THE MEASUREMENT, ESTERIFICATION AND BIOLOGICAL SIGNIFICANCE OF SERUM 7α-HYDROXYCHOLESTEROL

A dissertation submitted for the degree of
Doctorate of Philosophy
by

G. Louise Dowling, B.Sc. (Hons.)

August, 1995

Under the supervision of Dr. Rosaleen Devery

School of Biological Sciences
Dublin City University
Dublin 9, Ireland

Declaration

I hereby certify that the material, which I now submit for assessment on the programme of study leading to the award of Ph.D, is entirely my own work and has not been taken from the work of others save, and to the extent that such work has been cited and acknowledged within the context of the text.

Signed: G. Louise Dowling Date: 04/August/95

G. Louise Dowling

Acknowledgements

I wish to dedicate this thesis to my parents- Robert and Catherine, my sisters Margaret, Antoinette and Sandra- brothers Mark, Victor and Robert and my aunt Cabrini as my way of thanking them for their constant patience, support, encouragement and thoughtfulness throughout the years.

Thank you to Dr.Rosaleen Devery for giving me the opportunity to pursue this doctorate and for her help throughout its undertaking.

For blood samples- thank you to Professor Gerald Tomkin of The Adelaide hospital, Dublin and for excellent work carried out in electron microscopy, a sincere thank you to Pat Costigan of the Virus Reference Laboratory, U.C.D.

For financial support I would like to thank the Postgraduate Studies Committee, Dublin City University.

For their advice and friendship, I thank my laboratory colleagues both past and present- Ron, Jacqui, Sinead and Helen.

A very sincere and special thank you to all the friends that I have made in the biology department at D.C.U.- the list of which is endless! Special acknowledgement to Teresa and Sharon- thanks for your help and moreso for your friendship, long may it last!

My final acknowledgement goes to all my friends who have been a constant source of support and friendship throughout - a special mention to Nora for being so obliging and also to my housemates for being so tolerant of me- Mary, Andrea, Dean and Dee.

And who said that I never had a word to say for myself?

Abstract

Since bile acids are major excretion products of cholesterol in the body, changes in rates of production will affect the turnover rate of cholesterol in the body. 7α-hydroxycholesterol is the product of the rate-limiting reaction in the biosynthesis of bile acids and it has been suggested that its concentration in serum is a good parameter for establishing disorders involving the metabolic conversion of 7α-hydroxycholesterol towards bile acids.

The objectives of the project were to validate the methodology towards measurement of 7α-hydroxycholesterol in serum - to investigate serum levels of this analyte in human and rat sera and to develop a methodology based on discoidal complexes for esterification studies of 7α-hydroxycholesterol. The method involved the extraction of lipids from serum using the Folch method, hydrolysis of the sample to yield total amounts present, enzymatic conversion of 7α-hydroxycholesterol to its enone derivative which was then detected and measured at 240 nm using reverse phase HPLC.

A significant amount of 7α-hydroxycholesterol was found to be esterified, which resulted in studies being carried out on (a) its possible esterification in the liver by acyl coenzyme A : cholesterol-O-acyltransferase, or (b) in the plasma by lecithin : cholesterol acyltransferase, which was facilitated by the synthesis of discoidal complexes, containing 7α-hydroxycholesterol, by the sodium cholate dialysis method and which resembled native high density lipoproteins. Electron microscopy was carried out to examine the physical structures of these complexes.

The biological significance of serum 7α-hydroxycholesterol will be discussed in the thesis.

Abbreviations

Abs	absorbance
ACAT	acyl CoA : cholesterol acyltransferase
apo	apolipoprotein
ATP	adenosine triphosphate
AUFS	absorbance units full scale
BARE	bile acid response element
BCA	bicinchoninic acid
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
bp	base pairs
BSA	bovine serum albumin
°C	degrees celcius
cAMP	cyclic adenosine monophosphate
cm	centimetre
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
CHO	cholesterol
CV	coefficient of variation
conc.	concentration
CTX	cerebrotendinous xanthomatosis
Ci	Curie
δ	density
DTT	dithiothreitol
EDTA	ethylene diamine tetracetic acid
FC	free cholesterol
FH	familial hypercholesterolaemia
FFA	free fatty acids
g	grammes
GLC(-SIM)	gas-liquid chromatography (-selected ion monitoring)
hr	hour
H.P.V.	hepatic portal vein
HDL	high density lipoprotein

2-HPBCD	2-hydroxypropyl β-cyclodextrin
HPLC	high performance liquid chromatography
HMG-CoA reductase	3-hydroxy-3-methylglutaryl coenzyme A reductase
IDDM	insulin-dependent diabetes mellitus
IDL	intermediate density lipoprotein
k(Dal)	kilo(Dalton)
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LPL	lipoprotein lipase
ODS	octadecylsilane
m	metre
M	molar
MeOH	methanol
mg	milligramme
min	minutes
ml	millilitre
mm	millimetre
mM	millimolar
µg	microgramme
µl	microlitre
µm	micrometre
µM	micromolar
mRNA	messenger RNA
NIDDM	non-insulin-dependent diabetes mellitus
-OHCHO	hydroxycholesterol
PAP	phosphatidic acid phosphatase
PC	phosphatidylcholine
PL	phospholipid
psi	pounds per square inch
PTA	phosphotungstic acid
PUFA	polyunsaturated fatty acid
r	regression coefficient
Rf	retention factor
Rt	retention time

rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec, s	seconds
SER	smooth endoplasmic reticulum
sp. act.	specific activity
SRE	sterol response element
Tc	gel-to-liquid crystalline phase transition temperature
TEM	transmission electron microscope
TG	triglyceride
TLC	thin layer chromatography
TMS	trimethylsilyl
UC	unesterified cholesterol
UV	ultraviolet
VLDL	very low density lipoprotein
vol.	volume
v/v	volume per unit volume
w/v	weight per unit volume

Table of Contents

CHAPTER 1

INTRODUCTION

"Cholesterol is the most highly decorated small molecule in biology. Thirteen Nobel prizes have been awarded to scientists who devoted major parts of their careers to cholesterol. Ever since it was isolated from gallstones in 1784, cholesterol has exerted an almost hypnotic fascination for scientists from the most diverse areas of science and medicine. Cholesterol is a Janus-faced molecule. The very property that makes it useful in cell membranes, namely its absolute insolubility in water also makes it lethal". These are the celebrated words of Michael Brown and Joseph Goldstein who received the Nobel prize in medicine in 1985 for their pioneering studies of familial hypercholesterolaemia, a genetic disorder associated with defective cellular uptake.

Much has since been learned concerning cellular uptake of cholesterol in health and disease. Furthermore, much of the current perspective on atherosclerosis is now focussing on the role of oxysterols as species 'beyond cholesterol' that act as homeostatic signals for nuclear control of the many cholesterol utilising pathways in mammalian cells. In specialised tissues such as the adrenal glands, gonads and liver, cholesterol undergoes a variety of oxidation steps which increase its polarity and permit its transit through the aqueous fluids of the body. The parent sterol molecule lacks this facility and must therefore be solubilised by interaction with a number of amphipathic agents. In plasma, it exists in association with phospholipid and certain proteins to form the lipid-protein complexes that we call lipoproteins. In bile, on the other hand, bile salts and phospholipid act as its emulsifying agents, leading to the production of micellar aggregates. Bile acids are the major metabolites of cholesterol in humans being formed in the liver *via* pathways involving a large number of oxygenated steroid intermediates, many of which appear in blood also. The occurrence, identification and quantitation in serum of oxygenated steroid intermediates of both cholesterol and bile acid biosynthetic pathways is being seen to provide a diagnostic aid to liver and gastrointestinal diseases and during dietary, hormonal and pharmacological manipulation of plasma cholesterol levels.

The oxysterol at the centre of this study was 7α-hydroxycholesterol, the product of the rate-limiting reaction in bile acid biosynthesis. Most of our knowledge regarding the regulation of bile acid production has been gathered in the rat. The absence of a gall bladder in this species, coupled with an unusually long small intestine results in high levels of bile acid biosynthesis. Notwithstanding this however and despite the applications of modern chromatographic and spectroscopic methods to steroid analysis in serum, it appears that only one such investigation of levels of oxysterols in rat serum has ever been reported. The study, published earlier this year reported levels of 7α-hydroxycholesterol in portal blood (Fukushima *et al.*,). One of the objectives of this study was to validate a procedure for the accurate determination of 7α-hydroxycholesterol in rat serum. The second phase of the study compared the levels of

7α-hydroxycholesterol in serum of rat and man and to ascertain whether levels of this metabolite in serum reflected alterations in cholesterol metabolism. Increased flow through a metabolic pathway sometimes leads to an increased leakage of intermediates from the pathway into the circulation. It is well documented that the serum levels of different methyl sterols, intermediates in cholesterol biosynthesis are increased under conditions of increased biosynthesis of cholesterol. This study also investigated the possibility that the serum concentration of 7α-hydroxycholesterol may reflect the hepatic concentration of 7α-hydroxycholesterol formed by cholesterol 7α-hydroxylase. Unequivocable demonstration of the mode of transport of oxysterols in plasma has been elusive. However, several studies have reported and confirmed an oxidative nature to lipoproteins, 7α-hydroxycholesterol being one such oxysterol belonging to a broad spectrum of oxysterols identified within the major circulating lipoproteins. Differences in the metabolism of these oxidatively modified lipoproteins have been implicated in the initiation of atherosclerosis. Having investigated the extent of esterification of 7α-hydroxycholesterol in human and rat sera, the third and final phase of this study involved investigating serum as a possible site for its further metabolism. The reactivity of 7α-hydroxycholesterol with a serum cholesterol acyltransferase enzyme demanded development of an exquisite substrate vehicle and partially purified enzyme source devoid of contaminating acceptor lipoproteins and exchange/transfer proteins. The extent of its interaction with this enzyme was compared with that of its reaction with the hepatic equivalent sterol-esterifying enzyme.

The aim of this chapter is to introduce the elements of plasma lipoprotein metabolism prior to reviewing the occurence and biological effects of plasma oxysterols. Validation of the preparative and chromatographic procedures necessary for quantifying 7α-hydroxycholesterol in serum together with a species comparison survey of the sterol in serum paved the way forward for a study to determine a possible putative lipoprotein-mediated pathway for its metabolism. These three studies form the basis for the work described in this thesis.

1.1 Cholesterol and its metabolism:

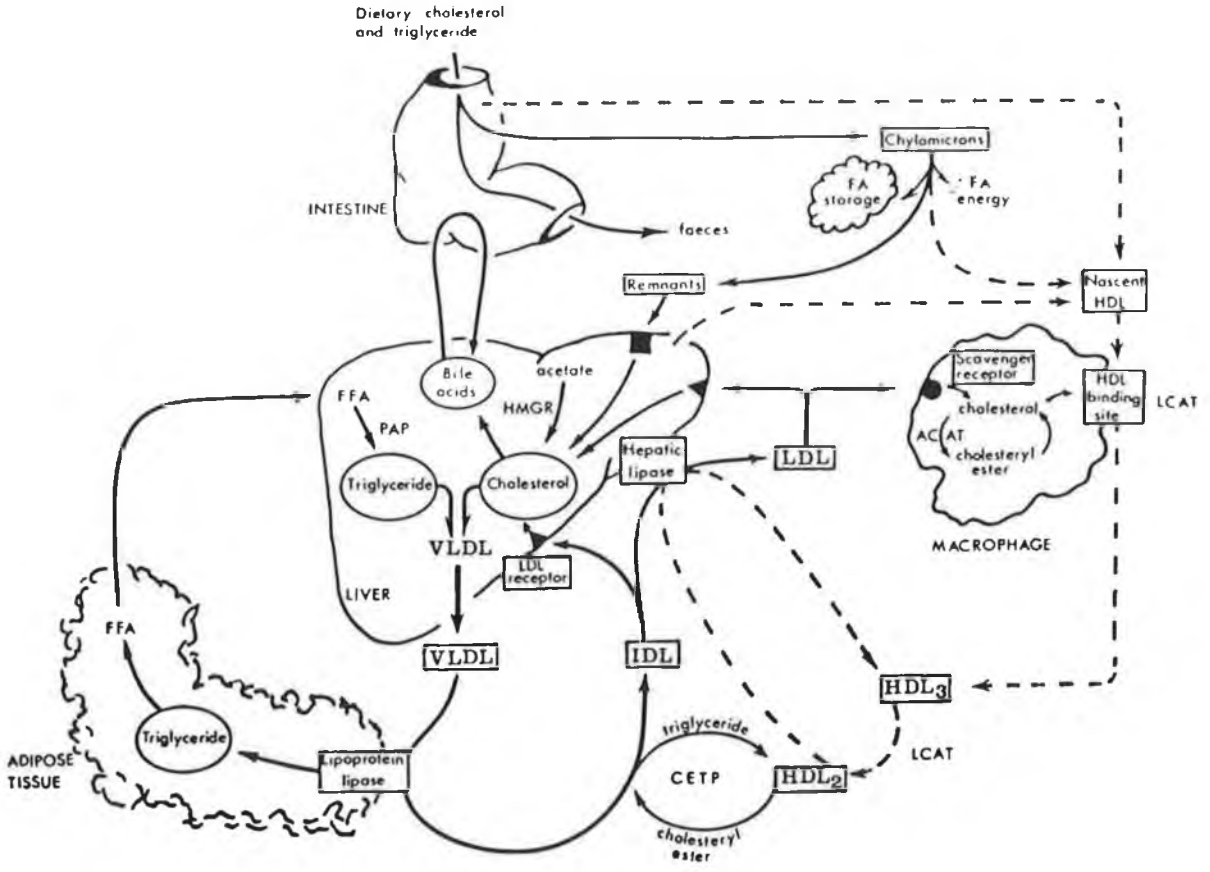
From a biochemical perspective, cholesterol is a necessary constituent for eukaryotic cell growth and development. The biosynthesis of cholesterol provides crucial building blocks for cell membranogenesis and membrane fluid regulation and for the synthesis of sterol and non sterol products that are important for normal cell function. Prevailing interest in cholesterol has revolved around its role in the development in atherosclerosis and cholelithiasis and much research energy has been directed toward identifying mechanisms to decrease elevated serum cholesterol levels. More recent developments have underscored the essential nature of cholesterol in cell growth and development and have begun to elucidate the mechanisms governing cholesterol homeostasis.

Cholesterol may be obtained for cellular metabolism either *via* uptake mediated by members of the low density lipoprotein (LDL) receptor family or through biosynthesis. The uptake pathway involving the lipoprotein receptors may be further subdivided into an exogenous (dietary) pathway and an endogenous pathway (**Fig 1.1**). Although uptake and biosynthesis are interdependent, cellular cholesterol requirements can be met equally well through either supply pathway. Cholesterol homeostasis in mammals is through the coordinate regulation of these supply pathways in the liver and by a third pathway in liver which involves the catabolism of cholesterol to hydrophilic bile acids. This latter pathway involving cholesterol conversion to bile acid accounts for approximately 50% of total cholesterol excreted from the body per day (Fielding and Fielding, 1985).

1.1.2: The plasma lipoproteins

Due to its insolubility in an aqueous medium, cholesterol is transported in plasma in lipoproteins, which are multimolecular complexes of triglycerides, phospholipids, free fatty acids, cholesterol free and esterified and protein. They are classified on the basis of their lipid or protein composition. They can be divided into five major classes, shown in **Table 1**. The larger lipoproteins usually have a higher content of lipids, especially neutral lipids and as a consequence, their density decreases with increasing size. The fatty acid composition of the lipids vary in the different human lipoproteins. Usually, a strong similarity exists between the fatty acid compositions of specific lipid classes (Gotto *et al.*, 1986). This may be attributed to the various lipid exchange proteins in plasma. The protein components of these complexes are termed apolipoproteins. Two major families of these proteins- apolipoprotein A and apolipoprotein B, are intimately involved in the transport of cholesterol (**Fig 1.1**). Apo A is the main protein of plasma HDLs, which by accepting cholesterol, is involved in the transport of cholesterol from peripheral tissues back to the liver in what is known as "reverse cholesterol transport". Apo B is an integral component of VLDL, IDL, LDL and chylomicrons. It is involved in the transport of cholesterol and triglyceride to peripheral parenchymal cells. These cells then utilise the cholesterol for structural and metabolic purposes. Thus, apolipoproteins stabilise the surface of the lipoproteins, while also providing "address labels" that govern which cells in the body receive and metabolise these lipoproteins (Fielding and Fielding, 1985).

Fig 1.1: Schematic representation of the major elements of human plasma lipoprotein metabolism illustrating the flow of exogenous/dietary and endogenous cholesterol. Abbreviations: ACAT = acyl CoA-cholesterol acyltransferase; LCAT = lecithin-cholesterol acyltransferase; HMGR = HMG CoA reductase; PAP = phosphatidic acid phosphatase; CETP = cholesteryl ester transfer protein; FA = fatty acids. (Shepherd and Packard, 1992).



The largest lipoproteins are the chylomicrons, which are synthesised in the gut during intestinal assimiliation of exogenous lipid and carry dietary triglycerides and cholesterol. They acquire apo E and C from HDL. The former apolipoprotein acts as a cofactor for lipoprotein lipase which is located on the surface of various extrahepatic tissues including adipose tissue, cardiac muscle and skeletal tissue. This enzyme is synthesised by the cells that underlie the capillaries of such tissues. It metabolises the chylomicrons such that they become depleted of triglyceride. The fatty acids which are released from triacylglycerol hydrolysis are used in storage or as a means of energy. The body can then direct chylomicrons and VLDL to particular organs through its tissue-specific control of lipoprotein lipase (Fielding and Fielding, 1985). Triglycerides are off-loaded into the adipose tissue and muscle while the cholesterol-containing remnants are taken up by the liver, so that 12-14 hours after fat digestion, chylomicra virtually disappear from plasma (Ireland *et al.*, 1980).

The remnant is formed as the chylomicron shrinks progressively, shedding its surface phospholipid and small molecular weight proteins into the HDL density interval, leaving a remnant which by virtue of the apo E on its surface, is rapidly cleared by the parenchymal cells of the liver (Shepherd and Packard, 1992). These remnants may be more completely hydrolysed to form LDL (Grummer and Carroll, 1988). This metabolism of remnant particles is extensive in humans, while in rats, VLDL remnants or IDL are largely cleared by the liver (Eisenberg and Levy, 1975).

Very-low-density lipoproteins (VLDL), although being slightly smaller, have a structure and composition similar to that of chylomicrons, but differ mainly due to apo B-100 of VLDL reacting with the apo B, E receptor, which is not the case with chylomicrons (Fielding and Fielding, 1985). This apolipoprotein activates lipoprotein lipase, thereby facilitating the triglyceride switch from carrier to tissue depot (Ireland *et al.*, 1980). This results in the formation of relatively cholesteryl-ester-rich, intermediate-density, remnant lipoprotein particles, IDL. These may be metabolised further to yield LDL, or they may be taken up directly by the LDL receptor, which reacts with apo B and apo E.

Low-density lipoproteins (LDL) are the main carriers of cholesterol, being cholesteryl ester-rich lipoproteins which are metabolised by the liver and peripheral tissues. Human LDL contains apo B-100 as the major or only apolipoprotein. This apoprotein, on the LDL particle's surface, facilitates the transport process by interacting with specific high-affinity receptors on cell membranes. The LDL receptor also binds apo E, which is present in certain subclasses of HDL in some species (Grummer and Carroll, 1988). Consequently, LDL reacts with the apo B, E receptor and its cholesterol is interiorised along with other components of the lipoprotein. The protein component of LDL is hydrolysed to amino acids and cholesteryl esters are hydrolysed by acid lipase, with free cholesterol being released for the cell's needs. They are, essentially, slimmed down

versions of VLDLs. After VLDLs have delivered triglyceride to peripheral tissue, they come in contact with HDLs thereby taking on cholesteryl esters in a mid-stream switch.

Table 1: Lipoprotein classification

Family name	Synonyms (based on electrophoretic mobility)	Density range (g/ml)	Composition			Major Function
			Protein (%)	Lipid (%)	Major Lipid	
Chylomicrons	-	< 0.95	0.5-2.0	98-99.5	TG	Transport exogenous TG
VLDL	Pre-beta lipoproteins	0.95-1.006	7.7	90.1	TG	Transport endogenous TG
IDL	Slow pre-beta lipoproteins	1.006-1.1019	19.0	81.0	TG	Intermediate in the transformation of VLDL-LDL
LDL	Beta lipoproteins	1.019-1.063	22.3	78.3	CHO	Transport CHO and PL to peripheral cells
HDL	Alpha lipoproteins	1.063-1.21	51.9	48.6	CHO and PL	Transport CHO and other lipids from tissues - liver

VLDL: Very low density lipoproteins
IDL: Intermediate density lipoproteins
LDL: Low density lipoproteins
HDL: High density lipoproteins

TG: Triglyceride
CHO: Cholesterol
PL: Phospholipid

High-density lipoproteins (HDL), which function as initiators of "reverse cholesterol transport", promote the clearance of sterol from peripheral tissues to the liver. They are considered to be the most important cholesterol carriers as low HDL cholesterol concentration has been established as a major risk factor for the development of coronary heart disease (Miller, 1990). Nascent disc-shaped HDL are secreted by the intestine and liver and are rich in cholesterol. The apolipoproteins of HDLs activate lecithin:cholesterol actyltransferase (LCAT) which esterifies cholesterol and allows for the further metabolism of lipids. Upon the occurrence of this enzymatic reaction, the HDLs become more spherical in appearance. HDLs are the only known system for transporting cholesterol out of peripheral tissue back to the liver for excretion in the bile (Ireland *et al.*,). This process where cholesterol from peripheral cells can be returned to the liver for catabolism is termed "reverse-cholesterol transport".

1.1.3: Cholesterol transport between the tissues in humans

Absorbed cholesterol, from food, enters the intestinal lymph as a component of chylomicrons (**Fig 1.1**). Upon entering the blood stream, lipoprotein lipase (LPL) hydrolyses the triglyceride and concomitantly, there is an interaction with HDLs, with the resulting transfer of some surface material and a change occurs in their apolipoproteins.The chylomicron remnant is then taken up by the liver.

On the surface of the liver, is found an Apo-E receptor, which allows for the uptake of the chylomicron remnant. This cholesterol then becomes available for cellular metabolism or recycling as plasma lipoproteins. Patients with the genetic disorder, familial lecithin:cholesterol acyltransferase deficiency, have a reduction in the clearance of these remnants. Cholesterol from the diet, which is not removed by the chylomicron remnant pathway, enters the pools of cholesterol in peripheral tissues, with cholesterol of LDLs.

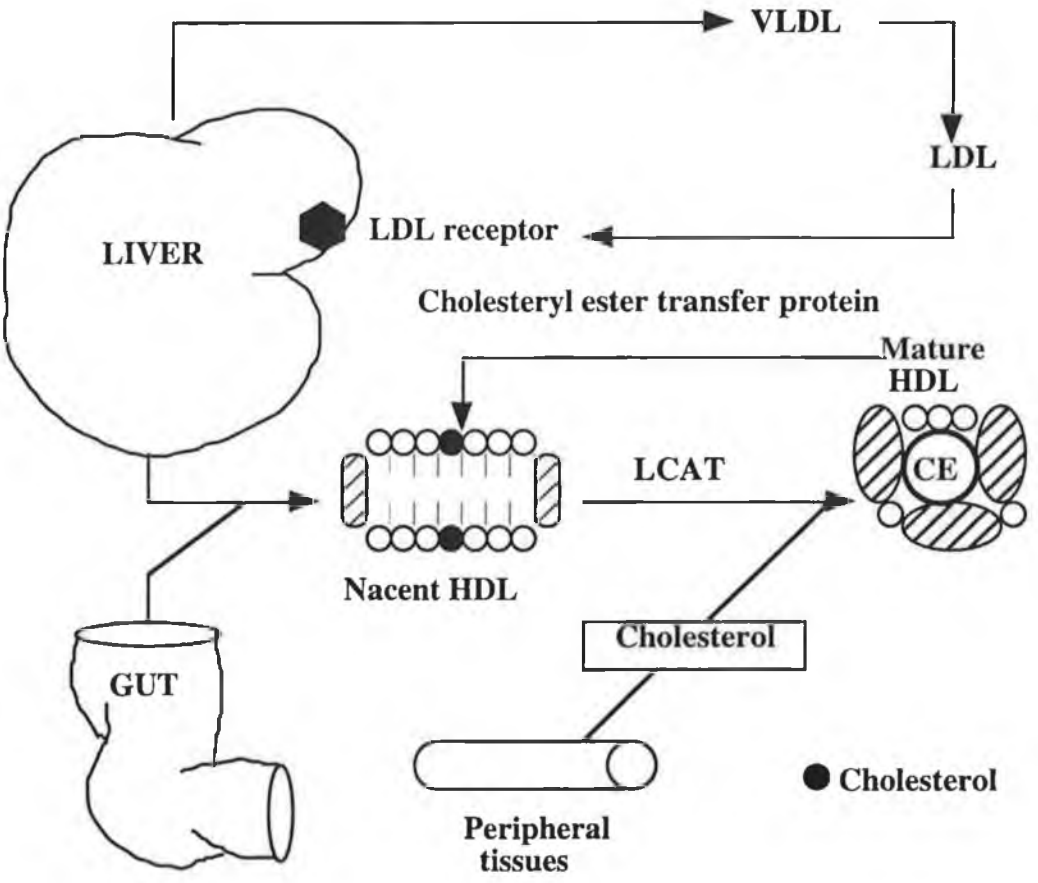
In order to prevent the build up of free and esterified cholesterol in cells, excess cholesterol must be recycled through the plasma to the liver, where it can be degraded. The mechanism by which those tissues not secreting lipoprotein particles return cholesterol to the liver is termed "reverse cholesterol transport", due to the opposing movement of cholesterol from peripheral cells to the liver. Cholesterol is mobilised from tissues when HDL binds to the cell membrane and incorporates excess unesterified cholesterol into its surface monolayer. Glomset (1964) proposed as free cholesterol in the cell became esterified outside (by LCAT), then this "reverse cholesterol transport" pathway was an active transport system (**Fig 1.2**). The cholesterol that is recycled is present in newly synthesised plasma lipoproteins, while part appears in the bile as bile acids or unesterified cholesterol. The cholesterol that passes through the intestine, is reabsorbed in the intestine and mixes with dietary cholesterol to be metabolised *via* the chylomicron degradation pathway (Fielding and Fielding, 1995).

The peripheral tissues involved in reverse cholesterol transport, transfer cholesterol only in its unesterified form (Fielding and Fielding, 1995). There is strong evidence that the net transport of cholesterol from nonhepatic cells into the circulation is maintained by a chemical reaction, that is catalysed by LCAT (Dobiasova, 1983). This enzyme is responsible for the maturation of nascent HDL, the transformation of smaller HDL subclasses (HDL_3) into larger ones (HDL_2), the removal of excess cholesterol and phospholipids from LDL, VLDL, chylomicrons and the flux of cholesterol from cell membranes into HDL (Jonas, 1991). As free cholesterol moves rapidly between different lipoproteins and between cell surfaces and lipoproteins, such different potential sources will compete for transfer of free cholesterol to HDL, down the chemical gradient generated by the LCAT reaction (Reichl and Miller, 1989). Cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) are two plasma lipid transfer proteins that act in the metabolism of plasma lipoproteins. Unlike enzymes, these proteins are energy-independent and they must work down preexisting concentration gradients.

The former facilitates both the exchange and net transfer of triglyceride and cholesteryl ester. The net transfer process proceeds by a reciprocal exchange of triglyceride and cholesteryl ester without net transfer of core lipids between lipoproteins, *i.e.*, net transfer is mediated by a hetero-exchange process. The latter is able to mediate transfer of a wide variety of phospholipids including phosphatidylcholines, diacylglycerol, phosphatidylethanolamine, phosphatidylserine and phosphatidylglycerol (Tall, 1986). These two lipid transfer proteins contribute to the further modelling of HDL. PLTP supplies lecithin to HDL, while CETP can move cholesteryl ester made by LCAT to other lipoproteins, particulary triglyceride rich lipoproteins and LDL (Fielding and Fielding, 1995).

Although cholesterol has been implicated in atherogenesis, much evidence now exists that cholesterol oxidation products may be more atherogenic than cholesterol itself. Thus, the study of cholesterol oxidation products is important- whether they result from cholesterol autoxidation or are metabolites of cholesterol itself. Studies on their identification and metabolism may provide further insight into problems such as disorders in bile acid metabolism or aid in studies on atherosclerosis.

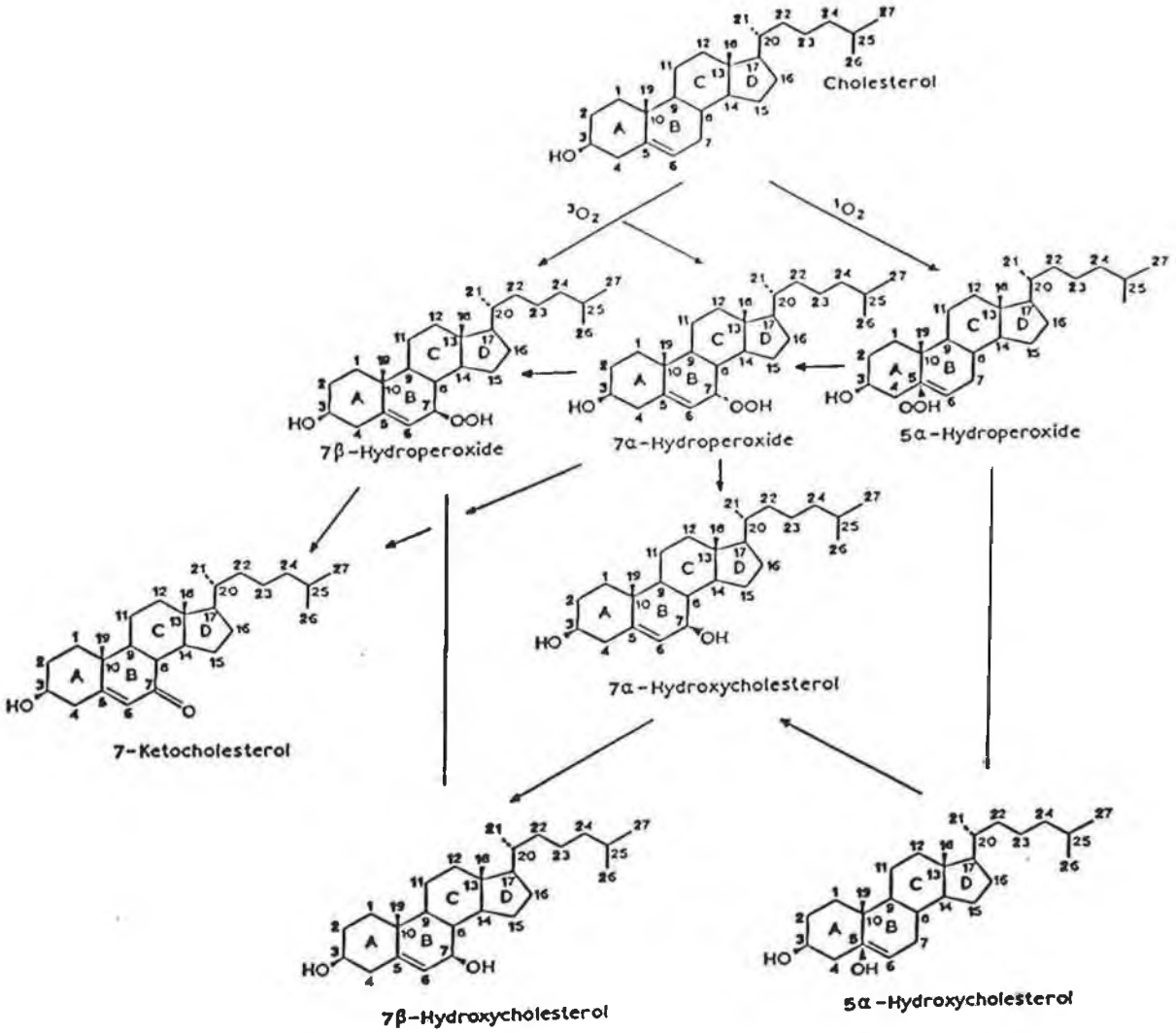
Fig 1.2 The role of HDL in reverse cholesterol transport. LCAT circulating in association with HDL, traps tissue cholesterol within the particle by esterifying the sterol, increasing its hydrophobicity and promoting its migration to the core of the lipoprotein. The mature HDL generated in the process, is then capable of transferring its cholesteryl ester load into chylomicrons and VLDL via the agency of CETP. These particles may thus represent the final pathway for sterol excretion into bile. Adapted from (Shepherd and Packard, 1992).



1.2: Cholesterol oxidation

Cholesterol, an unsaturated alcohol being insoluble in water, undergoes autoxidation in the presence of molecular oxygen through a free radical reaction, predominantly at position C-7. Many primary and secondary oxidation products are therefore formed (Nourooz-Zadeh, 1990). Such oxidation products are formed as a result of heat, light and radiation (Smith, 1981) and also by peroxidation *in vivo* and metabolism (Smith, 1987). They are also observed in various foodstuffs of animal origin after processing under a variety of conditions (Kumar and Singhal, 1991). Structural characteristics of cholesterol relevent to the process of its autoxidation are the B ring unsaturation and positions allylic to it and also the presence of two tertiary carbons in the side chain (Maerker, 1987). Due to the possible influence of the C_3 hydroxyl group and the tertiary C_5 atom, the C_4 (allylic) position rarely gets attacked by molecular oxygen. Therefore, it is the other allylic carbon atom, C_7, which is more sensitive to molecular attack by oxygen, resulting in the formation of a series of A and B ring oxidation products (Kumar and Singhal, 1991), following hydrogen abstraction at position C_7 (Maerker, 1987). Resulting products are hydroperoxides in the B-ring and the side chain and to a lesser extent, the dehydrogenation of the 3ß-alcohol group. Secondary processes then produce a multiplicity of derivatives (Bösinger *et al.*, 1993). Various hydroxylations take place on the side chain, such as at C_{20}, C_{25} and at C_7 on the B-ring (Bösinger *et al.*, 1993). The former two are seen in the autoxidation of solid or crystalline cholesterol, as such side chain oxidations are not observed in cholesterol in solution or in aqueous dispersions (Maerker, 1987). The abstraction of a hydrogen atom from position 7, results in the formation of epimers of 7-hydroperoxide (**Fig 1.3**) which, due to their instability, form 7-α and 7ß-hydroxycholesterol and also 7-ketocholesterol (Smith, 1987). The tertiary carbons, such as that at position 25, tend to yield the corresponding hydroxysterol upon reduction of peroxides which are formed at the position of the tertiary carbon atom. This is due to the fact that such structures tend to form relatively stable radicals (Finocchiaro and Richardson, 1983).

Fig 1.3: Mechanisms and major products of cholesterol oxidation.



1.2.1: Location of cholesterol oxidation products in animal tissues

Since cholesterol is found in many biological membranes and tissues, it is reasonable to expect to find its oxidation products in such tissues also (**Table 2**). Some of these oxidation products of cholesterol have been identified in the body and are important intermediates in the metabolism of cholesterol (Kumar and Singhal, 1991). It appears that some of them are products of lipid peroxidation reactions, while those from cholesterol metabolism are formed by enzymatic oxidation (Bernheimer *et al.*, 1987). The former involves the attack of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene ($-CH_2-$) group from a polyunsaturated fatty acid. Such radicals as the hydroxyl radical can carry this out (Halliwell and Gutteridge, 1989).

Table 2: Occurence of some cholesterol oxides in human and animal tissues (Bösinger *et al.*, 1993)

Cholesterol oxide	Found in
7α-hydroxycholesterol	Renal cortex, arteries, brain, human meconium, pregnant mare serum, bovine testes, pig liver, sheep's wool, rat faeces, rat liver, skin of toads
7ß-hydroxycholesterol	Renal cortex, brain, human meconium, pregnant mare serum, bovine liver and testes, foetal calf serum, pig spleen, rat faeces and liver, skin of toads
7-ketocholesterol	Human fatty tissue, aorta, brain, heart, erythrocytes, kidney, liver, lung, meconium, muscle, pancreas, spleen, urine, renal blood and aorta of baboons, renal vein blood in rhesus monkeys, equine aorta, bovine testes, aorta and brain, kidney, testes, fat, liver and blood of pigs, sheep's wool, feline aortas, rat kidney, aortas, faeces and liver, hen ovaries
25-hydroxycholesterol	Human fatty tissue, arteries, brain, heart, liver, lung, muscle, pancreas and spleen, baboon aorta, equine aorta, bovine renal cortex and aorta, feline aorta, rat aorta and liver mitochondria, pigeon aorta, porifera

Smith *et al.*, (1981) reported that 7α-hydroxycholesterol, 7ß-hydroxycholesterol, 7-ketocholesterol, 24-hydroxycholesterol, 25-hydroxycholesterol and 26-hydroxycholesterol were found to be most abundant in aortic tissue while hepatic levels of these oxides were much lower. They found these oxides to be esterified in such tissues. 24-hydroxycholesterol was found in significant amounts in the brain (Lin and Smith, 1974). 7α-hydroxycholesterol has been shown to increase, as has 25-hydroxylated bile alcohols, in patients of cerebrotendinous xanthomatosis (Oftebro *et al.*, 1980; Bjorkhem *et al.*, 1981).

27-hydroxycholesterol was found to be present in atherosclerotic human femoral arteries. This oxide is a potent inhibitor of HMG-CoA reductase (Björkhem *et al.*, 1994). Several reports have been published on the identification of cholesterol oxides in human atherosclerotic aortic tissues. There was found to be an association of levels of the oxides- 26-hydroxycholesterol and 7-ketocholesterol with an increase in the progression of atherosclerosis (Brooks *et al.*, 1966; Smith and Van Lier, 1970). Of the sixty cholesterol oxidation products identified (Smith, 1987), twelve oxidation products of cholesterol have been identified in human atheromata (Smith and Van Lier, 1970; Van Lier and Smith, 1967) which include the above mentioned.

Oxysterols (together with their fatty acyl esters) occur at appreciable levels distributed among the plasma lipoproteins (Smith and Johnson, 1989). Analysis of endogenous human plasma oxysterols suggest that 7-ketocholesterol and isomeric cholesterol 5α,6α-epoxide and the corresponding ß-epoxide, are present in equal amounts in LDL. Streuli *et al.* (1981), found that whenabout 7α-hydroxycholesterol was incubated with the major lipoprotein classes of human serum 40% was subsequently recovered in HDL and LDL, while 23% of 25-hydroxycholesterol added was carried by HDL and LDL.

1.2.2: Biological effects of cholesterol oxides

Several important biological activities may be attributed to cholesterol being present in cells and cell membranes. The cytotoxic effects of cholesterol autoxidative products have dominated studies of biological activities (Kumar and Singhal, 1991).

1.2.2.1: Effect on cell membranes

Oxygenated sterols have been shown to alter membrane morphology and function. They are taken up by skin fibroblasts (Khachadurian *et al.*, 1981) and are incorporated into the cell membrane of red blood cells (Hsu *et al.*, 1980). They have been found to be capable of altering the structure and function of mammalian cell membranes (Streuli and Chung, 1979). The structure of the red cell membranes changed shape to take on the "spiny" appearance of echinocytes. However, when selected oxysterols were incubated with red cells, in the presence of lipoproteins, there was an impedement of oxysterol

insertion into the cells, suggesting that lipoproteins bind oxysterols (Streuli *et al.*, 1981). Their mechanism of action is not clearly understood, however, it is thought that due to their inhibitory effect on HMG CoA-reductase, the rate-limiting enzyme controlling cholesterol synthesis, the cholesterol in the cell membrane is lacking, thereby resulting in it liquefying and having altered functions.

1.2.2.2: Effect on cholesterol metabolism/Cytotoxicity

The exact mechanism through which these compounds inhibit cell growth and eventually result in cell death is not well defined. The inhibition of the biosynthesis of cholesterol by oxygenated derivatives of cholesterol and lanosterol was demonstrated by Kandutsch *et al.*, (1978) to be a result of a decrease in cellular levels of HMG-CoA reductase activity. They found that oxysterols inhibited cholesterol synthesis, both *in vivo* and *in vitro* at concentrations between 10^{-9} and 10^{-6} M. This, in turn, affects the cholesterol content of membranes. Upon suppression of HMG-CoA reductase, the concentration of cellular sterol content declined as did the rate of DNA synthesis. The oxysterols seem to block cell division in the G1 phase of the cell cycle.

Smith and Johnson (1989) suggested that the process of peroxidation of sterols involving oxyl, peroxyl and carbon-centered radicals, as well as stable oxysterol products may contribute to toxic effects. The presence of cholesterol in cell surface membranes can influence their susceptibility to peroxidation, probably by intercepting some of the radicals present and also by affecting the internal structure of the membrane by interaction of its large hydrophobic ring structure with fatty acid side chains (Halliwell and Gutteridge, 1989).

Oxysterols mimic all the effects of LDL in cholesterol metabolism: the suppression of LDL receptor activity; the stimulation of acyl CoA:cholesterol acyltransferase (ACAT) and the suppression of other enzymes of cholesterol synthesis as well as HMG-CoA reductase (Fielding and Fielding, 1985). Also, a cytosolic oxysterol carrier protein has been identified. The affinities of oxysterols for this protein reflect their relative ability to suppress reductase. The reductase inhibitory activity of a large number of oxysterols is proportional to their affinity for the oxysterol carrier protein (Fielding and Fielding, 1985). A schematic representation of the inhibitory action of oxysterols on cholesterogenesis is depicted in **Fig 1.4**.

Work carried out by Taylor *et al.*, (1984) on a cytosolic binding protein (in mouse fibroblast cell cultures) being a mediator of the actions of oxysterols on HMG-CoA reductase indicated that many sterols did bind this protein. They reported that no sterol was found that would bind the binding protein with high affinity yet that would not repress HMG-CoA reductase activity.

Peng *et al.*, (1985) carried out studies on the effects of oxysterols on the uptake of

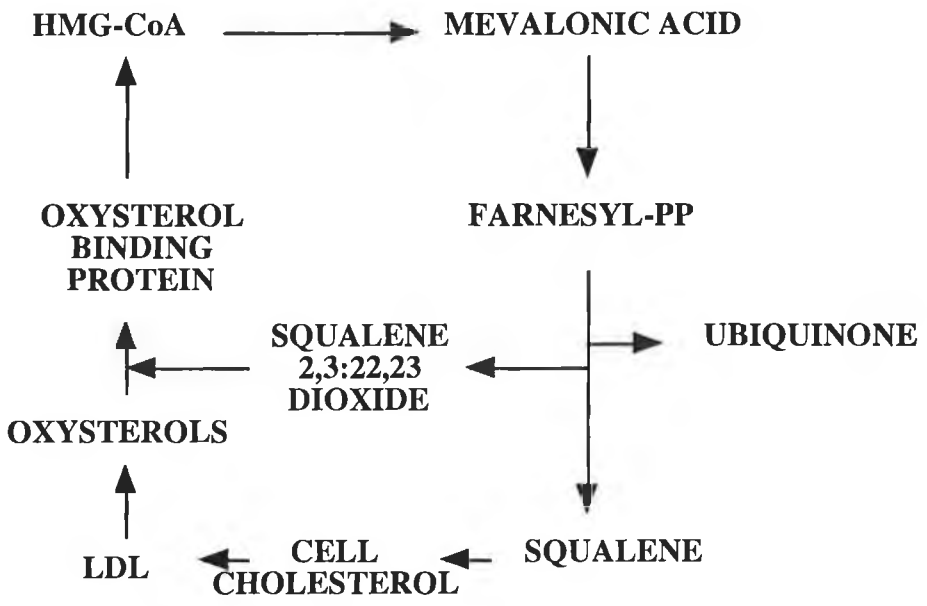
cholesterol, in aortic smooth muscle cells. They found that, in the presence of oxygenated derivatives of cholesterol, cholesterol uptake was severly impaired. Cholestane-3ß-5α,6ß-triol inhibited cholesterol uptake as much as 90% and other oxysterols tested showed inhibitions of between 30% to 60%. 7α-hydroxycholesterol was found to inhibit cholesterol uptake in these cultured cells by levels by approximately 40% when incubated at 50μg/ml medium, while at concentrations of ~100 μg/ml medium, 7α-hydroxycholesterol was found to be more potent in this inhibition of cholesterol uptake than 7-ketocholesterol.

It has been proposed that 25-hydroxycholesterol may influence ACAT activity, since its incubation with human fibroblasts resulted in an accumulation of cholesteryl esters (Brown *et al.*, 1975). Lichtenstein and Brecher (1983), later showed that 25-hydroxycholesterol itself was esterified by rat liver microsomes, in a similar reaction to that which esterified cholesterol, thereby proposing a stimulatory effect on ACAT.

The cytotoxicity of oxysterols on cultured aortic smooth muscle cells, may not be only due to their potent inhibition of cholesterogenesis, but may also be attributed to their capability for inhibiting exogenous cholesterol uptake, particularly in arterial cells, which synthesise cholesterol at a very slow rate (Peng *et al.*, 1985). A possible explanation for this event occuring is that cholesterol and oxysterols compete for identical and specific binding sites on the cell surface. More recent studies carried out by Zhang *et al.*, (1990) report that different oxysterols in oxidised LDL seem to have differing effects on cholesterol esterification in cultured macrophages. 7-ketocholesterol resulted in an enhancement of cholesterol esterification, whereas 7-hydroxycholesterol resulted in a much smaller effect. Depending on the extent of oxidation of LDL and the relative proportions of oxysterols used in the study, the net result of oxysterols on the esterification of cholesterol could be either inhibitory or stimulatory. Many reports are available on oxygenated sterols which are found to be suppressive of sterol synthesis and HMG-CoA reductase activity. Some are known metabolites of cholesterol, some are autoxidative products of cholesterol, while some precursors of cholesterol are also reported (Kudo *et al.*, 1989).

