts from the HDL fraction into the LDL fraction, while the increased triglyceride remains in the VLDL density range. The increased phospholipid appears in both the LDL range and in the cholesteryl ester-depleted HDL. In addition to the lipid compositional changes in galactosamine-induced hepatitis there is a marked alteration in the apolipoprotein composition of the plasma lipoproteins. These abnormal lipoproteins show a reduction in VLDL-associated apo-E and apo-C, and HDL-associated apo-A and apo-C and an increase in LDL and HDL
apo-E (Cartwright et al, 1982). The lipoprotein abnormalities in galactosamine-treated rats are similar to those described in patients with alcoholic hepatitis and LCAT deficiency (Weidman et al, 1982). For instance, a variety of heterogeneous HDL particles have been described in galactosamine-treated rats which resemble those from patients with familial LCAT deficiency. These particles demonstrate considerable variation in their apolipoprotein composition, e.g. the larger HDL subfractions in galactosamine-treated rats have been shown to be enriched in apo-E while the smaller particles contain predominantly apo-A1 (Matsuura & Swaney 1991). Furthermore, the apo-E-rich HDL are enriched in free cholesterol and phospholipid and have much lower percentages of protein and cholesteryl ester, while the apo-A1-rich HDL particles are enriched in phospholipid but not in free cholesterol (Matsuura & Swaney, 1991).

Our observation that apo-A1 mRNA was also reduced by galactosamine treatment suggests there may be common regulatory elements for both the LCAT and apo-A1 genes. There is evidence to support apo-A1 as a negative acute phase protein, i.e. apo-A1 is down-regulated during the acute phase response to injury and inflammation (Olpin & Price, 1988; Bausserman et al, 1989). This is thought to be achieved through the action of the cytokine, TGF-β which selectively induces a specific decrease in the levels of apo-A1 mRNA (Morrone et al, 1988). Other cytokines [human recombinant tumour necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1-β) and interleukin-6 (IL-6)] have since been implicated in the dose-related reduction in the concentrations of apo-A1, apo-B and LCAT activity (Ettinger et al, 1994). Other workers (Tape & Kisilevsky, 1990) have shown that the half-life of apo-A1 is reduced to approximately 25% of its normal value in mice with inflammation compared to control animals. A possible mechanism for this reduced half-life is the up-regulation of HDL receptors (Graham and Oram, 1987) during inflammation, facilitating an increased uptake of apo-A1-containing lipoproteins (Tape &
Kisilevsky, 1989). Since apo-A1 is a co-factor for LCAT, it is possible that in this case the observed decrease in apo-A1 mRNA may in turn affect LCAT activity. However, this seems unlikely since even small amounts of apo-A1 are sufficient to activate LCAT protein; this is not surprising as the ratio of apo-A1 protein to LCAT in the plasma is far in excess of that required to achieve maximum activation of LCAT. Furthermore, the LCAT reaction can be stimulated by other apolipoproteins (most notably apo-E, apo-A-1V and apo-C1); this explains essentially normal cholesterol esterification in the plasma of patients with Tangier disease (low HDL and apo-A1 ) or with familial apo-A1 deficiency (See Introduction).