In general, the overall structure of the oxysterol seems to determine the potency of its inhibitory effects. Greatest potency has been shown to be when the side chain is intact, while the presence of oxygen groups in the D-ring and the iso-octyl side chain also contribute to the inhibitory effect. Parish *et al.*, (1987) noted that as the distance between the second oxygen and the C-3, increased, so too did the activity.

Fig 1.4: Regulation of cholesterogenesis by external or internal oxysterols via the activity of oxysterol-binding protein. This scheme summarises results which suggest that a common signal derived from either external or internal oxysterols mediates HMG-CoA reductase activity (Fielding and Fielding, 1985).



1.2.2.3: Mutagenicity and carcinogenicity

The mutagenicity of 5,6-epoxycholestanols has been implicated in some studies. Sevanian and Peterson, (1984) demonstrated that 5,6-epoxycholestanols possessed mutagenic properties when incubated with V-79 Chinese hamster lung fibroblasts.

Carcinogenic properties have also been attributed to cholesterol oxides. Bischoff (1969) reported that, of numerous cholesterol analogues, 5α,6α- epoxycholestanol was the only cholesterol oxide that resulted in carcinogenic properties upon administration to rats and mice. This oxidation product was later found to be implicated in the development of colon cancer (Reddy *et al.*, 1977), where its breakdown product- cholestanetriol, was identified in the faeces of such patients. Hill *et al.*, (1971) reported that deoxycholic acid, which is recognised to be produced by bacterial degradation of bile salts, is in certain cases, a carcinogen.

1.2.2.4: Atherogenicity of oxysterols

Much evidence exists indicating that lipid oxidation products both initiate and promote atherosclerosis and that one particular group of these products- cholesterol oxidation products are far more atherogenic than cholesterol itself. There are two differing hypotheses to atherosclerosis- one which suggests that atherosclerosis results from hypercholesterolemia-induced deposition of lipid in the vessel wall, which is known as the "lipid hypothesis" the second theory is known as the "response-to-injury" hypothesis, where it is thought that cholesterol oxides are involved in the injury to the vessel wall. Cholesterol plays a major role in the former hypothesis, while oxysterols do not. The angiotoxic and cytotoxic properties of oxysterols may explain their role as initators of atherosclerotic lesions. Oxysterols have a reported role in the injury of the arterial wall (Imai *et al.*, 1980), thereby, initiating the atherosclerotic lesions.

Peng *et al.*, (1985) intravenously administered cholestanetriol and 25-hydroxycholesterol to New Zealand white rabbits. The injury to the endothelium was examined by means of transmission electron microscopy. The aorta of rabbits administered with 25-hydroxycholesterol showed 67% of the endothelium with some subendothelial edema. Those that received cholestanetriol indicated more damage to the endothelium of the aorta, approximately 90%.

On the contrary, Higley *et al.*, (1986) found that feeding cholesterol to rabbits resulted in six times more arterial lesions when compared to those fed a mixture of oxysterols (α- and ß-epoxides; 7α-hydroxyperoxide and 7-ketocholesterol). However, it must be noted that these oxysterols were different to those used in the previously described experiments, which were mainly 25-hydroxycholesterol and cholestanetriol. He thus concluded that cholesterol was more atherogenic than oxysterols. It would appear from these studies that oxysterols have varying atherogenic potential.

Faggiotto *et al.*, (1984), described the pathological sequence of events in the development of a mature atheromatous plaque. Methods used included scanning electron microscopy and transmission electron microscopy. The various stages included:

* The adhering of monocytes and endothelium; the migration of monocytes into the subendothelium; accumulation of lipid; foam cell formation- after twelve days.
* Formation of fatty streaks from foam cells; pathological changes due to discontinuity in the endothelium; the addition of monocytes to the fatty streaks in the intima and the final egression of foam cells into the circulation- after two months.
* A mural thrombus develops upon platelets and macrophages adhering to the denuded artery. Arterial smooth muscle cells proliferate due to stimulation caused by the secretion of a growth factor from platelets or cells which are local in the wall of the artery. Such smooth cells of the artery are converted into foam cells by LDL, which results in the wall of the artery thickening.

One area where lipid oxidation products may be affecting atherosclerosis is through oxidised LDL. Goldstein *et al.*, (1979) were the first to describe that a modified form of LDL could be taken up by macrophages to convert them into foam cells. They chemically modified LDL by acetylation which converted LDL to a form which could be recognised by the monocyte/macrophage and taken up at a rate several times that of native LDL. Thus, as this receptor does not recognise native LDL, it may be termed the "scavenger receptor". Macrophages express a specific scavenger receptor site reactive with LDL in which apo B has been chemically modified (Fielding and Fielding, 1985).

The molecular mechanisms of the oxidation processes and the reaction pathway leading to the modification of LDL is largely unknown. Aldehydes, such as malonaldehyde, generated by lipid peroxidation from PUFA contained in the phospholipids of LDL, interact with apo B and interfere with the binding of LDL with the apo B/E receptor (of fibroblasts) by modifing lysine residues of apo B. Evidence exists indicating that lipid oxidation by endothelial cells is responsible for the alteration of structural and biological properties of LDL (Esterbauer *et al.*, 1987), in human plasma. They found a surprisingly high resistance of the polyunsaturated fatty acids- 18:2 and 20:4, in LDL against oxidation.

It has been suggested that hydrolysed cholesteryl ester may be trapped in the lysosomal compartment of the macrophage, resulting in the accumulation of free cholesterol in the macrophage, when oxidised LDL are incubated with macrophages. This affects the amount cholesterol available, as substrate, for esterification. Native LDL, on the otherhand, unlike oxidised LDL, does not cause the accumulation of cholesterol in the macrophage. It binds and internalises to the cell by means of the LDL receptor, which is regulated by the levels of free cholesterol in the cell (Maor and Aviram, 1994).

The oxidative nature of LDL was examined recently by Hodis *et al.*, (1994) by determining the cholesterol and cholesterol oxide content of LDL. A similar array of oxysterols were identified in both types of LDL, thereby suggesting that a free radical mediated process was involved, in the oxidation of cholesterol. Again, some of the typical oxidative products of cholesterol were identified such as 7α-hydroxycholesterol, 7ß-hydroxycholesterol, 7-ketocholesterol, 25-hydroxycholesterol and 5α–cholestane-3ß,5α,6ß-triol. They concluded that LDL- (the more negatively charged LDL), an oxidatively modified lipoprotein, is a subfraction of total LDL, which is enriched in oxysterols. Upon separation from unmodified LDL, oxidised LDL shows cytotoxic effects on aortic endothelial cells, which was not the case with normal LDL.

It has well been established that LDL plays a major role in atherosclerosis, primarily by promoting the accumulation of lipid in the lesion. Thin-layer chromatography analysis of oxidised LDL lipids identified 7-ketocholesterol, 5,6-epoxycholesterol and 7-

hydroxycholesterol, in studies carried out on cholesterol esterification (Zhang *et al.*, 1990). Also, Bascoul *et al.*, (1990) reported that 7α-hydroxycholesterol was carried by LDLs. This may have serious implications in atherogenesis as oxidised LDLs are cytotoxic. This may induce functional changes in the endothelial cells that favour the penetration of circulating monocytes or favour the movement of LDL into the subendothelial space and thereby accelerate the formation of the fatty streak (Steinberg *et al.*, 1989).

Finally, atherosclerosis has been described as the basic lesion leading to the high morbidity and mortality in diabetes (Tomkin and Owens, 1991). Morel and Chislom (1989), were the first to demonstrate that a relationship existed between the *in vivo* oxidation of lipoproteins in diabetes and the potential for tissue damage as monitored by *in vitro* cytotoxicity, thus suggesting that the mechanism for the characteristic damage of tissues in the arteries and organs of diabetics, may be attributed, in part, to the activity of lipid peroxidation products.

1.2.3: Parameters influencing cholesterol autoxidation

As referred to in **1.2**, there are structural features of cholesterol that contribute to its autoxidation. Cadenhead *et al.*, (1982) reported the relative stabilty of crystalline cholesterol, as regards autoxidation, when compared to cholesterol in the liquid state. This was also reported by Kim and Nawar, (1993). Ozone, dioxygen species, superoxides and peroxides also result in the autoxidation of cholesterol.

Cholesterol oxidation is system dependent. Such factors as buffer type, form of substrate, presence of water, pH, temperature and oxidation time, influence the products of cholesterol oxidation and the relative amounts formed. In aqueous suspensions, the differences in the resistance of cholesterol to oxidation, were not significant within the pH range 6.0-7.4, except for the early stages of oxidation (Kim and Nawar, 1993). Their results also showed that only traces of side-chain derivatives, *i.e.*, 20- and 25-hydroxycholesterols and triols were detected in the dry-state oxidation.

1.2.4: Lipid oxidation and antioxidants

Oxidation of LDL causes multiple changes in both the lipid and protein constituents of the particles thereby facilitating recognition by the scavenger receptor. Peroxidation of the lipid components of LDL and the oxidative degradation of apo B occurs, results in the formation of several oxidation products (Esterbauer *et al.*, 1987 and Fong *et al.*, 1987). Lipid peroxidation has been reported in plasma of aging humans and also in subjects with diabetes (Nishigaki *et al.*, 1981).

As more than 75% of 7α-hydroxycholesterol is carried by LDLs, (Bascoul *et al.*, 1990) and as LDLs contain polyunsaturated fatty acids, (conjugated bonds rendering them

susceptible to free-radical-mediated peroxidation), the stability of such esterified form of oxysterols poses a worthwhile question. A second aspect of the possible suceptibility of cholesterol to oxidation, involves the fact that it is the C_7 position of cholesterol which is most sensitive to attack by molecular oxygen (Kumar and Singhal, 1991), thereby rendering cholesterol susceptible to autoxidation in air and peroxidation *in vivo*. However, free cholesterol has been reported to be more stable to autoxidation than its esters (Korahani *et al.*, 1982).

Smith (1981) found that polyunsaturated fatty acids were much more easily oxidised than cholesterol. The first step in the oxidative modification of lipoproteins is peroxidation of polyunsaturated, long-chain fatty acids (Step 1):

(1) $RH \longrightarrow R\bullet + H\bullet$
(2) $R\bullet + O_2 \longrightarrow ROO\bullet$
(3) $ROO\bullet + RH \longrightarrow ROOH + R\bullet$
(4) $ROOH \longrightarrow RO\bullet + \bullet OH$

This step is initiated by the abstraction of a hydrogen atom (adjacent to a double bond) from a polyunsaturated fatty acid (RH), to form a fatty acid free radical (Step 1). The double bond rearranges to form a conjugated diene, which combines with O_2 to produce a peroxyl radical (Step 2). This then can react with more polyunsaturated fatty acid to form hydroperoxide and another free radical (Step 3).

Steps 1-3 are self propagating. The primary reaction products are hydroperoxides, which are unstable and break down in several steps to produce numerous secondary breakdown products. Such secondary products include carbonyl compounds, both unsaturated and saturated fatty acids, aldehydes, ketones and alcohols.

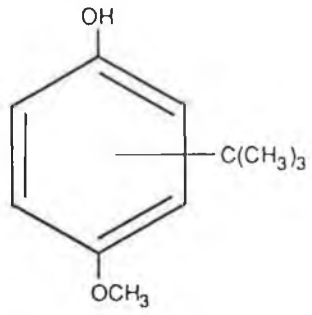
Such free radicals are formed in the reduction of oxygen to water during normal metabolism. Whether or not a free radical will act as a chain propagator (by attacking a stable molecule) or as a chain terminator (by combining with another free radical) depends on subtle numerical proportions and unpredictable affinities (Dormandy, 1969). Oxygen free radicals act on tissue unsaturated lipids, specifically on cholesterol (Smith and Johnson, 1989).

The cell has evolved many mechanisms to reduce the damage caused by prooxidants. Such enzymes as catalase, superoxide dismutase and glutathione transferase all act so as to inactivate the potentially damaging prooxidants which arise from the metabolism of biological materials. In addition, there are several natural dietary components, such as vitamins E and C and selenium, called "antioxidants". When present, they can reduce,

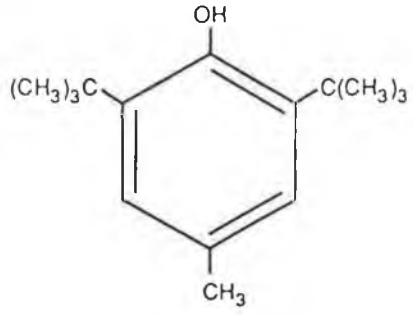
inhibit or significantly delay the oxidation of a substance that is susceptible to oxidation, by acting as "free-radical scavengers". Vitamin E, from the diet, acts preferentially by preventing the peroxidation of PUFA in cell membranes. Vitamin C, again adsorbed from the diet, acts by scavenging radicals in the water-soluble section of the cell. Selenium, combined in an amino acid, is an essential component of the prosthetic groups of several enzymes- particularly glutathione peroxidase. This enzyme acts in the protection of cells against the effects of hydrogen peroxide (Lehninger, 1982).

Synthetic additives to the diet, such as 2-(3)-tert-butyl-4-hydroxyanisole (BHA) and 3,5-di-tert-buytl-4-hydroxytoluene (BHT), have been shown to act effectively as antioxidants, having anticarcinogenic properties. The structure of these compounds are shown below (**Fig 1.5**).

Fig 1.5 Structures of BHA and BHT



BHA
(mixture of 2- and 3- tert-butyl- 4- methoxyphenonl)



BHT

BHT is frequently added as an antioxidant in sample biological preparations, where cholesterol is present and autoxidation is minimised (Björkhem *et al.*, 1987). Both of these compounds are phenolic antioxidants. They are commonly used as preservatives in the food and drug industry, owing to their usefulness to the comparative ease with which they undergo 2-stage oxidation. Between loosing the first and second electron, they pass through a free radical phase; and by combining with a chain-initiating or chain-propagating free-radical, this intermediate can block or break an autocatalytic chain (Dormandy, 1969).

As referred to above, lipid peroxidation has been reported in plasma of subjects with diabetes (Nishigaki *et al.*, 1981). Treatment of diabetic rats with the lipophilic antoxidants- vitamin E and probucol- after the establishment of diabetes, inhibited both the *in vivo* oxidation and the *in vitro* cytotoxicity of diabetic LDL and VLDL, without altering hyperglycemia (Morel and Chislom, 1989). This suggests that the frequently observed tissue damage in experimental diabetes may be explained, in part, by the mechanism of action of lipid peroxidation products and simultaneously highlighting the

importance of antioxidants in such studies.

Several therapeutic approaches have been tested in the attempt to inhibit the oxidative modification of lipoproteins *in vivo*, which include:

* Lowering of plasma LDL to reduce the amount of LDL available for oxidation
* Inhibition of cellular mediated mechanisms with 15-lipoxygenase inhibitiors
* Protection of LDL directly with lipid soluble antioxidants, such as Vitamin E or probucol
* Enhancement of the antioxidant capacity of endogenous antioxidants with ascorbate

etc., (Rosenfeld, 1991).

1.2.5: Metabolism of oxysterols

Human subjects have some capacity to absorb cholesterol oxidation products from food sources. Upon administration of these compounds in powdered egg meal to human subjects, plasma levels of the selected oxysterols were found to be elevated. Upon entering into the circulation, their presence was detected in chylomicrons. After a period of up to 30 minutes, the oxysterols had cleared from the chylomicrons, thereby suggesting their rapid transfer among lipoprotein fractions and/or clearance from plasma (Emanuel *et al.*, 1991).

The detection of oxysterols in various lipoprotein fractions was demonstrated upon incubation of oxysterols with red blood cells earlier in 1981 by Streuli *et al.*, where it was found that lipoproteins, by binding oxysterols, may act in a protective manner against the potential damaging effect which the latter have on cell membranes. Results from their experiments showed that upon incubation of human serum with oxysterols, namely 20-hydroxycholesterol, 25-hydroxycholesterol and 7α-hydroxycholesterol, the isolated lipoprotein fractions had the following constituents (% of oxysterols recovered are expressed with respect to the total amount of sterol recovered, as 60μg sterols were added to 3ml serum):

VLDL:	12.2±1.7%	20-OHCHO	**LDL:**	39.5±7.2%	7α-OHCHO
	8.0±1.1%	7α-OHCHO		38.2±2.1%	20-OHCHO
	4.5 ±2.7%	25-OHCHO		38.1%	CHO
	4.4%	CHO		21.6±2.7%	25-OHCHO
HDL:	40.8±5.9%	7α-OHCHO	**BOTTOM:**	49.6±10.6%	25-OHCHO
	29.7%	CHO	**FRACTION**	27.8%	CHO
	24.3±3.3%	20-OHCHO		25.3±2.7%	20-OHCHO
	24.3±5.1%	25-OHCHO		11.7±5.9%	7α-OHCHO

From these results, it is observed that approximately equal amounts of 7α-hydroxycholesterol (~40%) and 25-hydroxycholesterol (~23%) were present in HDL and LDL. This suggests that oxysterols are metabolised by transfer between plasma lipoprotein fractions, as suggested by Emanuel *et al.*, (1991), in a fashion similar to cholesterol. The percentage of 7α-hydroxycholesterol found in LDL differs significantly from more than 75% reported by Bascoul *et al.*, (1990) for circulating LDLs.

Other suggestions to the further metabolism of oxysterols indicate that cells, which have greater amounts of esterified cholesterol, may utilise oxysterols in preference to cholesterol as substrate for the enzyme- 7α-hydroxylase. This was shown in studies carried out by Dueland *et al.*, (1992) on transfected non-hepatic cells, which had an induction of LDL receptor mRNA. They concluded that 7α-hydroxylase indirectly induces the LDL receptor gene by metabolising/inactivating oxysterol repressors.

As regards the metabolism of esterified oxysterols, metabolic studies are also difficult to carry out, as oxidised cholesteryl esters apparently do form artifically in tissues subject to long term storage (Smith *et al.*, 1981). Very high levels of esterified cholesterol oxides have been reported in human liver which has been stored for years in the frozen state (Assmann *et al.*, 1975). Smith and Johnson (1989) recorded oxysterols to be subject to esterifications *in vivo* and *in vitro*, but that it is uncertain as to the functions of the storage form of these oxysterols, suggesting, perhaps, their role in transport.

More recent work by Kishinaka *et al.*, (1992) on the administration and subsequent metabolism of esterified 7α-hydroxycholesterol in the hamster model, revealed that this form of the oxide could be effectively taken up by the liver, hydrolysed by the enzyme-cholesterol esterase and further metabolised to taurine- or glycine-conjugated primary bile acids *via* the normal bile acid biosynthetic pathway, to bile acids. Their work was in agreement with that of Ogura and Yamasaki (1970), suggesting that 7α-hydroxycholesterol ester is taken up into the microsomal fraction for metabolism. The main metabolites in both cases were cholic acid.

7α-hydroxycholesterol-[^{14}C] (free) in rats was preferentially taken up by the mitochondrial fraction of liver cells (Ogura and Yamasaki, 1970). The main bile acid which formed from this oxide was shown to be chenodeoxycholic acid. Finally, the metabolism of labelled 7α-hydroxycholesterol differed to that of labelled cholesterol, much more radioactivity from the former was excreted in the bile than from the latter. Serum esterified 7α-hydroxycholesterol is considered to be under an equilibrium with a

rapid turnover, as the biological half-life of intravenously injected 7α-hydroxycholesterol-stearate is about 6 hours and hepatic uptake and conversion to primary bile acids are very efficient (Kuroki, 1995 Personal communication).

Although most of the literature referred to above discusses the adverse affects of oxysterols, there are also possible advantages to the action of these compounds. Such *in vitro* cytotoxicities of oxysterols may be of possible use if manipulated for control of tumor cell growth, proliferation, survival and also in the suppression of de novo sterol biosynthesis (Kandutch *et al.*, 1977) as a means of reducing plasma cholesterol levels *in vivo*. As 7ß-hydroxycholesterol was found to be toxic to cultured murine lymphoma cells but not to normal mouse lymphocytes, this may indicate that plasma oxysterols may, when present in plasma, act in order to protect against the survival of cancer cells (Smith and Johnson, 1989).

From the information presented here, it appears that oxysterols are of major importance in cholesterol metabolism and also in subsequent events which occur within the cell or plasma. As regards oxysterols which are metabolites of cholesterol- such as 7α-hydroxycholesterol which is to be examined in this study- its measurement in serum is important as a possible diagnostical marker for disorders of bile acid metabolism, as its concentration in serum has been reported to be indicative of the activity of hepatic cholesterol 7α-hydroxylase. Also, the presence of these oxysterols has been detected in varying fractions of plasma lipoproteins, suggesting the similar transport and metabolism to that of cholesterol. As they are present in both the free and esterified form, various reports have been issued on the possible reactivity of ACAT towards them. To our knowledge, no report exists to date, on the possibility of these oxides serving as substrates for LCAT in plasma. Such studies may prove worthwhile in the further metabolism of these products of cholesterol oxidation/metabolism, produced either endogenously or from exogenous foodstuffs, as much evidence exists for the atherogenicity of oxidised LDL.

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents

[4-^{14}C] Cholesterol (specific activty 52mCi/mmol) was purchased from Amersham Life Science, Buckinghamshire, U.K.

[1-^{14}C] oleoyl coenzyme A (specific activity 59.35 mCi/mmol) and [1,2,6,7-^{3}H(N)]-cholesteryl oleate (specific activity 75.7 Ci/mmol) were obtained from NEN Research Products, Du Pont, Dreiech, W.Germany

Ammonium peroxodisulphate and N, N' methylenebisacrylamide (Electran molecular grade) and phosphotungstic acid were obtained from BDH, Poole, U.K.

The following reagents: Acrylamide, apolipoprotein A-I (bovine source), bovine serum albumin, boric acid, bromophenol blue, butylated hydroxytoluene (BHT), cholic acid-sodium salt, cholesterol, cholesteryl oleate, cholestyramine, dithiothreitol, ethylene-diamine-tetra-acetic acid (EDTA), EGTA, 7ß-hydroxycholesterol, 7-ketocholesterol and 20α-hydroxycholesterol were purchased from Sigma (all oxides were made up in methanol (HPLC grade)), 2-hydroxypropyl-ß-cyclodextrin (2-HPBCD), glycine, lauryl sulfate, leupeptin, 2-mercaptoethanol, M.W-SDS-70L, SDS molecular weight markers (14000-70000), prestained molecular weight markers SDS-7B, potassium bromide, sodium chloride, sodium fluoride, sucrose, streptozotocin (2-Deoxy 2[([methylnitrosoamino]carbonyl)amino]-D-glucopyranose)- mixed anomers, tris (hydroxymethyl)methylamine), tyloxapol (triton WR-1339), L-α-egg yolk phosphatidylcholine (typical composition: 33% C_{16}, 31% C_{18}:1, 15% C_{18}:2, 13% C_{18} (Sigma, Technical Services, U.K.) were purchased form Sigma Chemical Co., Poole, Dorset, U.K.

7α-hydroxycholesterol was purchased from Steraloids, Wilton, NH.

Cholesterol oxidase from *Rhodococcus erythropolis* (*Nocardia erythropolis*, 25U/mg, 1mg/ml) was obtained from Boehringer Mannheim GmbH, .

Potassium chloride, potassium dihydrogen phosphate, di-potassium phoshate and sodium hydroxide were obtained from Riedel de Haën Chemical Co. (IRL).

All solvents: Acetonitrile, chloroform, diethyl ether, hexane, methanol and petroleum ether were HPLC grade and were purchased from Labscan Analytical sciences, Unit T26, Stillorgan Industrial Park, Dublin.

N, N, N', N',-Tetra-methylethylenediamine and sodium chloride were obtained from

Merck, FR Germany.

Sepharose CL 4B was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden while DE-52 anion exchange resin was purchased from while Whatman (USA).

IIG, Bluebell, Dublin, supplied Oxygen free nitrogen (OFN) and Ecolite was obtained from Medical Supply Co., Dublin.

Test kits:
Test-combination free cholesterol was purchased from Boehringer Mannheim.
Cholesterol CHOD-PAP (total) was obtained from Randox, Antrim, U.K.

Protein determination:
Biochoninic acid assay (BCA) protein kit was purchased from Pierce and Warriner (UK) Ltd., 44 Upper Northgate St., Chester, Cheshire, CH1 4EF, England.
BioRad protein assay reagent was purchased from BioRad Laboratories, BioRad House, MAylands Ave, Hemel Hempstead, Hertfordshire, HP2 7TD, UK

Beckman (IRE) supplied silica gel thin layer chromatography 60A LK6D prescored plates (20cmx20cm) and polyallomer Quick-Seal centrifuge tubes (25x89mm).

2.1.1 Equipment

A C-18 Partisil P10 ODS-2 (4.6x250mm, 10µm) column was supplied by Whatman (USA), while Guard-Pak Precolumn Cartridges- µBondapak C18 were purchased from Waters, Chromatographic division, Millipore Corporation, Milford, Mass 01757.
A density meter was supplied by DMA 35- Anton Paar K.G., Austria, Europe.
Labofuge 6000 (Hereus Christ).
LS-7500 Liquid Scintillation Counter (Beckman).
L8-70M Ultracentrifuge (Beckman).
Sorvall RC-5B centrifuge (Du-Pont Instruments).
Philips 201C transmision electron microscope.

2.1.2 Subjects-

Human-Healthy control subjects
Two females and two males served as controls

Hypercholesterolemic subjects
Blood samples from three females and three males being treated for hypercholesterolemia were analysed.
All of the above samples were obtained from The Adelaide Hospital, Dublin 8.

Healthy control rats

Male Wistar rats (200-300g) were housed in a light controlled room for at least 14 days under reversed 12 hour dark/light cycle and had access to food and water *ad libitum*..

Cholestyramine-treated rats

Male Wistar rats were fed a 3% cholestyramine (w/w) (Sigma) diet mixed with pulverised chow for 7 days. Animals were starved overnight prior to being sacrificed the following morning in the middle of the dark cycle.

Streptozotocin-induced diabetes

Male Wistar rats were injected intraperitoneally with 1% (w/v) streptozotocin in 0.1M sodium citrate buffer, pH 4.5 (150mg/kg body weight) i.e: 3 low dosages over a period of 5 days . Rats were housed as above, but received a restricted 20g chow daily. Diabetes was monitored daily, over a period of 11 days after the onset of diabetes, by urinary glucose measurements and for the absence of ketone bodies (Clinistix). Urinary glucose levels were between 10mM and 14mM. Rats were starved overnight and sacrificed the following morning, during the dark cycle.

All animals had blood samples taken from them while under anaesthetic prior to being sacrificed by cervical disslocation.

Bovine

Bovine plasma was obtained from Kepak abattoir, Clonea, Co.Meath.

2.2 Methods:

The methods described in **2.2.1** to **2.2.6** are illustrated in a flowchart (**Fig 2.1**).

2.2.1 Serum preparation

Blood was allowed to clot at 4°C and serum was isolated by centrifugation at 2500rpm for 10min. Samples had BHT (butylated hydroxytoluene) added to yield a final concentration of 50µg/ml, as an antioxidant.

2.2.2 Lipid extraction from serum - Folch method (1957)

0.5ml serum was mixed with 10ml chloroform-methanol (2:1). 2ml of 0.88% KCl was added and vortexed again. The upper phase and interfacial band, containing most non lipid material, were removed after centrifugation at 2500rpm for 10 min. The lower chloroform layer was dried down under oxygen free nitrogen.

2.2.3 Alkaline hydrolysis of lipids (total amounts present) - Oda *et al.*, (1990)

2.0ml of 2.5% KOH in 90% ethanol was added to the dried sample, vortexed and hydrolysed at 55°C for 45min. 1.2ml distilled water was added and the sterols were extracted three times by the addition of n-hexane (3mlx3). The hexane layer was then washed with 1ml water-ethanol (1:1 (v/v)) twice. The organic layer was then dried down under oxygen-free nitrogen.

2.2.4 Cholesterol-oxidase reaction - Hylemon *et al.*, (1989)

The residue was resuspended in 1ml 0.1M potassium phosphate buffer, pH7.4, containing 1mM DTT, 50mM NaF, 0.015% CHAPS and 1mM EDTA. To this, 30µl sodium cholate (20%) suspended in 10mM potassium phosphate buffer, pH7.4 containing 20% glycerol was added and preincubated at 37°C for 10min. Incubation with 40µl cholesterol oxidase (25U/mg, 1mg/ml) was then carried out at 37°C for 10min. Reaction was terminated with 2ml of 95% ethanol. Cholesterol metabolites were extracted by adding 6ml petroleum ether, vortexing for one minute, removing and drying down the upper layer under oxygen-free nitrogen. This ether extraction was repeated twice more and the final dried extract was resuspended in 100µl mobile phase. Samples were stored at 4°C until assayed.

2.2.5 HPLC analysis of cholesterol metabolites - Hylemon *et al.*, (1989)

Analysis was carried out by reverse phase HPLC, using a C-18 Partisil P10 ODS-2 (4.6x250mm, 10µm) Whatman column. HPLC was conducted on an LC-9A Schimadzu liquid chromatograph and an Axxiom 717 computing integrator. The mobile phase was acetonitrile:methanol (70:30 v/v) and elution was monitored at 240nm at a flow rate of 0.8ml/min for 20min, which was then increased to 2.0ml/min to elute off cholesterol. The U.V. detector was set at 0.1 AUFS. 20µl of sample was injected onto the column.

2.2.6 Standard curves for 7α-hydroxycholesterol and cholesterol:

Calibration curves for 7α-hydroxycholesterol and cholesterol were constructed, in the ranges 25.00 - 992 pmoles/20µl for the former and from 51.7 - 129.3 nmoles/20µl for the latter. Standards were brought through the whole procedure from the Folch extraction, together with 20α-hydroxycholesterol as internal standard, to the analysis by HPLC stage.

2.2.7 Recovery:

Control rat serum was spiked with varying amounts of 7α-hydroxycholesterol standard, ranging from 40-120pmoles/0.5ml. Analysis was as above, carried out in duplicate. The percentage C.V. was calculated to measure the degree of precision obtained.

2.2.8 Intra/Inter assay variation:

Analyses were carried out in quadruplicate and assayed as above. Again, the percentage C.V. was measured to determine the precision of the method.

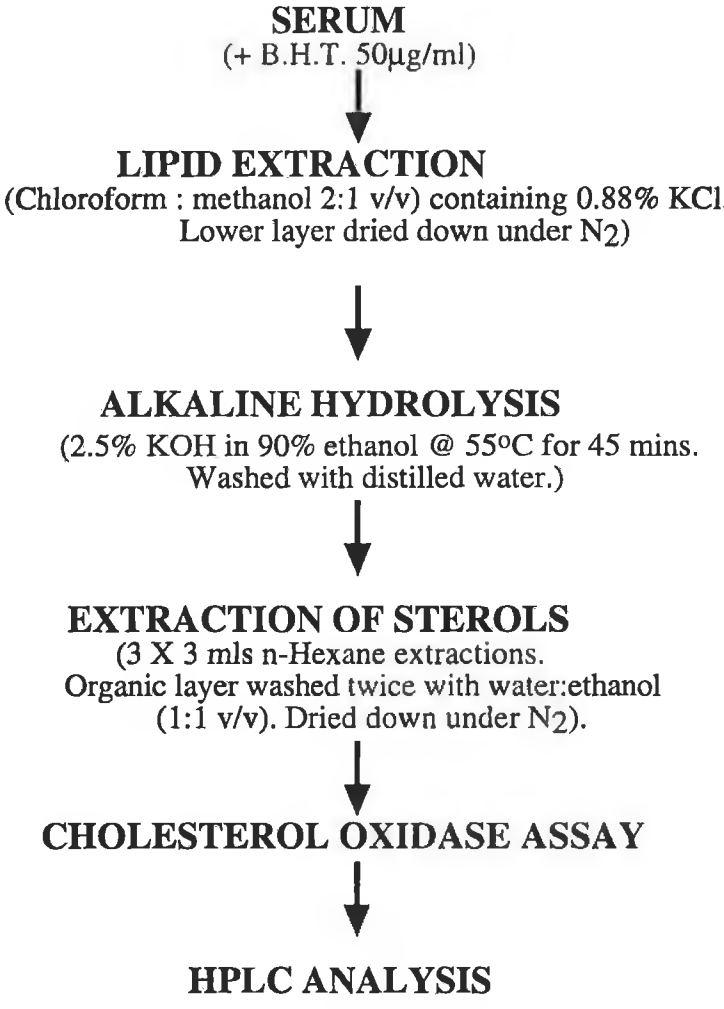
2.2.9 Measurement of Cholesterol 7α-hydroxylase in Rat Liver:

The preparation of rat liver microsomes and the subsequent measurement of Cholesterol 7α-hydroxylase activity was carried out by J.Quinn in the laboratory as described by Hylemon *et al.*, (1989):
The livers were cut up into small pieces prior to homogenisation in 100mM potassium phosphate buffer, pH7.2 containing 100mM sucrose, 50mM potassium chloride, 50mM sodium fluoride, 5mM EGTA, 3mM DTT, 1mM EDTA, 1mM PMSF and 100µM leupeptin. Each homogenate was centrifuged at 2500rpm fror 10min and the supernatant fluid was centrifuged at 10000rpm for 2h. A microsomal pellet fraction was obtained after centrifugation of the supernatant fluid at 28000rpm for 1h. The pellet was then homogenenised in the same buffer (4/1, w/v) and the protein content was determined as described by Lowry *et al.*, (1951).

One milligram of microsomal protein was added to 0.1M potassium phosphate buffer, pH7.4 containing 50mM NaF, 5mM DTT, 5mM EDTA, 20% glycerol and 0.015% CHAPS and incubated for 5min at 37^{0}C. The reaction was initiated by the addition of the NADPH regeneration system whose final concentration were 5mM sodium isocitrate, 5mM $MgCl_2$, 0.5mM NADPH and 0.075 unit of isocitrate dehydrogenase. The final reaction volume was 1ml in a 15x125mm glass tube. The reaction mixture was incubated at 37^{0}C for 20 min in a shaking water bath. The reaction was terminatd by adding 30µl of 20% sodium cholate and 1.9µg of 20α-hydroxycholesterol as an internal standard. Sterol conversion was initiated by the addition of 40µl of 0.1% cholesterol oxidase in 10mM

potassium phosphate buffer, pH7.4 containig 1mM DTT and 20% glycerol. This reaction mixture was incubated for 10min at 37^{0}C and the reaction was terminated and analysed by reverse-phase HPLC as described in **2.2.4** and **2.2.5**.

Fig 2.1 Flow chart of sample preparation and treatment for subsequent sterol analysis by reverse-phase HPLC.



2.2.10 Partial purification of lecithin-cholesterol acyltransferase from Bovine plasma.

Stage1: ∂ 1.21-1.25: Jonas, A., Personal communication

Bovine blood, containing 1mg/ml EDTA was spun down for 30 mins at 3500rpm/4^{0}C. Plasma obtained had its density adjusted to 1.21g/ml with solid KBr and was checked using a densitometer (DMA 35, Anton Paar). The Beckman Quick Seal tubes were heat sealed as they are considered to be bio-hazardous (39.0ml capacity) and spun at 54K/10^{0}C/30hours in a 70 Ti rotor. The use of such tubes eliminates many of the problems that are inherent in the use of tube caps. On ice, the three layers were separated and an aliquot was of each layer was reserved for further analysis. The uppermost layer contains lipoproteins, middle layer- contains LCAT, while the lowest layer contains plasma proteins. Middle layer was dialysed against 0.01M Tris/5mM EDTA and 50mM NaCl, pH 7.6, changing buffer four times.

Stage 2: Butanol-Ammonium sulphate treatment: Doi and Nishida, (1981)

Solid sucrose was added to the dialysate, while slowly stirring on ice, to a concentration of 100g/litre. Ammonium sulphate was then added to yield a final concentration of 40%. One volume of precooled butanol was added to two volumes of the solution and vortexed. Resultant mixture was centrifuged at 13000rpm/15mins/4^{0}C. The interface is collected, containing protein and suspended in precooled saturated ammonium-sulphate (40%) containing 10% sucrose (equivolume to middle dialysate of Stage 1). Precooled 1-butanol, (volume equal to half of middle dialysate), is added to the mixture, vortexed and centrifuged as above. This ammonium-sulphate/butanol treatment is repeated with one-half volumes of the solutions above. Resultant interface is resuspended in phosphate buffer, pH7.4 (containing 0.025% EDTA and 2mM NaN_3), containing 10% sucrose. The solution was then mixed for 30mins under nitrogen.The enzyme mix was dialysed against 10mMTris/5mMEDTA/50mMNaCl, pH7.6 (4 x 1litre) (Jonas, personal communication). The supernatant obtained by centrifuging the dialysate as above was treated as follows:

Stage 3: DE-52 Ion exchange chromatography: Jonas, A., Personal communication

An anion-exchange column was poured (2.5x6cm) with DE-52 anion resin, which had been prepared as follows:

Rinse resin with 100mM Tris/50mM EDTA/500mM NaCl, pH7.6 (buffer 1) and leave to settle. Decant and repeat. Rinse resin with 10mM Tris/5mM EDTA/50mM NaCl, pH7.6 (buffer 2). Repeat twice. Column was then poured and rinsed with buffer 2 prior to application of sample.

Sample was applied to column and the run-through peak was collected. Upon reaching a low, steady baseline, a linear gradient was applied (from 75mM NaCl-100mls to 200mM

NaCl-100mls, 10mM Tris/5mM EDTA buffer, pH7.6, containing the above concentrations of salt). Flow rate was maintained at 1ml/min and 2ml fractions were collected. This step was carried out at 4°C. Fractions were then assayed for LCAT activity.

2.2.11 SDS-PAGE (polyacrylamide gel electrophoresis) of steps in the partial purification of LCAT from bovine plasma.

Sample aliquots of each of the above stages in the partial purification of LCAT were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli (1970), as described by Bollag (1991). Proteins were separated on a resolving gel containing 7.5% (w/v) acrylamide, 0.20% (w/v) bis-acrylamide, 1.97M Tris-HCl pH 8.8, 1.25% (w/v) SDS, 0.15% (v/v) TEMED and 10% (w/v) ammonium persulphate. A stacking gel consisting of 2.85% (w/v) acrylamide, 0.08% (w/v) bis-acrylamide, 0.5M Tris-HCl, pH 6.8, 2.5% (w/v) SDS, 0.05% (w/v) TEMED and 0.07% (w/v) ammonium persulphate was used. The electrode buffer contained 25 mm Tris, 192mM glycine and 0.1% SDS prepared in distilled water. Samples for electrophoresis were dissolved at a concentration of 1mg/ml in solubilization buffer (equal ratio of sample to solubilization buffer) consisting of 60mM Tris-HCl, pH 8.8, 25% (v/v) glycerol, 2% (w/v) SDS, 14.4mM 2-mercaptoethanol and 0.1% (w/v) bromophenol blue were used when reducing conditions were required. Samples were boiled for 2-3 minutes and 10-20µl of these solutions were applied to the wells of the stacking gel. The gel was electrophoresed in electrode buffer, at 100mA for two gels, using an Atto vertical mini-system. Gels were stained for approximately 15 minutes in 0.25% (w/v) Coomassie Brillant blue in methanol:acetic acid:water (5:1:4) and destained in methanol:acetic acid:water (25:7:68) while rotating gently. Markers were run in Lane 1 and range from 32-195 kdal.

2.2.11.1 Concentration of DE-52 fraction for SDS-PAGE analysis:

T.C.A. (Trichloroacetic acid) precipitation: 20% T.C.A. was added in an equal volume to the sample. The mixture was left on ice for 30 mins. Sample is then centrifuged for three mins at 2000rpm (T.C.A. concentration is 10%). The supernatant is then removed, the pellet was washed twice with acetone and resuspended each time.

2.2.12 Synthesis and studies of discoidal complexes- containing A) cholesterol and B) 7α-hydroxycholesterol as substrates for LCAT.

A) <u>Cholesterol containing complexes</u>: Jonas (1986a).

Micellar complexes of apolipoprotein A-I:egg phosphatidylcholine:cholesterol, in the molar ratio 1:100:9.1, were prepared as described by Jonas, A. (1986), but by varying the ratios of the three components of the discoidal complexes, using the detergent-mediated (sodium-cholate) method. Sodium cholate micelles with phosphatidylcholine

form at a sodium cholate:phosphatidylcholine molar ratio of 1:2 to 2:1.

An aliquot of egg phosphatidylcholine was taken corresponding to 2.68mg (stock: 50mg/1ml ethanol). This, together with a total of 10 nmoles of cholesterol (comprising 2.289 nmoles of [4-^{14}C]Cholesterol (sp act, 52mCi/mmol) and 7.706 nmoles cold cholesterol,were dried down under a stream of oxygen-free nitrogen. Sodium cholate, in 10mM Tris-HCl, pH8.0, containing 150mM NaCl, 0.005% EDTA and 1mM NaN_3, was then added (2.68mg/total volume of complex=500µl) in order to disperse the lipids. The stock of sodium cholate is 30mg/ml. Such conditions result in the transformation of complex mixture from milky to translucent appearance. Incubation was carried out at 4°C for approximately 2 hours. If necessary, more sodium cholate may be added at this stage to ensure that the mixture becomes translucent, provided that the molar ratio of sodium cholate/phosphatidylcholine is no greater than 2/1. 870µg apolipoprotein A-I (was added 10mM Tris-HCl, pH 8.0, containing 150mM NaCl, 0.005% EDTA and 1mM NaN_3) next, in such an aliquot that the total volume of sodium cholate and apolipoprotein A-I is 500µl. The mixture was mixed thoroughly and incubated at 4°C for a period of 12-16 hours, usually about 13 hours. Dialysis of the product was then carried out against 6 changes of buffer, as above, in volumes of 500mls each in order to remove the sodium cholate. Such excessive dialysis was carried out over 3 days. Unreacted was removed from the mixture by centrifugation for 70 minutes, 10°C at 13000rpm. Supernatant was transferred to a vial and stored at 4°C until required.

B) 7α-hydroxycholesterol containing complexes:

Discoidal complexes of apolipoprotein A-I:egg phosphatidylcholine: 7α-hydroxy-cholesterol, in the molar ratio 1:100:9.1,were prepared as above, except that 10 nmoles 7α-hydroxycholesterol were dried down in the initial step, in place of cholesterol. The rest of the synthesis was as above.

2.2.13 Compositional analysis of discoidal complexes

a) Gel filtration of cholesterol-containing complexes- Chen and Albers, (1982)

The centrifuged preparation, containing 579µg apo A-I, 84.848µg cholesterol and 1.785mg phosphatidylcholine, was chromatographed on a Sepharose CL-4B column (2.2x50cm), which was equilibrated and eluted with 10mM Tris, 150mM NaCl, 1mM NaN_3 and 0.05% EDTA, pH 7.4 buffer. Fractions of 2ml were collected at a flow rate of 30ml/hour. Each fraction was assayed for each of its three components as described below. Markers run over the column were (Nichols *et al.*, 1986)

thyroglobulin- hydrated Stokes diametre 17.0nm, 6.69 x 10^5 daltons

ferritin- hydrated Stokes diametre 12.2nm, 4.4 x 10^5 daltons

catalase- hydrated diametre 10.4nm, 2.1 x 10^5 daltons

BSA- hydrated diametre 7.1nm, 6.7 x 10^4 daltons

Cholesterol elution was monitored by the addition of 100µl of fraction to 10ml scintillation fluid and carrying out radioactive counting.

Phosphatidylcholine was measured using a kit for L-α-lecithin by Boehringer Mannheim.

Apolipoprotein A-I was measured by Bio-Rad protein assay.

b) Native Gradient gel electrophoresis (3-20%)

A linear gradient acrylamide gel (3-20%), pH 8.3 was made according to the method of (Walker, 1984). Fractions (cholesterol complexes) were concentrated by reverse dialysis against PEG-8000, to yield a final concentration of approximately 1.75mg/ml. Equal volumes of sample and tracker dye solution (40% sucrose and 0.05% bromophenol blue) were mixed and applied to the gel. A voltage of 160 was applied and the gel was left to run for approximately 90 minutes, in 50mM Tris and 0.38M glycine, pH8.3. An Atto mini system was employed. Staining and destaining were as described by Bollag and Edelstein (1991). Markers used were the same as those for the Sepharose CL-4B.

7α-hydroxycholesterol complexes of equal concentration (approximately 10µg protein) were applied to the gel.

c) Electron microscopy of discoidal complexes

Electron microscopy was carried out by P.Costigan, Virus Reference Laboratory, University College Dublin. Transmission electron microscopy was used to determine the structure and approximate measure of diameter of the complexes. Samples were diluted to a final vlume of 4ml with "polished" water (reverse osmosis water which has been filtered and autoclaved), centrifuged at 2000g for 35min in an ultracentrifuge- Sorvall Combi. They were then negatively stained with 4% phosphotungstic acid, pH6.8, having been resuspended in 0.01% Bacitracin (a low molecular weight spreading agent which reduces the surface i.e., a "wetting" agent), for approximately 1min. A Philips 201C transmission electron microscope, operating at 80 kV, with an objective lens- aperature of 20 microns and a condenser of 100 was used in this study. Grids were scanned 20,000 times with a 9-fold magnification of the selected area (180,000- fold magnification). Prints were obtained for each sample. The diameters and widths were measured from the prints by utilisation of the bar indicated.

2.2.14 Reaction of LCAT with substrate complexes

2.2.14.1 Reaction of LCAT source with discoidal complexes containing cholesterol: Jonas and Matz (1982)

An aliquot of complexes, containing 10nmoles substrate (7α-hydroxycholesterol or cholesterol) incubated with 2mg defatted BSA, 4mM ß-mercaptoethanol and 10mM Tris-HCl, 0.01% EDTA, 1mM NaN_3 buffer, pH 8.0, to a final volume of 500µl. The mixture was preincubated for 30 minutes at 37°C. Then 100µl of the pooled DE-52 fraction was added and further incubated for 30mins (Jonas and Matz, 1982). Reaction was terminated by the addition of 10ml chloroform/methanol (2/1). Carriers added for TLC were as described by (Steinmetz, 1985), 10µg/ml each of cholesterol and cholesteryl oleate were added. Cholesterol and cholesteryl ester were separated by thin layer chromatography on scored precoated silica gel 60 plates (20cmx20cm Whatman). The TLC plate was spotted and run with a mobile phase of petroleum ether:diethylether:acetic acid (76:20:1) (v/v/v). Spots were visualised after placing iodine crystals in the tank for a few minutes. Bands were scraped off and placed in scintillation vials with 10ml scintillation fluid (EcoLite). Counts were taken and calculations carried out as below. Controls were run which contained no enzyme source and they contributed to a minimal amount of enzyme activity.

Enzyme activity was measured by the conversion of [4-^{14}C]cholesterol to [4-^{14}C] cholesteryl ester. The ratio of cholesteryl ester to free unesterified cholesterol is measured and the percentage esterification is calculated based on the number of moles of cholesterol employed in the reaction mixture.

$$= \frac{A.CE}{(CF+CE).v.t}$$

where A = number of mole substrate added to the incubation mix, CE = cpm in the cholesterol ester band of the plate, CF = cpm in the unesterified cholesterol band, t = incubation time and v = volume of enzyme used (Mahedevan and Soloff, 1983).

2.2.14.2 Reaction of LCAT source with discoidal complexes containing 7α-hydroxycholesterol:

The procedure is as above, except, due to the fact that non-radioactive 7α-hydroxycholesterol was employed in the assay, scintillation counting could not be used as a quantitative method of analysis. Thus, using the procedure described above for the hydrolysis and HPLC analysis of 7α-hydroxycholesterol, the following procedure was used:

LCAT assay with discoidal complexes containing 7α-hydroxycholesterol (10nmoles) was as described above.Upon termination of the reaction with 10ml chloroform/methanol

(2/1), 2ml of 0.88% KCl was added and vortexed. The upper phase and interfacial band, containing most non-lipid material, were removed after centrifugation at 2500rpm for 10 min. The lower chloroform layer was dried down under oxygen free nitrogen. Alkaline hydrolysis was carried out on one sample, to measure total 7α-hydroxycholesterol present, while a second sample was not hydrolysed, as the free amount of 7α-hydroxycholesterol was to be measured. Thus, the amount that was esterified was calculated as the difference between the total and the free amounts of 7α-hydroxycholesterol. Extraction with n-hexane, water:ethanol washing, cholesterol oxidase assay, petroleum ether extraction and finally, analysis by reverse-phase HPLC were carried out as in **2.2.5**. Visual analysis by development on a TLC plate was also undertaken. Enzyme/substrate blanks and LCAT source samples were analysed, the latter for endogenous 7α-hydroxycholesterol and cholesterol.

2.2.15 Preparation of liver microsomes- Einarsson *et al.*, (1989)

Liver samples were homogenised with a Teflon-pestle in nine volumes of 50mM Tris-HCl buffer, pH 7.4, containing 0.3M sucrose, 1mM EDTA, 50 mM NaF, 5mM EGTA and 50μM leupeptin after being minced finely. The homogenate was centrifuged for 15 min at 2500rpm. The supernatant fluid was centrifuged for 1 hour at 38000rpm. The microsomal pellet obtained was then resuspended in 0.1M phosphate bufer, pH 7.4, containing 1mM EDTA, to yield a final concentration of 10% (w/v). Protein content was determined by the method of Lowry *et al.*, (1951).

2.2.16 Removal of microsomal cholesterol using 2-HPBCD- Martin *et al.*, (1993)

Previous studies (De Caprio *et al.*, 1992) have indicated that 2-hydroxypropyl-ß-cyclodextrin, (2-HPBCD), is a molecule with a hydrophobic cavity capable of accomodating sterols and has been used in cell culture and *in vivo* studies. Results of Martin *et al.*, (1993) indicate that this is also a suitable vehicle for the removal of cholesterol from microsomal preparations.

100μl aliquots of microsomal suspension (diluted) containing 3.42μg cholesterol, 150μg protein in 100mM potassium phosphate buffer, containing 1mM EDTA, pH 7.4, were incubated at 37^{0}C for the duration of the experimental procedure. 2-HPBCD was added to the mixtures, in centrifuge tubes, to yield a final concentration of 30mM. Each assay was carried out at least in duplicate, while analysis was also carried out on replicates at time 45 (first incubation) and time 90 (second incubation), to measure the extent of cholesterol depletion. Upon completion of incubation of microsomal preparation with 30mM 2-HPBCD, the tube with its contents were immediately placed on ice and centrifuged at 150000g, at 4^{0}C for 40 min. Microsomal pellets were recovered and

resuspended in their original buffer- 100mM potassium phosphate buffer, containing 1mM EDTA, pH 7.4 (100µl) and subsequently used in the assay of ACAT activity.

2.2.17 Assay of ACAT activity- Sahlin *et al.*, (1994)

The above suspension, containing 150µg protein and 1mg of fatty acid-free bovine serum albumin, was made up a volume of 1ml (including the volumes of 7α-hydroxycholesterol). Preincubation was carried out at 37°C for 5min. Varying amounts of 7α-hydroxycholesterol, ranging from 4-24 nmoles/assay, were added to the mixture.[7α-hydroxycholesterol was made up as follows, using the principal as decribed by Billheimer *et al.* (1981):

Stock= 2.480mM 7α-hydroxycholesterol, in methanol. Aliquot dried down, under oxygen free nitrogen, corresponding to 300µg 7α-hydroxycholesterol (744.9nmoles). 9mgTriton WR-1339 was added in 90µl methanol (10% solution of Triton WR-1339 in methanol). Thus, the stock solution of 7α-hydroxycholesterol/Triton WR-1339=300µg/9mg in 90µl.

The working solution of 7α-hydroxycholesterol/Triton WR-1339 was as follows:

1ml assay buffer is placed in a test tube, weighed and warmed gently. The stock solution of 7α-hydroxycholesterol/Triton WR-1339 is also warmed gently, ensuring that the cholesterol oxide is dissolved completely. 24.2µl of the dissolved stock solution was then added to the buffer, with constant mixing. Oxygen-free nitrogen was used to evaporate off the solvent. If any loss in weight occured during the procedure, it was replaced at this stage with water, while vortexing. A transparent solution resulted, which contained 200nmoles 7α-hydroxycholesterol and 2.416mg Triton WR-1339 in a final volume of 1ml].

Thus, 20, 40, 60 and 120µl of the above mixture was added to the assay buffer, corresponding to 4, 8, 12 and 24 nmoles 7α-hydroxycholesterol per assay mix. These were further incubated for 20 min. 25nmoles (1.48µCi) of [1-^{14}C] oleoyl Co.A was added to initate the reaction. Reaction was terminated by the addition of 10ml chloroform/methanol [2/1(v/v)] after 6 min. [1,2,6,7-^{3}H] cholesteryl oleate[0.01µCi (0.132pmoles)] was added as internal standard, followed by the addition of 1ml 0.9% NaCl (w/v). Cholesteryl esters were recovered from the chloroform phase, which was evaporated under oxygen-free nitrogen. Having resuspended the residue in chloroform/methanol [2/1(v/v)], carried out thin layer chromatography on precoated Whatman silica gel 60 plates (20cmx20cm), with hexane:ethyl acetate 95:5 (v/v) as mobile phase, the resulting spots were visualised with iodine vapour. Cold cholesterol and cold cholesteryl oleate were also run as markers. Resulting bands for analysis were

scraped off and placed directly in scintillation vials with 10ml EcoLite scintillation fluid for radioactive counting. A substrate blank was also included in the assay. Enzyme activity was expressed as pmoles/min/mg protein.

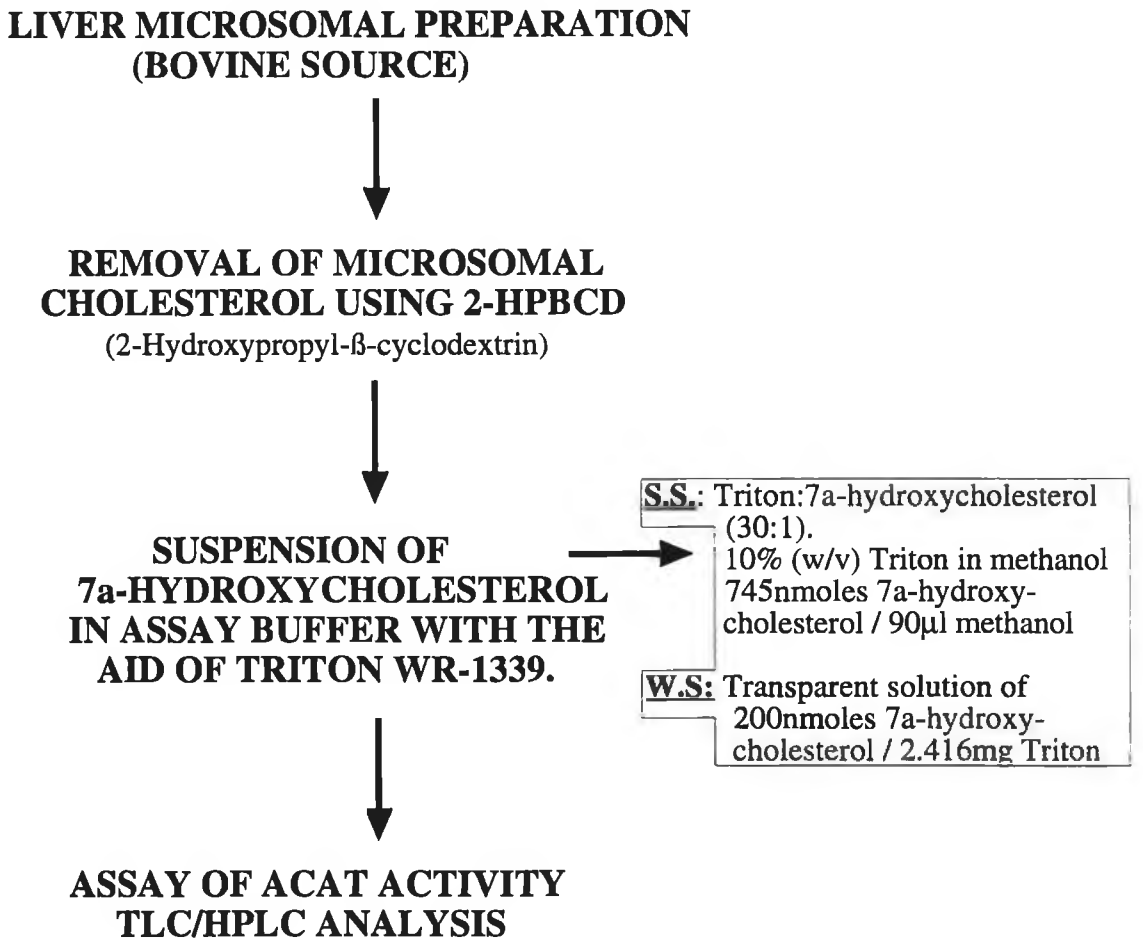
A replicate of this procedure containing 24 nmoles 7α-hydroxycholesterol was produced and run on TLC. However, instead of being analysed quantitatively by radioactive counting, the bands of the TLC plate were scraped off, eluted 3 times with 2.5 ml diethyl ether as described by Nimmannit and Porter (1980). The combined extracts were subjected to hydrolysis, n-hexane extraction, cholesterol oxidase assay, petroleum ether extraction and finally, reverse HPLC analysis as described in **2.2.4** and **2.2.5**. Each band of the TLC lane was analysed for its composition.

The amount of product formed was calculated using the following equation:

nmoles esterified 7α-hydroxycholesterol produced/6min/mg protein=

$$\frac{\text{dpm } [^{14}C]7\alpha\text{-hydroxycholesterol recovered}}{\text{specific activity } [^{14}C] \text{ oloeyl CoA (dpm/nmole)}} \times \frac{\text{dpm } [^{3}H]\text{cholesterol oleate added}}{\text{dpm } [^{3}H]\text{cholesterol oleate recovered}}$$

Fig 2.2 Schematic representation of the procedure involved in the preparation and assay of ACAT, with respect to 7a-hydroxycholeserol as a potential substrate.



S.S: Stock solution

W.S: Working solution

2.2.18 Protein assays

2.2.18.1 Bicinchonic acid (BCA) protein assay

Cu^{2+} reacts with protein under alkaline conditions to yield a coloured product upon reaction with the BCA reagent. 200µl of commercial working BCA reagent (Pierce Chem.Co.,) was added to 10µl test sample or protein standard solution (10-2000µg/ml), in 96-well microtitre plate wells. The plate was agitated to ensure thorough mixing and incubated for 30min at 37^{0}C. The absorbance was read at 560nm using a Titertek Twinreader® Plus microtitre plate reader. Protein concentrations were determined from a standard curve. Typically, BCA standard curves for protein determination gave r values of 0.999.

2.2.18.2 Bio-Rad assay

The Bio-Rad protein assay is based on the fact that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595nm, when binding to protein occurs. 40µl of commercial Bio-Rad reagent (Bio-Rad Laboratories) was added to 160µl of test sample or standard protein solution (5-25 µg/ml). Again, 96-well microtitre plates were used. Typically, BSA was the standard protein employed. The samples and reagent were mixed thoroughly using a micropipette as the reagent is viscous and does not mix readily with the sample or standard. Absorbance readings were measured on the Twinreader® Plus microtitre plate reader, at 620nm- 5-60min after sample mixing. Protein concentrations were obtained from a standard curve. Typical r values were 0.998

CHAPTER 3

VALIDATION OF PREPARATIVE AND CHROMATOGRAPHIC PROCEDURES FOR THE MEASUREMENT OF SERUM 7α-HYDROXYCHOLESTEROL

3.1 Introduction

This chapter discusses the concepts behind an efficient preparation of an analyte for subsequent analysis by a suitable chromatographic method. One of the problems that continually faces biochemists is the separation, purification and identification of one or more biological compounds from a mixture of such compounds. One of the most convenient methods for achieving such separations is the use of chromatographic techniques. The feature that distinguishes chromatography from most other physical and chemical methods of separation is that two mutually immiscible phases are brought into contact; one phase is stationary and the other mobile. It is possible to rationalise to some extent the type of system most likely to be applicable to the separation of compounds when the physical characteristics are known. An example of this would be the applicability of reverse-phase chromatography to the analysis of a polar compound such as 7α-hydroxycholesterol upon extraction from an aqueous environment, whereby the use of a mobile phase which is more polar relative to the stationary phase results in analytes being eluted from the column in order of decreasing polarity. Stationary phases which are frequently used in this form of chromatography are chemically bonded such as octadecylsilane (ODS), an alkane with 18 carbon atoms. C8, shorter alkyl chains and also cyclohexyl and the more polar phenyl groups provide other alternatives.

Water is often described as the strongest elution medium for chromatography, but in fact this is only true for adsorption processes. Water may interact strongly with the active centres in silica gel and alumina, so that adsorption of sample molecules becomes highly restricted and they are rapidly eluted as a result. Exactly the opposite applies in reversed-phase systems: water cannot wet the non-polar alkyl groups and does not interact with them in any way. Hence, it is the weakest mobile phase of all and gives the slowest sample elution rate (Meyer, 1988). Before discussing HPLC applicability and methodology in detail, an introduction to sample cleanup will be given.

3.1.1: Sample collection

The first step in such biological analysis involves the collection of the sample. Such information as the expected concentration of the analyte to be found in the sample, the stability of the analyte, for example, to light, temperature, pH and biochemical changes due to enzymic reactions and the physiological variations of the analyte due to physical activity, stress or biological rhythms is required in order to maximise the efficiency of sample collection.

Plasma is obtained by centrifuging whole blood in a heparinised tube or tubes containing salts of EDTA to prevent clotting. It is rich in lipids and lipoproteins, since these compounds cannot be removed by centrifugation. Care must also be taken not to haemolyse the blood cells, as many compounds are present in different concentrations between red cells and plasma.

Serum samples, which are produced by the natural clotting of fibrinogen, results from blood being drawn into tubes containing no anticoagulant. The clot is allowed to form after standing for 10-15 mins at room temperature or longer if left at 4^0C. A wooden stick may be used to remove the clot, if it adheres to the walls of the container. The clot is normally left to retract, before centrifugation, so as to release as much volume of serum as possible.

3.1.2: Sample storage

If the sample requires being transported from the site of collection to the site of analysis, then note should be taken of the possible effects of transport and storage on the analyte (Uihlein, 1986). Unless otherwise specified, the appropriate conditions for the sample during transport are freezing or freeze-drying. If required, an antioxidant such as butylated hydroxytoluene should be used. Under all conditions, stability is a time-dependent property. Care should also be taken if the sample is light sensitive, as it is well documented that certain substances, such as cholesterol are known to be light sensitive and break down under light. Containers themselves may also interact with the sample (lipids are known to react/adhere to glass), migration of plasticisers from the container into the sample may interfere with or adsorb the analyte (Steyn and Muller, 1980).

3.1.3: Sample preparation

3.1.3.1: Deproteination

The choice of sample preparation method is dependent on the sample matrix, size availability, concentration of the analyte, stability of the analyte, whether or not the analyte is bound to proteins, sensitivity and specificity of the detector used and whether a qualitative or a quantitative analysis is to be performed. Such pretreatments of the sample include the disintegration of the sample where the matrix structure is destructed by homogenisation, grinding and blending in an appropriate solvent and secondly by deproteination which is necessary where high concentrations of protein are present which may form emulsions during organic extraction. Deproteination of a blood sample is generally associated with pH adjustment for the following extraction step into an organic solvent. Thus the method selected for deproteination depends on the pK_a of the material to be analysed. Common solvents used include acetone, acetonitrile and alcohols, which lower the solubility of proteins in aqueous solution. By deproteination and extraction, protein bonds must be broken in order to increase the recovery of the analyte (Christians and Sewing, 1989). Removal of protein is required whenever there is a hint of irreversible binding of the analyte to proteins. Extraction by means of solid-phase seems to avoid the formation of emulsions.

Proteins may also be precipitated by the formation insoluble salts. Acidic agents such as trichloroacetic acid and perchloric acid can form insoluble protein salts with the cationic form of proteins at low pH, thus precipitating them of solution. Alternatively, proteins,

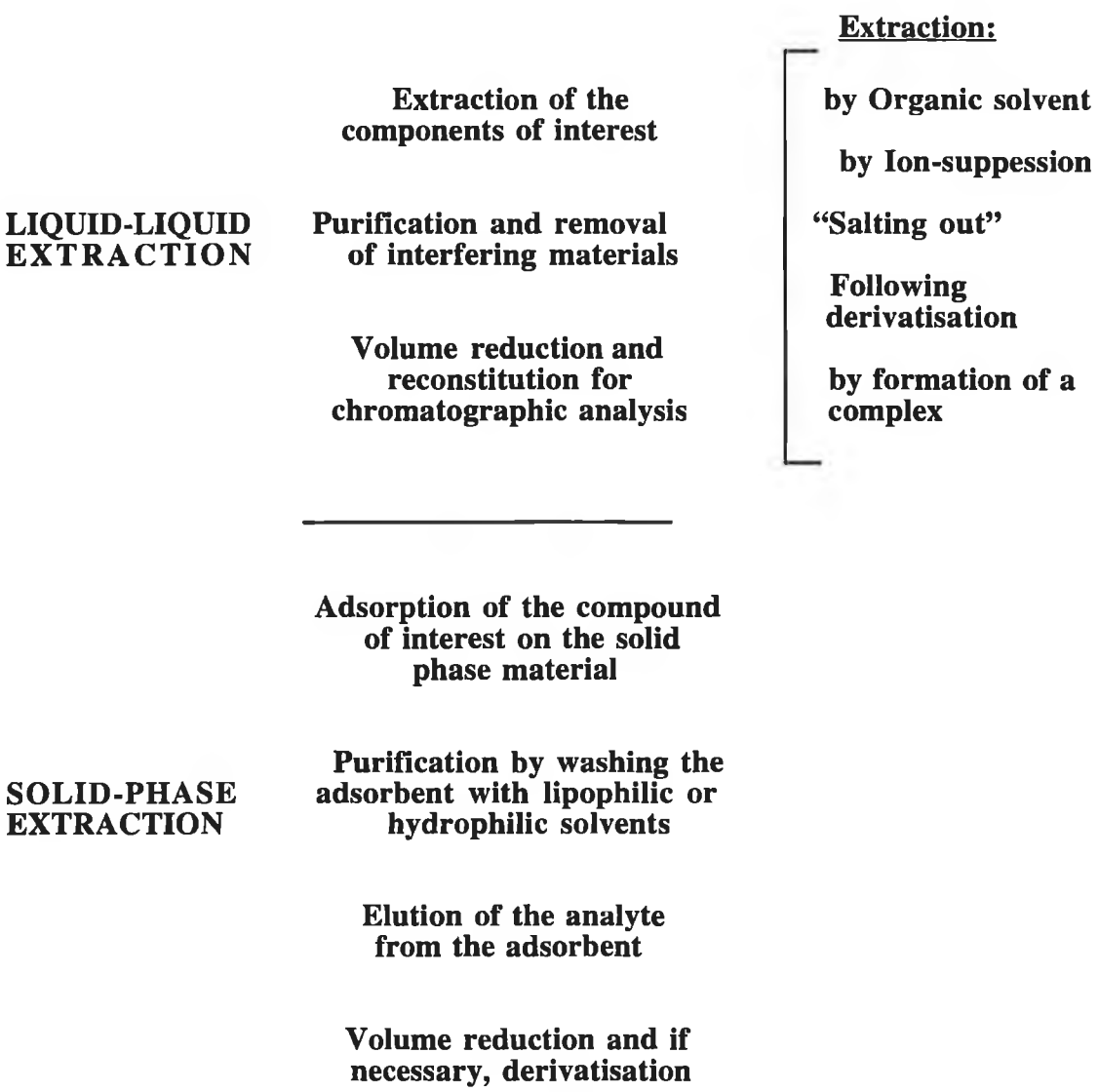
being negatively charged in strongly alkaline solution, may be precipitated by the formation of insoluble salts with cationic precipitants such as Cu^{2+} and Zn^{2+}ions.

Ultrafiltration may also be used to separate protein from an analyte. Here, filtration through a size-selective semipermeable membrane under pressure or by centrifugation in a membrane cone, results in a protein-free ultrafiltrate. An advantage of its applicability to small volumes over that of protein precipitation methods is that no dilution occurs during filtration.

3.1.3.2 Extraction procedures for blood samples

Many cleanup procedures for samples exist and include filtration, centrifugation, precipitation, freeze-drying, evaporation, extraction of solids/liquids by liquids and the extraction of liquids by adsorption on solids (**Fig 3.1**).

Fig 3.1: Methods involved in sample preparation

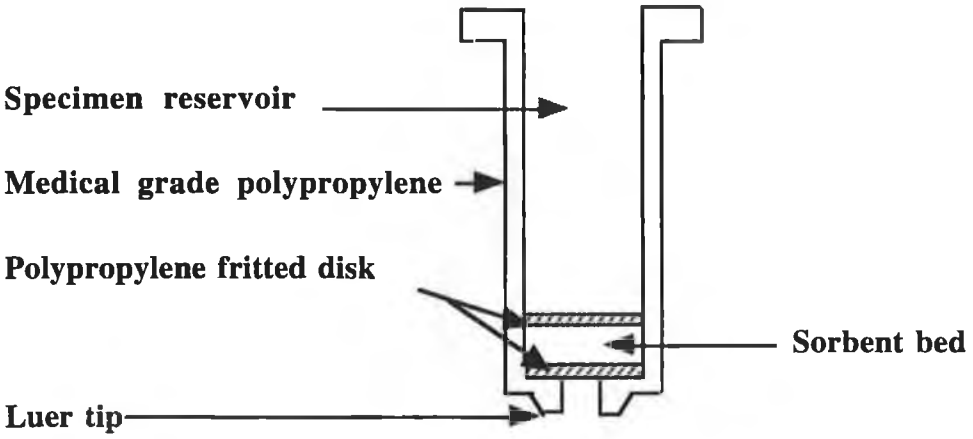


Liquid-liquid extractions are usually followed by evaporation of the extraction solvent to concentrate the extracted analyte. Extraction is usually rapid, relatively simple and mostly results in high purification (Uihlein, 1986). The solvent is selected in order to provide maximum efficiency of extraction with the minimum amount of contamination. The least polar solvent that sufficiently extracts the analyte is selected for the extraction. By increasing the polarity of the extracting media one also increases the range of compounds extracted. Ideally, the solvent chosen should be volatile, as most samples are recovered, after solvent extraction by evaporation.

Ion-pair formation involves the extraction of samples into an organic solvent, by the suppression of their ionisation in the aqueous phase, by means of addition of acid, base, or a buffer of controlled pH. "Salting-out" results in the extraction of polar compounds into an organic phase, after decreasing their affinity for the aqueous phase, by the addition of buffer salts to the aqueous solution. Derivatisation is commonly used to improve the detectability of certain analytes. It may also be used in the analysis of compounds which are insufficiently soluble in organic solvents, but may be extracted after being derivatised to more organically-soluble derivatives. Formation of a complex allows the extraction of compounds which are hydrophilic, from the aqueous phase, by the formation of a hydrophobic complex with an appropriate complexing agent.

Solid-phase extraction involves the adsorption of the analyte onto the solid phase material. The adsorbent needs to be of suitable selectivity as does the solvent in which the analyte can be quantitatively recovered. Solid phases include- silica, silicic acid, alumina, Celite, talc, charcoal or ion-exchange, hydrophobic or hydrophilic resins. Silica gels have also been bonded with a wide variety of functional groups such as alkyl (C_1, C_8 and C_{18}), phenyl, aminopropyl and diol groups. Thus, these allow for the specific interaction between the analyte and the support. The cartridges (**Fig 3.2**) are disposable and the use of such a procedure results in the concentration of the sample on the cartridge as well as effecting at least a partial clean-up.

Fig 3.2 A disposable sorbent extraction cartridge

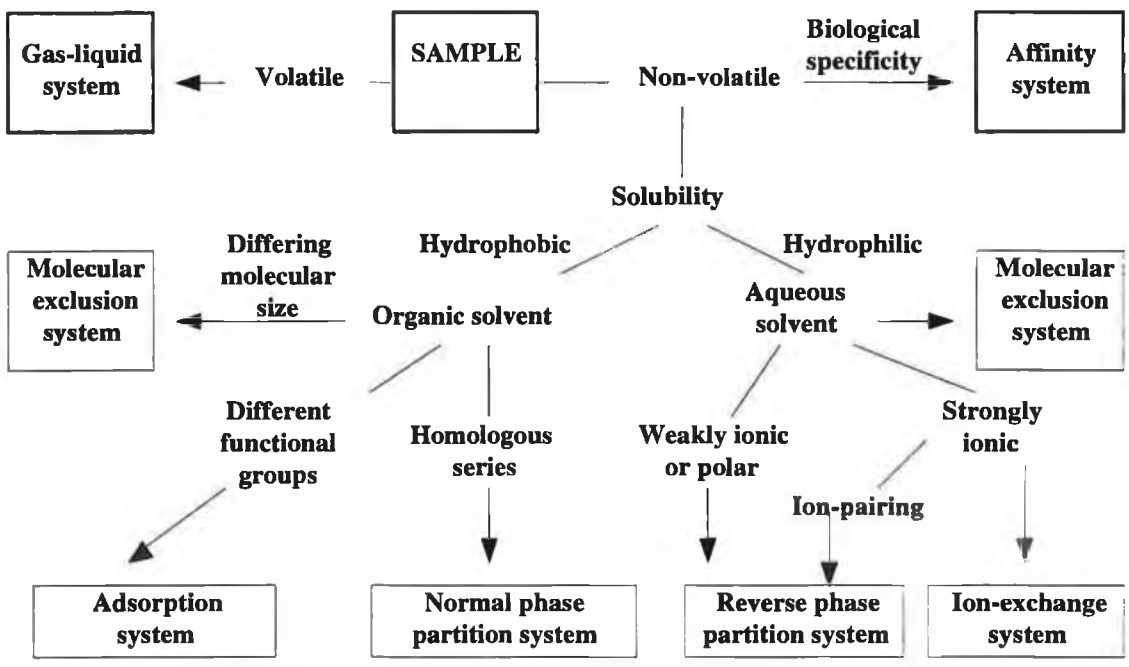


3.1.4 Modes of separation

As mentioned earlier it is possible to rationalise to some extent the type of system most likely to be applicable to the separation of compounds, based on such characteristics as their molecular weight, solubility in aqueous/non-aqueous systems and ionisability. The different modes of separation by HPLC may be divided into classes such as ion-exchange, reverse-phase, normal-phase (adsorption), partition and gel-permeation chromatography (**Fig 3.3**). In brief, these are discussed below:

Ion-exchange chromatography describes separation that is based on the relative molecular charges of the compounds being separated, i.e., the attraction between oppositely charged molecules. The charge on the stationary phase is opposite to that of the ion. The ion-exchange columns for HPLC include such packing material as silica bonded with ionic groups (cationic or anionic). Ion-exchange chromatography may also be performed on bare silica with methanol containing an ammonium salt, e.g., ammonium perchlorate (Law *et al.*, 1984; Flanagan and Jane, 1985), as eluent.

Fig 3.3 Rationale for the choice of a chromatographic system (Wilson and Goulding, 1986)



Normal-phase chromatography also known as adsorption chromatography, allows the separation of components based on differences both in their degree of adsorption by the adsorbent and solubility in the solvent used for separation. The molecular structure of the compound governs these features. The eluents here are usually relatively non-polar organic solvents. Thus, the analytes are eluted off in order of increasing polarity, while retention decreases with increasing solvent polarity. Compounds that are highly soluble in organics, such as lipids and fat-soluble vitamins, are usually separated by normal-phase HPLC.

Partition chromatography especially in the high performance form, is used for the separation of a very wide range of biological compounds. It is based on the partition of the solute between the liquid stationary phase and the mobile phase. In normal phase partition chromatography, the stationary phase is water supported by a matrix. In reverse phase partition chromatography, the stationary phase is a non-polar compound such as liquid paraffin supported by a matrix similar to those employed in normal phase systems (cellulose, starch or silicic acid).

Gel permeation chromatography or size-exclusion chromatography, separates compounds on the basis of molecular size. Large molecules, outside the molecular weight cut-off range of the gel, are excluded from the gel, as they cannot enter the pores of the stationary phase. Elution occurs in order of decreasing size, the smallest molecules being eluted lastly.

Reverse-phase chromatography as the name suggests, is the opposite to normal-phase chromatography. Thus, the mobile phase is more polar than the stationary phase. The solute is retained mainly as a result of hydrophobic interactions between the solutes and the hydrophobic stationary phase surface. Solutes are normally eluted in order of decreasing polarity (increasing hydrophobicity) and increasing the content of the non-polar component in the mobile phase, results in the decrease of the retention of the solutes. Various additives can be incorporated into the mobile phase to give special selectivities. Thus, reverse-phase chromatography may be extended to include ion-pairing, ionisation or ion-suppression.

3.1.5 Instrumentation for HPLC

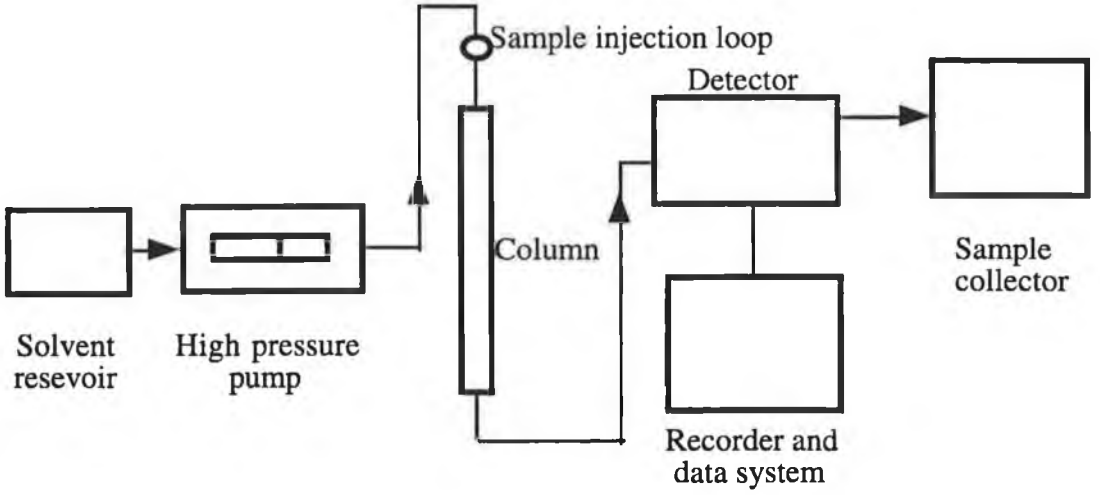
The instrumentation for HPLC components for HPLC includes the following (**Fig 3.4**).

3.1.5.1 Solvent reservoir for the mobile phase:

The mobile phase (eluent) selected will depend on the type of separation that is to be achieved. Eluents for HPLC may comprise water, aqueous buffer solutions, aqueous/organic mixtures, organic liquids or mixtures of organic liquids. Only HPLC-quality products should be purchased or solvents must be subjected to preliminary

purification procedures.

Fig 3.4 Taken from Wilson & Goulding (1986)



Stabilisers may alter the solvent polarity completely, e.g. chloroform stabilised with ethanol is much more polar than the pure solvent. Solvents should be degassed or filtered prior to use. A gradient elution system is where the composition of the mobile phase changes continuously by use of a gradient programmer. This involves the use of two pumps. An isocratic system is where a single solvent , or two or more solvents mixed in fixed proportions are used. It is essential that the solvents used in mobile phases are specially purified of any impurities, which may interfere with the detection system or affect the column. If absorbance monitoring is below 200nm, these effects become noticable. The presence of air bubbles in the mobile phase may alter the resolution of the column and interfere with the effluent monitoring, thus the mobile phase must be degassed. This may be carried out by stirring the solvent with a magnetic stirrer, warming it, subjecting it to a vacuum, ultrasonic vibration or by bubbling helium gas through the solvent resevoir. The eluent viscosity is normally between 0.2×10^{-4} (pentane) and 2×10^{-3} mN s m^{-2} (50:50 water:methanol). **Table 3.1** lists such properties of commonly used organic solvents in chromatography as viscosity, refractive index and the solvent strength parameter, described by Snyder as Σ^0. The latter is determined empirically and recorded numerically and is the hypothetical "energy of adsorption" of the eluent. The series of weak, medium strength and strong solvents thus derived is referred to as the eluotropic series. The table lists the solvents in order of increasing solvent strength- pentane being considered the weakest and water being the strongest. TLC may also be used in an attempt to determine the best mobile phase polarity for a specific separation.

Another factor to be considered in the selection of a suitable phase is the UV cut off

value. Such values vary at random with the solvent strength- acetonitrile and methanol having relatively low UV cut off values of 190 and 205nm respectively when compared to carbon tetrachloride which has a cut off value of 265nm (Knox, 1978).

Table 3.1:

SOLVENT STRENGTH PARAMETRE, Σ^o, AND PHYSICAL PROPERTIES OF SELECTED SOLVENTS

Solvent	Σ^o (SiO_2)	Σ^o (Al_2O_3)	Viscosity, 20^0C (mN sec m^{-2})	Refractive index,20^0C
Pentane	0.00	0.00	0.230	1.358
Hexane		0.00	0.313	1.375
Cyclohexane	-0.05	0.04	0.980	1.426
Carbon disulphide	0.14	0.15	0.363	1.628
Carbon tetrachloride	0.14	0.18	0.965	1.460
1-Chlorobutane		0.26	0.470	1.402
Diisopropyl ether		0.28	0.379	1.368
2-Chloropropane		0.29	0.335	1.378
Benzene	0.25	0.32	0.650	1.501
Diethyl ether	0.38	0.38	0.230	1.353
Chloroform	0.26	0.40	0.570	1.443
Methylene dichloride		0.42	0.440	1.425
Methylene isobutyl ketone		0.43		1.394
Tetrahydrofuran		0.45	0.550	1.407
Acetone	0.47	0.56	0.320	1.359
1,4-Dioxane	0.49	0.56	1.540	1.422
Ethyl acetate	0.38	0.58	0.450	1.370
1-Pentanol		0.61	4.100	1.410
Acetoniltrile	0.50	0.65	0.375	1.344
Methanol		0.95	0.600	1.329
Water		Large	1.000	1.333

Σ^o, solvent strength parameter values from Snyder (1968)

Table reproduced from (Willard *et al.*, 1988).

3.1.5.2 High pressure pump

The mobile phase must be delivered to the column over a wide range of flowrates and pressures. The pump, seals, all connections must be made from materials which are resistant to the mobile phase. A pump should, ideally, be able to operate to at least 100 atm (1500 psi) which is a pressure suited to less expensive chromatographs. However, 400 atm (6000 psi) is a more desirable pressure limit. There are different types of pumps-

constant pressure, constant displacement and reciprocating piston-pumps.

The first of these, the constant pressure pump, operates by the introduction of high pressure gas into the pump. The gas forces the solvent from the pump chamber onto the column. These pumps result in pulseless and continous pumping and high flowrates through the column, for preparative purposes. However any decrease in the permeability of the column will result in lower flow rates which the pump will not compensate for.

With constant displacement a constant flow rate is maintained through the column, irrespective of changing conditions within the column. One type of constant displacement pump is a motor-driven syringe-type pump. These pumps work by means of a piston, which is driven mechanically at a constant rate. The rate of solvent delivery is controlled by the voltage being changed on the motor.

Reciprocating piston pumps are the most commonly used form of constant displacement pump. These pumps have advantages of permitting a wide range of flowrates and are relatively inexpensive. A small motor-driven piston moves rapidly back and forth in a hydraulic chamber that varies in size. On the backward stroke, the piston pulls in solvent from the mobile-phase reservoir, by means of check valves. The outlet to the separation column is closed at this stage and a forward stroke causes the pump to push solvent onto the column and the inlet from the reservoir is closed. All constant displacement pumps have built-in safety cut-out mechanisms so that if the pressure within the chromatographic system changes from the preset limits, then the pump is inactivated.

3.1.5.3 Sample injection loop / application of sample

Ideally, the sample should be introduced onto the column as an infinitely narrow band. The injection system itself, should have no dead volume. This is achieved by either of two methods:

1: Stop flow injection. This method can be used up to very high pressures. Here the pump is turned off until the inlet column pressure becomes essentially atmospheric. The sample is injected through a septum in an injection port, either onto a plug of inert material immediately above the column or directly onto the column. Once the injection is made, the pump is switched on again.

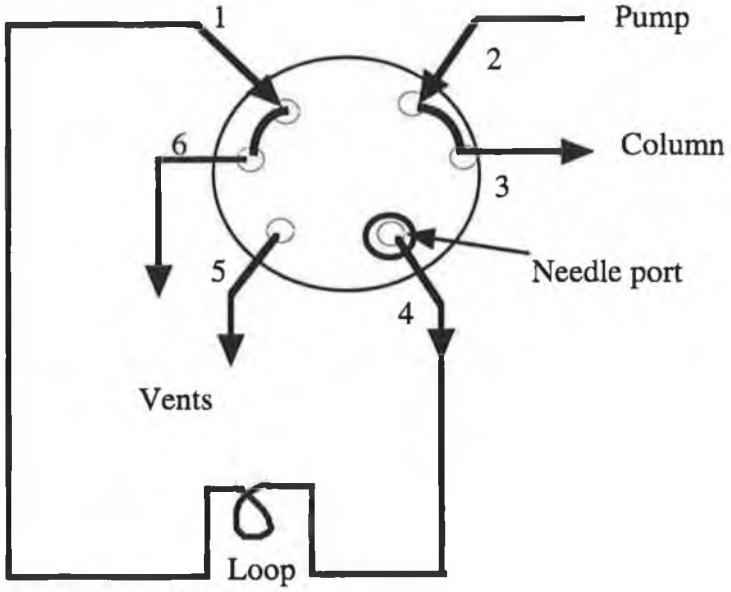
2: Loop / valve injection. This method is rapid and can operate at high pressures. Microsampling valves are the most widely used. An unnecessary solvent peak is avoided by dissolving the sample in the mobile phase. The sample is applied onto the metal loop, by means of a syringe. The valve, as shown in **Fig 3.5**, is then rotated, placing the sample-filled loop into the stream of mobile phase, resulting with the application of sample onto the top of the column. The interruption of flow is non-significant.

3.1.5.4 Columns

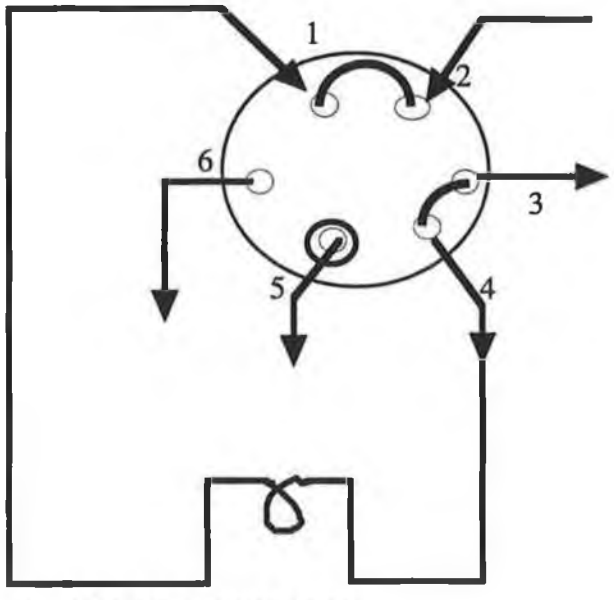
These are genarally made of stainless steel, to withstand the high pressures of HPLC

and also the chemical action of the mobile phase. The preferred columns are precision bored with an internal mirror finish in order to allow efficient packing of the column. The packing material is retained within by the action of porous plugs of stainless steel/teflon.

Fig 3.5 Flow diagram of a six-port sample injection valve



A: LOAD POSITION



B: INJECT POSITION

Radial compression columns have wider diameters. This allows the lowering of flow rates, due to lower pressures, which in turn allows the flow rate to be increased, resulting in the overall shortening of run-times. However, there is an associated decrease in response.The column is compressed by means of a radial-compression module. Separations are carried out when the column is under compression. After analysis, the column is decompressed and removed from the module. The internal diameter of standard columns is normally 4-5mm. Particles for packing are uniformly sized and mechanically stable (discussed below). By decreasing the internal diameter of the narrow bore column by a factor of two, there is an increase in the signal of the sample component by a factor of four. Packed columns with internal diameters of 1.0mm or less impose limitations on extra-column effects. Columns of 3-6cm, being short columns, can save solvent costs and result in high sensitivity (higher than conventional columns). In brief, there are three types of packing- microporous where micropores ramify through the particles (5-10μm in diametre), pellicular where porous particles which are coated onto an inert solid core e.g., glass bead (40μm diametre) and bonded phases- where the stationary phase is chemically bonded onto an inert support. All forms of HPLC column packing are characterised by their regular spherical shape which distinguishes them from conventional materials.

Reverse-phase chromatography uses a hydrophobic bonded packing, usually with an octadecyl (C-18) or octyl (C-8) functional group (**Table 3.2**) and a polar mobile phase, frequently a partially or fully aqueous mobile phase. Generally, the lower the polarity of the mobile phase, the higher it's eluent strength.

Table 3.2 Silica-based column material and mobile-phase solvents for reverse-phase chromatography

	Sample	Column packing	Mobile phase	
Low	Low/moderate polarity (soluble in aliphatic hydrocarbons)	Bonded C-18	Methanol/ water	**High**
POLARITY ↑	Moderate polarity soluble in methyl ethyl ketone)	Bonded C-8	Acetonitrile/ water	**POLARITY** ↓
↑ **High**	High polarity (soluble in lower alcohols)	Bonded C-2	1,4-Dioxane/ water	↓ **Low**

3.1.5.5 Detector system The most common detecting system is a variable wavelength uv-visible spectrophotometer, fluorimeter, a refractive-index monitor or an electrochemical detector. Detectors based on the adsorption of UV-VIS radiation are virtually insensitive to variations in the temperature of the test liquid. However, such detectors are relatively inexpensive and are sensitive for such compounds that absorb in the ultraviolet or visible region of the spectrum. Detection by fluorimetry offers both sensitivity and selectivity. The former is improved as the signal produced is measured against a low background (assuming that the mobile phase does not fluoresce itself). The latter is enhanced as not all compounds absorb radiation fluoresce. A refractive-index monitors is limited by its poor detection sensitivity, lack of selectivity and its sensitivity to changes in flow and temperature. Finally, electrochemical detectors are used mainly when polar phases are employed. Sensitivity is very good and selectivity is also good as only certain compounds are likely to be electroactive.