Cytokines have been shown to reduce LCAT activity in HepG2 cells (Skretting et al, 1995). The observed reduction in LCAT activity by galactosamine may be related to changes in HDL during acute inflammation. One such change in acute-phase HDL is the acquisition of apo serum amyloid A (apoSAA) (Benditt, 1979; Benditt & Erickson, 1977), which has the ability to displace apo-A1 from HDL. The apoSAA family of lipoproteins (in mice apoSAA1 and apoSAA2) is among a group of acute phase reactants synthesised by the liver and circulates primarily in association with HDL (Benditt, 1979). Normally found in trace amounts, the circulating level of apoSAA increases by 500-1000-fold within 24h of an inflammatory stimulus (McAdam & Sipe, 1976; McAdam et al, 1978) and may be the most abundant HDL apolipoprotein under these conditions. Thus, galactosamine treatment could lead to a reduction in HDL-bound apo-A1; and, by altering the composition of HDL particles may change the affinity of HDL for LCAT, resulting in an apparent loss of LCAT enzymic activity. This may explain why plasma LCAT activity is negatively correlated with SAA levels (Steinmetz et al, 1989). However, in this study an efficient exogenous substrate was used to assay LCAT activity which should have circumvented the problem of a poor endogenous HDL substrate.
4-APP is a purine analogue (adenine) that lowers endogenous plasma lipoprotein levels by reducing hepatic secretion of lipoproteins (Henderson et al, 1963; Shiff et al, 1971). This reduction in plasma cholesterol level is associated with an enhancement in HMG-CoA reductase activity and cholesterol synthesis in at least two non-hepatic tissues of the rat, namely the kidney and the lung (Balasubramaniam et al, 1976; Anderson & Dietschy, 1976). Treatment of mice with 4-APP resulted in virtually total loss of both hepatic LCAT and apo-A1 mRNA levels at the lowest accumulative dose used (50mg kg⁻¹). However, LCAT activity did not follow this pattern. The lowest dose of 4-APP, caused a slight increase in LCAT activity, although this was not significant. Subsequent doses of APP resulted in a dose-dependent reduction in LCAT activity. Thus, 4-APP appeared to be acting at the transcriptional level to reduce LCAT activity although there appeared to be a 24h delay between the action of APP on transcription of the LCAT gene and the effect on LCAT activity.

Lipopolysaccharide or endotoxin, administration, which mimic infection have previously been used as experimental models because they stimulate cytokine production. Furthermore, administration of LPS to monkeys appears to prevent normal intravascular metabolism of lipoproteins and results in the accumulation of immature forms of lipoproteins that resemble those from the plasma of patients with familial LCAT deficiency (Auerbach & Parks, 1988). Our study demonstrates that LPS treatment of mice may increase LCAT mRNA (Figure 5.11), although the apparent increase did not appear to be significant. This was not reflected in plasma LCAT activity or in the percentage of esterified cholesterol in plasma, which though significantly unchanged, tended towards reduced levels. This is in contradiction to work by Ettinger et al (1990) who demonstrated that administration of LPS led to reduced plasma LCAT activity in cynomolgus monkeys, and, more recently, by Ly et al (1995) who showed that a reduction in LPS-induced LCAT mRNA levels in Syrian hamsters
accompanied this fall in plasma LCAT activity. However, the reported changes in plasma cholesterol concentration due to the effects of acute inflammation are variable and appear to be species-specific; in rabbits, cholesterol levels increase during the acute phase response (Feingold & Grunfeld, 1987; Cabana et al, 1989), while in non-human primates and humans, infection and inflammation almost always lead to hypocholesterolaemia (Alvarez & Ramos, 1986; Sammalkorpi et al., 1988; Auerbach & Parks 1989).

An increase in LCAT mRNA would be expected to result in an increase in plasma LCAT activity; but this was unchanged after 24h in the LPS-treated mice (Figure 5.11). A similar result was shown with dexamethasone in HepG2 cells (Skretting et al, 1995), where an increase in LCAT mRNA did not lead to enhanced LCAT activity. This may be because changes in LCAT mRNA occur more rapidly than changes in LCAT activity, as was demonstrated in syrian hamsters by Ly et al (1995) and, since the acute phase of the inflammatory response usually lasts between 48 to 72h (Fey & Fuller, 1988), it may be that a further measurement taken at a later time interval would show an increase in plasma LCAT activity. Low dose endotoxin increases serum triglycerides primarily by stimulating hepatic triglyceride production and VLDL secretion (Chajek-Shaul et al, 1989; Feingold et al, 1992). In whole animals endotoxin has also been shown to stimulate lipolysis, thus raising free fatty acid levels (Chajek-Shaul et al, 1989), which may contribute to the cytokine-induced increase in hepatic VLDL production. Higher doses of endotoxin increase serum triglyceride levels by delaying the clearance of triglyceride-rich lipoproteins and decreasing lipoprotein lipase activity in adipose tissue, post-heparin serum and muscle (Feingold et al, 1992). Thus in our study the observed increase in LCAT mRNA in mouse liver may be in response to increased VLDL levels due to the action of cytokines.
During these studies one common result to emerge is the eventual reduction of both LCAT mRNA and activity by hepatotoxic drugs. A reduction in LCAT activity should lead to a decline in esterification of plasma cholesterol (and indeed has been shown to occur in galactosamine-injected mice - see Figure 5.7). Furthermore, a fall in apo-A1 concentration may reflect a decrease in HDL, which could slow down the reverse cholesterol transport process (Chapter 1.2.3). The physiological significance of this is not known. However, during acute illnesses, delivery of cholesterol to the liver for excretion may be altered to enable cholesterol to remain in the tissues for repair and regeneration of damaged membranes (Cabana et al, 1989).
CHAPTER 6