In order to study the metabolism and biological significance of 7α-hydroxycholesterol in serum, it was necessary to validate the method of measurement of this sterol. Thus, the objective of the first phase of this study was to develop an efficient extraction for cholesterol and 7α-hydroxycholesterol from serum samples of animals and human subjects. Upon extraction, measurement of 7α-hydroxycholesterol was carried out using a reverse-phase HPLC method. Finally, the complete procedure, as described in the following section, was validated in terms of recovery, linearity and inter/intra assay variation.

Aims of Chapter 3 "Validation of preparative and chromatographic procedures for the measurement of serum 7α-hydroxycholesterol":

* To develop an efficient extraction for 7α-hydroxycholesterol and cholesterol from human and animal sera

* To validate the developed method

* To measure levels of 7α-hydroxycholesterol in the selected sera samples

3.2 Results

Prior to commencing a study to determine levels of 7α-hydroxycholesterol in human and animal sera, it was decided to carry out a preliminary study on the efficiency of solvent systems to extract cholesterol and 7α-hydroxycholesterol from serum and to determine the performance characteristics of the chromatographic procedure used to assay 7α-hydroxycholesterol in extracts of serum.

Therefore, organic solvent mixtures varying in degrees of polarity were compared with respect to their ability to extract cholesterol and 7α-hydroxycholesterol from a fixed aliquot of rat serum. This was followed by determining the optimal conditions for converting the sterols into derivatives having characteristic UV absorption profiles. Finally, this chapter will describe the characteristics of the chromatographic method of assay with respect to precision, linearity and recovery.

3.2.1 Efficiency of solvents in the extraction of 7α-hydroxycholesterol and cholesterol from rat serum.

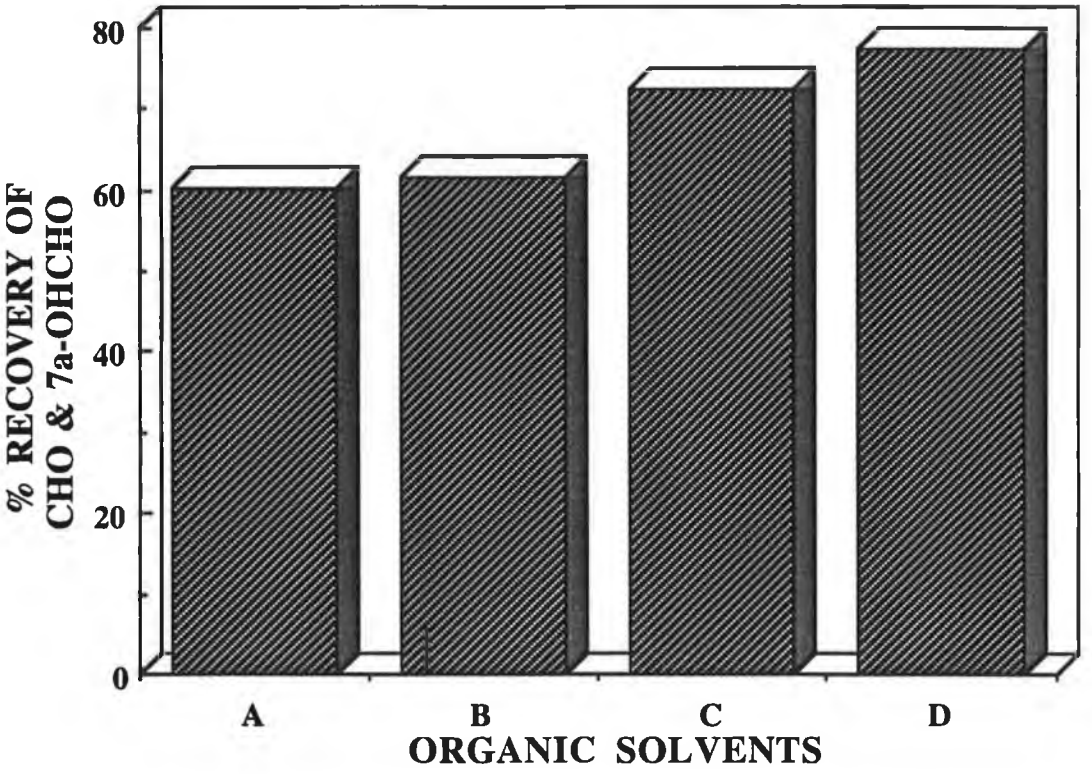
0.5ml aliquots of rat serum, spiked with known amounts of cholesterol and 7α-hydroxycholesterol which ranged in concentrations across the standard curves, were mixed with 10ml of one of the following solvent mixtures: diethyl ether:methanol (2:1 (v/v)), chloroform:methanol:diethyl ether (1:1:1 (v/v/v)), acetonitrile:methanol (2:1(v/v)) or chloroform:methanol (2:1 (v/v)). After centrifugation at 2500rpm for 10min as described in Chapter 2, the non-lipid material was removed from the biphasic system. The lower lipid-containing layer was dried down under nitrogen and subjected to alkaline hydrolysis treatment, in order to enable total amounts of lipid present (both free and esterified) to be quantitated using HPLC, as described previously in Chapter 2.

In order to estimate the amount of sterol recovered at each of the preparative stages, it was necessary to compare HPLC peak areas of each of these steps with those obtained when the corresponding standard was injected directly onto the HPLC column. Thus, serum samples were spiked with 7α-hydroxycholesterol and cholesterol and at the various stages of sample preparation as in **Fig 2.1** a 20 µl aliquot was analysed by HPLC and the resulting area units were compared with those obtained by direct injection onto the column. Absorption of 7α-hydroxycholesterol and cholesterol was monitored at 214nm and 205nm respectively. The overall efficiency was calculated for the extraction of 7α-hydroxycholesterol and cholesterol from spiked serum samples using the above selection of organic solvents and are as illustrated in **Fig 3.6**.

Fig 3.6: Efficiency of various solvents in the extraction of cholesterol and 7α-hydroxycholesterol from serum samples. Cholesterol and 7α- hydroxycholesterol, in the range of physiological concentrations, were added to samples of 0.5ml rat serum. Efficiencies of their extraction were quantified by comparing peak area HPLC units obtained, with those of corresponding standards injected directly onto the HPLC column.

The following organic solvents were tested in this study:

A: Diethyl ether : Methanol (2/1 (v/v))
B: Chloroform : Methanol : Diethylether (1/1/1 (v/v/v))
C: Acetonitrile : Methanol (2/1 (v/v))
D: Chloroform : Methanol (2/1 (v/v))



It was apparent that the extent of lipid extraction (cholesterol and 7α-hydroxycholesterol) using these solvent mixtures, varied between 60.22 and 77.33%, the maximum being achieved using chloroform:methanol in the ratio 2:1 (v/v).

More in depth analysis of each preparative stage, indicated that approximately 17% of the standards was lost in the initial Folch extraction step, another 2% at the alkaline hydrolysis stage (this form of hydrolysis was preferred to the enzymatic hydrolysis by cholesterol esterase, as it resulted in slightly higher, though not significant, levels of total sterol) and finally a further 3.2% at the final cholesterol oxidase step. Thus, it would appear that the most significant loss occured at the inital extraction stage.

3.2.2 Optimisation of conditions for cholesterol oxidase activity

The conversion of 7α-hydroxycholesterol into 7α-hydroxy-4-cholesten-3-one having an intense absorption at 240nm by cholesterol oxidase, was deemed critical in achieving a sensitive assay for 7α-hydroxycholesterol. Hence, optimal conditions with respect to length of incubation time of substrate with cholesterol oxidase and extraction of 7α-hydroxy-4-cholesten-3-one were next determined. Authentic 7α-hydroxycholesterol, (62 pmoles in a final 20µl HPLC injection) was incubated with 40µl cholesterol oxidase (25U/mg, 1mg/ml) in a medium containing 0.6% (v/v) sodium cholate as described in Chapter 2 for 5, 10, 15, 20 and 25 min at 37°C. **Fig 3.7 (a)** shows the typical chromatographic profile of the extract of the mixture after a 10 minute incubation while **Fig 3.7 (b)** shows a time course relating peak area to length of incubation time. Conversion of 7α-hydroxycholesterol (62pmoles/20µl) into peak area units of 7α-hydroxy-4-cholesten-3-one was linear up to 10 min incuation with cholesterol oxidase, approximately. The time chosen for the assay - 10 min, where upon, the reaction has reached completion.

The optimal number of washes/extractions for recovering the cholesterol-oxidase generated metabolites from the reaction mixture was next established. After incubation of 730 pmoles 7α-hydroxycholesterol/20µl final resuspension, with 40µl cholesterol oxidase (25U/mg, 1mg/ml) in a medium containing 0.6% (v/v) sodium cholate for 10 min at 37°C, the 7α-hydroxy-4-cholesten-3-one product was extracted from the assay medium with varying volumes of petroleum ether (HPLC grade) as described in Chapter 2. Extraction of products of the cholesterol oxidase reaction was shown to be dependent on the number of washes with petroleum ether. The optimum volume of petroleum ether required for the final extraction of 7α-hydroxycholesterol from the assay buffer was found to be 18ml (**Fig 3.8**).

Fig 3.7 (a) A typical chromatographic profile obtained upon conversion of 7α-hydroxycholesterol (62 pmoles/20µl) and cholesterol (400nmoles/20µl) to 7α-hydroxy-4-cholesten-3-one (16.48min) and 4-cholesten-3-one (36.8min), respectively. Assay conditions as in Chapter 2. The peak observed at 14.06 min is that of 20α-hydroxycholesterol, the internal standard.

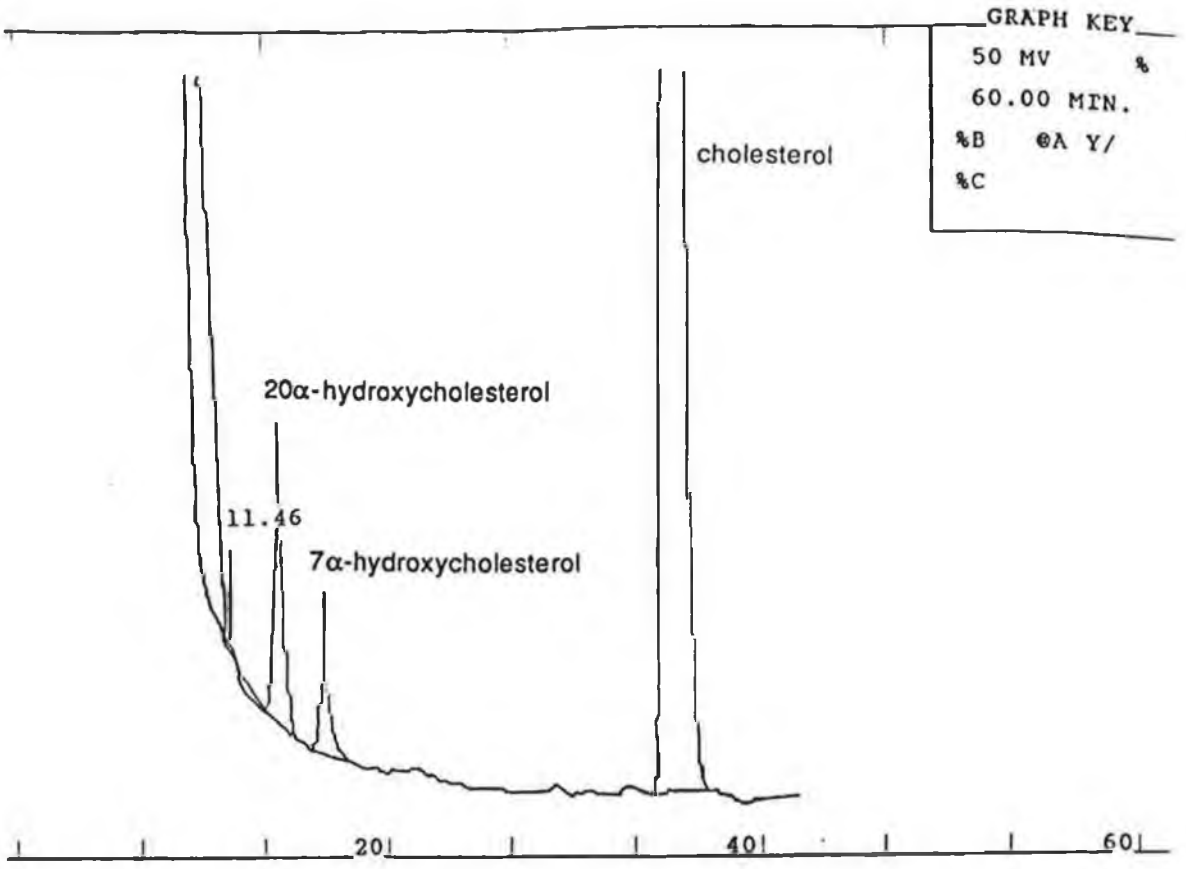


Fig 3.7 (b): Time course asssay for the conversion of 7α-hydroxycholesterol (62 pmole/20µl) to 7α-hydroxy-4-cholesten-3-one using 1 unit Cholesterol oxidase (*Nocardia erythropolis*). Conditions for assay were as described in Chapter 2.

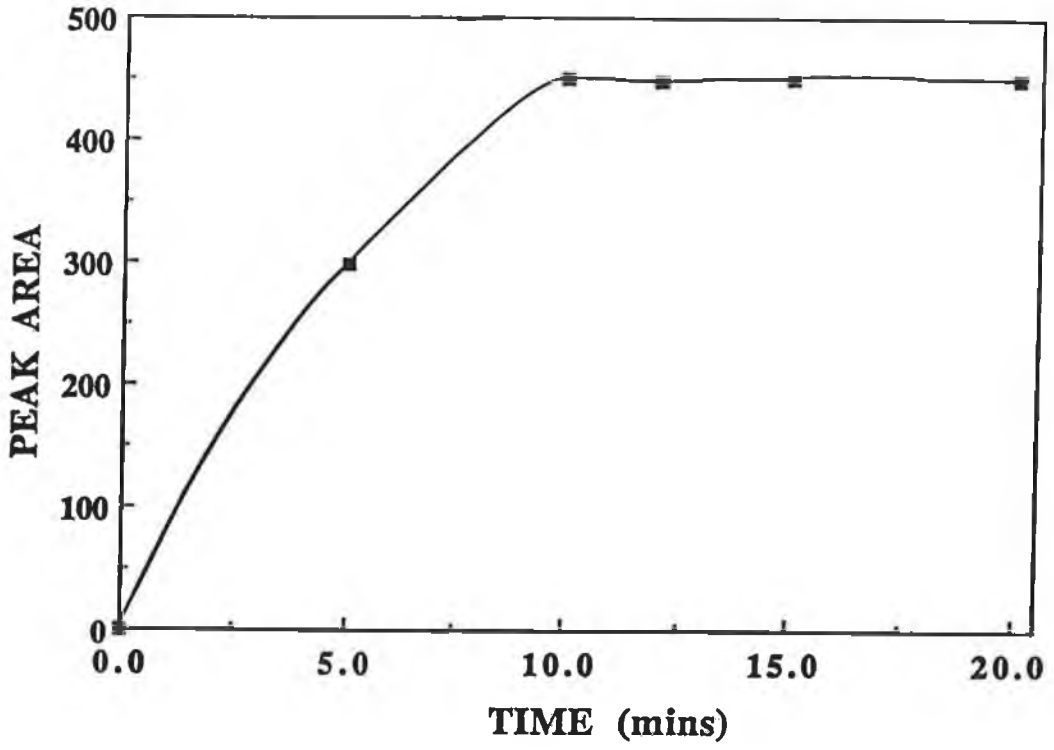
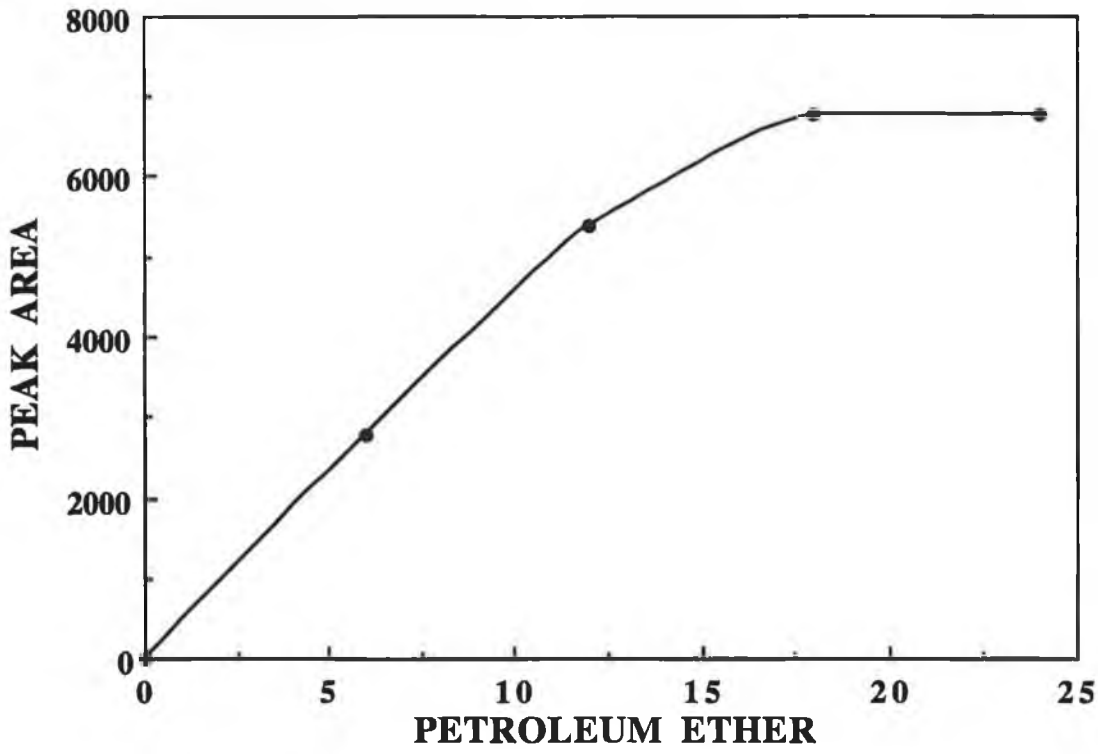


Fig 3.8: The optimal volume of petroleum ether required for the final extraction of 7α-hydroxycholesterol (730 pmoles/20µl) from the assay buffer. Assay conditions as described in Chapter 2.



3.2.3 Measurement of performance characterisitics of HPLC assay

Method validation is a very important aspect of method development. This section describes the criteria for assessing validation of the HPLC procedure for 7α-hydroxycholesterol assay.

3.2.3.1 Linearity:

Fig 3.9 is a calibration curve for the conversion of 7α-hydroxycholesterol to its enone derivative - 7α-hydroxy-4-cholesten-3-one. Varying amounts of 7α-hydroxycholesterol (25 to 992 pmoles in final resuspension of 20µl) were incubated with 1 unit of cholesterol oxidase at 37oC for 10 min. After termination of the reaction, the product was extracted with 3x6 ml aliquots of petroleum ether (HPLC grade). An aliquot (20µl) was injected onto a column and monitored at 240nm. It is apparent from **Fig 3.9 (a)** that the method was able to produce a response proportional to the concentration of 7α-hydroxycholesterol. Regression analysis for this sterol was Y = -112.49 + 9.8200x, with a correlation coffecient of= 0.999. **Fig 3.9 (b)** shows the calibration curve for the above conversion, but in the concentration range of 25.00 to 62.00 pmoles 7α-hydroxycholesterol/20µl injection. Regression analysis yielded the equation :
Y = 0.87887 + 7.7746x and correlation coffecient 0.999. Linearity of the method was also established for the conversion of cholesterol to 4-cholesten-3-one. **Fig 3.10** is a standard curve relating peak area to cholesterol concentration in the range 51.7 to 129.3 nmoles/20µl injection. Regression analysis for this curve yielded the equation:
Y = -248.69 + 1810.8x, r= 0.997.

Fig 3.11 compares the selected method of analysing 7α-hydroxycholesterol (by its conversion to its enone derivative and subsequent detection and measurement at 240nm) to a method whereby 7α-hydroxycholesterol is injected directly onto the HPLC column and measured at 214nm. Peak areas of 7α-hydroxy-4-cholesten-3-one after enzymatic conversion by cholesterol oxidase are greater than those for 7α-hydroxycholesterol injected directly and monitored at 214nm, by about 4.4 fold. Regression analyses were
Y = 1.3143 e-2 + 1.7460x, r = 1.000 (A_{240nm}) and
Y = 1.2716 + 7.769x, r = 0.999 (A_{214nm}).

Fig 3.9: Standard curve for the conversion of 7α-hydroxycholesterol to 7α-hydroxy-4-cholesten-3-one using 1 unit Cholesterol oxidase (*Nocardia ertythropolis*)

(a): Standards ranging from 25.00to 992.00 pmoles/20µl

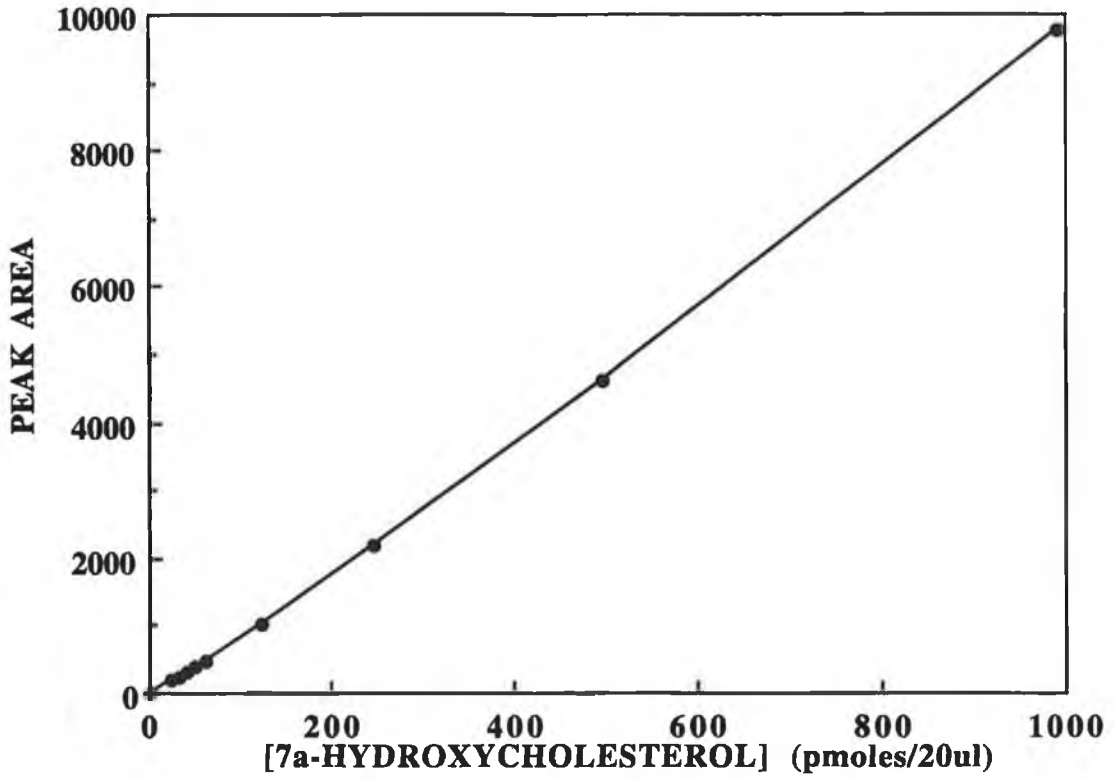


Fig 3.9 (b) Standards ranging from 25.00 to 62.00 pmoles/20µl

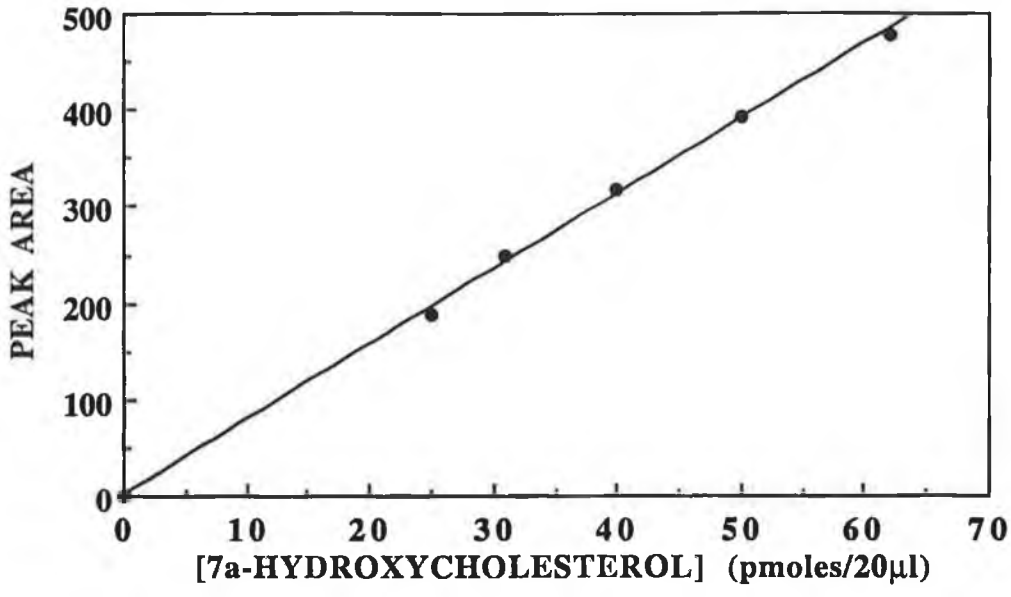


Fig 3.10: Standard curve for the conversion of cholesterol to 4-cholesten-3-one, using 1 unit of Cholesterol oxidase (*Nocardia erythropolis*), in the range of 51.7 to 129.3 nmoles/20µl. Assay conditions as in Chapter 2.

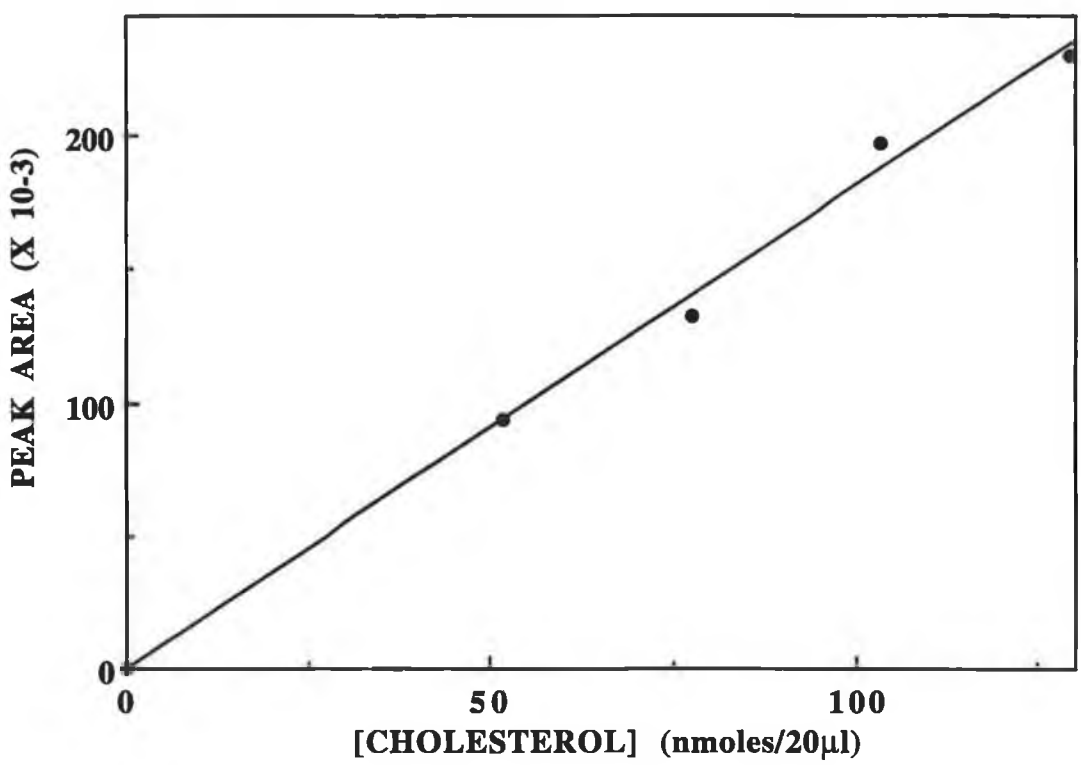
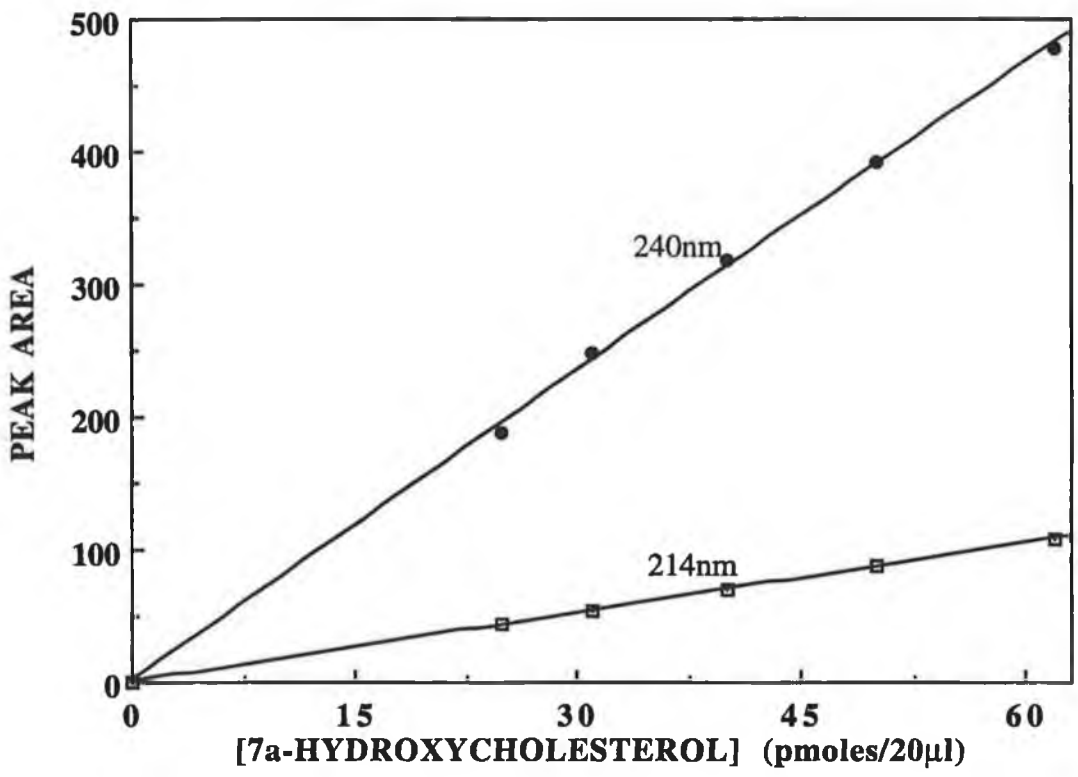


Fig 3.11: Standard curves for 7α-hydroxycholesterol monitored at

a) 214nm, after direct injection onto the C18 column

b) 240nm, after conversion to 7α-hydroxy-4-cholesten-3-one.



3.2.3.2 Recovery:

This is an important part of the validation procedure as it gives the analyst some idea of the efficiency of the procedure. **Table 3.3** demonstrates the % recovery of 7α-hydroxycholesterol from rat serum across the range 40 to 120 pmoles. Concentrations of 7α-hydroxycholesterol in a series of spiked serum samples (containing 40, 80 and 120 pmoles), taken through the full procedure described in Chapter 2, were extrapolated from the standard curve (**Fig 3.9**). Mean ± SD concentration of 7α-hydroxycholesterol in two unspiked serum samples was 121.3±1.2 pmoles/0.5ml serum. Addition of 40 pmoles of 7α-hydroxycholesterol standard to the two samples resulted in a 1.25 fold yield (151.6±0.5 pmoles) representing a 75.6% recovery. Addition of 80 and 120 pmoles 7α-hydroxycholesterol to the serum samples resulted in a 1.5 fold (183±3.4 pmoles) and 1.78 fold (216.4±4.7 pmoles) return respectively. % recoveries on adding 80 and 120 pmoles 7α-hydroxycholesterol were 77.1 and 79.3% respectively. **Fig 3.12** demonstrates that the slope of the processed standard curve for the recovery of 7α-hydroxycholesterol from rat serum was Y=120.55+0.79179x; r=1.00.

3.2.3.3 Precision:

Precision, made up of two components, repeatability and reproducibility, was determined by means of an intra assay (**Table 3.4**) and inter-assay (**Table 3.5**). 0.5, 0.75 and 1ml aliquots of bovine serum were analysed in quadruplicate for intra assay (within day) variation and the concentration determined from a standard curve, constructed on the same day. The coefficient of variation was shown to range from 1.59 to 3.69%. These low values are indicative that the method is excellent for same day repeat analysis. Quadruplicate analysis of 0.5ml serum yielded 231.8±3.7 pmoles/ml while quadruplicate analysis of 0.75ml and 1ml aliquots returned mean concentrations of 224.9±8.3 pmoles and 219.4±6.6 pmoles/ml respectively.

Repeatability of the chromatographic procedure as assessed by quadruplicate analysis of different volumes of serum was shown to be satisfactory. Inter-assay (between day) variation was also examined and evaluated by analysis of 0.5, 0.75 and 1ml aliquots of bovine serum, in quadruplicate over a period of four days. The concentrations were determined from a standard curve constructed daily and results (**Table 3.5**) once again show that due to low coefficients of variation obtained that this is an excellent method for repeated analysis of 7α-hydroxycholesterol. Mean±SD concentration of 7α-hydroxycholesterol in 0.5ml serum measured over 4 days was found to be 227.9±2.3 pmoles/ml. This compares with similar values obtained when 0.75ml and 1ml aliquots of serum were assayed similarly over 4 days (223.9±3.76 and 222.5±5.48 pmoles/ml) respectively.

Table 3.3: Percentage recovery of 7α-hydroxycholesterol from rat serum was determined across the linear range, following the extraction as described in Chapter 2. Xo represents the amount of endogenous 7α-hydroxycholesterol present and Na the amount added to 0.5ml control rat serum (n=6)

SAMPLE	AMOUNT ADDED (pmoles)	AMOUNT FOUND (pmoles)	RECOVERY (%)
Xo	0	120.12	
Xo	0	122.45	
Xo + a	40	151.08	74.38
Xo + a	40	152.08	76.87
Xo + 2a	80	186.35	81.33
Xo + 2a	80	179.61	72.90
Xo + 3a	120	221.13	83.20
Xo + 3a	120	211.65	75.30

Fig 3.12 Plot of amount of 7α-hydroxycholesterol recovered after spiking 0.5ml rat serum with known concentrations of 7α-hydroxycholesterol standard, from 40 to 120 pmoles per sample. Assay conditions as described in Chapter 2.

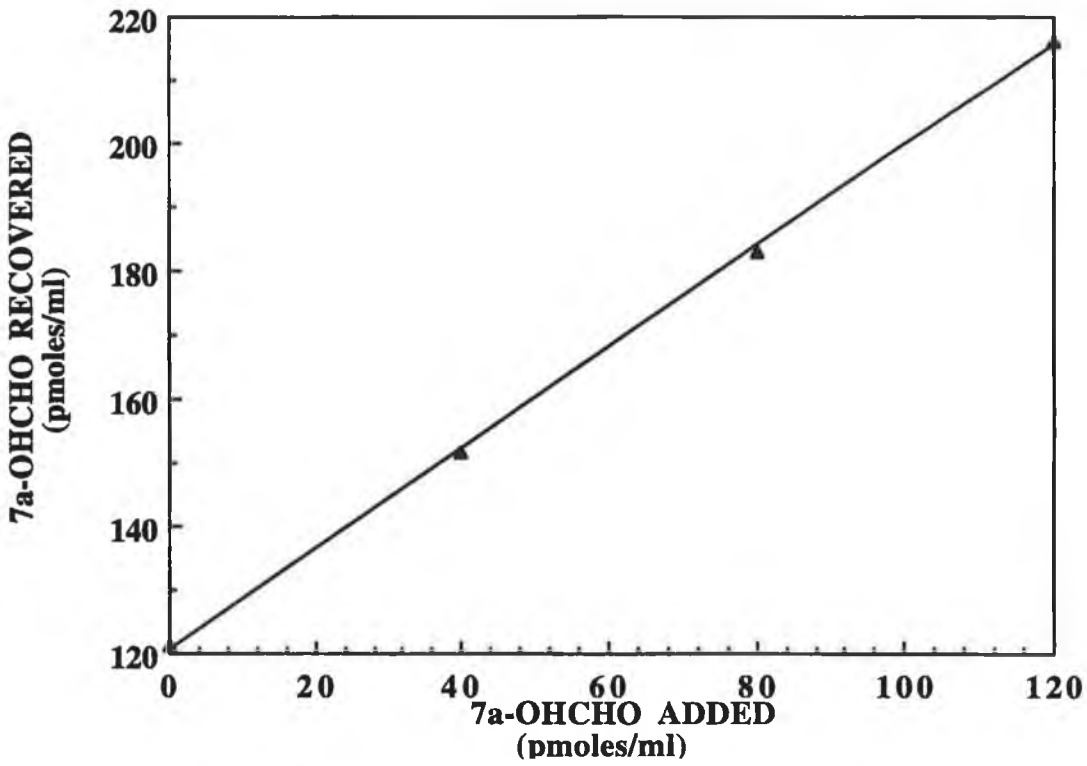


Table 3.4: Intra-assay variation for 7α-hydroxycholesterol following extraction from serum (n=4). Analyses were carried out in quadruplicate for volumes of control bovine serum ranging from 0.5-1.0ml, over one day. % C.V.obtained is indicative of precision.

SERUM VOL. (mls)	CONC. (pmoles/ml)	CONC. MEAN±S.D (pmoles/ml)	C.V. (%)
0.50	229.94 236.22 227.80 233.02	231.75 ± 3.67	1.59
0.75	230.26 212.57 227.83 229.10	224.94 ± 8.31	3.69
1.00	225.73 211.15 217.16 223.65	219.42 ± 6.61	3.01

Table 3.5: Inter-assay variation for 7α-hydroxycholesterol following extraction from serum (n=4). Analyses were carried out in quadruplicate for volumes of control bovine serum ranging from 0.5-1.0ml, over a period of 4 consecutive days. % C.V.obtained is indicative of precision.

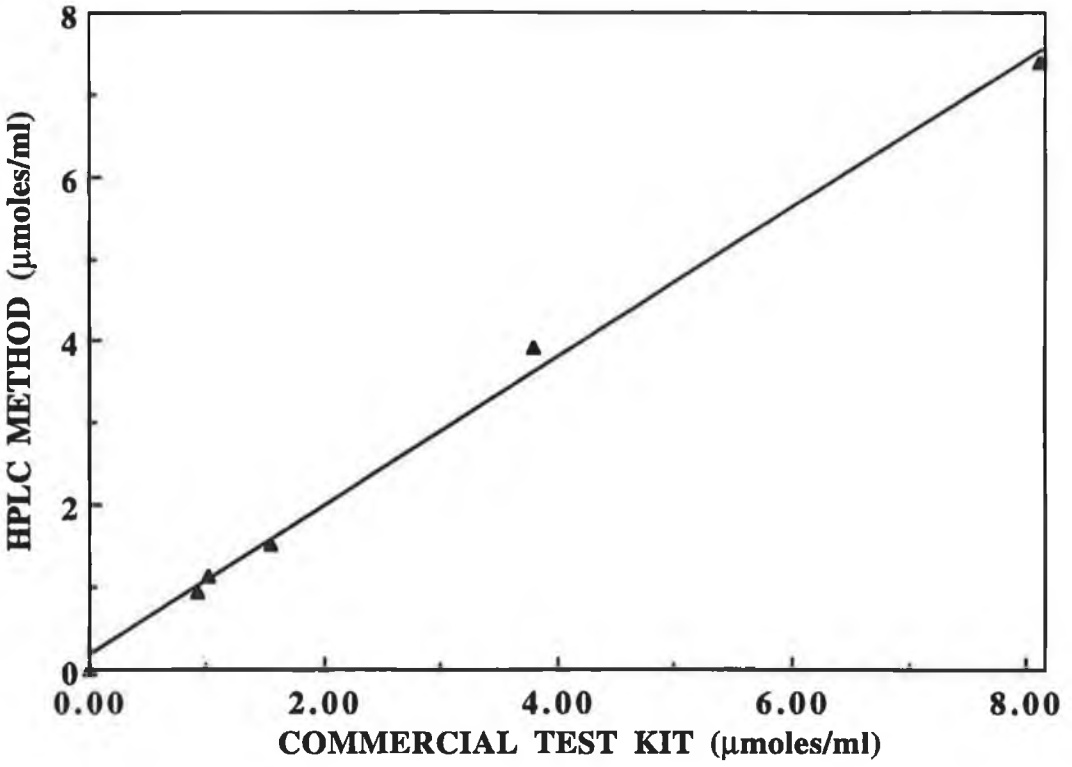
SERUM VOL (mls) / CONC 7a-OHCHO (pmoles/ml)	0.50	0.75	1.00
DAY 1	231.75	224.94	219.42
DAY 2	224.86	222.30	230.40
DAY 3	228.68	219.93	218.28
DAY 4	226.40	228.70	221.93
C.V. (%)	2.62	3.43	4.30

The HPLC procedure was also suitable for measurement of serum cholesterol, which was apparent from each chromatogram obtained. Retention time of 4-cholesten-3-one was approximately 34 min. The accuracy of the described method was further tested, by comparing serum cholesterol levels as measured by HPLC with those obtained using the standard cholesterol assay kit (Randox). **Table 3.6** tabulates the values obtained using both methods for serum cholesterol in control and hypercholesterolemic human sera and in control, diabetic and cholestyramine-treated rat sera. Excellent agreement (r=0.998) between values was found in all sera, when comparing both methods of analyses, as can be seen in **Fig 3.13**, where the equation obtained from plotting the information in **Table 3.6** is Y=0.14696+0.91148x, thereby further validating the use of the HPLC analytical procedure for the assay of cholesterol and also of 7α-hydroxycholesterol.

Table 3.6: Comparison in the values obtained for the measurement of serum cholesterol, using the above described reverse HPLC method to those obtained using a standard assay kit (Randox).

SUBJECT	CONC. CHOLESTEROL (μmoles/ml) TEST KIT	CONC CHOLESTEROL (μmoles/ml) HPLC
RAT:		
CONTROL	1.544±0.021	1.509±1.02
CHOLESTYRAMINE-TREATED	0.915±0.061	0.953±0.96
DIABETIC	1.014±0.731	1.121±0.90
HUMAN:		
CONTROL	3.790±0.423	3.911±0.96
HYPERCHOLESTEROLEMIC	8.110±1.01	7.400±1.09

Fig 3.13 Comparison between the described procedure for the extraction and analysis of serum cholesterol and that of a commercial kit (Randox) for the measurement of cholesterol in biological samples.



3.3 Discussion

HPLC is a popular method for steroid separation, due to its rapidity and non-destructive nature (Heftmann and Hunter, 1979). By contrast, TLC may not be considered a method of choice because it allows the samples to become susceptible to potential oxidation due to large surface area and also be deemed unsuitable due to low loading capacity (Csallany *et al.*, 1989). It has been reported that extraction followed by gas chromatography produced a number of peaks suspected to be oxidation products (Van Lier and Smith, 1967); however some of them were found to be artifacts (Smith and Van Lier, 1970) due to the exposure of the samples to air and high temperature. Koopman *et al.*, (1987) reported difficulties in setting up a GC determination for hydroxycholesterols, which he reported to be a result of the close structural similarity and the large concentration differences in these compounds (*i.e.* hydroxycholesterols) in comparison with cholesterol. He therefore removed cholesterol from the assay.

The reverse-phase HPLC method described here is that of Hylemon *et al.*, (1989), where it was used in the analysis of liver samples. We used this method in the analysis of 7α-hydroxycholesterol in serum, having set up an efficient method for its extraction and hydrolysis (in samples where the total amount of 7α-hydroxycholesterol is to be measured).

The first step in the analysis of lipids is the selective extraction of the lipids out of the biological matrix (Nissen, 1990). As natural lipids possess a high proportion of polyunsaturated fatty acids and are subject to autoxidation, appropriate precautions were taken during the analysis. These included the addition of an anti-oxidant (BHT) to serum and the work was carried out as much as much as possible under an atmosphere of oxygen-free nitrogen.

The procedure described here uses reverse phase HPLC for the analysis of 7α-hydroxycholesterol and cholesterol in sera samples. The mobile phase employed is acetonitrile:methanol (70:30 (v/v)). It has previously been found that eluent systems containing an alcohol (methanol, ethanol, 2-propanol) resulted in broader chromatographic peaks, while an eluent based on acetonitrile gave a satisfactory chromatogram (Kasama *et al.*, 1987) in the analysis of sterols in serum by HPLC. However, as observed in the obtained chromatograms, the peaks obtained were well separated and sharp.