TISSUE DISTRIBUTION AND DEVELOPMENTAL EXPRESSION OF LCAT IN GUINEA-PIG
6. Tissue Distribution and Developmental Expression of LCAT In the Guinea-pig

6.1. Tissue distribution and developmental expression of LCAT

6.1.1 Introduction

The guinea-pig was initially chosen for a developmental study on LCAT expression because of its manageable size and physical maturity at birth compared to rats and mice; this made the isolation of different organs and subsequent extraction of mRNA easier, especially in pre-natal animals (viable offspring can be delivered up to three days before expected parturition). Furthermore, for the later study on fat-feeding, the guinea-pig was a preferable animal model to the rat (see 6.2.1), although the rat was also examined for comparison. The aim of this study was to assess whether LCAT might be developmentally regulated, by identifying any changes in hepatic LCAT mRNA in guinea-pigs of different ages using northern blot analysis. The presence of LCAT mRNA in other guinea-pig tissues and organs was also analysed. Tissues were removed and snap-frozen in liquid nitrogen from animals of the following age: day -2 (e.g. Figure 6.3, 2 days prior to expected parturition and 65 days after mating), day 0 (birth), days 2, 3, 4, 6, 8, 10, 13, 19 and day 29 (adult). The tissues were stored at -70°C until RNA was extracted and analysed by northern blotting. Serum was frozen at -70°C for subsequent measurement of plasma LCAT activity.

6.1.2 Results

LCAT mRNA was detected in the liver and duodenum of adult guinea-pigs (Figure 6.1) but was barely detectable in the brain and testes (Figure 6.4). The LCAT probe also detected a signal in the guinea-pig kidney, lung and heart (Figure 6.5). LCAT was not detected in the spleen (data not shown) or ileum (Figure 6.1). The LCAT probe detected a single hybridising mRNA species 1.5Kb long in all tissues expressing LCAT except in the kidney, where two
closely hybridising species were detected (Figure 6.5). Different organs at different ages were also examined to see whether LCAT was developmentally expressed.

**Liver**

There was no hepatic LCAT expression in foetal liver or at birth (Figure 6.2). However, by day 3 following parturition, LCAT mRNA was detected.

**Duodenum**

There was some weak LCAT expression in the duodenum; which was most apparent at day 19 (Figure 6.3).

**Brain and Testes**

LCAT expression was very faint and the signal was not strong enough for any changes to be detected (Figure 6.4)

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**Figure 6.1** Northern blot using RNA from adult guinea-pig liver, brain, duodenum and ileum, hybridised to the LCAT probe.
Figure 6.2  Autoradiograph of northern blot using guinea-pig liver RNA from animals of different ages, hybridised to the LCAT probe

Figure 6.3  Autoradiograph of northern blot; RNA was extracted from the ileum and duodenum of the guinea-pig of different ages and hybridised to the LCAT and apo-A1 probes. (Lower blot shows actin).
Figure 6.4 Autoradiograph of northern blot using brain and testes RNA from guinea-pigs of different ages, hybridised to the LCAT and apo-A1 probes

Figure 6.5 Autoradiograph of northern blot using kidney, lung and heart RNA from guinea-pigs of different ages, hybridised to LCAT
**Kidney**

There was a strong double-banded LCAT signal in the kidney at each stage of development, up to the adult (day 29). Only a weak second band was visible at day 0 (Figure 6.5)

**Lung and heart**

LCAT mRNA levels were faint in both the lung and heart (Figure 6.5).

**Plasma LCAT activity**

LCAT activity also changed with age in the guinea-pig. However, while LCAT mRNA was expressed at day 3 after parturition, and showed an increase at day 19 followed by a decline in the adult (31 days and over), plasma LCAT activity was not apparent until day 13. Plasma LCAT activity increased dramatically from day 13 to day 19, peaking at day 29 and then fell sharply in the adult. (Figure 6.6)

![Figure 6.6](image-url)

**Figure 6.6** Plasma LCAT activity in guinea-pigs of different ages (5 days prenatal through to adult). Plasma samples were taken from frozen sera and LCAT activity measured using the proteoliposome method (see 2.5.2) using 10 samples per age increment. Vertical lines show the SEM.
6.1.3. Discussion

LCAT has now been shown by Warden et al (1989) to be expressed in tissues other than the liver, namely the brain and testes of the rat. However, expression has not previously been demonstrated in other tissues and organs. Thus, the presence of LCAT in guinea-pig tissues other than the liver, brain and testes may reflect a species difference. The LCAT gene is expressed in vivo in the hepatocyte at relatively high levels, while in the kidney, heart, lungs, brain, testes, ileum and duodenum it is expressed at much lower levels. Recent work carried out on the LCAT gene promoter (Meroni et al, 1991) has defined elements required for an ubiquitous low level type of transcription, which may be comparable to that level of transcription occurring in the kidney, heart, lungs, brain, testes and duodenum.

The developmental study on LCAT mRNA levels in the guinea-pig showed that LCAT expression in the liver begins approximately 3 days after birth. Plasma LCAT activity also showed changes in the guinea-pig with age (Figure 6.6), with a sudden rise in activity approximately two weeks after birth and falling again in the mature adult. These changes largely follow the mRNA pattern (Figure 6.2), implying that plasma LCAT enzymic activity reflects the hepatic mRNA level. Changes in expression of proteins such as LCAT during development into the adult may be potentiated by hormonal influences. For example, Archer et al (1986) have demonstrated that apo-A1 mRNA is increased by oestrogen treatment of HepG2 cells and Strobl et al (1990) have shown the stimulation of apo-A1 gene transcription by thyroid hormones. Guinea-pig hepatic parenchymal cells contain particles thought to be precursors of lipoproteins (Bohmer et al, 1972). Towards the end of gestation, these particles enlarge to the size range characteristic of chylomicrons secreted from the intestinal mucosa after fat ingestion, which can be interpreted as evidence of intense hepatic synthesis and secretion of VLDL; this hyperlipidaemia falls after birth (Bohmer et al, 1972). The blood plasma of the
guinea-pig also contains moderate amounts of LDL and HDL, however the latter decreases to barely detectable levels during the first two weeks of post-natal life (Bohmer et al., 1972). In our study, LCAT activity continued to be suppressed during the first two weeks after birth; this may be linked to a decline in HDL. Free fatty acids appear to be the source of hepatic triglycerides in the guinea-pig foetus (Hershfield et al., 1968). After birth, secretion of triglyceride-rich lipoproteins may be related to the switching on of LCAT expression; as indicated in Chapter 1, LCAT may play a role in initiating the hepatic stimulus which produces the secretion of these lipoproteins (Quarfordt et al., 1993) or it may function in the catabolism of these particles (see Chapter 1).

Another factor that could influence gene expression is diet (see Clarke & Abraham, 1992). Dietary fat may alter a regulatory pool of cellular cholesterol or a metabolite in the sterol biosynthetic pathway to indirectly alter expression of the LCAT gene. To test this hypothesis experiments were carried out to investigate the response of LCAT mRNA to dietary fat (see 6.2).

6.2 Dietary regulation of LCAT expression
6.2.1 Introduction

The tissue distribution study revealed that LCAT mRNA was present in the guinea-pig duodenum, albeit at a very low level compared to hepatic LCAT expression (Figure 6.1). To investigate whether the LCAT message was being actively translated, a further study was carried out with isolated duodenal enterocytes (see methods Chapter 2) to detect the secretion of any LCAT protein by the duodenum, using an antibody to human LCAT (for results see 6.2.2).