The method involved the Folch extraction, which was found to be the most efficient, having examined the efficiences of different solvents in the extraction of cholesterol and 7α-hydroxycholesterol from serum. An enormous problem connected to deproteinisation is the possibility of the analyte adsorbing on - or clustering of the analyte in - the precipitate. This effect has been reported to be minimised by addition of the homogenised

sample to the deproteinising agent (solvent) and not the reverse way. With certain compounds, it was found that a further increase in recovery could be obtained by agitating the solvent in an ultrasonic bath during the addition of the homogenised sample (Uihlein, 1986). Thus, this may be of due consideration in future work, as our results show that the main loss that occured during the developed procedure was at the initial step, the Folch extraction.

The solvent system chosen- chloroform / methanol (2:1 v/v), was selected as the analyte was found to be highly soluble, thus this should help avoid adsorption on or coprecipitation with the protein during the organic solvent - protein precipitation step. It has been suggested (Lim, 1991) that sometimes it may be necessary to use a mixture of two solvents to attain a recovery which is quantitative. Other workers also found that the chloroform/methanol (2:1, v/v) was the most suitable solvent for the extraction of total lipids and cholesterol (Kaneda *et al.*, 1980). Methanol has an additional advantage of inactivating many phosphatases and lipases present in animal tissues, but it also extracts contaminants such as sugars, salts, amino acids etc. However, these are removed by washing the crude lipid extract. The sample analyte was not found to adhere to the glass tube, as results indicate that there was no significant difference between recoveries of 7α-hydroxycholesterol from tubes which had been silanised with SigmaCote, to those which had not been silanised.

The sample was then subjected to alkaline hydrolysis in order to hydrolyse the esters of cholesterol and 7α-hydroxycholesterol, when total amounts of each was required. Koopman (1987) and Oda (1990) reported the stability of 7α-hydroxycholesterol during alkaline hydrolysis. Sample was re-extracted with n-hexane three times and then washed twice with water/ethanol (1:1, v/v). Such a step is carried out because although hexane is nonmiscible with water, there is a possibility that polar compounds may easily be transferred into the organic phase together with the dissolved water (possible solubility of water). Thereby, the extract is washed several times with small volumes of aqueous phase.

Other extractions and washes were carried out, the sample extract was then reacted with cholesterol oxidase (an enzyme which is specific for the 3ß-hydroxyl group) to convert cholesterol and 7α-hydroxycholesterol to enone derivatives, which were detected more sensitively at 240nm, as opposed to 214nm direct measurement. Cholesterol oxidase (EC 1.1.3.6) can modify certain sterols by converting them into enones which are conjugated (Smith and Brooks, 1976). The 3ß-hydroxyl group of cholesterol or related sterols is oxidised to a keto group by dioxygen and the isomerisation of the Δ^5-bond to yield this conjugated double bond which, in turn acts as a chromophoric group (α,ß-unsaturated

ketone; E_{240nm}=16,000 M^{-1} cm^{-1}) (Lee and Biellmann, 1988). This results in an increase in sensitivity in the detection of the oxide, as they absorb UV light more readily in this form. This enzyme conversion allows the separation of closely related sterols. This increase in sensitivity was demonstrated and shown to be more sensitive than direct detection at 214nm. This is an advantage if using an enzyme reagent system, as they allow sensitivity and specificity (Flegg, 1973).

The method developed was validated and the coefficient of variation for inter- and intra-assay were determined. Also, the percentage recovery was determined by the addition of known amounts of 7α-hydroxycholesterol standard to sera samples. In general, extraction methods do not yield 100% recoveries. Over a chosen concentration range yields may vary. Thus, it is necessary to construct standard curves which are based on known amounts added to the sample and are carried through the whole extraction and analytical procedure as well as the sample. Quantities added should (and did) cover the expected range of analysis, as extrapolations from the standard curve may not result in true values.

The overall values obtained for the validation suggest that this described method is a suitable, reliable and reproducible method for the analysis of serum cholesterol and 7α-hydroxycholesterol. When cholesterol concentrations measured were compared to those measured by means of a commercial kit- there was a highly significant correlation found, r=0.998. Such a value is greater than that reported by Dadgar and Smyth (1986), who reported that the correlation of the developed method to another reliable method (commercial kit) by linear regression analysis ($r \geq 0.98$) is the most acceptable form of validation.

20α-hydroxycholesterol was added as internal standard and brought through the complete procedure. However, it should be noted that the extraction of the internal standard may not be exactly the same as for the analyte, even if it is a homologue. A suitable internal standard minimises errors by compensation. In theory, the best internal standard should elute closely with the analyte band, which was observed here- 20α-hydroxycholesterol eluted at approximately 14 min, while 7α-hydroxycholesterol eluted just after that, with a retention time of 16.5 min.

Altogether, the results obtained for the validation of this method indicate that it is a reliable, sensitive and precise method (comparing well to a standard commercial kit when measuring serum cholesterol content), for the simultaneous measurement of serum cholesterol and 7α-hydroxycholesterol. Others have reported problems related to autoxidation during analysis. We however did not experience this, as results indicate that

upon addition of BHT and working under the conditions stated, there was no significant increase in the levels of 7ß-hydroxycholesterol or 7-ketocholesterol, which are the reported major autoxidative products of cholesterol (Smith, 1987, Bergström and Wintersteiner, 1961).

Having set up and validated this method, sera samples from human control subjects and from subjects being treated for hypercholesterolemia were analysed, as were sera samples of control, diabetic and cholestyramine-treated rats. As will be illustrated in later chapters, the method was found to be satisfactory for an investigation of 7α-hydroxycholesterol metabolism.

CHAPTER 4

SERUM 7α-HYDROXYCHOLESTEROL IN HUMAN AND ANIMAL MODELS OF ALTERED CHOLESTEROL METABOLISM

4.1 Introduction

The most important pathway for the metabolism and excretion of cholesterol in mammals is the formation of bile acids. Over the last decade the enzymes and proteins involved in this pathway have been major points of research focus. In 1989, cDNAs for two key enzymes of bile acid biosynthesis were isolated and several more have been reported since. These advances have provided a wealth of insight and tools for examining this pathway. Here, I have reviewed the main regulatory step in the pathway of bile acid biosynthesis. Also included is an overview of defects in bile acid biosynthesis and of alterations in cholesterol metabolism in hyperlipidaemic conditions.

Modern advances in spectroscopy and chromatography have provided new tools for studying the complex chemistry and physiology of these bile acids. The final part of this section will review the instrumentation that has been in use lately for monitoring alterations in bile acid flux in the various human and animal models of cholesterol metabolism.

4.1.1 Bile Acid Metabolism

There are considered to be two pathways in the formation of bile acids from cholesterol. The first of these is that which involves the enzyme- sterol 27-hydroxylase in the initation step, the other involves cholesterol 7α-hydroxylase. The former pathway differs mainly to the latter in that it is a widely distributed mitochondrial enzyme with high activity in vascular endothelial cells (Javitt, 1994), while cholesterol 7α-hydroxylase is a liver enzyme.

Sterol 27-hydroxylase has an important role in the degradation of the side-chain of cholesterol. The product of this reaction, 27-hydroxycholesterol, is a potent inhibitor of the overall regulation of cholesterol biosynthesis. Recently, it has been suggested, by Björkhem *et al.*, (1994) that conversion of cholesterol into 27-hydroxycholesterol and 3ß-hydroxy-5-cholestenoic acid represent a general defense mechanism for macrophages and for other peripheral cells which are exposed to cholesterol. Such cholesterol oxides are efficiently converted into bile acids in human liver.

When the enterohepatic circulation is disrupted, the cholesterol 7α-hydroxylase pathway is preferred Javitt (1994). The pathway which is initiated by sterol 27-hydroxylase, originates in vascular endothelium and thus provides a mechanism for reverse cholesterol transport, ending with the synthesis of chenodeoxycholic acid, predominately. The pathway which is discussed and examined below is that of hepatic cholesterol 7α-hydroxylase.

The conversion of cholesterol into bile acids involves almost all of the conceivable mechanisms for conversion of a lipophilic compound into an excretable water-soluble

product. Of the more than 14 different enzymes participating in the conversion, hydroxylases, oxidoreductases and conjugating systems are of particular importance for increasing the polarity. The major primary bile acids synthesised in the human, the hamster and the rat are cholic acid and chenodeoxycholic acid (Danielsson and Sjövall, 1975), however the synthesis and metabolism of bile acids may differ significantly between different vertebrate species. There are two main categories of steps which lead to their formation: those reactions in the endoplasmic reticulum and cytosol which modify the cyclopentanoperhydrophenanthrene ring structure or nucleus (**Fig 4.1**) and those in mitochondria and peroxisomes that oxidise and shorten the 8 carbon atom side chain of the sterol. These divisions are for explanatory convenience only as many of the enzymes exhibit substrate promiscuity leading to uncertainty with regard to the exact order of biosynthetic steps (Bjorkhem, 1985).

In reaction 1 (**Figure 4.1**) a hydroxyl group is inserted in the axial (α) configuration at position C-7 of cholesterol, resulting in the formation of 7α-hydroxycholesterol. This reaction is catalysed by a unique cytochrome P-450 enzyme, microsomal cholesterol 7α-hydroxylase (EC 1.14.13.17), one of three cytochrome P450s (the others being sterol 12α-hydroxylase and sterol 27-hydroxylase) that participate in bile acid biosynthesis and belongs to a class of enzymes known as "mixed-function" oxidases. This term, introduced by Mason in 1965, refers to a reaction, catalysed by an enzyme, whereby an atom of molecular oxygen is incorporated into the substrate molecule, while the other is reduced to water. The reduction requires a coenzyme, usually NADPH.

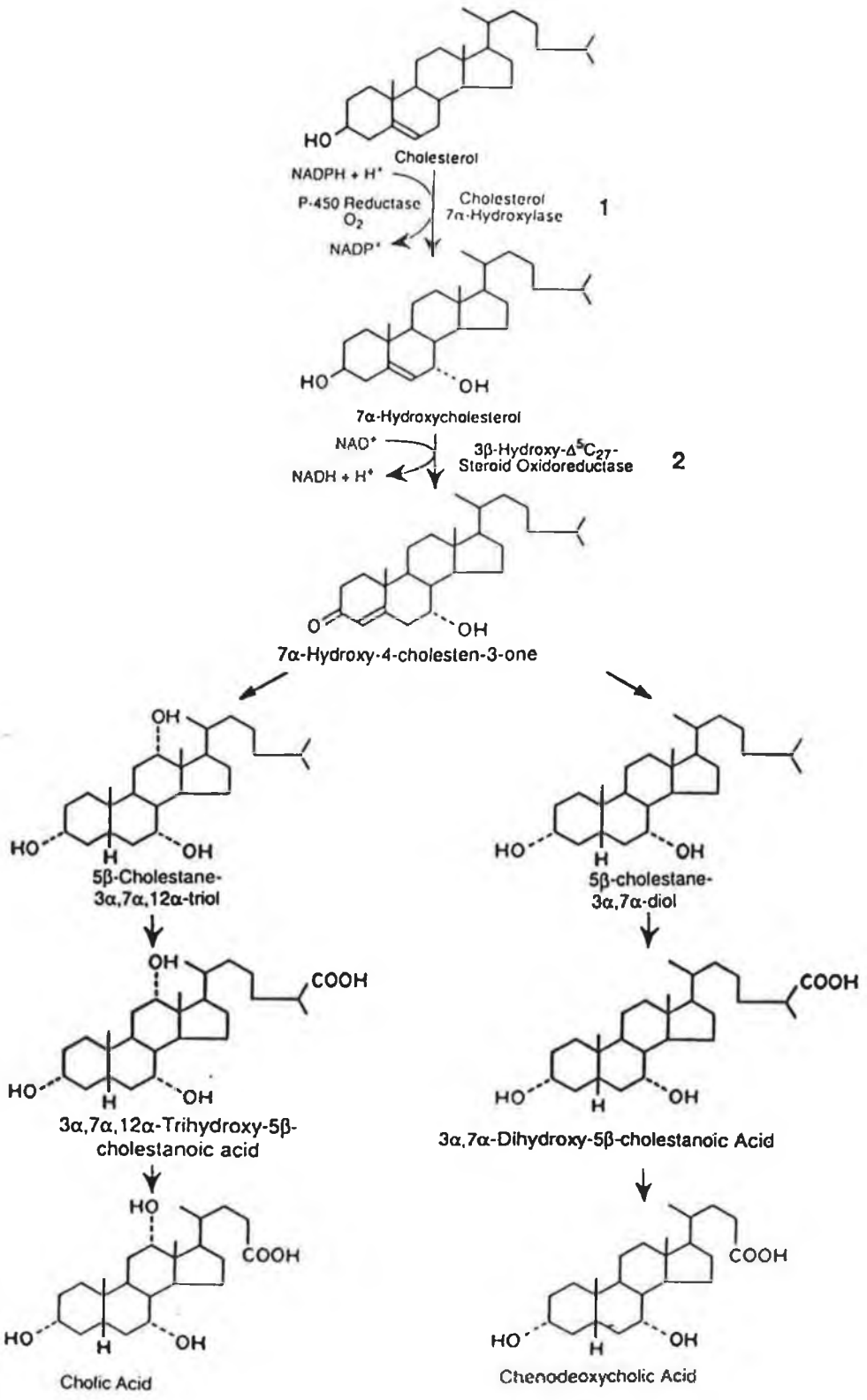
RH + NADPH (or NADH) + H^+ + O_2

$\longrightarrow$ **ROH + $NADP^+$ (or NAD^+) + H_2O** where RH is the substrate.

This enzyme requires oxygen as its activator and cytochrome P450 as a substrate. The 7α-hydroxylation of cholesterol resembles other hepatic microsomal mixed-function oxidation reactions in that it does not require adrenodoxin (an iron-sulphur protein), which is essential for cytochrome P450-dependent hydroxylations occuring in the adrenal mitochondria (Myant and Mitropoulos, 1977).

This first step involves the formation of a reversible complex between cytochrome P450 in the oxidised form (Fe^{3+}) and the substrate. The Fe^{3+} of the cytochrome P450-substrate complex is then reduced to the Fe^{2+} state, by NADPH transferring one electron. Molecular oxygen then reacts with this reduced complex, resulting in the formation of a ternary complex of Fe^{2+} cytochrome P450, substrate and O_2.

Fig 4.1 Routes of synthesis of cholic and chenodeoxycholic acids, the major bile acids of human bile acids. Steps 1 and 2 are numbered in bold type.



Another electron is then transferred to the ternary complex, an atom of oxygen is introduced into the substrate, water is expelled from the complex and the resulting hydroxylated substrate and oxidised cytochrome P450 are released (Myant and Mitropoulos, 1977). Cholesterol 7α-hydroxylase catalyses the rate-limiting step in bile acid synthesis and the expression and activity of this enzyme are subject to multiple regulatory inputs which will be discussed below.

The second step in this pathway results in the reduction of 7α-hydroxycholesterol to 7α-hydroxy-4-cholesten-3-one. This step (reaction 2 **Fig 4.1**) is catalysed by a microsomal 3ß-hydroxy-steroid oxidoreductase isoenzyme specific for C-27 steroids This reaction uses NAD+ as a cofactor. Although this 3ß-hydroxy-C-27-steroid oxidoreductase was the first bile acid biosynthetic enzyme purified to homogeneity by Wikvall in 1981, a cDNA encoding this isoenzyme has not yet been reported.

It is at this stage of the bile acid biosynthetic pathway that a divergence occurs. Such a branching point is thus, of potential regulatory importance (Russell and Setchell, 1992). The two primary bile acids formed are cholic and chenodeoxycholic acid. These two acids, differing in only a hydroxyl-group at C-12 have never been shown to be interconverted. The 7α-hydroxy-4-cholesten-3-one product of 3ß-hydroxy-C-27-steroid oxidoreductase can take one of two paths in bile acid biosynthesis (**Fig 4.1**). If this intermediate is acted upon by the second microsomal cytochrome P-450 enzyme of the pathway, sterol 12α-hydroxylase then the resulting product is ultimately converted to cholic acid. 7α-hydroxy-4-cholesten-3-one can alternatively serve as a substrate for a soluble Δ4-3-oxosteroid 5ß-reductase to yield a steroid intermediate that is ultimately converted into chenodeoxycholic acid. This enzyme also catalyses the identical reaction on 7α,12α-dihydroxy-4-cholesten-3-one, the product of 12α-hydroxylation. Cholic acid and chenodeoxycholic acid, as well as their secondary and tertiary metabolites, have different physicochemical and physiological properties (Carey *et al.*, 1982).

The idea that 7-hydroxylation commits cholesterol to bile acids seems sound (Fielding and Fielding, 1985). However, while the later intermediates for synthesis of the common bile acids are well known, there is little agreement on the regulation of the steps that produce the intermediates. An example of this is where 12-hydroxylation commits the sterol nucleus to the synthesis of cholic rather than chenodeoxycholic acid (**Fig 4.1**).

Once the nucleus has been hydroxylated at positions C-7 and C-12, the hydroxyl group at C-3 is inverted from the ß- to the α- orientation. This inversion seems to precede the saturation of the double bond. Once the nucleus has been elaborated, the side chain

degradation may proceed leading to C-24 acids. The enzymes responsible for converting cholesterol into 3α, 7α, 12α-trihydroxycoprostane are located in the microsomal fraction of rat liver cells. They require cofactors such as ATP, NAD and glutathione for optimal activity. Synthesis of bile acids begins in the endoplasmic reticulum and from here, intermediates follow a cytoplasm-->mitochondria-->cytoplasm -->endoplasmic reticulum-->peroxisome-->endoplasmic reticulum-->bile route (Russell and Setchell, 1992).

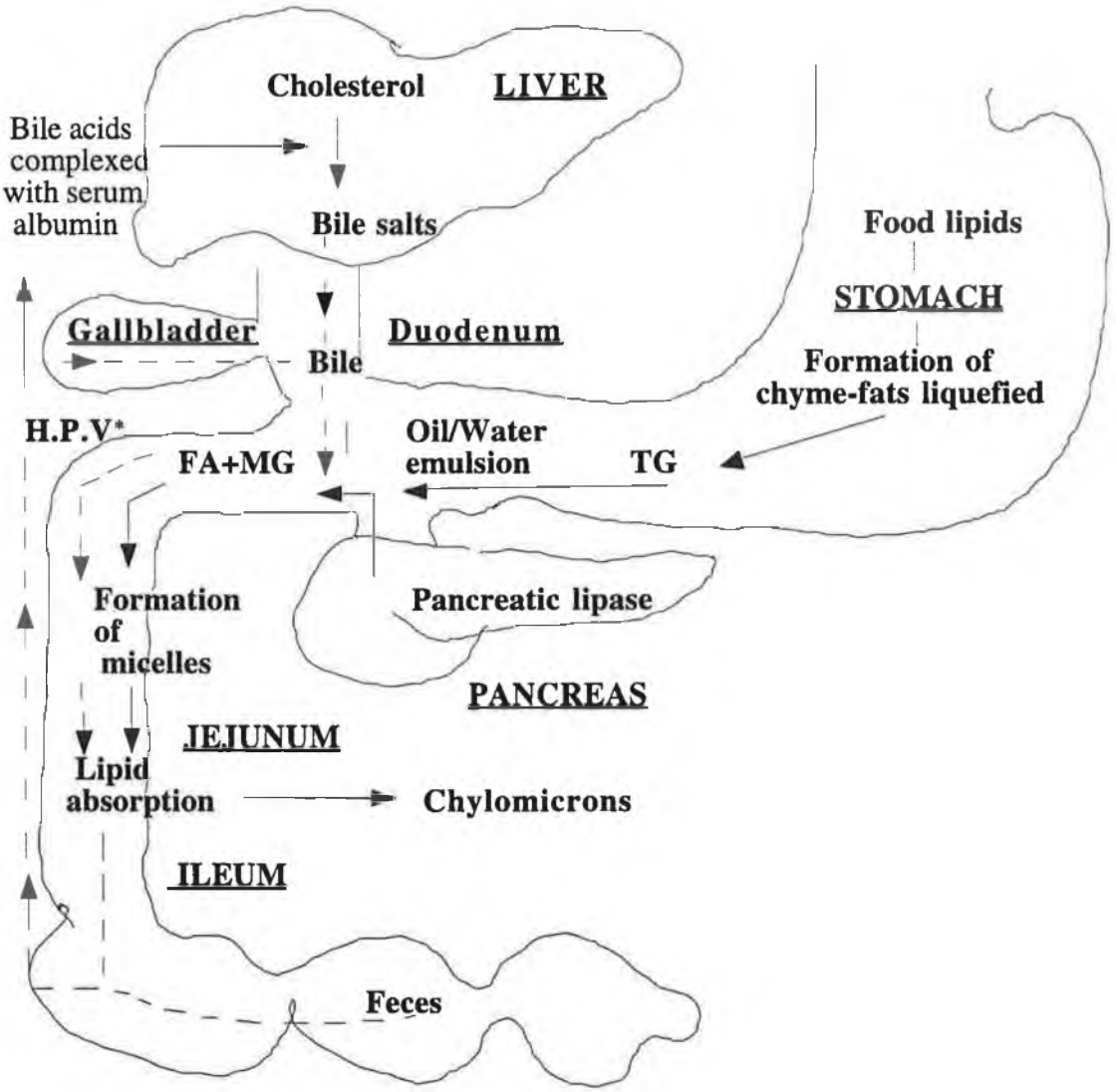
4.1.2 The enterohepatic circulation of bile salts

The two bile acids primarily formed in human liver are cholic acid and chenodeoxycholic acid, the ratio between the synthesis rates being about 1.5-2. Bile salts are secreted by the liver in conjugated form, together with free cholesterol. In animals possessing a gall bladder, most of the bile secreted by the liver during fasting is diverted into the gall bladder via the cystic duct where it is concentrated and stored until the gall bladder contracts in response to feeding. When this occurs, the bile is secreted into the duodenum through the common bile duct. In the lower ileum and colon, the conjugated bile acids are acted upon by enzymes of the intestinal flora, with deconjugation and 7α-dehydroxylation as the major reactions, resulting in the formation of deoxycholic from cholic acid and of lithocholic acid from chenodeoxycholic acid. After participating in the adsorption of lipids in the jejunum, the bile salts pass down the ileum, where at least 95% of the total secreted is reabsorbed, the unabsorbed fraction escaping into the faeces (**Fig 4.2**).

The bile acids reabsorbed from the intestine enter the portal vein and are rapidly and efficiently extracted from the portal blood by the hepatocytes; only a minor proportion passes through the liver into the peripheral blood circulation. Within the hepatocytes, the unconjugated bile acids are reconjugated with glycine and taurine. Fasting bile acid concentrations in the systemic circulation at 2-3μM are around 6-fold higher than those in the portal circulation, reflecting the efficient extraction of bile acids by the liver under normal physiological conditions (Ceryak *et al.*, 1993). In some species, including rats, deoxycholic acid is 7α-hydroxylated to cholic acid, however, in man the 7α-hydroxylation of deoxycholate is incomplete, resulting in the presence of conjugated deoxycholic acid in human bile.

The enterohepatic circulation of bile salts is so efficient that each day the relatively small pool of bile acids (about 3-5g) can be cycled through the intestine 6-10 times with only a small amount lost in the feces (approximately 1% per pass through the enterohepatic circulation). Each day, an amount of bile acid equivalent to that lost in the feces is produced from cholesterol by the liver, so that a pool of bile acids of constant size is maintained. This occurs as a result of a feedback control system.

Fig 4.2 Enterohepatic circulation of bile salts and the digestion of lipids.



Dashes represent enterohepatic circulation of bile salts. TG, triacylglycerol; MG, monoacylglycerol; FA, long chain fatty acids (Harper, 1982).
H.P.V* : Hepatic portal vein.

4.1.3 Regulation of Cholesterol 7α-hydroxylase

The rate-limiting step in bile acid biosynthesis was shown in the late 1960's to be catalysed by cholesterol 7α-hydroxylase (Danielsson *et al.*, 1967; Shefer *et al.*, 1970). These and subsequent investigations have shown that once 7α-hydroxycholesterol is formed in liver primary bile acid synthesis soon results. Various studies have demonstrated that cholesterol 7α-hydroxylase activity is induced by blocking the return of bile acids to the liver either by surgical interruption of the enterohepatic circulation

(Danielsson *et al.*, 1967) or by dietary administration of anion-exchange copolymers that bind bile acids in the small intestine and thereby prevent their uptake. Conversely, infusion or dietary administration of bile acids leads to a decrease in enzyme activity (Shefer *et al.*, 1970). Thyroid hormone was shown to induce the amount of cholesterol 7α-hydroxylase mRNA and protein in the liver (Ness *et al.*, 1990). Other physiologic up-regulators of this enzyme include glucocorticoids, cholesterol (Straka *et al.*, 1990) and metabolites of cholesterol. Recent work by Doerner *et al.*, (1995) found that cholesterol and not oxysterols regulate cholesterol 7α-hydroxylase. Expression is also governed by a hormone-mediated circadian rhythm (Chiang *et al.*, 1990; Sundseth & Waxman, 1990).

Recent studies by Chiang *et al.*, (1990) using reverse phase HPLC and a highly sensitive immunoblot procedure showed parrallel changes in enzyme activity and concentration of cholesterol 7α-hydroxylase in microsomes of rats maintained under different lighting conditions, in rats treated with cholestyramine-supplemented and cholestyramine-depleted diets, in genetically obese Zucker rats and in groups of rats treated with different cytochrome P-450 inducers. The study provided the first direct evidence that the bile acid feedback regulation and circadian rhythm of microsomal cholesterol 7α-hydroxlase activity involved the induction of cholesterol 7α-hydroxylase enzyme level.

It is now widely believed that regulation of cholesterol 7α-hydroxylase activity is mainly controlled at the transcriptional level. Cholesterol 7α-hydroxylase activity, protein mass and mRNA levels have been shown to increase in response to partial and complete biliary diversion; conversely, continuous intraduodenal infusion of taurocholate in rats with chronic bile fistula leads to a decrease in cholesterol 7α-hydroxylase specific activity, protein mass, mRNA levels and transcriptional activity (Pandak *et al.*, 1991). These findings provided direct evidence that taurocholate, a relatively hydrophobic bile salt regulates cholesterol 7α-hydroxylase at the level of gene transcription. It has been shown in some studies that posttranscriptional regulation of the enzyme may occur (Shefer *et al.*, 1991) adding a possible additional level of control.

In 1988 Sundseth *et al.*, established that cholesterol 7α-hydroxylase is regulated in response to long-, mid- and short term control factors, referred to above, at the pretranscriptional level and suggested that this regulation was of greater importance than the allosteric effects of bile acids on the P-450 cholesterol 7α-hydroxylase protein, reversible phosphorylation, or the availability of cholesterol as substrate, which are other proposed mechanisms. Long-term regulation is mediated by feedback inhibition of bile

acids, mid-term regulation through the diurnal cycle and finally, the short-term regulatory factors are hormones and dietary factors.

Short term regulation of the enzyme has also been well documented.The inhibition of cholesterol 7α-hydroxylase by phosphatase and stimulation by cAMP-dependent protein kinase has been demonstrated in a reconstituted enzyme system (Tang and Chiang, 1986).

Dietary factors, such as cholesterol feeding are also known to influence the regulation of cholesterol 7α-hydroxylase. The activity and specificity of cell surface bile acid transport proteins in the small intestine and the liver may also contribute to the regulation of cholesterol 7α-hydroxylase (Russell and Setchell, 1992). The transcription factors and *cis*-acting regulatory elements involved in the regulation of this enzyme have not been fully elucidated. Although there is about 70-80% homology in the first 250 bp of the 5′ flanking sequence of rat, hamster and human gene, there is much divergence in the upstream sequence- thereby possibly accounting for the differences in regulation of cholesterol 7α-hydroxylase between different species. It is clear that the regulation of cholesterol 7α-hydroxylase, and hence cholesterol catabolism, occurs at multiple levels and may even rival that of HMG-Co A reductase, the other major regulatory enzyme of cholesterol metabolism in its complexity.

4.1.4 Regulation of bile acid synthesis:

As a result of these extensive studies on cholesterol 7α-hydroxylase activity in the rat, it has been generally accepted that bile acid synthesis is regulated by bile acids returning to the liver. However, recent studies *in vitro* and *in vivo* are not in agreement with the classical concept of negative feedback of bile acid and so, regulation of bile acid synthesis has become an area of controversy. For example, several groups of investigators could not demonstrate the inhibition of bile acid synthesis in bile fistula rats (whereby there is a diversion of bile acids from the bile duct by means of a fistula which results in the upregulation of bile acid biosynthesis), suggesting that bile acids may not regulate cholesterol 7α-hydroxylase directly (Davis *et al.*, 1988). Intravenous (unlike intraduodenal) infusion of taurocholate did not down regulate cholesterol 7α-hydroxylase (Pandak *et al.*, 1994). However the degree to which bile acids repress cholesterol 7α-hydroxylase activity does appear to parallel the relative hydrophobicity of bile acids in portal blood; specifically, hydrophobic but not hydrophilic bile acids are effective down regulators. A recent study in primary cultures of rat hepatocytes has indicated that the repression of cholesterol 7α-hydroxylase transcription by hydrophobic bile acids may be

mediated by protein kinase C isozymes (Stravitz *et al.*, 1995). Another example of discrepancy is the failure to demonstrate negative bile acid biofeedback in cultured or freshly suspended hepatocytes following the addition of high concentrations of different bile salts to the culture medium (Kubaska *et al.*, 1985). By contrast, manipulations that increase or decrease hepatocyte cholesterol were observed to up- or down-regulate bile acid synthesis *in vitro*. These observations suggest that bile acids may regulate their own synthesis indirectly by altering the concentration of cholesterol in a critical microsomal regulatory pool. A recent publication by Fukushima *et al.*, (1995) reported that bile acid synthesis in the rat model was not regulated directly by the portal bile acids returning to the liver, which differ to the biliary bile acids, the former being more concentrated when in circulation.

4.1.5 Cholesterol supply for catabolism

The role of cholesterol in the regulation of cholesterol 7α-hydroxylase and the interrelationship between the cholesterol and bile acid biosynthesis pathways has also been an area of concentrated research. There are two alternative sources of cholesterol available for bile acid synthesis: cholesterol that is newly synthesised in the adjacent hepatic rough endoplasmic reticulum and the free and esterified cholesterol of the plasma lipoproteins, interiorised via different pathways. Cholesterol may be supplied from varying lipoproteins such as that from the HDL fraction, LDL and also possibly from the uptake of cholesterol form chylomicron remnants (Fry *et al.*, 1990).

Early studies carried out on rats indicated that newly synthesised cholesterol was the preferred (but not the only) substrate for bile acid biosynthesis (Balasubramaniam *et al.*, 1973), as their results indicated that the pool of cholesterol that 7α-hydroxycholesterol was synthesised from did not equilibrate rapidly with the plasma cholesterol. Björkhem *et al.*, (1975) provided further evidence which was in agreemeent with this theory. However, in favour of the argument that cholesterol substrate for cholesterol 7α-hydroxylase is derived from plasma lipoproteins, whole-body turnover experiments with human subjects reported that such a pool of cholesterol provided about two-thirds of the cholesterol substrate for the enzyme (primarily free cholesterol of lipoproteins). (Schwartz *et al.*, 1982).

Under most experimental conditions, the activity of the rate limiting enzyme in cholesterol biosynthesis, HMG Co A reductase changes in parallel with the activity of the cholesterol 7α-hydroxylase. This linkage is most important for cholesterol homeostasis and an increased synthesis of cholesterol always causes a compensatory increase in degradation of cholesterol.

Evidence for transcriptional regulation of the enzyme by cholesterol or related sterol

came from *in vivo* studies with lovastatin and mevalonate. Under circumstances in which cholesterol is present in excess, cholesterol 7α-hydroxylase transcriptional activity is up-regulated and removal of cholesterol from hepatocytes is facilitated by an increase in bile acid synthesis. When cholesterol availability is decreased, cholesterol 7α-hydroxylase transcriptional activity is down- regulated leading to a decreased elimination of cholesterol via bile acid synthesis. In both instances, hepatic cholesterol homeostasis is effectively maintained via the link between the pathways of cholesterol supply and catabolism.(Jones *et al.*, 1993).

However the nature of the link between these two enzymes is still unknown. The fact that bile acids are able to downregulate both enzymes in a similar way may be important for the coordination of the two enzymes. Coordinate regulation of the two pathways can be readily demonstrated in the rat by dietary manipulation. Feeding animals bile acid binding resins leads to a decrease in the return of bile acids to the liver and a consequent increase in cholesterol 7α-hydroxylase and of *de novo* bile acid synthesis. This increase in turn depletes intracellular cholesterol pools, causing an increase in the synthesis of enzymes in the cholesterol biosynthetic pathway and of low density lipoprotein receptor. Conversely, when cholesterol is included in the diet, enzymes in the supply pathway are suppressed and cholesterol 7α-hydroxylase is induced. This induction in turn leads to an increase in bile acid production and to an increase in excretion in this species (Bjorkhem, 1991). When primary bile acids are fed, both cholesterol 7α-hydroxylase and enzymes in the supply pathways are suppressed. This response of the supply pathways can be explained by an increased absorption of dietary cholesterol mediated by cholic or chenodeoxycholate and a subsequent increase return of the sterol to the liver in the form of chylomicron particles (Spady *et al.*, 1986). In addition, down regulation of cholesterol 7α-hydroxylase decreases *de novo* synthesis of bile acids and further increases the suppression of cholesterol synthesis and uptake.

4.1.6 Disorders associated with defects in the bile acid biosynthetic pathway.

Several liver and intestinal diseases are associated with a changed production of bile acids (Axelson *et al.*,1991). Since bile acids are major excretion products of cholesterol (Björkhem, 1985) such changes will affect the turn-over rate of cholesterol in the body (Axelson *et al.*, 1991). The liver employs many strategies in the coordinate regulation of cholesterol metabolism, in an attempt to prevent the pathological consequences of a disruption in overall cholesterol homeostasis. These tacts include physiological occurrences that link the activities of different regulatory enzymes in each of the pathways and also a shared negative feedback regulatory mechanism at the transcriptional level. Many inherited diseases have been discovered, in which an enzyme deficiency is present

in the synthetic pathway of bile acids (Russell and Setchell, 1992). Three of these inherited diseases have been described to date.

The first was identified by Salen in 1974. It was observed that subjects with the rare autosomal recessive disease, cerebrotendinous xanthomatosis (CTX), which results in a defect in the degradation of the side-chain of cholesterol, excreted abnormal C27 bile alcohols (urinary and faecal) and reduced levels of C24 bile acids, notably chenodeoxycholic acid (Setoguchi *et al.*, 1974). This disorder is characterised by an increase in the level of cholestanol and a decreased level of 26-hydroxycholesterol (Javitt *et al.*, 1982). Such products are then found to build up in the tissues of the affected individual and when accumulation occurs in the central nervous system, progressive neurological dysfunction and eventual death result (Menkes, 1968). The molecular basis of CTX has recently been established (Cali *et al.*, 1991) by cloning the normal sterol 27-hydroxylase cDNA. The gene was localised to the distal long arm of chromosome 2. Diagnosis, if made at early stages, may result in treatment by oral primary bile acid therapy, which act by feedback inhibition, thus preventing further accumulation of cholesterol and bile alcohols (Salen *et al.*, 1975). 27-hydroxylase results in the conversion of 5ß-cholestane-3α,7α,12α-triol to 5ß-cholestane-3α,7α,12α,27-tetrol.

The second disorder recorded by Clayton *et al.*, (1987) was identified as a defect in the enzyme- 3ß-hydroxy-C27-steroid oxidoreductase, which oxidises 7α-hydroxycholesterol to 7α-hydroxy-4-cholesten-3-one.

Finally, the third defect has been identified in infants with neonatal hepatitis and cholestasis, by Setchell *et al.*, 1988. The enzyme that is defective here is Δ4-3-oxosteroid 5ß-reductase (involved in the step whereby 7α-hydroxy-4-cholesten-3-one is reduced to 7α-hydroxy-5ß-cholesten-3-one, which subsequently proceeds with the side chain oxidation of cholesterol). This results in the elevation in the levels of C24 bile acids that retain the Δ4-3-oxo configuration of the sterol substrates. The cause of liver disease in such patients is thought to be the accumulation of potentially cholestatic and hepatotoxic atypical bile acids, worsened by the fact that primary bile acids, which are required for the generation of bile acid-dependent bile flow, are lacking. Treatment of such disorders by bile acid therapy results in the normalisation of liver dysfunction (Russell and Setchell, 1992).

The pathogenesis of human cholelithiasis has been ascribed to abnormal hepatic cholesterol metabolism. An important discovery was that bile acid pools were diminished in such a disease (Vlahcevic *et al.*,1970). Gallstone subjects have been shown to have a decreased activity in cholesterol 7α-hydroxylase (Salen *et al.*, 1975). Other reports have

described changes in bile acid metabolism which may contribute to hypocholesterolemic (Kern, 1991) or hypertriglyceridemic (Angelin, 1987) states.

4.1.7 Hereditary hyperlipoproteinemias

Polymorphism and mutations at the numerous sites of metabolic control in lipoprotein homeostasis impart a distinct biochemical individuality. Thus, there is a wide range of serum lipoprotein profiles in the general population. For clinical convenience, the systems of classifications are based on combinations of the following: HDL cholesterol, plasma apoprotein levels, total levels of serum cholesterol and triglycerides, lipoprotein phenotype, family history, mode of transmission and presence of other disorders known to affect the metabolism of lipoproteins. Such classifications are represented in the following two tables, **Table 4.1** and **Table 4.2.**

The major aims when treating hyperlipidaemias are the prevention of *de novo* development of coronary heart disease and other atherosclerotic disorders, as well as retardation and regression of such disorders, if already manifest (Tikkanen, 1990).

Pharmacologic intervention with the liver plays an important role in the treatment of such patients, as the liver possesses more than half the total body complement of LDL receptors. So, any event leading to a perturbation of hepatic cholesterol metabolism would be expected to have a profound effect on plasma LDL cholesterol levels (Shepherd and Packard, 1992). The sterol pools of hepatocytes may be depleted by the inhibition of cholesterol biosynthesis or by stimulating the conversion of cholesterol to its oxidation products, the bile acids.

Lipid-lowering drugs, used in such treatments, are usually differentiated by their actions. Such drugs can be divided into two main groups- the HMG-CoA reductase inhibitors, which reduce lipid production by the liver and the bile acid sequestrants, which prevent dietary lipids from being absorbed.

4.1.8.1 HMG-CoA reductase inhibitors/Statins:

These agents act by suppressing cholesterol synthesis in the liver cell, by acting as competitive inhibitors of HMG-CoA reductase. Alkaloids were isolated from *Penicillium citrinum* and found to be powerful competitive inhibitors of the rate-limiting enzyme in cholesterol biosynthesis, HMG-CoA. They are capable of inhibiting the enzyme when present in very low concentrations. As a result of decreasing the concentration of intracellular cholesterol, there is an associated increase in the number of LDL receptors which are produced and expressed on the surface of hepatocytes. Thus, the removal of lipoprotein cholesterol from the plasma is promoted. They include such drugs as lovastatin, pravastatin and simvastatin. They lower plasma triglyceride by reducing the circulating level of VLDL.

Tables 4.1 & 4.2: Typical classifications of hyperlipoproteinemia (Khachadurian, 1990)

Table 4.1 PHENOTYPIC CLASSIFICATION OF HYPERLIPOPROTEINEMIAS	
PHENOTYPES	**LIPOPROTEINS THAT ARE ELEVATED**
II	LDL
III	IDL
IV	VLDL
V and I	Chylomicrons and VLDL

TABLE 4.2 LIPOPROTEIN PHENOTYPES IN MAJOR FORMS OF HYPERLIPOPROTEINEMIAS				
PRIMARY HYPER-LIPOPROTEINEMIAS	**Phenotypes**			
	II	**III**	**IV**	**V & I**
Lipoprotein lipase deficiency	-	-	±	+
Familial Dysbeta lipoproteinemia	-	+	±	-
Familial Hypercholesterolemia	+	±	±	-
Familial Combined Hyperlipidemia	+	±	+	-
SECONDARY HYPER-LIPOPROTEINEMIAS				
Diabetes		+	+	+
Hypothyroidism	+	+	+	+
Dysglobulinemias		+	+	+

+ = frequent
± = occasional
- = absent

Pravastatin and simvastatin are new HMG CoA reductase inhibitors that have promise in the treatment of elevated serum cholesterol. Lovastatin has a very high affinity for HMG-CoA reductase (K_i of ~1nM while the K_m of ~ 10μM). Lovastatin and simvastatin are administered as prodrugs. They are in a lactone form that is inactive and become active, as the ß-hydroxyacid form, upon metabolism in the liver. However, pravastatin is delivered as an open acid. The most frequent reason requiring that therapy with pravastatin be stopped was asymptomatic elevation of liver transaminase levels and non-specific gastrointestinal complaints with a frequency similar to that seen with lovastatin (Milander *et al.*, 1992). The efficiency of statins in FH (apart from the rare homozygous patients who have no functioning LDL receptors) is explained by this mechanism. In milder (non-FH) forms of hypercholesterolaemia another mechanism may be of importance. The increase of LDL receptor activity also causes enhanced removal of VLDL and VLDL remnants (precursors of LDL) via these receptors in the liver (Grundy, 1988), resulting in a decreased production of LDL as in **Fig 4.3.** (Tikkanen, 1990).

Overall, lovastatin and the other HMG-CoA reductase inhibitors are very well tolerated with few known adverse side-effects. The major being abnormalities in liver function tests (Shepherd *et al* , 1992) and also the occurrance of myopathy. As referred to above, statins reduce the circulating levels of VLDL, thus, the moderate elevation in HDL cholesterol observed in several patients during statin treatment may be partly explained by the reciprocal relationship commonly observed between HDL and VLDL. It is not caused by alterations in lipoprotein lipase or hepatic lipase activities (Tikkanen, 1990).

Recently developed drugs which act in the suppression of cholesterol biosynthesis include the squalestatins- developd by Glaxo and the zaragozic acids- developed by Merck Sharp and Dohme, in 1992. They act as potent inhibitors of mammalian and fungal squalene synthese, an enzyme involved in the biosynthesis of cholesterol (Kelly and Roberts, 1995).

4.1.7.2 Bile acid sequestrants:

Previous studies have shown that treatment with bile-acid-binding resins, which bind bile acids in the intestine and prevent their reabsorption, increases fecal bile acid excretion (Grundy, 1988), thereby, stimulating bile acid synthesis. Cholestyramine and colestipol are unabsorbable resins, the former acts as a potent inducer of HMG-CoA reductase and cholesterol 7α-hydroxylase (Cighetti *et al.*, 1983). The interruption of the recirculation of bile acids results in a triggered production of LDL receptors and accelerated receptor-mediated removal of LDL from circulation. However, the therapeutic response is blunted by a compensatory stimulation of hepatocellular cholesterol synthesis **(Fig 4.4)** (Tikkanen, 1990)

Fig 4.3 Mechanism of action of HMG CoA reductase inhibitors

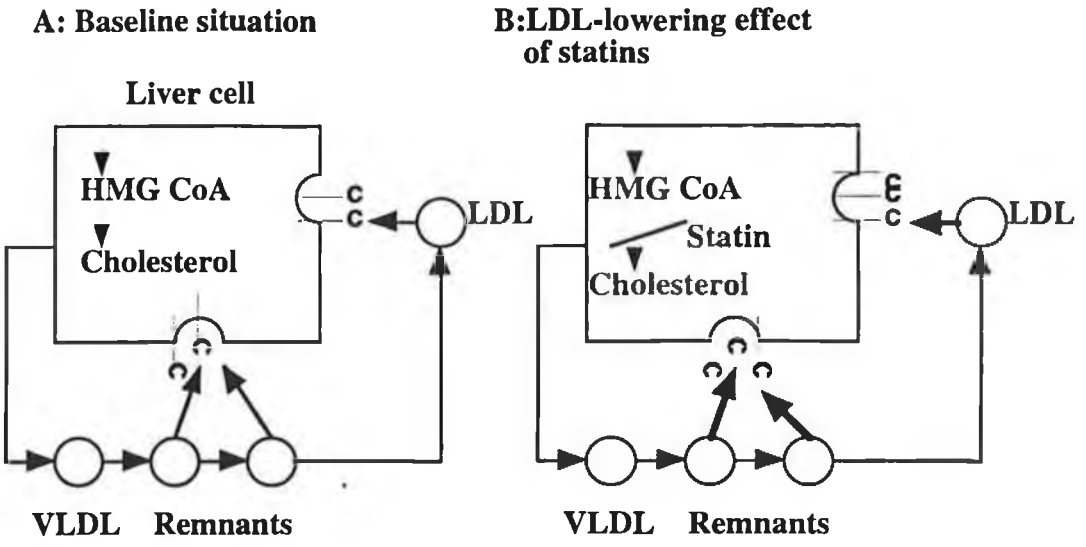
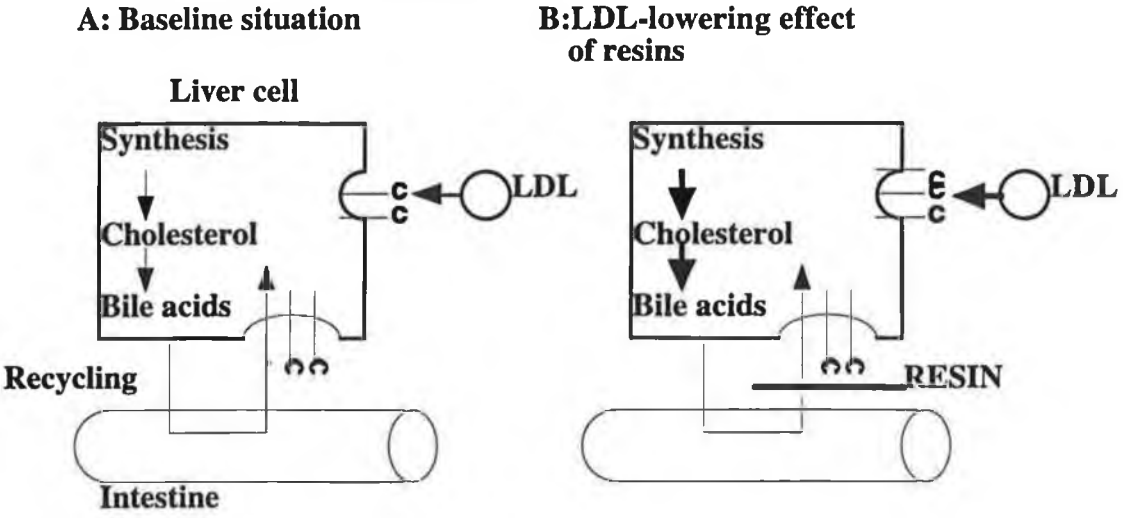


Fig 4.4 Mechanism of action of resins



Brown and Goldstein (1984 and 1986) suggested that as statins and resins stimulate LDL receptor activity and that the resulting increase in cellular cholesterol synthesis is blocked by the statin, that a combination of both the statin and resin would be ideal in the treatment of severe cases of hypercholesterolaemia (Tikkanen, 1990). Thus, combined drug therapy is thus warranted in such circumstances and can be used to control the high LDL cholesterol levels seen in the worst affected heterozygotes (Shepherd and Packard, 1992).