Furthermore, since LCAT plays a central role in cholesterol transport, and is also required for the clearance of cholesterol following hydrolysis of dietary
chylomicrons, it was of interest to determine whether the translational mechanisms in this tissue could be 'switched on' by a fatty diet stimulus, i.e. whether LCAT gene expression was increased following a fatty meal. In the following study, olive oil containing predominantly monounsaturated (oleic acid) was used. Much of this would be absorbed and secreted as chylomicrons which, after lipolysis, would provide redundant surface material rich in cholesterol and lecithin that is a potential substrate for the LCAT reaction (see Chapter 1). The effect of cholesterol feeding on the guinea-pig duodenum was analysed by northern blotting (Figure 6.7). The effect of an oral fat load on rat LCAT mRNA was also examined for comparison.

6.2.2. Results

Detection of LCAT from guinea-pig duodenum using the LCAT antibody
The human LCAT antibody did not detect any secreted LCAT protein from the duodenal enterocytes, although the culture medium of HepG2 cells treated in the same way was found to contain $^{35}$S-LCAT, indicating active protein secretion from these cells. This suggests that while LCAT mRNA is present in the guinea-pig duodenum, the message is not translated into protein, although a possible explanation for this negative result may be related to the duration of fat-feeding (see discussion).

Northern and Slot Blot Analysis of fat-fed guinea-pig
Although LCAT mRNA was just detectable in the duodenum of the control guinea-pig, it was not found in the rat. By contrast, apo-A1 was readily detected in the duodenum of both the guinea-pig and the rat and, in both animals, responded to fat-feeding by an increase in mRNA concentration. In addition, this faint signal for LCAT was unchanged by fat-feeding (Figure 6.7).
**Guinea Pig**  
**Rat**

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**Figure 6.7** Northern blot of guinea-pig and rat duodenal tissue from fat-fed animals, hybridised to the LCAT and apo-A1 probes.
6.2.3. Discussion

The results demonstrate that LCAT mRNA in the guinea-pig duodenum was unchanged by exposure to dietary fat, whereas duodenal apo-A1 mRNA increased. Moreover, although there is a steady-state level of LCAT message in the duodenum, it is not translated into protein. LCAT expression has not previously been reported in the small intestine. Therefore the presence of LCAT mRNA in the guinea-pig duodenum questions its role in this tissue and the possible contribution of duodenal LCAT to normal lipoprotein metabolism.

Since the catabolism of the excess surface lipids generated by lipolysis of chylomicrons involves the action of LCAT, it is possible that in the guinea-pig LCAT is co-secreted with the chylomicrons produced by the duodenum in response to a fatty meal into the circulation where it has its site of action. This appears plausible in light of studies such as those by Quarfordt et al (1993), where plasma LCAT was shown to increase in cholesterol fed rats, along with an increase in plasma cholesterol. Furthermore, Ostwald and Shannon (1964) demonstrated a six-fold increase in liver lipid content of the guinea-pig after fat-feeding; this was accompanied by a doubling of plasma triglycerides and an increase in total plasma cholesterol content, mostly due to a rise in free cholesterol but also due to a large increase in cholesteryl ester content.

However, in these studies a fat diet was unable to alter expression of LCAT in the guinea-pig duodenum. This ties in with the earlier data showing that the guinea-pig duodenum does not secrete LCAT protein and suggests that the necessary transcription factors are absent in this tissue, although it is possible that hepatic LCAT mRNA may have responded to this treatment. However, other workers have demonstrated that fat-feeding of guinea-pigs over a number of weeks rather than hours causes changes in HDL lipoproteins (Lin et al, 1995) and in some cases can produce anaemia-like symptoms, with lipoproteins that resemble those observed in secondary LCAT deficiency (Ostwald and Shannon, 1968). Thus in this study, it would have been of
interest to fat-feed over a longer period of time, as this may have shown changes in duodenal LCAT mRNA levels and/or activity.

An increase in apo-A1 was observed after fat-feeding in both the guinea-pig and rat; this agrees with a previous report that fat-feeding stimulates the synthesis of apo-A1 in the human intestine (Glickman et al, 1978).