Cholestyramine has been frequently used in treating human patients for hypercholesterolaemia and begins to reduce cholesterol and LDL after 4 to 7 days of administration. However, the maximum effect is not noted until two weeks after therapy has started (Milander and Kuhn, 1992). It has also been used in studies carried out in rats (Song *et al.*, 1991, Kogaire *et al.*, 1992), whereby the latter group reported that cholestyramine feeding stimulated pancreatic growth and exocrine pancreatic secretion in rats. In parallel to a reduction in LDL cholesterol concentrations, there is a fall in apolipoprotein B values, the former being much greater than the latter, as a result of change in lipoprotein composition (Shepherd and Packard, 1992).

As resins are not absorbed from the intestine, they are thought to be among the safest lipid-lowering drugs. However, due to their ion-exchange properties, they may interfere with the absorption of other anionic drugs, such as thyroxine, warfarin and pravaststin. In addition, gastrointestinal symptoms and problems such as bloating are reported (Tikkanen, 1992 and Milander and Kuhn, 1992). Based on the efficacy of these sequestrants and also due to the lack of any long-term adverse effects, bile acid sequestrants remain the drugs of first choice in the initiation of therapy of hypercholesterolemia after failure of dietary restriction (The Expert Panel, 1988).

4.1.7.3 Fibrates

Gemfibrozil, Bezafibrate and fenofibrate are commonly used fibrates whose mechanism of action has not been clearly understood. Various studies have shown that these act by accelerating the catabolism of VLDL and VLDL remnants. Such remnants are produced by the action of lipoprotein lipase (whose activity is known to increase during fibrate therapy) on VLDL (Tikkanen, 1990). Lipoprotein lipase cleaves the triglyceride molecules from VLDL and IDL (Milander and Kuhn, 1992). Thus, the major action of the fibrates is to reduce triglyceride levels.

HDL cholesterol levels are found to be elevated in patients being treated with fibrates due to two main reasons; firstly, there is an increased flow of liberated surface components from VLDL (degraded by lipoprotein lipase), which may be utilised in the synthesis of HDL (Nikkilä, 1984 and Tikkanen, 1989). Secondly, there is less of a transfer of triglyceride from VLDL to HDL (reduction in VLDL levels), thereby less esterified cholesterol from HDL to VLDL.

Bezafibrate therapy in hypertriglyceridaemics results in an increase in the average size of LDL and a change in the conformation of apolipoprotein B, so that it exhibits a higher affinity for receptors. A possible explanation for these results is that the drug reduces the proportion of LDL-I to LDL III (differing sizes in LDLs) in the patient's plasma.

Side effects of fibrates include an associated increase in size and number of gallstones (Millander *et al.*, 1992). This may be attributed to an increase in conversion of cholesterol to bile acids in the liver. They result in slight gastrointestinal disturbances.

Thus, from results of various trials, it has been suggested that gemfibrozil may be recommended as a first-line agent in the treatment of hypertriglyceridemia and as a secondary agent for several other hyperlipidemias (Shepherd and Packard, 1992).

4.1.7.4 Nicotinic acid

This is a lipid-lowering agent, an effect that is separate to its effect as a vitamin (Milander and Kuhn, 1992). Again, there is an associated drop in VLDL levels, which are thought to be responsible for a corresponding drop in triglyceride levels. This acid works primarily in adipose tissue, where it causes a rapid suppression in lipolysis with a fall in circulating free fatty acids. Receptors on the cell surface facilitate the mode of action of nicotinic acid, which in turn are coupled to inhibitory G proteins. Thus, its mode of action involves the inhibition of adenylate cyclase and decreasing accumulation of cAMP.

In treatment, it is rapidly absorbed from the intestine, as it is part of the water-soluble vitamin B complex. It is the only hypolipoproteinemic agent to date that has been associated with a decrease in overall mortality (Shepherd and Packard, 1992). Its use in therapy may result in elevated levels of alkaline phosphatase and transaminase (hepatic dysfunction)- which may in turn be associated with jaundice, which are just a few of the many adverse effects of the use of this agent. It results in a cutaneous flush within a short period of its administration. A deterioration of glucose tolerance is also observed in patients, with preexisting impaired glucose tolerance (Shepherd and Packard, 1992).

4.1.7.5 Probucol

Due to the fact that it lowers HDL more than it does LDL, this drug is not as prescribed as frequently as others (Milander and Kuhn, 1992). Its mode of action is by decreasing the amount of apolipoprotein A-I and A-II which results in a decline in HDL cholesterol. Studies have indicated that the probucol-induced fall in LDL cholesterol is due to increased catabolism of LDLs *via* non-receptor mediated pathways. Such pathways may not effect the production of LDLs. It is a synthetic lipophilic antioxidant, which is incorporated into lipoproteins, thereby preventing their oxidation. It also distributes into adipose tissue. Such properties may have important implications in the therapy of atherosclerosis. Although its mode of action does not involve affecting the production or

degradation of LDL, it does, however, alter the ratio of protein:cholesterol of HDL. Due to its former effect on LDL, probucol ie presently regarded as a secondary agent in the treatment of hypercholesterolemia. It has few side effects, some of which include-gastrointestinal disturbances, mainly diarrhea and nausea (Milander and Kuhn, 1992).

4.1.8 Abnormalities in cholesterol metabolism in diabetes mellitus

Although diabetes is by definition a disorder of carbohydrate metabolism, it has been apparent for some time that abnormalities of lipid metabolism occur commonly in patients with this syndrome. Furthermore, it has been generally assumed that the defects in lipid metabolism seen in diabetes help explain why these individuals are at increased risk of developing atherosclerosis (Chen *et al.*, 1987). High levels of plasma cholesterol are frequently found in human diabetes and also develop in the alloxan diabetic rat (Wong and Bruggen, 1960). Such associations with diabetes mellitus- as hyperlipidaemia and premature atherosclerosis, may result due to alterations of plasma lipoprotein composition and/or to changes in cholesterol synthesis (Devery *et al.*, 1987). Kraemer (1986) reported that abnormal cholesterol metabolism resulted due to a deficiency in insulin in animal models such as the mouse, while O'Meara *et al.*, (1986) reported such results for the rabbit model.

The liver can direct fatty acids into one or other of two fundamentally different pathways: triglyceride synthesis for transport to the periphery and fuel storage and oxidation to ketone bodies as instant energy source for muscle or brain (Ireland *et al.*, 1980). Hormones determine which pathway will be taken. Insulin has a large role in the balance between storage and release of fat and it promotes the conversion of carbohydrate into triacylglycerols. When insulin is lacking, as in severe diabetes, ketogenesis will be the major metabolic pathway. VLDLs are frequently increased in mild diabetes due to a combination of hepatic synthesis and lack of sufficient insulin in the periphery to allow lipoprotein lipase to metabolise them. Abnormalities of HDL metabolism also exist in patients with diabetes. Obesity and hypertriglyceridemia have been shown to be associated with low plasma HDL-cholesterol levels (Albrink *et al* ., 1980, Philips *et al.*, 1981 and Schaeffer *et al.*, 1978).

Non-insulin-dependent diabetes mellitus patients (NIDDM- Type 2) have high levels of VLDL and LDL and low levels of HDL. Insulin therapy leads to raised HDL-cholesterol levels in patients with type 1 diabetes, the predominant rise occuring in HDL_2-cholesterol (Bergman *et al.*, 1986). Although a fall in lipoprotein lipase activity has been reported to be responsible for the low plasma HDL concentration with insulin deficiency, it has also been suggested that the evidence is circumstantial and that decreased lecithin:cholesterol acyltransferase (LCAT) activity could also be involved (Chen *et al.*, 1987). Cholesteryl esters are transferred to LDL and VLDL by cholesterol ester transfer protein, after LCAT has esterified cholesterol in HDL_3, thereby converting these particles to HDL_2 (Glomset

and Norum, 1973 and Eisenberg, 1984), which is cholesterol ester rich.

Varying results have been reported for the amount of LCAT activity in diabetic subjects. Misra *et al.*, (1974) reported the activity of LCAT was increased in experimental diabetes. Their study showed that administration of insulin did not have any effect on the activity of the enzyme. However, it must be noted that the substrate used in their experiments was that obtained from normal individuals pooled together. Finally, they showed that the enzyme activity was depressed in certain cases where diabetic ketosis or uraemia occured. Upon correction of acidosis however, the activity of the enzyme was found to be normal.

In relation to the use of suitable substrate for the LCAT assay, Chen *et al.*, (1987) suggested that in studies on type 1 diabetics, that are poorly controlled, the use of the subjects own plasma as source of substrate would aid in the study of LCAT activity in such individuals. Meanwhile, Weight *et al.*, (1993), found that there was no significant difference between LCAT activities in control subjects and poorly controlled type 1 diabetics. The activity of the latter was shown to increase with improved metabolic control. Other reports in support of the fact that LCAT activity is unchanged in diabetics include Schernthaner *et al.*, (1983), who reported that the activity did not change irrespective of age, sex or type of diabetes.

On the contrary, Mattock *et al.*, (1979) reported that LCAT activity was slightly, but significantly, higher in IDDM and NIDDM subjects studied, than in controls. Regarding diabetic rat models and LCAT studies, Kudchodkar *et al.*, (1988), reported that upon feeding dietary caesin to diabetic rats, there was a decreased fractional rate of plasma cholesterol esteification, which may be indicative of changes in the nature of substrate lipoproteins, as the plasma LCAT activity measured using an exogenous substrate was not affected. This result, in the case of the caesin fed rat, may be attributed to the fact that there was an associated increase in the levels of apo-E rich HDL subclass, which is of a lower substrate potential than apoA-rich HDL (Kudchodkar *et al.*, 1988).

LCAT is one enzyme associated with the esterification of cholesterol, which is found in plasma- another esterifying enzyme is acylcoenzyme A:cholesterol-O-acyltransferase (ACAT), which is found in the liver and intestine. As the latter enzyme is present in the intestine, chylomicrons secreted from the intestine are relatively low in free cholesterol and have a high cholesteryl ester content proportional to the rate of uptake of cholesterol from the intestinal lumen. The level of ACAT in the liver in some species (including humans) is very low (Fielding and Fielding, 1985). Thus, it is likely that human hepatic lipoproteins contain little, if any cholesteryl esters when secreted (Fielding and Fielding, 1985).

The activity of ACAT is affected by a number of factors including dietary fat, cholesterol and ethanol, the availability of its cholesterol substrate, the concentrations of

LDL, progesterone, 25-hydroxycholesterol and possibly the phosphorylation / dephosphorylation mechanism (Suckling and Stange, 1985). ACAT activity is reported to increase in rabbit aorta during diet induced hypercholesterolaemia (Brecher and Chan, 1980), while in humans being treated for gallstones, its activity has been shown to be reduced (Smith *et al.*, 1986). Devery *et al.*, (1987), reported intestinal ACAT activity, while reduced in diabetic rabbits, was unchanged in diabetic rats. Their results also propose that the decrease in hepatic ACAT activity of the poorly controlled diabetic rats (by 10% , $P<0.01$), may reflect a decreased esterification of free cholesterol within the liver or a greater ability of liver to secrete cholesterol out of the liver in the form of VLDL particles. Owens *et al.*, (1991) also reported that ACAT levels were found to be significantly increased (55±18%) in mononuclear leukocytes of diabetic patients after treatment with simvastatin. The significance of this decrease in enzyme levels in diabetics may suggest that more free cholesterol is available for transport leading to a higher pool of circulating cholesterol (Tomkin and Owens, 1991).

4.1.9 Methods for the measurement of 7α-hydroxycholesterol

Recent advances in spectroscopy and chromatography have permitted measurement of various cholesterol biosynthetic pathway intermediates, which covary with rates of cholesterol biosynthesis. Many of these methods have also been used to measure intermediates of the bile acid biosynthetic pathway, including 7α-hydroxycholesterol not only in biological samples but also in foodstuffs. Such methods include the following:

4.1.9.1 Incorporation of [^{14}C] cholesterol.

Here, radioactive cholesterol is incubated with liver microsomes. The enzyme, cholesterol 7α-hydroxylase converts cholesterol into 7α-hydroxycholesterol, the first step in the bile acid biosynthetic pathway. The product of this reaction is 7α-hydroxy[^{14}C]cholesterol. The radioactive 7α-hydroxycholesterol is then isolated from the reaction medium and assayed for radioactivity. The initial methods developed for the analysis of 7α-hydroxycholesterol used the principle of this method. Thin-layer chromatography is frequently used to isolate the product as decribed by Balasubramaniam *et al.*, (1975) and more recently, Bascoul *et al.*, (1988). However, one cannot be sure that the behaviour of the enzyme towards exogenous substrate is analogous to that towards endogenous cholesterol.

4.1.9.2 Colormetric assay

7α-hydroxycholesterol may be detected and estimatedby the Lifschutz colour reaction, as decribed by Bergström and Wintersteiner (1942). As 7α-hydroxycholesterol is known as an azurosteroid, the presence of a hydroxy group at position at C-7 results in an

intense blue colour being produced in acidic or dehydrating medium (SO_4H_2/H_2O, (5:5, v/v)), or in the presence of antimony chloride (Bascoul *et al.*, 1990). 7α-hydroxycholesterol is also detectable in the ultraviolet-visible region of the spectrum (Hutton and Boyd, 1966).

4.1.9.3 Isotope dilution-mass spectrometry

A sensitive and accurate assay was developed by Björkhem *et al.*, (1981) for the measurement of various intermediates in the bile acid biosynthetic pathway, including 7α-hydroxycholesterol. The radioactive extracts from the assay were subjected to HPLC (preparative), peaks of interest were collected, converted to trimethylsilyl ether and subjected to analysis by gas-liquid chromatography-mass spectrometry.

4.1.9.4 Capillary gas-liquid chromatography-selected ion monitoring (GLC-SIM)

Oda *et al.*, (1990) described a method for the analysis of 7α-hydroxycholesterol using this method. Samples which were cleaned up using Sep-Pak columns were treated with TMSI-H, 1% pyridine-n-hexane and analysed by GLC-SIM. In the SIM mode, the selected ions were scanned for TMS ether derivatives of 7α-hydroxycholesterol.

4.1.9.5 HPLC

This technnique has been used frequently in recent years. Efficient HPLC separations of mixtures of oxygenated sterols has been described (Ansari and Smith, 1979) and HPLC separation in combination with enzymatic detection has been suggested (Ógren *et al.*, 1980). This is reported to improve the detection of sterols in the low UV absorption range. Both normal (Ogishima and Okuda, 1986) and reverse-phase HPLC (Hylemon *et al.*, 1989) are reported for such analysis.

Aims of "Serum 7α-hydroxycholesterol in human and animal models of altered cholesterol metabolism":

* To measure levels of 7α-hydroxycholesterol in the serum of humans- control and hypercholesterolaemic

* To measure levels of 7α-hydroxycholesterol in the serum of rats- control, cholestyramine-treated and streptozotocin-induced diabetics

* To examine the possible correlation between the levels of 7α-hydroxycholesterol in the serum and liver of the rat models as an indicator of hepatic bile acid synthesis

4.2 Results

Having shown that the HPLC method is a reliable, sensitive and precise method for the measurement of 7α-hydroxycholesterol and cholesterol in serum, a species survey of its levels in both human and animal sera, its biological significance in serum and its route of metabolism required further investigation.

This chapter describes data obtained when serum samples from normolipidaemic and hypercholesterolaemic human subjects were analysed for 7α-hydroxycholesterol. Since the liver plays a major role in the regulation of whole body cholesterol metabolism and is the sole organ for bile acid synthesis in the body, it was decided that 7α-hydroxycholesterol, the bile acid precursor metabolite should also be determined in liver of experimentally-induced animal models of altered cholesterol metabolism using essentially the same HPLC analytical technique as was used for the analysis of 7α-hydroxycholesterol in serum.

4.2.1 Levels of 7α-hydroxycholesterol in human sera

0.5ml aliquots of serum from 4 control normocholesterolaemic subjects and 6 hypercholesterolaemic patients were analysed for 7α-hydroxycholesterol as described in Chapter 2. Typical HPLC chromatographs obtained from these samples are depicted in **Fig 4.5(a) and (b)**. **Table 4.3** depicts the range, mean and standard deviation in levels of 7α-hydroxycholesterol obtained in these two groups. No overlap was observed in the range of serum levels in normal subjects (153.3-216.6 pmoles/ml) and hypercholesterolaemic (260-563 pmoles/ml) patients. Hypercholesterolaemic patients showed significantly higher levels of 7α-hydroxycholesterol (371.1±126.5 (6) pmoles/ml) ($p<0.05$) than control subjects (185.4±26.4 (4) pmoles/ml). The relatively large standard deviation in the level of analyte obtained in the hypercholesterolaemic group, may in part, be attributed to the fact that the patients from which serum was obtained were being treated for varying medical disorders such as diabetes, hypothyroidism, obesity, hypertension, colitis and euthyroidism. Drugs treatments in some of the above patents included simvastatin, pravastatin and probucol. The serum levels of cholesterol ranged from 4.6mM to 10.3mM, averaging 7.26±2.4mM.

Fig 4.6 represents the profile of serum which has not been enzymatically treated with cholesterol oxidase. This was carried out in order to determine whether or not endogenous amounts of 7α-hydroxy-4-cholesten-3-one were present.

Fig 4.5(a) and 4.5(b) Typical HPLC chromatographs of (a) normal human serum aliquot (0.5ml) and (b) hypercholesterolaemic human serum (0.5ml). Procedure for sample preparation and analysis is as in methods.

The positions for 20α-hydroxycholesterol, 7α-hydroxycholesterol and cholesterol are indicated below.

Fig 4.5(a)

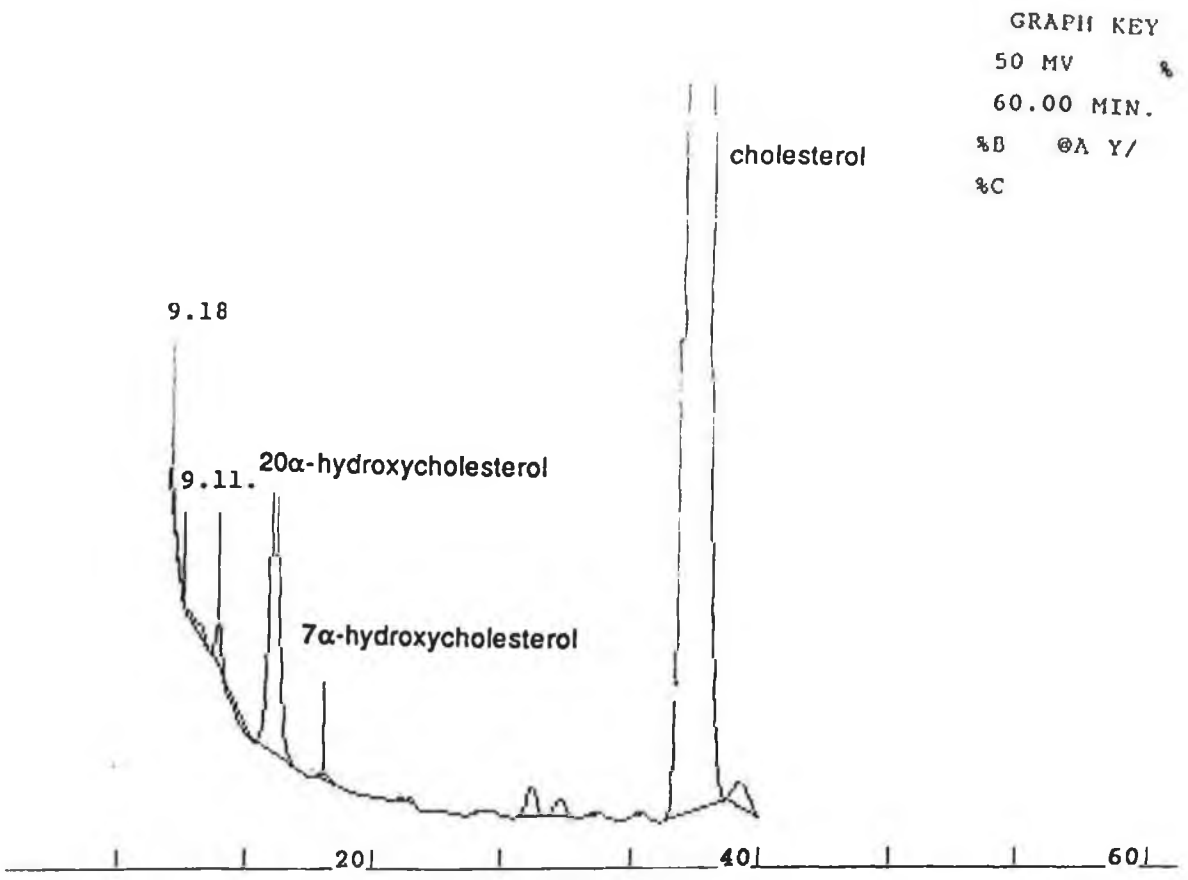


Fig 4.5(b)

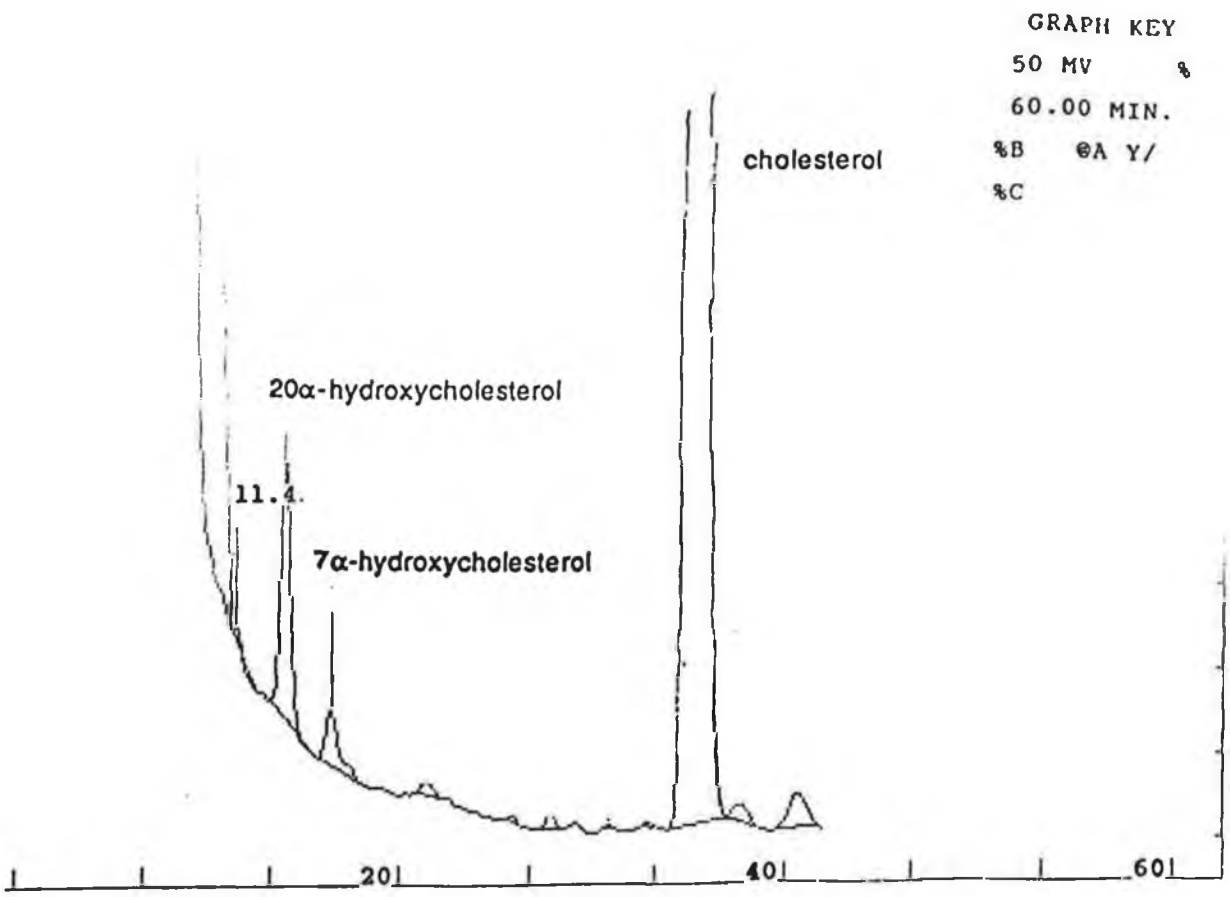
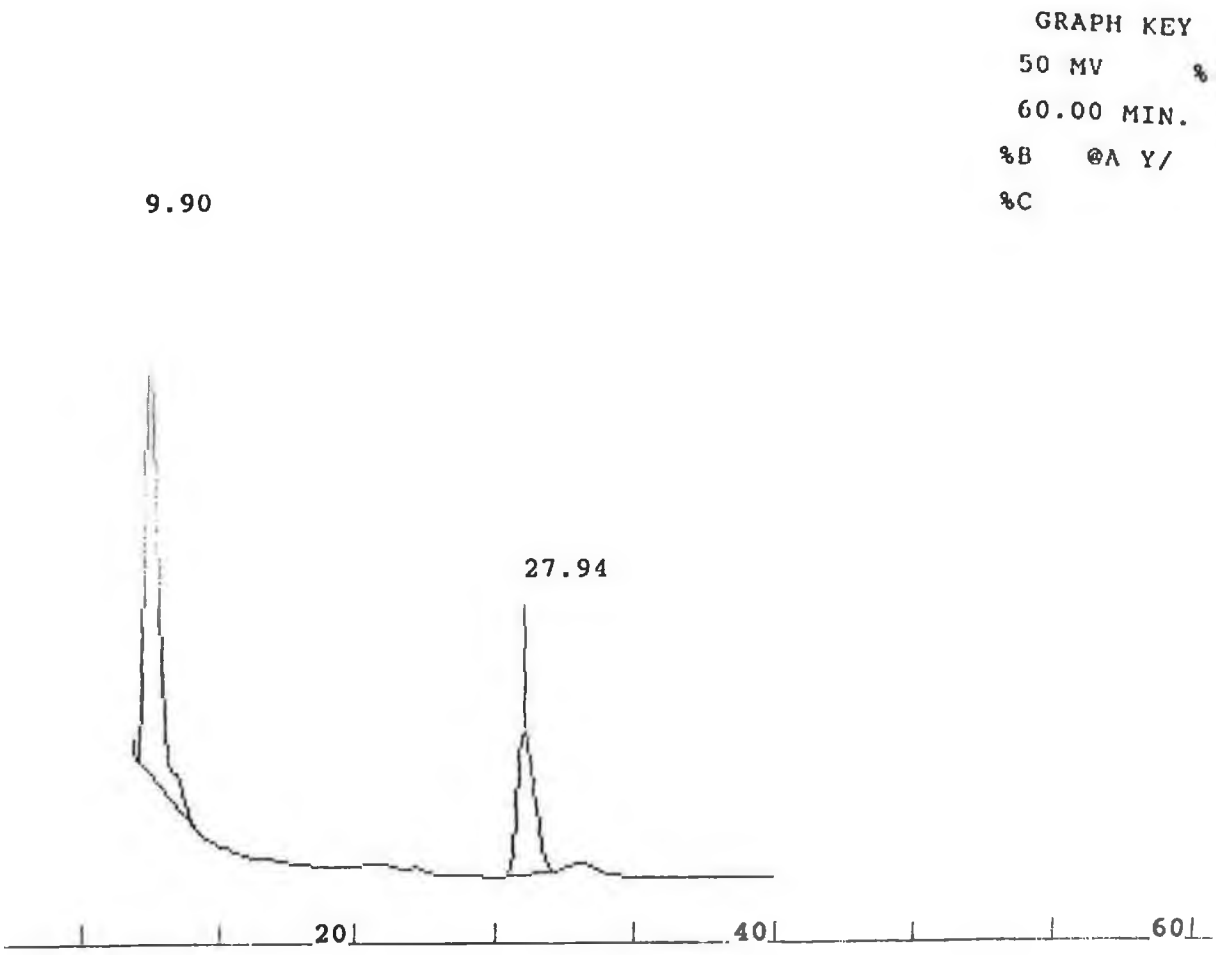

GRAPH KEY
50 MV %
60.00 MIN.
%B @A Y/
%C
cholesterol
20α-hydroxycholesterol
11.4
7α-hydroxycholesterol
20
40
60

Table 4.3: Concentrations of 7α-hydroxycholesterol in serum of control human subjects and subjects being treated for hypercholesterolemia. Data represents: The range of values obtained and Mean ± S.D.

SUBJECT	SERUM LEVEL OF 7a-OHCHO (pmoles/ml)
CONTROL	**153.30 - 216.60** **185.43 ± 026.40 (n=4)**
***HYPERCHOLESTEROLEMIC**	**260.00 - 563.00** **371.06 ± 126.49 (n=6)**

*** p<0.05**

Fig 4.6 HPLC chromatographic representation of human serum, which has not been treated with cholesterol oxidase, indicating the presence of endogenous enone derivatives of cholesterol.



4.2.2 Levels of 7α-hydroxycholesterol in rat serum

Serum 7α-hydroxycholesterol was determined in 6 control rats (**Fig 4.7(a)**), 7 cholestyramine-treated rats (**Fig 4.7(b)**) and 6 diabetic rats (**Fig 4.7(c)**). **Table 4.4** and **Fig 4.8** show the concentration of serum 7α-hydroxycholesterol in the three rat groups. The levels of the sterol in serum were similar in control rats and in diabetic rats (mean concentration 259.3±63 (n=6) and 202.1±66 (n=6) pmoles/ml respectively) but were significantly elevated ($p<0.01$) in the cholestyramine-treated rat (591.4±143.9 (n=7) pmoles/ml). The range of 7α-hydroxycholesterol showed no overlap with either the control (186-335 pmoles/ml) or the diabetic group (115-279.9 pmoles/ml), while only one of the cholestyramine levels (257.22 pmoles/ml) overlapped with the control values.

For reasons of comparison, hepatic levels of 7α-hydroxycholesterol, the product of endogenous cholesterol catabolism by cholesterol 7α-hydroxylase, were determined by J.Quinn, another post-graduate student in this laboratory. As expected, the levels of 7α-hydroxycholesterol were elevated in rats treated with 3% cholestyramine. Treatment with cholestyramine resulted in a 2.7 fold increase in the concentration of hepatic 7α-hydroxycholesterol (17.2±3.7 (n=3) nmol/ml) relative to control rats (6.2±1.6 (n=6) nmoles/ml). Streptozotocin-induced diabetes resulted in a significance decrease ($p<0.05$) in the concentration of hepatic 7α-hydroxycholesterol (2.8±0.8 (n=6) nmoles/ml).

Paired data for liver and serum levels of 7α-hydroxycholesterol in 6 diabetic and 3 cholestyramine-treated rats and random matched data for control rats showed that there was a correlation between hepatic and serum levels of 7α-hydroxycholesterol (**Fig 4.9**). This correlation was shown to be significant (even in the absence of control data) ($r=0.67$, $p<0.05$). Although the % of 7α-hydroxycholesterol in rat serum relative to liver in all groups was <10%, there were indications that leakage of 7α-hydroxycholesterol from liver into serum was higher in the diabetic group (7.3%) relative to the cholesytyramine-treated group (2.3%) and control group (4.2%).

Fig 4.7 HPLC chromatograms of serum obtained from (a) control (b) cholestyramine-treated and (c) streptozotocin-induced diabetic rats.

Fig 4.7 (a) Control rat serum

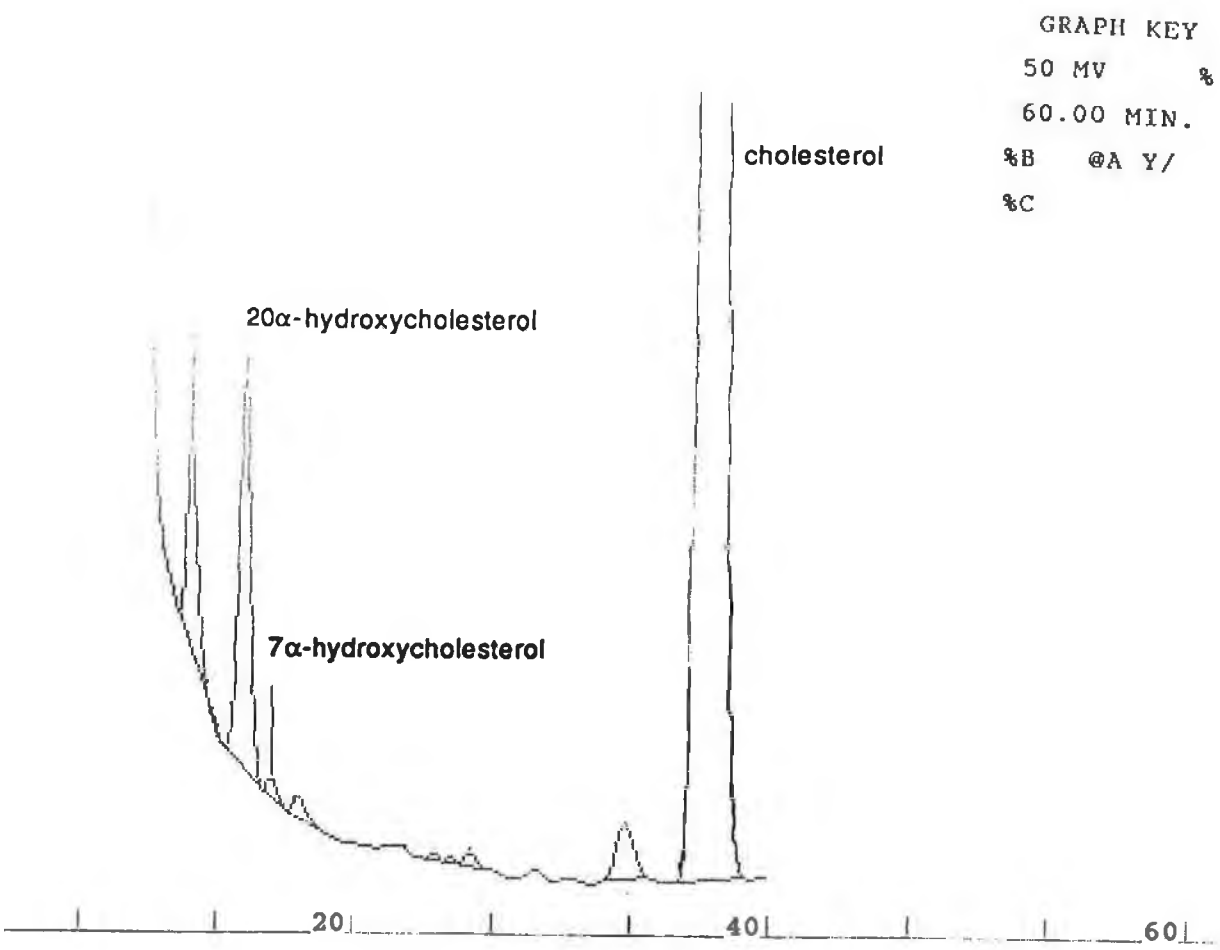


Fig 4.7 (b) Cholestyramine-treated rat serum

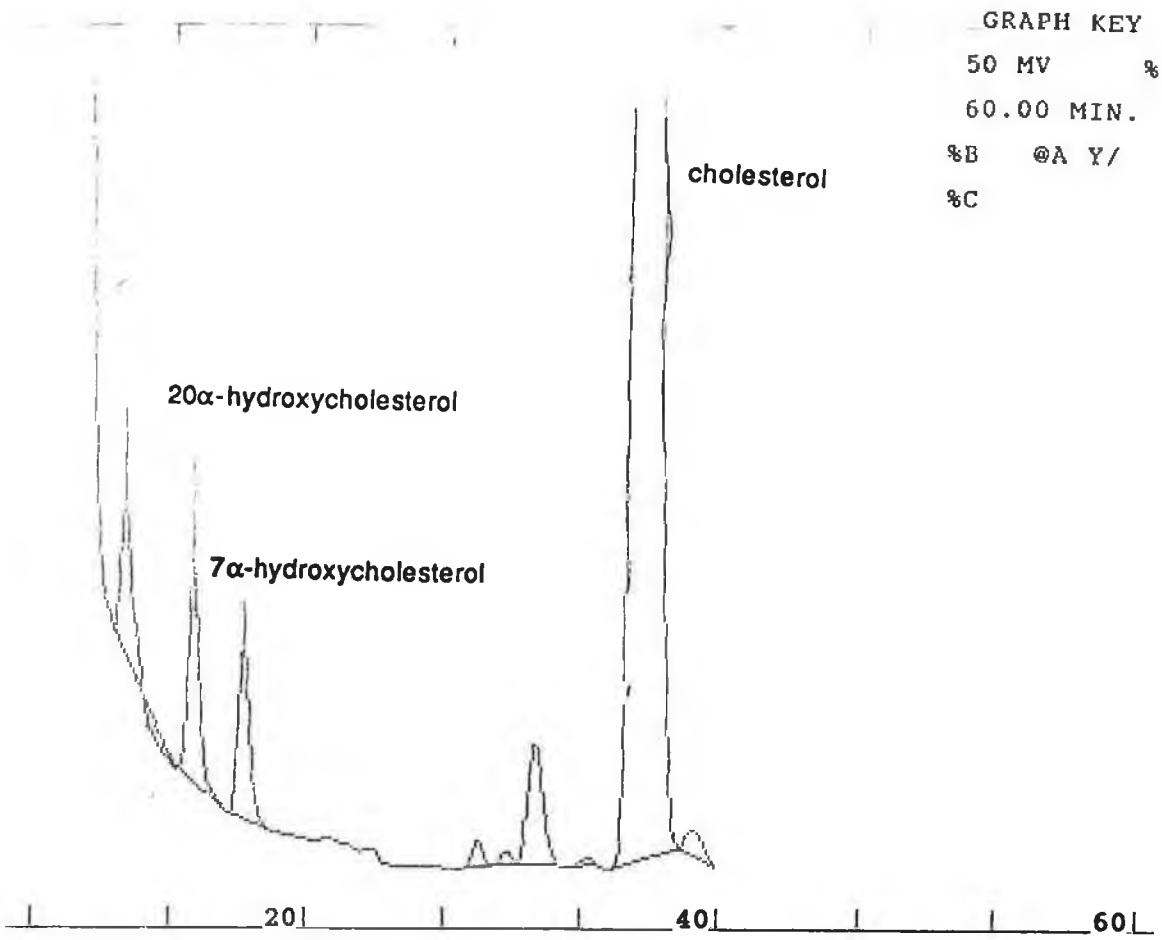


Fig 4.7 (c) Streptozotocin-induced diabetic rat serum

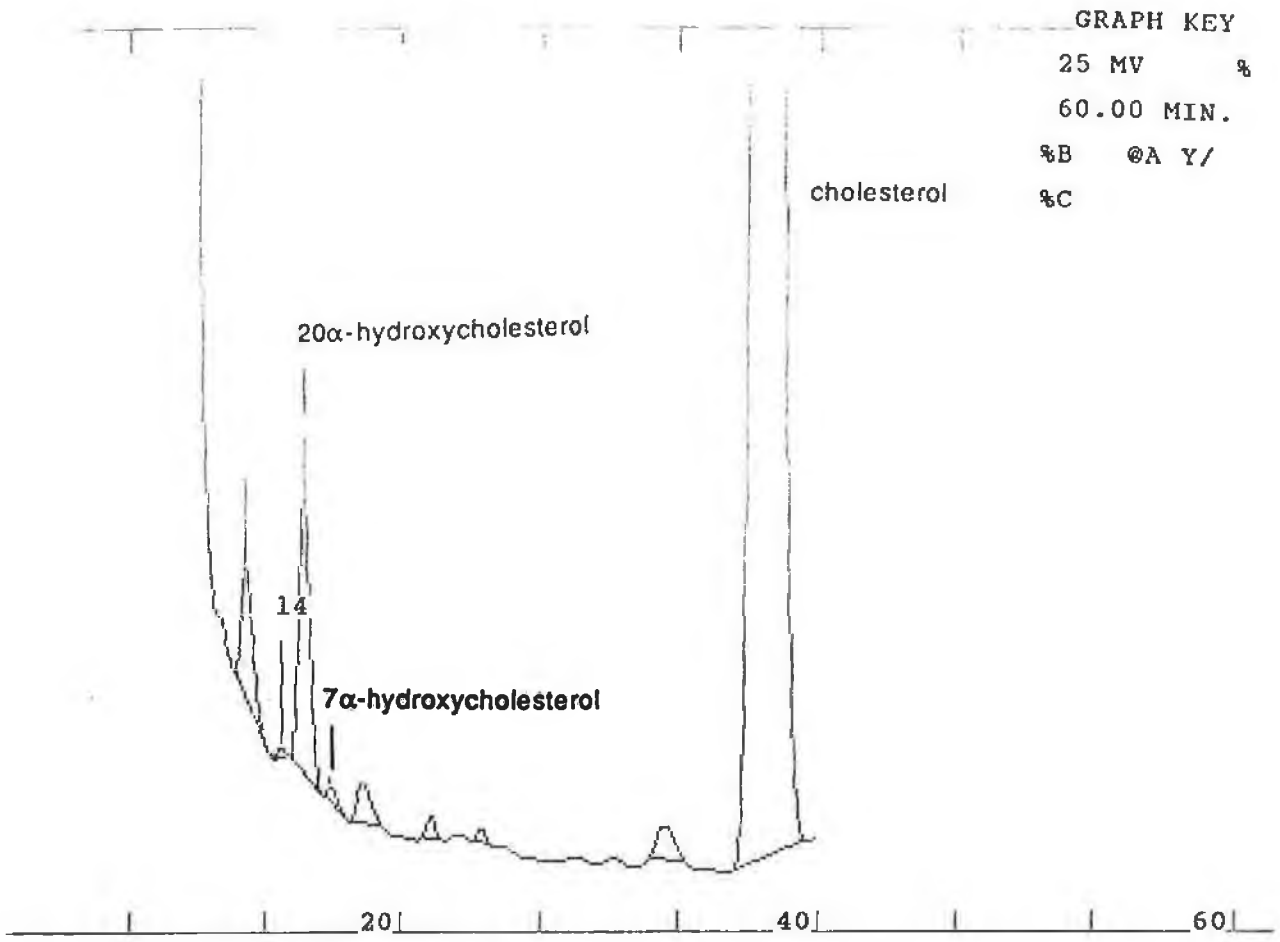


Table 4.4 & Fig 4.8: Serum levels and hepatic levels of 7α-hydroxycholesterol in the control, streptozotocin-induced diabetic and cholestyramine treated rat models.

$p<0.05$ for hepatic 7α-hydroxycholesterol levels in the streptozotocin induced diabetic rats with respect to controls; and also in the cholestyramine-treated rats with respect to controls.

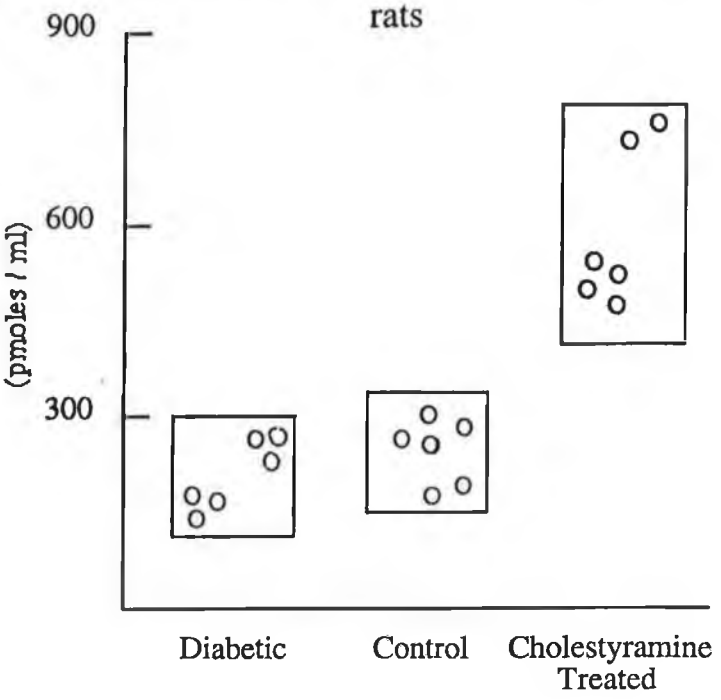
$p<0.01$ for serum 7α-hydroxycholesterol levels in the cholestyramine-treated rats with respect to the controls.

Table 4.4:

		Control	Streptoz-otocin induced diabetic	Cholesty-ramine treated
Serum 7a-hydroxy-cholesterol (pmoles/ml)	Value range	186.0 - 335.0	115.0 - 279.9	433.9 - 791.0
	Mean ± S.D	259.3 ± 63.9 (n=6)	202.1 ± 66.5 (n=6)	591.4 ± 143.9 (n=6)
Hepatic levels of 7a-hydroxy-cholesterol (nmoles/ml)	Value range	4.62-8.61	1.55-3.63	14.87-21.5
	Mean ± S.D	6.18±1.57 (n=6)	2.83±0.71 (n=6)	17.1±3.72 (n=3)

Fig 4.8 RAT SUBJECTS

Serum Levels of 7α-Hydroxycholesterol in control, diabetic and cholestyramine-treated rats



Hepatic Levels of 7α-Hydroxycholesterol in control, diabetic and cholestyramine-treated rats

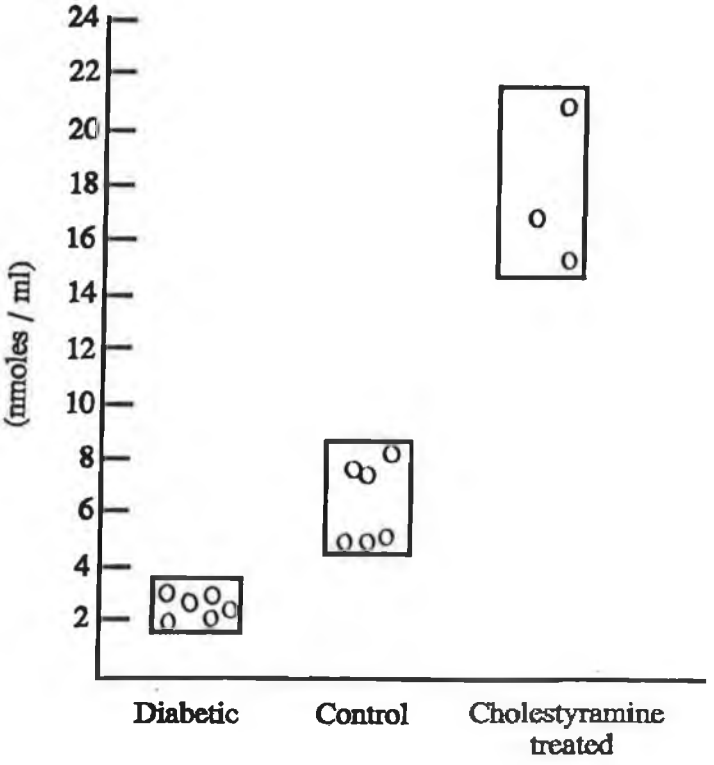
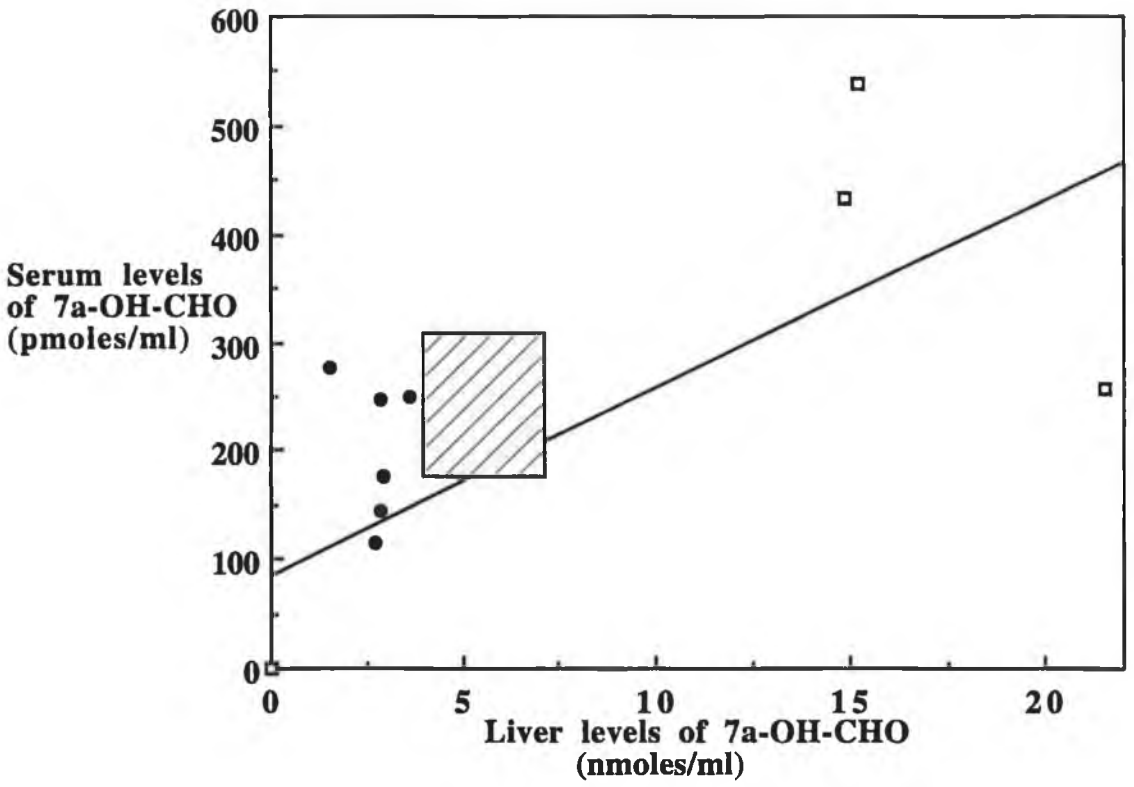


Fig 4.9: Correlation between levels of 7α-hydroxycholesterol in serum (total) and liver (free) of rats. Rat models included a streptozotocin-induced diabetic model and also the cholestyramine treated rat.(r=0.67). Hatched area indicates values obtained for random matched hepatic and serum data from control rats (n=6).

Diabetic (n=6)

Cholestyramine (n=3) treated models.



4.2.3 Free and esterified levels of 7α-hydroxycholesterol in human and animal serum

0.5ml aliquots of sera from 3 control and 3 hypercholesterolaemic human patients, 3 control and 3 experimentally-induced diabetic rats were assayed for both free and esterified 7α-hydroxycholesterol. Esterified 7α-hydroxycholesterol levels were computed as the difference between free and total 7α-hydroxycholesterol, the former being determined by the analytical procedure described in Chapter 2 but in the absence of alkaline hydrolysis. The procedure facilitated simultaneous measurement and comparison of free and total levels of both 7α-hydroxycholesterol and cholesterol. **Table 4.5** demonstrates that a large proportion of total 7α-hydroxycholesterol in normal human serum is esterified (78.12%). Mean free 7α-hydroxycholesterol levels in control sera was found to be 42.93±4.21 pmoles/ml. A slightly higher level of esterified 7α-hydroxycholesterol was observed in hypercholesterolaemic patients (83.72%) with respect to controls (78.12%). Mean free 7α-hydroxycholesterol level in the latter was 61.50±14.38 pmoles/ml. The % esterification of cholesterol was slightly lower than that of 7α-hydroxycholesterol (71.46% in control sera and 72.37% in hypercholesterolaemic sera).

Levels of free and esterified 7α-hydroxycholesterol were calculated for rat sera also (**Table 4.5**). Free 7α-hydroxycholesterol levels were 41.95 pmoles/ml in control and 27.22 pmoles/ml in diabetic rats. However, the total levels of 7α-hydroxycholesterol in the former group were 247.83±55.29 pmoles/ml and 145.0±30.64 pmoles/ml for the latter. Thus, as can be calculated, the % esterification of 7α-hydroxycholesterol in controls was 74.91%, while in diabetics was found to be 81.23%. The % esterification of cholesterol was slightly lower than that of 7α-hydroxycholesterol in control rats (64.12%) and diabetic rats (77.32%).

In summary, this chapter provided a comparison of the levels of 7α-hydroxycholesterol in human and rat sera and indicated a species difference in levels of both total and esterified 7α-hydroxycholesterol. In addition, there is some evidence to support the current literature that analysis of serum 7α-hydroxycholesterol may be a convenient method for evaluating the relative rate of bile acid synthesis *in vivo*.

Table 4.5 Percentage esterification of cholesterol (CHO) and 7α-hydroxycholesterol (7α-OHCHO) in the various groups studied. Results obtained are those by HPLC analysis (n=3). Free and total measurements tabulated below are expressed as pmoles/ml serum for 7α-OHCHO, while CHO figures are as μmoles/ml serum. (Hypercholest*: hypercholesterolaemic subjects).

SUBJECT	7a-OHCHO			CHO		
	free	total	% esterified	free	total	% esterified
HUMAN:						
CONTROL	42.93	196.2	78.12±7.5	1.27	4.46	71.46±10.57
HYPER-CHOLEST.*	61.50	377.8	83.72±22.4	2.28	8.26	72.37±9.00
RAT:						
CONTROL	41.95	247.8	74.91±16.1	0.431	1.20	64.12±0.83
DIABETIC	27.22	145.0	81.23±12.7	0.235	1.04	77.32±1.01

4.3 Discussion

It has been suggested that the 7α-hydroxycholesterol concentration in serum is a good parameter for establishing disorders involving the metabolic conversion of 7α-hydroxycholesterol towards bile acids (Koopman *et al.*, 1987), whether acquired, inherited or brought about by medical treatment. Previous methods for the identification and quantitation of 7α-hydroxycholesterol in serum and in the liver have utilised TLC , Gas-liquid chromatography (Bascoul, 1990), a combination of these with mass spectrometry (Bjorkhem and Kallner 1976; Einarsson *et al.*, 1986) and recently reverse phase HPLC for liver analysis (Hylemon *et al.*, 1989).

Having set up and validated this method, serum samples from human control subjects and from subjects being treated for hypercholesterolemia were analysed. It was found that the range of measures found in control samples were from 153.3 - 216.6 pmoles/ml, which compares well with values of 49 - 410 nmoles/l - (Koopman *et al.*, 1987)- analysis by gas chromatography. There was a significant increase in the levels of 7α-hydroxycholesterol in the serum of the patients being treated for hypercholesterolaemia, with respect to controls ($p<0.05$). This is in accord with other findings (Koopman *et al.*, 1987) and (Bascoul *et al.*, 1990). The latter reported on Familial hypercholesterolemia which is associated with a defect in LDL receptor activity. Elevations in both enzymes-HMG-CoA reductase and cholesterol 7α-hydroxylase activity were reported in Yoshida rats, which are animal models of spontaneous hyperlipidemia (Fantappie *et al.*, 1992). As referred to in the introduction (**4.1.7: Tables 4.1** and **4.2**), diabetes and hypothyroidism are classified as secondary hyperlipoproteinemias, having altered lipoprotein patterns. All the hypercholesterolaemic patients in this study had cholesterol levels above 7.2mM.

Results of 7α-hydroxycholesterol measurement in rat serum, indicate that the levels found were higher than those of control humans (rat values = 259.33 ± 63.86 pmole/ml, human values = 185.43 ± 26.4 pmoles/ml). This is in accord with the report by Einarsson *et al.*, (1986), who showed that the activity of cholesterol 7α-hydroxylase in human liver was found to be lower than in the rat (one magnitude). However, the observation that rats do not possess a gall bladder and have an unusually long small intestine, may account for high levels of bile acid metabolism in these animals. Such anatomical and biochemical differences coupled with the different manner by which the rat metabolises endogenous and exogenous cholesterol (Turley and Dietschy, 1982) suggest that not all of the results obtained in the rat may be extrapolated to other species. Nevertheless, most mammals share the same rate-limiting step in bile acid biosynthesis and in this regard, the rat is an exemplary species (Russell and Setchell, 1992).

Studies carried out on rats included the administration of cholestyramine, an anion

exchange resin (a gritty polymer carrying many positively charged chemical groups), in the diet. As the resin is not absorbed from the intestine, it is excreted, carrying the bound bile acids with it. This drug, as it binds negatively charged bile acids in the intestine and prevents their reabsorption, releases the feedback inhibition of cholesterol 7α-hydroxylase activity by enterohepatic recirculating bile acids (Myant and Mitropoulos, 1977), thus stimulating the enzymatic conversion of cholesterol towards bile acids. Cholestyramine treatment resulted in a significant elevation ($p<0.01$) in circulating levels of 7α-hydroxycholesterol in serum of treated rats. A corresponding increase in the hepatic levels of 7α-hydroxycholesterol were also observed (a 2.7 fold increase relative to controls: Quinn, unpublished results). These results are in accord with others (Song 1991), who reported a 5- to 6-fold increase in enzyme activity in rat liver microsomes of animals treated with cholestyramine. The animals studied by Song et al., had 3% cholestyramine administered for at least 2 weeks *ad lib* , compared to the same concentration for a period of 7 days, as with our models.

It also has been reported (Balasubramaniam *et al.*, 1972 and Mayer, 1972) that there is a marked fall in the activity of cholesterol 7α-hydroxylase during fasting, the amplitude of the diurnal rhythm in activity being much reduced but not completely abolished. Einarsson *et al.*, (1991), reported results of a study carried out on gallstone patients treated with cholestyramine for three weeks and also of untreated patients. They concluded that the significant increase in the activity of cholesterol 7α-hydroxylase in such treated patients, was probably not explained by an increase in substrate availability, but possibly due to changes in the turnover of specific species of cytochrome P-450 of the enzyme. Chiang *et al.*, (1990) indicated that microsomal cholesterol 7α-hydroxylase enzyme levels were increased in parallel with cholesterol 7α-hydroxylase activity upon the treatment of rats with diet supplemented with cholestyramine.

Results here indicate that an increase in circulating levels of 7α-hydroxycholesterol mirrored an increase in the level of hepatic 7α-hydroxycholesterol. The measurements of serum 7α-hydroxycholesterol reported here are for total amounts. This matter has been previously examined in human patients undergoing treatment for cholelithiasis, where Oda *et al.*, (1990) found that esterified and total 7α-hydroxycholesterol were more highly correlated with the enzyme activity than the free form, even when cholesterol 7α-hydroxylase is not induced, i.e. bile acid synthesis is decreased by treatment with chenodeoxycholic acid. Björkhem *et al.*, (1987) showed that serum free 7α-hydroxycholesterol reflected cholesterol 7α-hydroxylase activity when bile acid synthesis

was induced by cholestyramine. Yoshida *et al.*, (1993), recently reported a significant correlation between plasma-free 7α-hydroxycholesterol levels and cholesterol 7α-hydroxylase activity in patients with cholelithiasis or early cancer of the gastrointestinal tract.

The liver has been found to be capable of converting 7α-hydroxycholesterol to 7α-hydroxy-4-cholesten-3-one, which may be an intermediate formed during the epimerisation of the 3ß-hydroxyl group to 3α-hydroxyl with migration of the double bond in its biosynthesis (Hutton and Boyd, 1966). It has also been suggested by Axelson *et al.*, (1991) that the circulating levels of 7α-hydroxy-4-cholesten-3-one, present in plasma, reflect both the total production of bile acids in man and the activity of hepatic cholesterol 7α-hydroxylase. Literature findings indicate that the circulating levels of 7α-hydroxycholesterol are higher than those of 7α-hydroxy-4-cholesten-3-one. A recent study by Pettersson and Eriksson (1994), found that the concentration range of serum 7α-hydroxy-4-cholesten-3-one (2-35 ng/ml (~5-87pmoles/ml)) was considerably lower than the levels of 7α-hydroxycholesterol (19.7-165ng/ml (50-410 pmoles/ml)) reported by Koopman *et al.*, (1987). As presented by means of a HPLC chromatograph in **Fig 4.6**, such a form of 7α-hydroxycholesterol was not detected in this study.

Studies carried out on the cholestyramine-treated rat suggest that an increase in the activity of hepatic cholesterol 7α-hydroxylase accounts for the increase in newly synthesised 7α-hydroxycholesterol in the liver. The significant positive correlation between the serum and hepatic levels of this oxide suggests that when 7α-hydroxycholesterol accumulates in liver, it may subsequently leak from hepatocytes into the systemic circulation.

Although there was a significant decrease in the levels of 7α-hydroxycholesterol in liver of control and diabetic rats, there was, however, no significant difference between the levels of 7α-hydroxycholesterol in the serum of diabetic rats with respect to controls. As there are no corresponding liver and serum data for control rats, this study may only conclude that a significant correlation is only found when cholesterol 7α-hydroxylase activity is stimulated as referred to by Björkhem *et al.*, (1987).

Although rats, the most commonly used animal model in diabetes research, are normally resistant to dietary cholesterol-induced hypercholesterolemia and atherosclerosis (Mahley

and Holcomb, 1977), they become susceptible to this dietary stress following streptozotocin-induced diabetes (Russell *et al.*, 1962). However, it has been reported that the restriction of food intake by diabetic rats prevents hypercholesterolemia (Young *et al.*, 1982). The levels of cholesterol in the plasma of rats has been shown to be proportional to the fraction of cholesterol in plasma originating in the diet (Young *et al.*, 1985). Recently, work carried out by Maechler *et al.*, (1992), disagreed with this theory, saying that hyperphagia and intestinal hypertrophy could be excluded as triggering factors for the hyperresponsiveness of diabetic rats to cholesterol feeding.

The level of circulating cholesterol in the diabetic model here, was found to be lower than that of non-diabetic rats. This may be due to the fact that the daily diet was monitored and rationed in order to prevent hyperphagia. Levels of 7α-hydroxycholesterol in the liver, being lower than non-diabetics, will be discussed elsewhere and may be attributed to changes in size of the endogenous cholesterol substrate pool used in the assay (Quinn, unpublished results) .

Morel and Chislom (1989), reported increased peroxidation products in VLDL and LDL of streptozotocin-induced diabetic rats. However, this was not observed in the present study, as it is evident from the chromatographs that there was no significant difference between the levels of 7α-hydroxycholesterol in the serum of control and diabetic rats. In addition, there was no significant increase in the serum levels of 7ß-hydroxycholesterol or 7-ketocholesterol, which are two other prevalent oxidative products of cholesterol.

Oxysterols have been reported to have known effects on enzymes involved in cholesterol metabolism: Kandutsch *et al.*, (1977), reported that oxidised cholesterols at a concentration of between 10^{-6} and 10^{-9} M *in vitro* and *in vivo* inhibit cholesterol synthesis (i.e., inhibits HMG-CoA reductase), consequently, the cholesterol concentration in cells and their membranes decreases resulting in "leaky" cells. 22-hydroxycholesterol has been shown to block cholesterol esterification and cholesterol synthesis (Bates *et al.*, 1983).

That more than 75% of 7α-hydroxycholesterol is carried in LDL raises the question of whether an increase in the circulating level of 7α-hydroxycholesterol affect all cells with LDL receptors? (Bascoul *et al.*, 1990). Such effects may result in inhibition of HMG-CoA-reductase activity, the principal enzyme in the biosynthesis of cholesterol and may also have an effect on the synthesis of LDL receptors (Dueland *et al.*, 1992). Alternatively, it may affect cholesterol metabolism as a result of its cytotoxic nature

(Crastes de Paulet *et al.*, 1988).

7α-hydroxycholesterol in diabetic rats was found to be esterified only to a slightly greater extent than in control rats. The sterol was also found to be only slightly more esterified in human hypercholesterolaemic patients than in control subjects. As regards the esterification of cholesterol, there was no significant difference in the amount esterified between the two human groups, however, there was a significant difference between the rat groups. No result was obtained for the esterification of 7α-hydroxycholesterol in cholestyramine-treated rats.

As referred to above, Bascoul *et al.*, (1990) reported that more than 75% of 7α-hydroxycholesterol, found in circulation, was found carried by LDLs, with approximately 75% being esterified at the 3ß-position (Oda *et al.*, 1990). Little is known about the metabolism of esterified 7α-hydroxycholesterol or its formation- although studies have been carried out on the metabolism of the free form *in vitro* (Anaki and Yamasaki, 1970) and *in vivo.* (Anderson *et al.*, 1972). Recently, metabolic studies were carried out on the further metabolism of 7α-hydroxycholesterol-3ß-stearate in the hamster, by Kishinaka *et al.*, (1992), which had been intravenously infused and it was effectively taken up by the liver, to be excreted in the bile. However, some of this esterified 7α-hydroxycholesterol may be hydrolysed prior to hepatic uptake. Such studies suggest that the microsomes might be the first subcellular organelle in which esterified 7α-hydroxycholesterol is metabolised within the hepatocyte.

The formation of esterified 7α-hydroxycholesterol also poses questions-is it esterified by LCAT or ACAT, or does it result from the hydroxylation of esterified cholesterol? Part of 7α-hydroxycholesterol which, when formed in the hepatocyte and is not oxidised further to 7α-hydroxy-4-cholesten-3-one, may be esterified by ACAT (Sakamoto 1968). Free 7α-hydroxycholesterol may leave the hepatocyte and enter the systemic circulation and be esterified by LCAT in the plasma, along with free 7α-hydroxycholesterol which may be the result of the action of cholesterol esterase on esterified 7α-hydroxycholesterol. Thus, the results of such studies of the esterification of 7α-hydroxycholesterol, *in vitro*, will be discussed in Chapter 5.

In summary, the developed method proves to be an efficient, reproducible and precise method for the analysis of serum 7α-hydroxycholesterol. The results suggest that the

analysis of 7α-hydroxycholesterol in serum is a sensitive and convenient method to determine relative rates of bile acid production, as there was shown to be a positive correlation ($p<0.05$) between levels of 7α-hydroxycholesterol in serum and liver in the two treated groups. Furthermore, despite an interspecies difference in levels of both total and esterified 7α-hydroxycholesterol, it is apparent that the % esterification of 7α-hydroxycholesterol was similar in both species.

CHAPTER 5

REACTIVITY BETWEEN 7α-HYDROXYCHOLESTEROL AND CHOLESTEROL ACYLTRANSFERASE ENZYMES IN SERUM AND LIVER

5.1 Introduction

Interest in the hydrolysis of cellular cholesteryl esters is relevant to both normal and pathologic situations since hydrolysis of cholesteryl ester is a necessary prerequisite to further metabolism of the cholesteryl moiety, either intracellulary, for steroid synthesis or membrane biosynthesis, or *via* efflux to the plasma compartment for delivery to the liver. Apart from cells that secrete lipoproteins and the flux of intact cholesteryl ester from cells to plasma cholesteryl ester transfer protein, most other cells do not secrete intact cholesteryl esters (Glick, 1990). Thus, due to the fact that cholesteryl esters may be deposited and have pathological consequences- as seen in atherosclerosis, characterised by the retention of cholesterol within the peripheral vascular bed (Reichl and Miller, 1989), where cellular cholesteryl esters are deposited in macrophages and smooth muscle cells of the vessel wall (Small, 1988; Ross, 1984)- there has been much interest shown in the metabolism of such esters.

5.1.1 Lecithin-cholesterol acyltransferase (LCAT)

An enzyme central in the extracellular metabolism of plasma lipoproteins is lecithin-cholesterol acyltransferase (LCAT- E.C. 2.3.1.43). It is secreted by the liver, into the plasma, bound to lipoproteins or in the free form (Jonas, 1991). Early data showed cholesteryl esters in all the major plasma lipoprotein fractions. Fielding and Fielding (1985), reported that due to the low level of hepatic acyl CoA-cholesterol acyltransferase (ACAT) in humans, the major part of cholesteryl ester of VLDL and LDL is derived from the LCAT reaction. The absence of almost the entire plasma cholesteryl ester moiety in congenital LCAT deficiency indicated that cholesteryl esters in human plasma are derived from LCAT activity. This enzyme has become of interest for several reasons, which include the following:

Biochemically- LCAT is a serine hydrolase, whose three dimensional structure and mechanism have been studied. It has both phospholipase (or esterase) and acyltransferase activities. Both such activities are dependent on the presence of an apolipoprotein coprotein (Fielding, 1990).

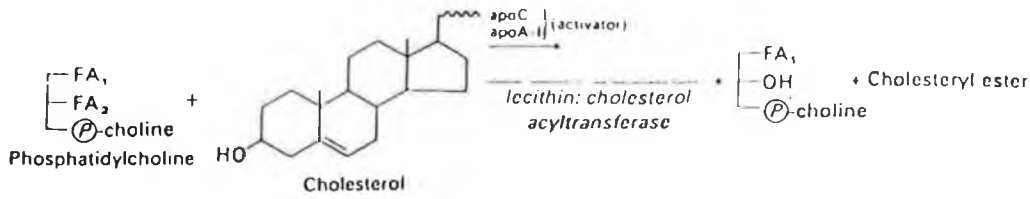
Physically/chemically- the substrate specificity of LCAT, as a function of the physical properties of the lipids, has been studied. Its transferase activities result in the transfer of acyl groups to many alcohols, including sterols and long-chain alcohols (acceptors with free -OH groups).

Due to the role of LCAT in the metabolism of plasma lipoproteins and subsequently in the process of "reverse cholesterol transport", whereby peripheral cell cholesterol can be returned to the liver for catabolism, LCAT studies have been of major importance. This is also evident in the number of reports on its clinical significance in various diseases (Jonas, 1991).

5.1.1.1: MECHANISM OF ACTION OF LCAT

The reaction catalysed by LCAT is depicted in **Fig 5.1** below.

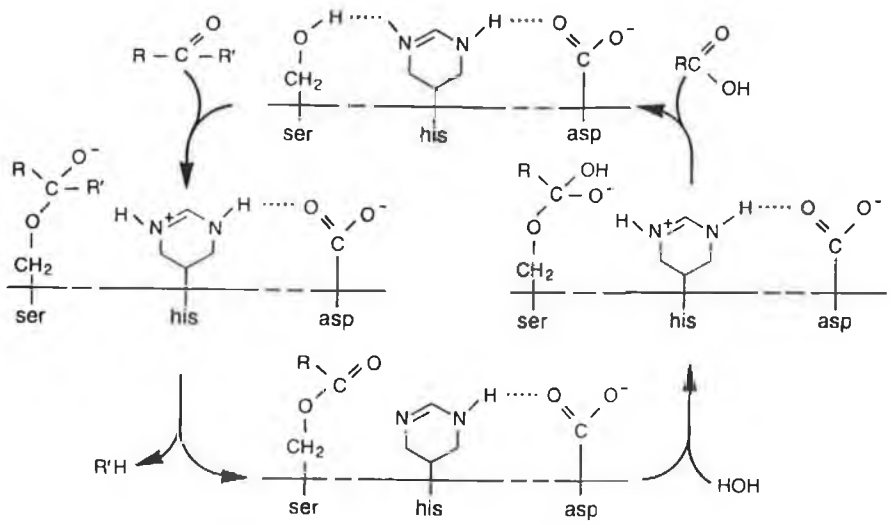
Fig. 5.1 Reaction catalysed by LCAT



An acyl group is transferred from phosphatidylcholine (PC) to free cholesterol. Under physiological conditions LCAT reacts with cholesterol derived from the membranes of blood cells or vascular cells, or with that from the plasma lipoproteins (Fielding, 1990). Jonas (1986b) found that, in general, mixed chain PC particles are the preferred substrates of LCAT, followed by disaturated PCs of increasing chain length and by long chain polyunsaturated-PC particles. Much published work on the acyltransferase activity of LCAT reports that it is specific for the sn-2 position of PC, however, recent work by Subbaiah *et al.*, (1994) showed that LCAT derived a significant percent of acyl groups from the sn-1 position of certain PC species. They also found that the positional specificity was determined at the formation of acyl-enzyme intermediate and that such specificity is influenced by the structure of PC, especially the chain length of the sn-2 acyl group.

The mechanism of action of LCAT is as for chymotrypsin and trypsin, as it is considered to belong to the same family of serine hydrolases. Fielding (1990) proposed such a mechanism, which is shown below in **Fig 5.2.**

Fig 5.2 Mechanism of action of LCAT



The proposed triad comprises of Asp-Ser-His which are linked by a charge relay system. This triad, according to Fielding (1990), is involved in the deacylation of phospholipid substrates, formation of a Ser-O-acyl intermediate and subsequent transfer of the acyl chain to cholesterol or water. Jauhianinen and Dolphin (1986), demonstrated that a single serine and histidine residue within the active site of human LCAT, catalysed the cleavage of lecithin with the formation of a fatty acyl-enzyme intermediate (oxyester) involving serine, that is- the phospholipase A_2 step of the reaction. They also postulated that the two Cys residues participate in the cholesterol esterification step only (forming a thioester), **Fig 5.3**. Various results conclude that the rate-limiting step is in one involving the phospholipase activity (Yogoyama *et al.*, 1980).

Although the reaction involving LCAT occurs on the surface of HDL particles, the reactants (PC and cholesterol) and the products (lyso-PC- which binds serum albumin for removal and cholesteryl esters) of this reaction are widely distributed (Jonas, 1986b). This results from HDLs interacting with other lipoproteins- where free cholesterol and phospholipids are transferred into HDLs from LDL and VLDL and also the exchange of triglycerides in the other direction- into VLDL and LDL from HDL. This overall catalytic reaction is considered to be only partially reversible.

Once synthesised, cholesteryl ester moves into the interior of the HDL particle and some of it is exchanged for triglyceride in other lipoproteins by cholesteryl ester transfer protein. This form of cholesterol accumulates in LDLs and cells which require free cholesterol take up the contents of these LDL particles via the LDL receptor pathway. Thus, LCAT plays an important role in the metabolism of cholesterol by helping HDL to accept additional free cholesterol from other lipoproteins or from cell membranes. Larger HDL particles then deliver their cholesteryl ester content to the liver for metabolism. This concept is explained in more detail in Chapter 1.

5.1.1.2: Interaction of LCAT with HDLs

As referred to above, this reaction takes place on the surface of HDLs. The number of apolipoprotein A-I molecules that are present on the lipoprotein has been reported to be proportional to their activating effect (Yokoyama *et al.*, 1980). LCAT requires apo A-I and apo A-IV for optimal activity and seems to act preferentially on the smaller high-density lipoprotein particles, including pre-beta HDL and HDL_3 (Gillett and Owen, 1992). It is also stimulated, to a lesser extent, by apo C-I and apo-E. Chen and Albers (1986) found that apo A-II must be incorporated together with apo A-I into lecithin-cholesterol liposomes to exert its stimulatory effect on LCAT activity and that apo A-II in HDL may play an important role in the regulation of LCAT activity. Present in high concentrations, apo A-II and serum amyloid A inhibit the LCAT reaction by apparently displacing apo A-I from the liposome substrates or from HDL in plasma (Steinmetz *et al.*, 1989).

Fig 5.3 LCAT triad (Jauhianinen and Dolphin, 1986)

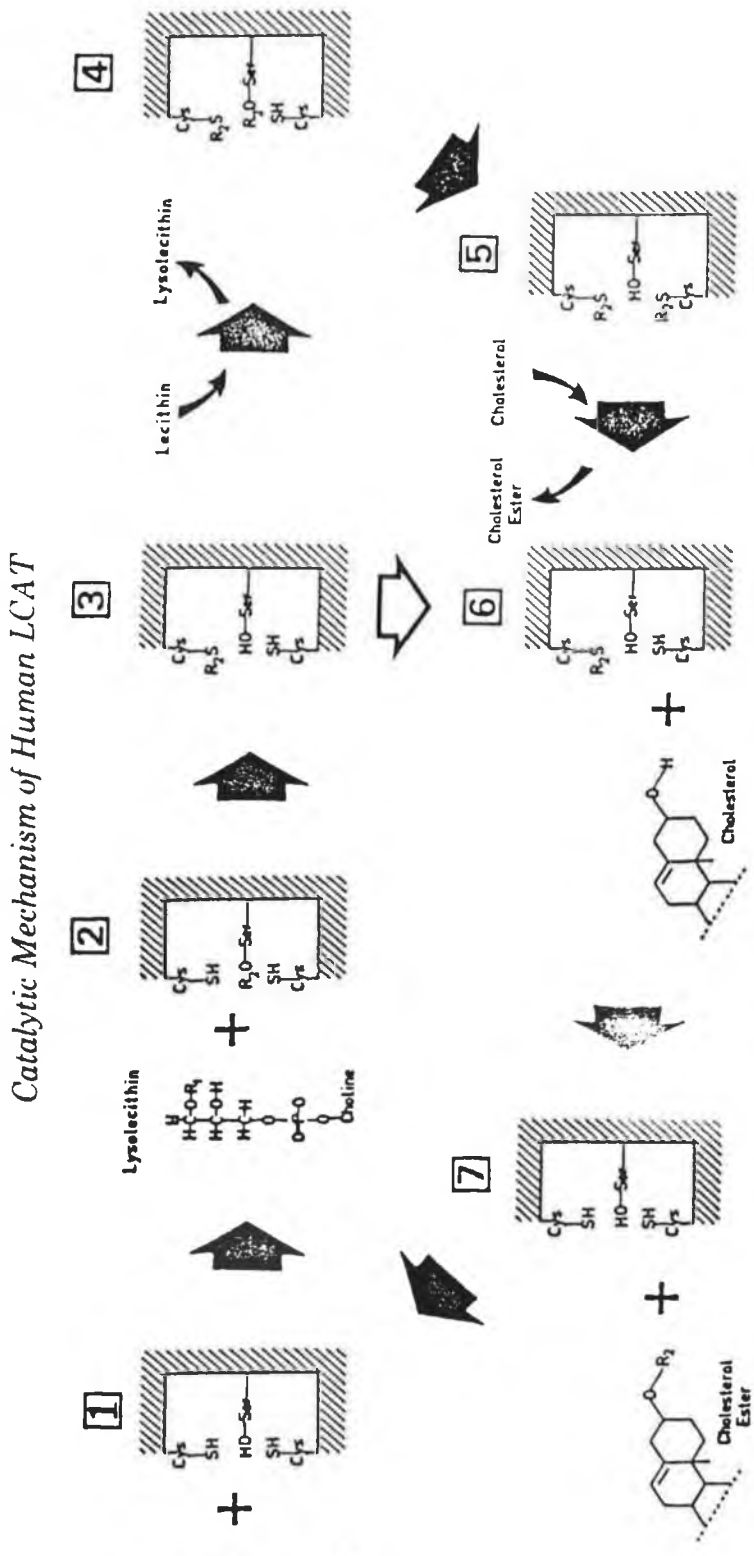


Fig 5.3. A proposed chemical mechanism of action for lecithin-cholesterol acyltransferase. The LCAT reaction is viewed as a series of transesterification reactions with chemical mechanisms similar to that of the initial cleavage of the lecithin *sn*-2 ester bond and formation of the serine oxyester (2). The fatty acyl group (R_2) esterified to serine is then transesterified to either of the 2 cysteine residues forming a thioester (3). The enzyme may then either cleave a second lecithin molecule forming a second serine oxyester (4) and then a second cysteine thioester (5) or transfer the first fatty acyl group attached to cysteine to cholesterol (6). The di-thioesterified enzyme (5) transfers both fatty acyl groups to cholesterol molecules in a sequential fashion, thereby regenerating the fully reduced enzyme. Histidine (not shown) is viewed as participating in the initial hydrolysis of lecithin and formation of the serine-fatty acyl intermediate and possibly in the subsequent transacylation reactions.

It appears that the interaction of apolipoproteins with different PCs produces different interfaces which are recognised differently by the enzyme. Evidence which highlights the importance of a) the structure and conformation of apolipoprotein and b) the interface between it and the lipid, comes from various results and observations, which include the following:

* chemical modification of certain lysine residues in apo A-I, which did not affect the overall structure of apo A-I, did not lower the overall LCAT activation process. However, charge interactions and structural changes of apo A-I were found to be responsible for the observed decrease in activating capacity. Such results indicate that the activation of LCAT by apo A-I *in vivo* depends on the structure and charge of the apolipoprotein, which may be altered by mutations, changes in HDL size and composition, or by interactions with macromolecules or ions (Jonas *et al.*, 1985).
* Various apolipoproteins of HDL, reacted with LCAT resulted in different levels of activation of the enzyme
* Synthetic substrates for LCAT may be synthesised in different sizes, thus having varying structures of apolipoproteins associated with the complex.The smallest sized particle, as studied by Jonas and McHugh (1984), was found to be the most reactive toward LCAT.

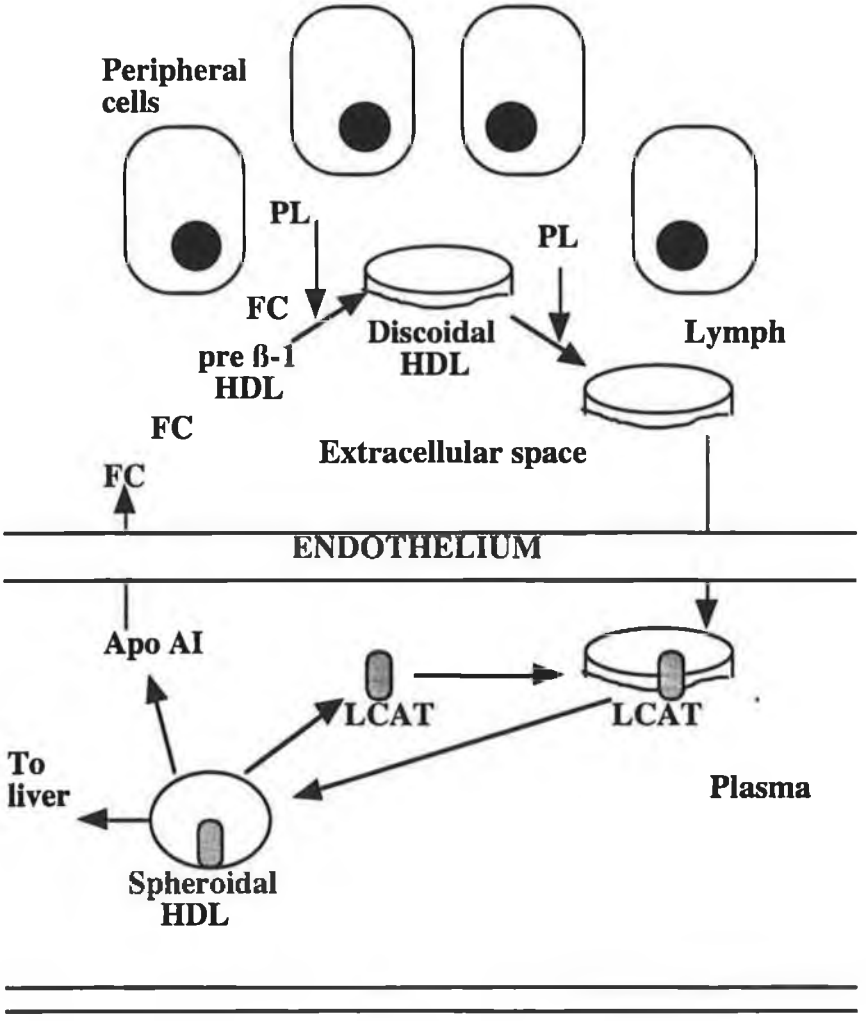
5.1.1.3: Transformation of lipoprotein by LCAT

Upon LCAT interacting with apolipoprotein on the surface of the HDL particle, morphological changes occur in the appearance of the latter. It changes from being discoidal in shape to taking on a spherical appearance, forming small, mature HDL particles. Observations made by Jonas (1991), upon reaction of LCAT with rHDL, containing two or more apo A-I molecules, involves the formation of cholesteryl esters, their accumulation in the central hydrophobic core of the complex, rearrangement of the surface components and also major fusion and disproportionation occurences which involve the protein and lipids of the complex.

When nascent HDLs are synthesised by the liver (**Fig 5.4**) and intestine (the HDLs of the latter contain only apolipoprotein A, while the former also contain apolipoprotein C) they are discoidal phospholipid bilayers containing apolipoprotein and free cholesterol. They acquire unesterified cholesterol on efflux from peripheral cells. After reaction with LCAT, the nonpolar cholesteryl esters move into the hydrophobic interior of the bilayer, the reaction continues to generate a nonpolar core that pushes the bilayer apart until a spherical pseudomicellar HDL is formed, covered by a surface of polar lipids and apolipoproteins.

Thus, it is evident that due to the fact that there are several different sizes and shapes of HDL particles, it is extremely difficult to study the reactivity of LCAT toward them, therefore the synthesis of particles of defined chemical composition, having the overall size and density of HDL was developed (Jonas, 1986), to study LCAT activity.

Fig 5.4 Synthesis of discoidal HDL in extracellular fluid.
PL: phospholipid, FC: free cholesterol , LCAT: lecithin-cholesterol acyltransferase (Fielding and Fielding 1995)



5.1.2: Measurement of serum LCAT activity

Various methods have been used in the analysis of serum LCAT activity, which use natural substrates (Glomset and Wright, 1964) and also synthetic substrates (Chen and Albers, 1982 and Jonas and McHugh, 1984). These methods are:

* the endogenous self-substrate method and
* the exogenous common-substrate method.

5.1.2.1: The endogenous self-substrate method

Such experiments use the subjects plasma as a substrate and enzyme source in measuring the esterification of cholesterol. Early studies carried out on plasma esterification involved measuring the extent of esterification occuring over a prolonged period of incubation. A decrease in unesterified cholesterol is measured by GLC after incubation of the plasma itself or LCAT activity is measured upon incubation of sample with [^{14}C]cholesterol and subsequently measurement of the incorporation of radioactivity into cholesteryl esters (Stokke and Norum, 1971). Nagasaki and Akanuma (1977), developed a colorimetric method for measuring LCAT activity, using cholesterol oxidase and peroxidase.

The above are a few examples of self-substrate methods and have certain draw-backs. Long preincubation and equilibration times may result in an alteration of the substrate properties of the plasma and labelled exogenous cholesterol may not be in complete equilibration with endogenous cholesterol (Chen and Albers, 1982).

5.1.2.2: The exogenous common-substrate method

The substrates- artificial liposomes (Batzri and Korn, 1973) or heated plasma (Glomset and Wright, 1964) are used in the measurement of LCAT activity, where radiolabelled cholesterol ([^{14}C] or [^{3}H]) is converted into cholesteryl ester. A small quantity of sample plasma is used in such assays. However, it has been reported by Blomhoff (1974) that when different inactivated substrate sources are used, the resulting LCAT activity measurements differ. Also, the use of heated plasma is not ideal (Albers *et al.*, 1981) as it is a less efficient substrate than unheated serum and cannot be readily standardised.

5.1.2.2.1: Reconstituted high-density lipoproteins/Micellar complexes Vs vesicles

The main components for most reconstitution experiments have been purified apolipoproteins (A-I, A-II, E or Cs), synthetic or natural phosphatidylcholines and free cholesterol (Jonas, 1986b). Micellar, discoidal complexes (non-aqueous internal compartment) were prepared as substrates for LCAT in this study. Preparation of such complexes and their formation is controlled, kinetically, by such factors as the temperature at which the synthesis is carried out; the concentration of cholesterol in the lipid bilayer; the type of apolipoprotein and PC; the PC vesicle size and composition

(Jonas, 1986a).

The temperature at which the PC is incubated with sodium cholate (in discoidal complexes prepared by detergent-mediation), equilibrated with apolipoprotein and the various stages of dialysis should be as close as possible to the gel to liquid-crystalline phase transition temperature (T_c) of the PC. 4^0C is an acceptable temperature to work with when using PC such as egg-PC. Matz and Jonas (1982), described a method for the synthesis of discoidal complexes based on the dispersal of lipids in sodium-cholate (which allows for the incorporation of many types of phospholipids into the complexes) and the subsequent removal of detergent in the presence of apolipoprotein.

Much evidence exists on the surfactant-like action of apolipoprotein A-I on multilamellar or unilamellar vesicles to produce micellar complexes, resembling HDLs (Swaney, 1980 and Atkinson *et al.*, 1976). Apolipoprotein may intercalate with lipid vesicles by: cosonication of all the components (products are not very reproducible); spontaneous interaction of the lipid vesicles and the apolipoprotein (this method may only be used with certain PCs and there are restrictions due to the nature of the lipid vesicles) or finally, by the commonly used detergent-mediated method. The last method has the added advantage over the "spontaneous" method as it allows, due to the dispersion of the lipids, apolipoprotein to penetrate the bilayer of the complex. The resultant product consists of a bilayer disc surrounded by a peripheral ring of protein which is mostly present in the α-helical structure. Micellar complexes, formed when apolipoprotein interacts with vesicles, differ to the latter in that vesicles have aqueous inner compartments, their inner phospholipids being protected from external influences, while the phospholipid head groups of micellar, discoidal complexes are fully exposed. Another main difference between these two substrate vehicles is that there are associated problems with vesicle stability, while discoidal complexes are reportedly more stable. Differently sized particles may be synthesised by varying the proportion of PC to apo A-I.