In conclusion, duodenal LCAT mRNA was unchanged by exposure to dietary fat in the guinea-pig, although apo-A1 increased in response to a fat load.
CHAPTER 7
GENERAL DISCUSSION
7. General Discussion

The aim of these studies was to determine whether it is possible to alter the expression of the LCAT gene, through one or more of 3 different manipulations: both *in vitro* and *in vivo*, by stimulating the production of triglyceride-rich particles; by altering apo-A1 mRNA or HDL levels; or by the use of hepatotoxic agents. This would lead to a greater understanding of the factors influencing the regulation of this gene.

Although oleic acid supplementation produced the anticipated increase in triglyceride secretion in both HepG2 and Hep3B cells, this treatment resulted in different responses from the two cell lines; whereas in the HepG2 cell line, a rise in triglyceride secretion was accompanied by a reduction in LCAT mRNA level, in Hep3B cells a similar rise in triglyceride resulted in a corresponding increase in LCAT mRNA. Therefore, LCAT mRNA appears to be linked to the secretion of VLDL particles in the Hep3B cell model, but not in the case of the HepG2 cell line. One explanation for this difference may be that HepG2 cells carry less triglyceride in the VLDL fraction than do Hep3B cells (Forte et al, 1989); it is possible therefore that the stimulation of VLDL triglyceride from basal levels by oleic acid in HepG2 cells was not great enough to upregulate steady-state LCAT mRNA. The HepG2 cell line may therefore not be a suitable model for the human hepatocyte.

Duodenal LCAT expression was detected in the guinea-pig. However, in agreement with previous work (McLean et al, 1986; Warden et al, 1989), LCAT mRNA was not detected in the rat duodenum. LCAT expression thus appears to be both tissue-specific and species dependent. Many eukaryotic genes are inactive in most tissues but are activated in a specific tissue in response to a specific signal(s); it appears more economical to have a system in which the gene is constitutively inactive and requires a particular factor(s) to bind to its promoter. The observation that LCAT mRNA levels were unchanged in the
guinea-pig duodenum after a fatty meal suggests that the factors required for transcription of the LCAT gene were not available and thus excludes fatty acids as one such factor. LCAT mRNA and activity of the enzyme did change in various tissues in the developing guinea-pig, thus other factors (e.g. hormonal) may be important in mediating these changes.

The manipulation of apo-A1 mRNA levels in HepG2 cells was also attempted using three different treatments: the use of gemfibrozil to stimulate apo-A1 mRNA synthesis; glucose deprivation to reduce apo-A1 mRNA synthesis and protein secretion, and cholesterol loading using LDL and free cholesterol to stimulate apo-A1 mRNA. In each case, LCAT mRNA levels were monitored in order to obtain a comparison with apo-A1 mRNA concentrations and to identify any possible similarity in the pattern of both responses. Glucose deprivation led to a decrease in both apo-A1 and LCAT mRNA levels, although the reduction in apo-A1 mRNA levels was more dramatic than that seen for LCAT mRNA. LDL-cholesterol loading led to a six-fold increase in apo-A1 mRNA; this corresponded to a similar (approximately three-fold) increase in LCAT mRNA. However, gemfibrozil treatment of HepG2 cells, which produced a two-fold stimulation of apo-A1 mRNA, resulted in a 41% reduction in LCAT mRNA levels. Thus, while there is some evidence that LCAT and apo-A1 at the mRNA level may be co-ordinately linked, this is not always the case. Hence, with gemfibrozil, where a stimulation of apo-A1 mRNA occurs parallel to a reduction in LCAT mRNA, it appears possible to dissociate the two responses. It may be possible that an immediate response to the drug occurs at the translational level and that more transcripts are made only if needed as a secondary response.

Various models for hepatocyte injury were set up in an attempt to answer the question of whether LCAT gene expression is altered in liver disease. In HepG2 cells exposed to paracetamol, galactosamine and hydrogen peroxide,
LCAT mRNA levels were dramatically reduced. When plasma LCAT activity was analysed in the guinea-pig following galactosamine injection, the activity of this enzyme was found to follow a similar pattern to the hepatic LCAT mRNA levels. A point of interest was the recovery of both plasma LCAT activity and LCAT mRNA 24 hours after galactosamine injection. Furthermore, plasma LCAT activity appeared to be slower to respond to recovery than LCAT mRNA levels; after 24 hours the LCAT mRNA value had exceeded the control value by five-fold, whereas plasma LCAT activity had still not reached control values. This suggests a rebound effect whereby the hepatocytes are able to respond to hepatotoxic insult by an eventual increase in the steady-state level of LCAT mRNA. It would therefore be interesting to follow the plasma LCAT activity after a longer incubation period with galactosamine, in order to elucidate whether the activity eventually reflects the pattern of recovery of LCAT mRNA. However it is not possible to define at this stage whether the increase in LCAT mRNA reflects a change in gene transcription or mRNA stability.

The reductions in LCAT mRNA in the human diseased liver samples suggest that the plasma LCAT deficiency observed in patients with liver disease may be a consequence of defective gene expression, rather than reduced protein secretion. Although the studies with hepatotoxic drugs and human diseased liver suggest that the observed reductions in LCAT protein mass and activity are partly due to a defect at gene level, future work must concentrate on establishing whether regulation of this gene is due to transcriptional or post transcriptional mechanisms, or, as is more likely the case, to both.
REFERENCES


References


References


References


## GUINEA PIG DIET FD1

**SUITABLE SPECIES AND APPLICATIONS:**
Guinea pigs for breeding and maintenance.

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<td>Micro-elements</td>
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**BENEFITS:**
- □ This is the original Frant Diet formula which has proved very successful, over a period of many years, for both the breeding and maintenance of guinea pigs.
- □ Fortified with 2500mg per kg of vitamin C, sufficient to make further supplementation unnecessary during the shelf-life of the diet (providing storage conditions are suitable).

**FEEDING GUIDE:**
Ad-lib feeding is recommended. Although supplementary hay is not necessary, it is beneficial to provide it for breeding stock.

**AVAILABLE AS:**

<table>
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<tr>
<th>Diet Code</th>
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<tr>
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<tr>
<td>FD1 (P)</td>
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**PACKAGING VARIATIONS:**
For Packaging Variations see Product Reference List.
## CALCULATED ANALYSIS

<table>
<thead>
<tr>
<th>NUTRIENTS</th>
<th>NÄHRSTOFFE</th>
<th>TENEURS NUTRITIVES</th>
<th>SUPPLEMENTATION</th>
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<tbody>
<tr>
<td>Crude Oil</td>
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<td>Huile Crue</td>
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<td>Crude Protein</td>
<td>Rohprotein</td>
<td>Protéine Brute</td>
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<td>Crude Fibre</td>
<td>Rohfasche</td>
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<td>N-freie Extraktstoffe</td>
<td>Glucides</td>
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<td>Verdauliches Rohfett</td>
<td>Huiles Crues Digestibles</td>
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<tr>
<td>Dig. Crude Protein</td>
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<td>Fibre Diététique Total</td>
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<td>Bruttoenergie</td>
<td>Energie Digestible</td>
<td>MJ/kg</td>
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<td>Verdauliche Energiefette</td>
<td>Energie Metabolique</td>
<td>MJ/kg</td>
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<tr>
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<td>Acide Myristoléique</td>
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<tr>
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<td>AMINOACIDES</td>
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<tr>
<td>Taurine</td>
<td>Taurin</td>
<td>Taurine</td>
<td></td>
</tr>
</tbody>
</table>

Note 1. All values calculated to nominal 10% moisture content.
Note 2. Values on left are total calculated values.
Note 3. Values on right are amounts added via supplementation.
Note 4. 1 μg Retinol = 3.3 i.u. Vitamin A activity.
Note 5. Total Retinol content includes the Retinol equivalent of Carotene.
Note 6. 1 μg β-carotene = 16 i.u. Vitamin A activity.
Note 7. 1 μg Cholecalciferol = 400 i.u. Vitamin D₃ activity.
Note 8. 1 mg Tocopherol = 11 i.u. Vitamin E activity.
Note 9. 1 MJ = 239.23 Calories.
## Appendix

### NUTRIENTS

<table>
<thead>
<tr>
<th>AMINO ACIDS</th>
<th>NÄHRSTOFFE</th>
<th>TENEURS NUTRITIVES</th>
<th>SUPPLEMENTATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>Glykokoll</td>
<td>Glycine %</td>
<td>1.26</td>
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<td>Aspartic Acid</td>
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<td>Acide Aspartique %</td>
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<td>Serin</td>
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<td>Hydroxyproline</td>
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<td>Hydroxylysine</td>
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<td>Alanine</td>
<td>Alanin</td>
<td>Alanine %</td>
<td>0.07</td>
</tr>
</tbody>
</table>

### MAJOR MINERALS

| Calcium           | Kalzium          | Calcium %           | 1.15 0.47       |
| Total Phosphorus  | Phosphor         | Phosphor %          | 0.86 0.22       |
| Phyate Phosphorus | Phytin Phosphor  | Phytate de Phosphore % | 0.24 0.22     |
| Available Phosphorus | Verfügbarer Phosphor | Phosphate Disponible % | 0.62 0.22 |
| Sodium            | Natrum           | Sodium %            | 0.28 0.21       |
| Chlorine          | Chloir           | Chlor %             | 0.43 0.32       |
| Magnesium         | Magnesium        | Magnésium %         | 0.34            |
| Potassium         | Kalium           | Potassium %         | 1.27            |

### TRACE MINERALS

| Iron              | Eisen            | Fer mg/kg           | 537.0 335.0     |
| Copper            | Kupfer           | Cuivre mg/kg        | 16.0 6.0        |
| Manganese         | Mangan           | Manganese mg/kg     | 173.0 125.0     |
| Zinc              | Zink             | Zinc mg/kg          | 69.0 53.0       |
| Cobalt            | Kobalt           | Cobalt µg/kg        | 720.0 700.0     |
| Iodine            | Jod              | Iode µg/kg          | 1257.0 700.0    |
| Selenium          | Selen            | Sélenium µg/kg      | 281.0           |
| Fluorine          | Fluor            | Fluor µg/kg         | 42.0 22.0       |

### VITAMINS

| Retinol           | Retinol          | Retinol µg/kg       | 68891.0 1020.0   |
| Vitamin A         | Vitamin A        | Vitamin A µg/kg     | 227340.0 3366.0  |
| Cholecalciferol   | Cholecalciferol  | Cholecalciferol µg/kg | 45.0 20.6 |
| Cholecalciferol   | Cholecalciferol  | Cholecalciferol µg/kg | 1800.0 824.0 |
| Vitamin D₃        | Vitamin D₃       | Vitamin D₃ µg/kg    | 877.0 424.0     |
| α-Tocopherol      | α-Tocopherol     | α-Tocopherol µg/kg  | 67.0 24.0       |
| Vitamin E         | Vitamin E        | Vitamin E µg/kg     | 73.7 26.4       |
| Vitamin B₁        | Vitamin B₁       | Vitamin B₁ µg/kg    | 8.3 3.3         |
| Vitamin B₂        | Vitamin B₂       | Vitamin B₂ µg/kg    | 14.2 8.3        |
| Vitamin B₆         | Vitamin B₆       | Vitamin B₆ µg/kg    | 11.4 8.3        |
| Vitamin B₁₂       | Vitamin B₁₂      | Vitamin B₁₂ µg/kg   | 12.6 4.2        |
| Vitamin C         | Vitamin C        | Vitamin C µg/kg     | 2678.0 2500.0   |
| Vitamin K₁        | Vitamin K₁       | Vitamin K₁ µg/kg    | 134.5 41.6      |
| Folic Acid        | Folsäure         | Folsäure µg/kg      | 8.8 8.3         |
| Nicotinic Acid    | Nicotinsäure     | Acid Nicotinque µg/kg | 70.9 20.8   |
| Pantothenic Acid  | Pantothenäsäure  | Acide Pantothenique µg/kg | 30.9 12.3 |
| Choline           | Cholin           | Choline µg/kg       | 1621.0 670.0    |
| Inositol          | Inosit           | Inosit µg/kg        | 2110.0          |
| Biotin            | Biotine          | Biotine µg/kg       | 376.0 85.0      |
| p-Aminobenzoic Acid | p-Aminobenzoësäure | Acide p-Aminobenzoïque µg/kg | 148.2 |
| β-Carotene        | β-Carotene       | β-Carotene µg/kg    | 148.2           |

### PIGMENTS

| Xanthophyll       | Xanthophyll      | Xanthophyle µg/kg   |                |

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