However, it must be remembered that there are certain ratios that must be selected carefully, such as the molar ratio of sodium cholate/PC, which ideally should be from 1/1 to 2/1, yielding a suggested sodium cholate concentration of 3 mg/ml (Jonas, 1986a). Work carried out by Matz and Jonas (1982), on the synthesis of micellar complexes, consisting of egg-PC, apo A-I and cholesterol, showed that when their results were examined in light of others, that when the molar ratio of PC/cholate was below 1/2, the major resultant products were small spherical micelles; ratios from 1/2 to 2/1 yielded bilayer discs of PC and cholate which were stabilised by an annular arrangement of cholate molecules and finally, ratios above 2/1 yielded PC mutilamellar compexes which allowed the intercalation of cholate. This, combined with the use of the correct T_c, usually improves the micellisation of the process.

Physical and chemical analysis may be carried out on these complexes. Determination of their size and structure by gel filtration, transmission electron microscopy, gradient gel electrophoresis, analytical ultracentrifugation, static fluorescence polarisation and quasielastic light scattering have been described, in brief by Jonas (1986a).

5.1.3: Requirements for LCAT activity

The enzyme, when studied with artificial substrates, seems to show a preference for the sn-2 position of phosphatidylcholine, while recent studies also indicate that LCAT derived a significant percent of acyl groups from the sn-1 position of certain PC species (Subbaiah *et al.*, 1994). On the basis of their work, it was postulated that the active site of human (and pig) LCAT was not sufficiently large to accomodate the long chain polyunsaturated acyl groups (PC 16:0-20:4), thereby utilising the group at position sn-1 instead, whereas rat and mouse LCAT preferentially derived their acyl groups from the sn-2 position. Subbaiah *et al.*, (1994), concluded that the substrate and positional specificities of LCAT are controlled by the central domain of LCAT protein, corresponding to the amino acid residues 130-306.

Kitabatake *et al.*, (1979) found LCAT specificity to be broad, whereby sterols and long chain primary alcohols exhibited acceptor activity. Tilvis and Miettinsen (1980), concluded that sterols without the methyl group at position 4 of the steroid nucleus were esterified by LCAT. Piran and Nishida (1979), recorded that LCAT required that the sterol acyl acceptor possess a 3ß-hydroxyl group and a *trans* configuration of the A/B rings.

5.1.4: Acyl CoA:cholesterol acyltransferase: Intracellular cholesterol esterification

The regulation of cholesterol homeostasis in cells appears to be vital for their proper functioning. The cholesterol of lipoproteins entering the cell, the rate of intracellular cholesterogenesis, also the rate of cholesterol excretion from the cell by means of bile acid synthesis and by lipoproteins, are all processes which are balanced within liver cell. Acyl-coenzyme A:cholesterol acyltransferase, (ACAT), is an intracellular enzyme responsible for the esterification of cholesterol, the storage form.

The most important function of this enzyme is to act in conjunction with hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and the low-density lipoprotein (LDL) receptor system to minimise free cholesterol fluctuations in the cell (Szanto *et al.*, 1994). ACAT is an integral protein of the rough endoplasmic reticulum. The metabolic role of ACAT was first studied in cultured skin fibroblasts. When cholesterol uptake by receptor-mediated endocytosis take place, the result is that cholesterol synthesis is reduced and its esterification with fatty acids, catalysed by ACAT, takes place, accompanied by down-regulation of receptor expression and decreased synthesis of free cholesterol.

5.1.4.1: Location of ACAT

ACAT has been identified in various tissues, apart from the liver. These tissues include the adrenal cortex (and other steroidogenic tissues); the arterial and venous tissue and finally, in the intestine (Suckling and Stange, 1985). Billheimer and Gillies (1990), concluded that the activity of ACAT in these tissues is found to vary greatly, with specific activities being greatest in those organs which play a major role in cholesterol metabolism. The tissue with the highest activity was the liver, which was similar to the intestine, the decreasing order was ovary, mammary gland, aorta, placenta, endometrium and finally, the gallbladder.

5.1.4.1.1: ACAT activity in the steroidogenic tissues

The importance of the intracellular pool of esterified cholesterol varies between steroid hormone-producing tissues (Suckling and Stange, 1985). Relatively little ester is found in the Leydig cell of the testis, while much ester is located in the adrenal cortex of the rat. However, measurements of the cholesteryl ester pools in such tissues is somewhat distorted by the presence of cholesteryl ester hydrolase. Most of the tissues referred to here, utilise cholesterol from lipoproteins, which is thought to be the main source for long term steroidogenesis, but the intracellular pool of esterified cholesterol also partakes, when the trophic hormone stimulates the tissue (Boyd *et al.*, 1983). Thus, the above steroid-hormone producing tissues demonstrate a complex metabolism of cholesterol. The resulting stored cholesteryl esters in these tissues may be hydrolysed by a hormone-sensitive cholesteryl ester hydrolase and the mobilised free cholesterol transferred to mitochondria where steroid hormone synthesis is initiated (Gillett and Owen, 1992).

5.1.4.1.2: ACAT activity in the venous and arterial tissues

Cholesterol metabolism in the arterial wall is predominantly a function of three enzymes, ACAT, and acidic lysosomal cholesteryl ester hydrolase and a neutral cholesteryl ester hydrolase (Billheimer and Gillies, 1990). Other enzymes include LCAT and cholesterol esterase (Ross and Glomset, 1973), ACAT being the one of most significance, quantitatively (Billheimer and Gillies, 1990). In atherosclerosis, there is a documented increase in the accumulation of cholesteryl esters in the arterial wall. Some argue this statement, suggesting that the increase in ACAT activity and subsequent increase in cholesteryl ester deposition is just a consequence of the increased availability of cholesterol, as a substrate for ACAT. The arterial tissue is also capable of synthesising its own cholesterol. The activity of aortic ACAT is similar to the intestinal and liver forms in that it is preserved by reducing agents and inhibited by detergents (Proudlock and Day, 1972; Morin *et al.*, 1974).

From the order of ACAT activities in varying tissues, referred to above, it is noted that the activity of ACAT in arterial tissue is not very high. This may be attributed, in part, to the fact that ACAT, assayed microsomally, in liver preparations having high levels of endoplasmic reticulum, is of a high activity, whereas aortic microsomal preparations

which are rich in plasma membrane and poor on endoplasmic reticulum, have significantly lower activities of the enzyme (Billheimer and Gillies, 1990).

5.1.4.1.3: ACAT activity in the intestine

ACAT is known to catalyse the esterification of cholesterol in the intestine of many different animals (Field and Salome, 1982) with highest activity found in the jejunum and proximal ileum of the small bowel (Field *et al.*, 1982). Human and rat activities are both highest in this region, while rabbit intestine is of a higher ACAT activity than rat intestine (Devery *et al.*, 1987). Its activity parallels cholesterol absorption in the intestine and suggests that the differing activities observed in various intestinal segments is due to substrate supply . In contrast to the esterification of dietary or lipoprotein cholesterol, there seems to be limited esterification of endogenously synthesised cholesterol by the intestinal muscosa (Billheimer and Gillies, 1990). Intervention in the adsorption of cholesterol by giving cholestyramine (which increases cholesterogenesis) to experimental rats, was shown not to have any effect on the activity of ACAT in the rat intestine (Stange *et al.*, 1983). This may be due to cholesterol being preferentially used for bile acid synthesis.

The activity of ACAT in the intestine of 3-week old diabetic rats was reported to be increased with respect to controls (Jiao *et al.*, 1988). When intestinal ACAT activity was suppressed by compound 58-035, there was a resulting malabsorption of cholesterol, both in the cholesterol-fed rabbit and in the lymph-fistula rat with a cholesterol bolus or infusion into the stomach or duodenum (Stange *et al.*, 1981 and Bennett and Tercyak, 1984). In the intestine, cholesteryl esters that are formed become associated with chylomicrons (Burrier *et al.*, 1994). Thus, intestinal ACAT activity is actively involved in the esterification and in the absorption process of cholesterol.

5.1.4.1.4: ACAT activity in the liver

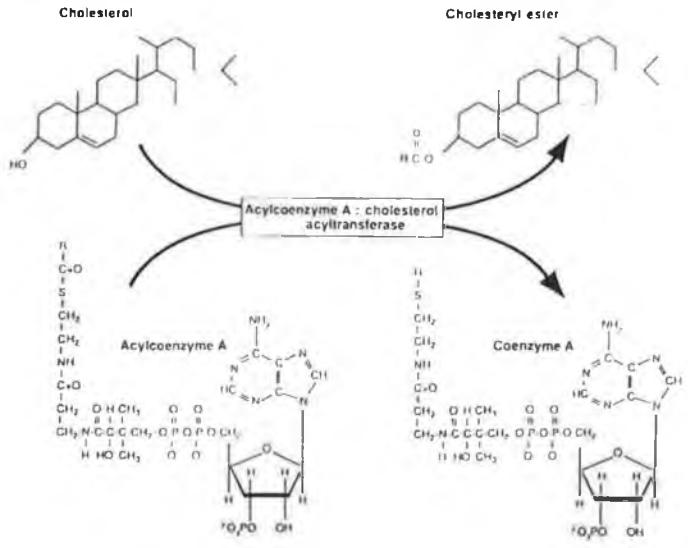
ACAT has been shown to be located in the rough endoplasmic reticulum (RER) (Reinhart *et al.*, 1987). The lack of ACAT activity in the smooth endoplasmic reticulum (SER), has been suggested by Billheimer and Gillies (1990), not to be due merely to the loss of ribosomes since *in vitro* agents, which remove the ribosomes from the RER result in an increase in ACAT activity. The RER has less cholesterol than SER, thus ACAT may play a part in the biogenesis of SER and in protein synthesis by regulating the cholesterol levels of the RER. It is an intrinsic protein, which although it has been solubilised, has not been purified (Erickson *et al.*, 1994).

Balasubramaniam *et al.*, (1979) demonstrated, with human liver ACAT, that the preferred substrate was endogenous cholesterol and that exogenously added cholesterol was not well utilised by ACAT. Others report that ACAT, in human liver microsomes, can utilise exogenous cholesterol as substrate (Einarsson *et al.*, 1989). In hepatocytes, cholesteryl esters synthesised by ACAT are incorporated into VLDL and secreted.

5.1.5: Mechanism of action of ACAT

The mechanism of action of ACAT is depicted in **Fig 5.5**.(Gillett and Owen, 1992) where an acyl group is transferred to the 3ß-position of cholesterol, from acylcoenzyme A, resulting in the formation of cholesteryl ester and free coenzyme A.

Fig 5.5 Mechanism of action of ACAT



5.1.6: Substrate specificity of ACAT

Rat liver ACAT has a high degree of specificity for cholesterol. The ability of sterols to be esterified by ACAT declines sharply with either the reduction or extension of the length of the side-chain from that normally observed in cholesterol (Billheimer and Gillies, 1990), thereby, indicating that the side chain is very important in the binding of the substrate in the active site of the enzyme. This would suggest that sterols with an oxygenated side-chain, would not be esterified at an appreciable rate by ACAT. However, work carried out by Lichtenstein and Brecher, (1983) demonstrated that 25-hydroxycholesterol was esterified by a microsomal enzyme with properties similar to ACAT. Intestinal ACAT was found to esterify 25-hydroxycholesterol (Field and Mathur, 1983). Sterols with the molecular length of cholesterol, were shown to be optimal in the esterification by ACAT (Tavani *et al.*, 1982). Goodman *et al.*, (1964) found that oleoyl-CoA was the best substrate followed by palmitoyl-, stearoyl- and linoleoyl-CoA. A part of this selectivity may be due to the presence of acyl-CoA hydrolase in the microsomal preparations (Billheimer, 1985). The latter enzyme hydrolyse oleoyl-CoA at a rate an order of magnitude greater thatn its esterification (Berge, 1979).

ACAT is known to esterify precursors of cholesterol in the liver (Brady and Gaylor, 1971). Sterols that contain a *gem*-dimethyl group at position C-4 (e.g., lanosterol) are not esterified by ACAT. The lack of esterification in such a case, may be attributed to steric hindrance produced by the positioning of the methyl groups at C-4 on the reactivity of the 3ß-hydroxyl group (Tavani *et al.*, 1982). Although these sterols were not found to be substrates for ACAT, they had no inhibitory effects on the enzyme. Neither the presence

of unsaturation in the B-ring, nor the positioning of a double bond at C-5 (as in cholesterol) seem to be critical in the esterification by ACAT (Tavani *et al.*, 1982). But, deformation of the B-ring, as is observed in 7-ketocholesterol (conjugated ketone), reduces the esterification of the substrate by ACAT.

5.1.7: Regulation of ACAT activity

Relatively little is known on the regulation of ACAT, due to limited information on such matters as the organisation of the various movements of cholesterol within the cell and the communication between processes taking place such as the movement of cholesterol that has been newly synthesised from the endoplasmic reticulum; the movement of exogenously derived cholesterol out from the lysosome or the transfer of cholesterol among various intracellular pools. (Billheimer and Gillies, 1990).

5.1.7.1: Diurnal regulation of hepatic ACAT

Recent work by Szanto *et al.*, (1994) presents evidence for the first time that hepatic ACAT exhibits a characteristic low amplitude diurnal rhythm. Their work was inconclusive as to whether or not the diurnal rhythm was a direct result due to the changes in the availability of endogenous cholesterol substrate or to changes in the protein expression of the enzyme. This rhythm of hepatic ACAT activity showed an inverse relationship to that of HMG-CA reductase, under physiological conditions. Conflicting views argue that these rhythms may, or may not, be dependent on the cellular level of free cholesterol.

5.1.7.2: Substrate availability

It is believed that ACAT may not be saturated with cholesterol in the microsomal membrane. Experiments carried out on measuring ACAT activity, found that cholesteryl ester formation could be significantly increased upon addition of exogenous cholesterol. The supply of cholesterol, the effects of cholesterol on the enzyme itself by a possible allosteric machanism, the opposing effects of progesterone (Suckling and Stange, 1985) are also factors to be considered. Einarsson *et al.*, (1989) concluded from experiments on ACAT in human liver microsomes, that the effect of freezing on ACAT activity, which resulted in an increase, may be attributed to the fact that the freezing procedure made more endogenous cholesterol available to ACAT.

The amount of cholesterol available for esterification is determined by the amount of cholesterol in the metabolic pool and also on the movement of this cholesterol by various routes. These routes include the delivery of:
Cholesterol synthesised *de novo*, cholesterol acquired from plasma lipoproteins, that from the plasma membrane and that involved in the bile acid/steroidal hormone pathways.

The endogenous pool of cholesterol may be supplemented with exogenously added substrate, by means of detergent or organics. The use of Triton WR-1339 was reported by Billheimer *et al.*, (1981). More recently, Martin *et al.*, (1993) used cyclodextrin

vehicles to deliver substrates to microsomal enzymes. Such methods will be discussed in **5.1.8**

5.1.7.3: Other possible regulating factors

Lichtenstein and Brecher, (1983) found that 25-hydroxycholesterol was esterified by a microsomal enzyme (in rat liver), which when compared to the esterification of cholesterol, seemed to indicate that this enzyme was ACAT. Another oxide of cholesterol, 22-hydroxycholesterol was examined by Bates *et al.*, (1983). On the contrary, 25-hydroxycholesterol was found to stimulate cholesterol esterification, where Drevon *et al.*, (1980) postulated that the effects of this oxide may be due to its direct effect on ACAT. Neither hydrophobic nor hydrophilic bile salts were found to inhibit ACAT (Heuman *et al.*, 1988).

The possible short-term regulation of ACAT by phosphorylation/dephosphorylation has been documented. Suckling and Stange (1983), provided data which was consistent with a short-term, *in vivo* regulation of hepatic ACAT activity, while work carried out by Einarsson *et al.*, (1989) were not in accordance with this finding, as no activation/inactivation of ACAT by phosphorylation/dephosphorylation was observed.

5.1.8: Assay of ACAT activity

ACAT activity, in microsomes, is frequently measured by a radiochemical assay in which [^{14}C]oleoyl-CoA is converted to [^{14}C]cholesteryl ester. Upon completion of the reaction, the products are separated by means of TLC. Radioactivity of the cholesteryl eser is counted in a scintillation counter.

Assays which involve exogenous cholesterol as substrate, suffer from problems such as incomplete knowledge of the extent to which the exogenous cholesterol has mixed with the endogenous substrate. Increasing concentrations of fatty acyl-CoA initally promote formation of cholesteryl esters but, upon reaching the plateau region, the further increase in the concentration of this fatty acid rapidly inhibit esterification (Suckling *et al.*, 1985). Serum albumin is employed in order to minimise the inhibiting effect of high concentrations of oleoyl-CoA on ACAT activity, as this fatty acid is a detergent, which may disrupt the microsomal membrane.

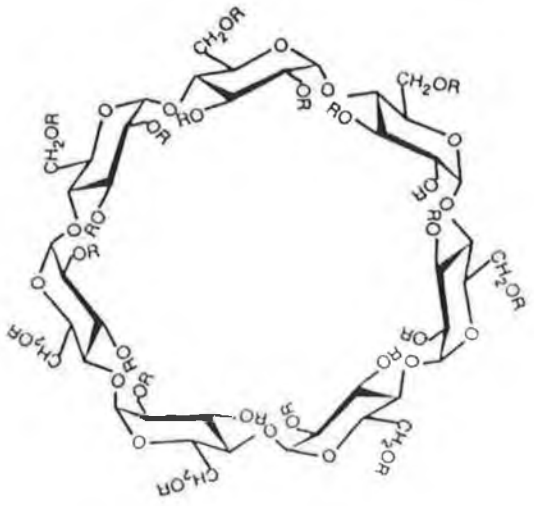
The most commonly used assay procedure for ACAT activity requires a [^{14}C]-labelled fatty acyl coenzyme A as substrate. The addition of a radiolabelled internal standard, such as [^{3}H] cholesteryl ester, should be added to the assay mixture at the extraction of the products stage, to account for losses in the procedure and estimate recovery. The products are then run on TLC plates, under suitable conditions to separate cholesteryl ester from free cholesterol, but also from triacylglycerol- which is a potential contaminant (Suckling *et al.*, 1985).

As referred to above, it is believed that ACAT is not saturated with cholesterol in the microsomal membrane. The membrane may be dissociated with a detergent, thereby, allowing the addition of exogenous cholesterol. This procedure allows for the removal of the constraining environment around the enzyme. The exogenous substrate may be added dissolved in an organic, such as acetone (Billheimer *et al.*, 1981), where no stimulation of the enzyme was observed, while cholesterol, being added under the same experimental conditions, except suspended in Triton WR-1339, resulted in a significant stimulation of ACAT. Cholesterol may also be added as cholesterol-rich liposomes (Gillett and Owen, 1992).

5.1.8.1: The use of Cyclodextrin in studies on cholesterol metabolism

Such "carrier" molecules of fat soluble compounds (e.g., vitamins and hormones) are frequently used to facilitate dissolution. Cyclodextrins are cyclic oligosaccharides consisting of 6,7, or 8 glucopyranose units, usually referred to as α-, ß- or χ-cyclodextrins respectively. They are naturally occuring compounds which have relatively rigid doughnut-shaped structures and act as natural complexing agents (Sigma). The overall structure of ß-cyclodextrin is shown in **Fig 5.6**. The arrangement of C6-hydroxyls opposite the hydrogen bonded C2- and C3-hydroxyls forces the oxygen bonds into close proximity within the cavity. The number of glucopyranose units which make up the cyclodextrin determine the size of the hydrophobic cavity.

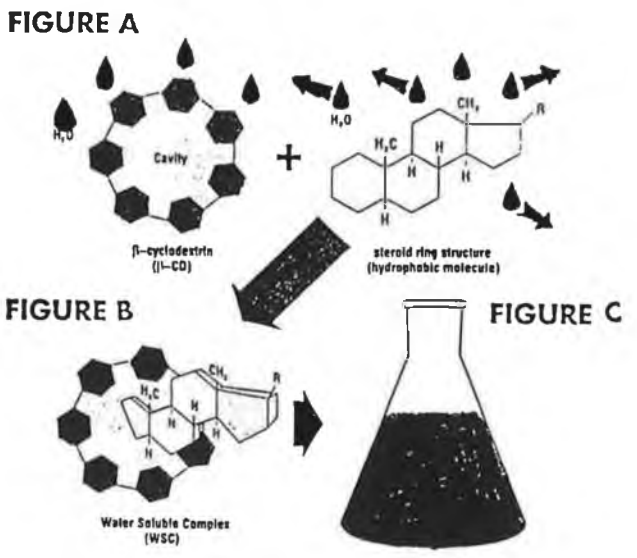
Fig 5.6 Structure of ß-cyclodextrin. In hydroxyethyl ß-cyclodextrin (HEBC), some H's of the OH groups are replaced by CH_2CH_2OH, in hydroxypropyl ß-cyclodextrin (HPBCD) by $CHCHOHCH_3$ (Sigma information leaflet, 1995).



The mechanism of action of these agents is simple. The hydrophobic molecules are incorporated into the central cavity by the displacement of water molecules. This is represented in **Fig 5.7**. The repulsion by water, of the molecule, results in the encapsulation of the desired molecule- rendering the molecule water-soluble.

Both bile acids and cholesterol form complexes with nondegradable derivatives of cyclodextrins (Gerloczy *et al.*, 1994). ß-cyclodextrin has been successfully employed in the solubilisation of microsomal cholesterol and also in the delivery of cholesterol, as substrate, in the enzymatic study of Cholesterol 7α-hydroxylase (Martin *et al.*, 1993). Thus, ß-cyclodextrin, due to the size of its cavity diameter, is suitable for use with molecules the size of hormones or vitamins (Sigma).

Fig 5.7 Schematic representation of incorporation of hydrophobic molecule and ß-cyclodextrin (**Fig A**), giving rise to encapsulated hydrophobic molecule in low-energy cavity of ß-cyclodextrin (**Fig B**). The encapsulated molecule is released when the water-soluble complex is dissolved in a large volume of free H_2O molecules (**Fig C**) (Sigma information leaflet, 1995).



From chapter three, it is noted that a substantial percentage of 7α-hydroxycholesterol was found to be esterified in the serum samples examined. This is in accord with other reports (Streuli *et al.*, 1981 and Oda *et al.*, 1990). Thus, there have been studies carried out on the possible mechanism of its esterification. Such studies and their findings will be discussed in detail in the following chapter (chapter 6). Earlier work was carried out on the possible hydroxylation of cholesterol esters, which indicated that this did not result in the formation of 7α-hydroxycholesterol esters. Thus, work was then carried out on the hepatic cholesterol esterifing enzyme- ACAT. However, to date, it appears that no study has been carried out on the possible esterification of 7α-hydroxycholesterol by the plasma cholesterol esterifing enzyme- LCAT.

In the following section, the partial purification of LCAT from a bovine source is described. Synthetic substrates, resembling HDLs were synthesised, containing cholesterol or 7α-hydroxycholesterol as substrates for the enzyme. Work was also carried out on the microsomal enzyme- ACAT, with respect to its possible contribution to the presence of esterified 7α-hydroxycholesterol in plasma. Again, a bovine source of enzyme was used. The method of analysis of 7α-hydroxycholesterol, described in chapter 3, is utilised here also. The results of our findings are reported in this chapter, while the significance of our results will be discussed in the overall discussion.

Aims of "Reactivity between 7α-hydroxycholesterol and cholesterol acyltransferase enzymes in serum and liver":

* To synthesise a suitable substrate carrier, containing 7α-hydroxycholesterol or cholesterol for reaction with LCAT (lecithin-cholesterol acyltransferase)

* To partially purify this enzyme from bovine plasma

* To examine the possible reactivity of ACAT (acyl CoA:cholesterol acyltransferase) with 7α-hydroxycholesterol

5.2 Results

The demonstration that an appreciable amount of 7α-hydroxycholesterol was esterified in both human and rat serum prompted a study to determine the origin of its esterification. The previous chapter provided evidence that serum 7α-hydroxycholesterol may be hepatic in origin. A possible route of metabolism might be esterification by acyl CoA cholesterol acyltransferase (ACAT) within the liver of any 7α-hydroxycholesterol which had escaped from the bile acid biosynthetic pathway prior to secretion into the circulation. Another route may be the esterification of any free sterol form in serum by lecithin:cholesterol acyltransferase (LCAT).

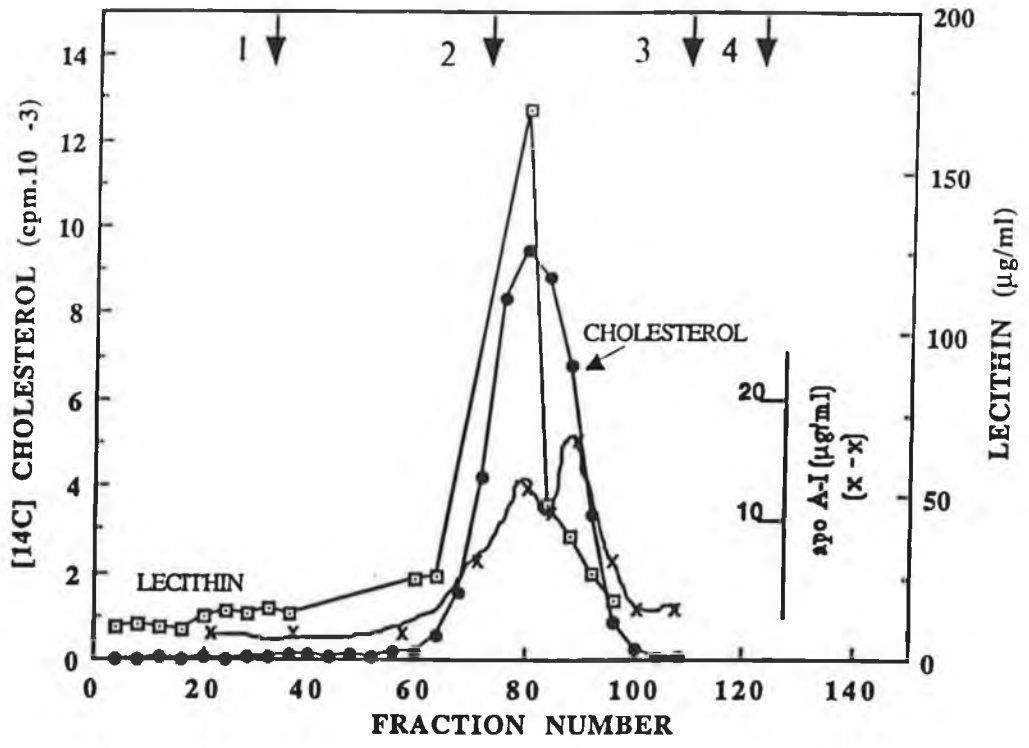
In order to investigate the esterifying potential of the serum enzyme LCAT, a suitable substrate vehicle needed to be developed. Thus, proteoliposome complexes containing apo A-I, lecithin and either cholesterol or 7α-hydroxycholesterol were prepared and characterised. Furthermore, a partially purified form of the enzyme, LCAT, was deemed a prerequisite for any study of the specificity of this enzyme with respect to acyl group acceptors. This chapter describes progress made in the partial purification of LCAT from bovine blood and in determining its reactivity with artificial substrates during incubations *in vitro*. The reactivity of hepatic ACAT activity towards 7α-hydroxycholesterol will be discussed later.

5.2.1 Characterisation of synthetic substrates for LCAT

5.2.1.1 Characterisation of cholesterol-containing complexes by gel filtration on a Sepharose CL-4B column

In order to develop a well defined, standardised and effective common artificial substrate proteoliposomal vesicles containing apo A-I, phosphatidylcholine and cholesterol (including [^{14}C]-labelled cholesterol) in the molar ratio of 1:100:9.1, were prepared by the cholate dialysis method as described in Chapter 2. A 2ml aliquot of this preparation containing 579µg apo A-I, 1.785mg phosphatidylcholine and 84.85µg cholesterol was applied to a Sepharose CL-4B column (2.2 x 50cm) column equilibrated with 10mMTris-HCl buffer pH 7.4, containing 150mM NaCl, 1mM NaN_3 and 0.05% EDTA. 110x2ml fractions were collected and analysed for protein, lecithin and cholesterol as described in Chapter 2. Gel filtration of these vesicles gave a predominantly single peak containing all of the micellar components initially introduced to the column (**Fig 5.8**). The elution profile showed peaks for both phosphatidylcholine and cholesterol at fraction 79. However, although there was a corresponding peak in the protein concentration at fraction 79, there was also a second higher peak observed at fraction 89. This may indicate the presence of a slightly smaller secondary proteoliposomal complex. Overall, the three components eluted over the same fraction numbers, indicating that complexes formed which contained apo A-I, phosphatidylcholine and cholesterol.

Fig 5.8 Elution profile of apo A-I, phosphatidylcholine (PC) and cholesterol complexes (molar ratio 1:100:9.1) from a Sepharose CL-4B (2.2 x 50cm) column. 2mls of the preparation, containing 579µg apo A-I, 1.785mg PC and 84.848µg cholesterol were applied to the column. The column was eluted with 10mM Tris, 150mM NaCl, 1mM NaN_3 and 0.05% (w/v) EDTA, pH 7.4 buffer. 2ml fractions were collected at a flow rate of 0.5ml/min and assayed for protein, cholesterol and PC as described in the methods. Arrows indicate the following calibration markers:
(1) Thyroglobulin (2) Ferritin (3) Catalase and (4) BSA.



The Stokes radius of the complexes was calculated to be 58Å, by extrapolation of a calibration plot as in **Fig 5.9**. A corresponding molecular weight of approximately 3.71 x 10^5 daltons was determined, again by extrapolation of V_e from the calibration curve, **Fig 5.9**.

5.2.1.2: Characterisation of cholesterol- and 7α-hydroxycholesterol-containing complexes by gradient gel electrophoresis of proteoliposomal complexes

The micellar complexes of apo A-I, phosphatidylcholine and cholesterol/7α-hydroxycholesterol, in the molar ratio 1:100:9.1 were analysed by electrophoresis on a native 3-20% polyacrylamide gradient gel, (both complex types, containing 10 nmoles sterol, were prepared by the sodium cholate dialysis method as described in Chapter 2). The cholesterol containing complexes, having passed through the Sepharose CL-4B gel for analytical purposes, were reversed dialysed against PEG-8000 in order to concentrate them for electrophoretic studies. Such complexes were run in Lane 1 (**Fig 5.10**). The protein markers- thyroglobulin (6.6 x 10^5), ferritin (4.4 x 10^5), catalase (2.1 x 10^4) and bovine serum albumin (6.7 x 10^4) were run in Lane 2. Finally, complexes containing 7α-hydroxycholesterol were run in Lane 3. It is apparent from the electrophoretogram (**Fig 5.10**) that both of the proteoliposomal complexes showed similar electrophoretic behaviour. Two electrophoretic bands of different intensities (Rf=0.28 and 0.51) were observed in the cholesterol-containing complexes. Their molecular weights were calculated by means of a calibration curve of relative mobility with molecular weight of each protein marker (**Fig 5.11**). Molecular weights of the upper and lower bands approximated 6.60 x 10^5 and 3.85 x 10^5 daltons respectively (**Fig 5.11**). The band of higher Rf (Mol. Wt., 3.85 x 10^5) was the more intense. Two bands (Rf=0.25 and 0.49) were also observed in the 7α-hydroxycholesterol-containing complexes. Molecular weights approximated 7 x 10^5 and 4 x 10^5 daltons. As observed in the cholesterol-containing complexes, the band of higher Rf (Mol.Wt., 4 x 10^5) was the more intense.

5.2.1.3 Electron microscopy studies

Transmission electron microscopy, using negative staining with 4% phosphotungstic acid (pH 6.8) was carried out in The Electron Microscopy Unit, University College Dublin. Prints of different fields were made using 3 fold magnification of the negatives. The complexes seen in **Fig 5.12** are those containing apo A-I, egg phosphatidylcholine and cholesterol in the molar ratio (1:100:9.1). The preparation of complexes was quite dilute so only relatively few complexes were examined. It is apparent that these micellar complexes are quite heterogenous in size. Arrows indicate diameter sizes: 10.908nm, 14.540nm, 18.180nm and 25.452nm.

Fig 5.9 Calibration curve for the measurement of molecular weight and corresponding Stokes radius of micellar complexes from their elution volume, upon passing through a Sepharose CL-4B column. Markers for calibration as in Fig 5.8- parenthesis refer to Stokes radius).

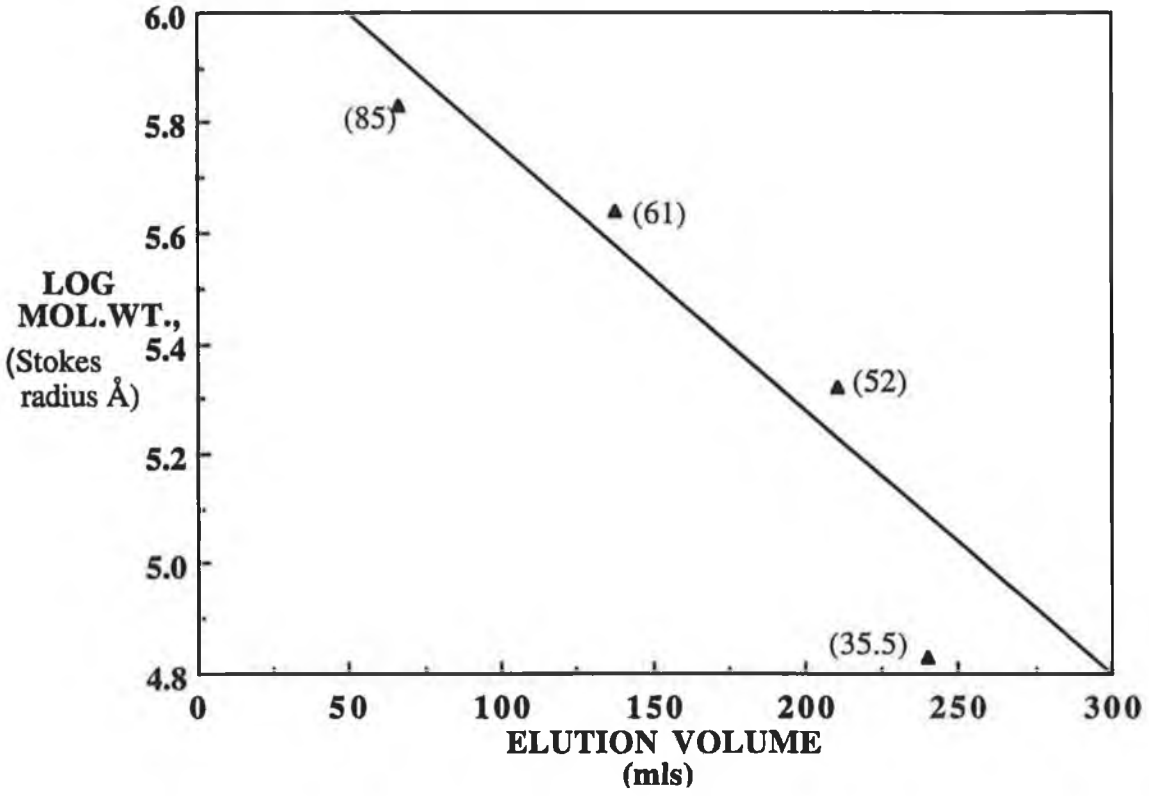


Fig 5.10: A 3-20% native gradient-gel electrophoresis of micellar complexes. The markers are indicated in Lane 2 and range from 68-669 kdaltons. Complexes stained with Coomassie Brillant Blue appear to be of two heterogenous sizes.

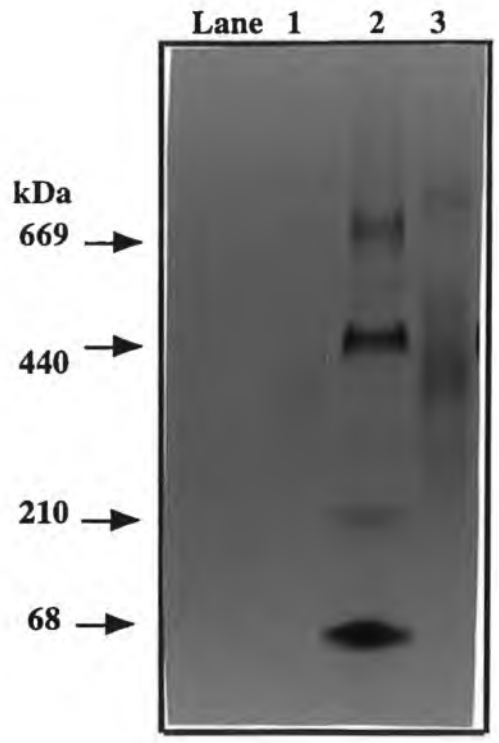


Fig 5.11 Logarithmic plot of molecular weight of standards versus their relative mobilty through a non-denaturing gradient polyacrylamide gel (3-20%).

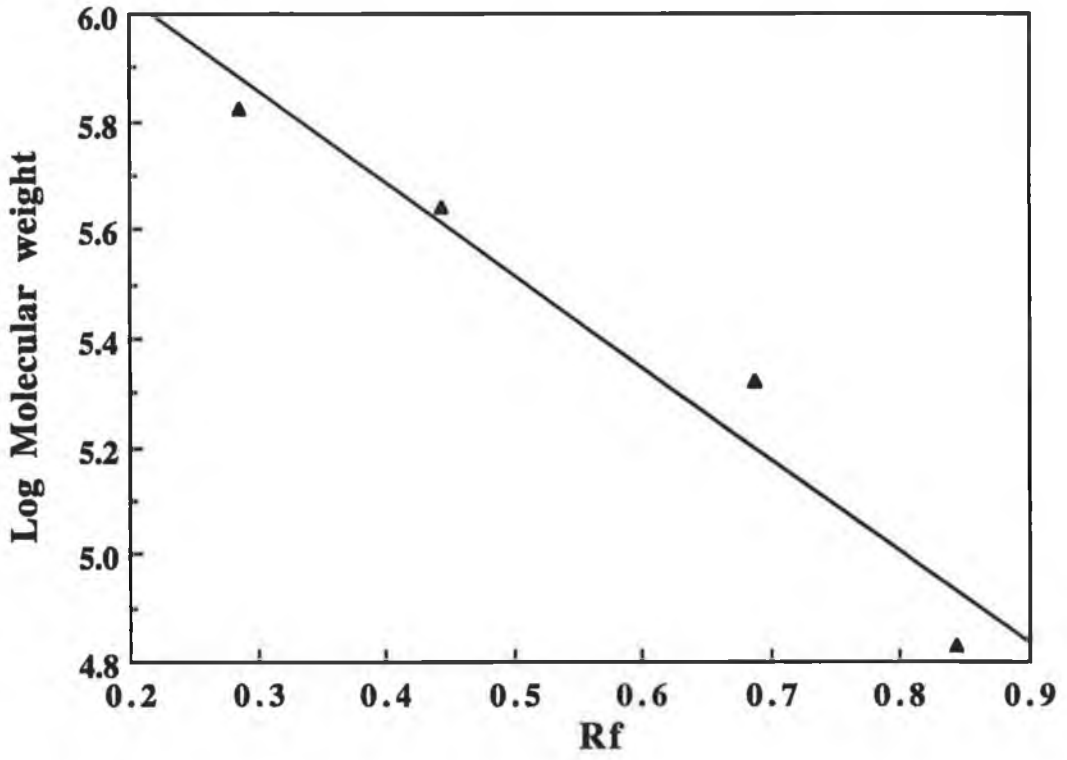


Fig 5.12: Electron micrograph of discoidal complexes. Discoidal complexes of bovine apolipoprotein A-I, egg phosphatidylcholine and cholesterol were prepared using a molar ratio of 1:100: 9.09. Microscopic analysis was performed as described in the experimental section. The bar represents 100nm.

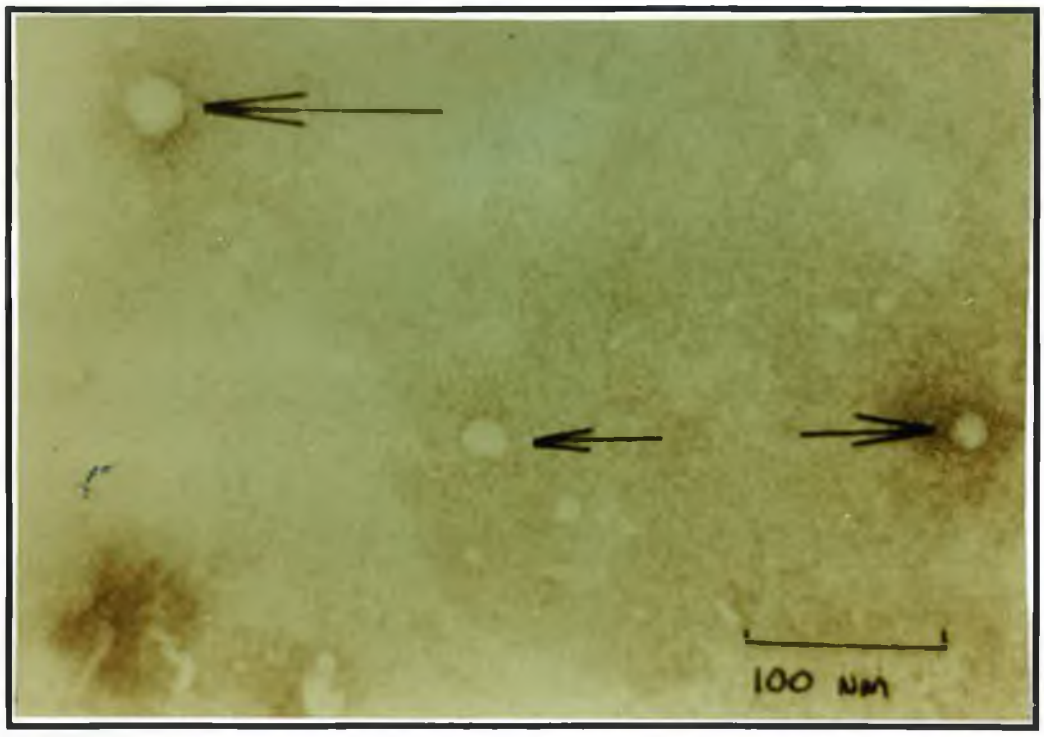


Fig 5.13(a) and **Fig 5.13(b)** show complexes of apo A-I, egg-phosphatidylcholine and 7α-hydroxycholesterol in the molar ratio (1:100:9.1). Again, this preparation was as for the cholesterol containing complexes above. The discoidal appearance of the complexes is more evident here. **Fig 5.13(a)** shows that slight stacking of the complexes has occured, while **Fig 5.13(b)** illustrates this stacking effect from the side. These complexes measure 14.54nm in diameter by 9.09nm (side view) and the spherical complexes were 10.908nm, the more disc shaped complexes being 10.908 x 18.18nm. Thus, size heterogeneity was also a characteristic of the 7α-hydroxycholesterol-containing preparations.

5.2.2 Partial purification of lecithin:cholesterol acyltransferase

Since most of the LCAT in plasma has been shown to be associated with the HDL fraction, the strategy used in the present purification (partial) was to first dissociate LCAT from lipoproteins by potassium bromide gradient ultracentrifugation. At an overall density of 1.21g/ml, lipoproteins float to the top of the tube, plasma proteins precipitate to the bottom while LCAT, dissociated from HDL, remains in the middle layer. Butanol/$(NH_4)_2SO_4$ treatment of the resulting enzyme lipoprotein fraction was followed by ion-exchange chromatography on DE-52, as described in Chapter 2. Activity of LCAT was measured using proteoliposomal complexes containing [^{14}C]-cholesterol as the substrate. 30μl of the proteoliposomal preparation, containing 10nmoles of free cholesterol were incubated with source of LCAT for 30mins at 37^{0}C. The reaction, lipid extraction, separation of [^{14}]C-cholesterol and [^{14}C]-cholesterol ester and determination of radioactivity were done as described previously in Chapter 2. The rate of cholesterol esterification is given as nmole of cholesterol esterified/hour and unit of enzyme designates the esterification of 1nmol of unesterified cholesterol/hour at 37^{0}C under the standard assay conditions in presence of apo A-I.

The results of a typical procedure for the partial purification of LCAT from 168ml bovine plasma are summerised in **Table 5.1**. Upon ultracentrifugation of the density adjusted plasma (∂=1.21g/ml), the middle fraction contained 72.7% of the LCAT, some activity was also found associated with the lower protein layer. Most of the protein was found to be in the bottom layer while the LCAT enriched middle layer contained only about 2.12% (336.8mg) of the total plasma protein (15.85g).Thus, most of the contaminating plasma lipoproteins were removed in this initial purification step. A significant 34- fold increase in the specific activity (20.15 units/mg) was always observed after dialysis of the middle ultracentrifugation fraction (∂ 1.21-1.25g/ml). After treatment with 1-butanol in the presence of ammonium sulphate, the specific activity was increased almost 100- fold (58.14 units/mg). This step, as may be seen from the SDS-PAGE gel shown later (**Fig 5.15**) removes a significant amount of the lower molecular weight proteins.

Figs 5.13 (a) & 5.13 (b): Electron micrograph of discoidal complexes of bovine apolipoprotein A-I, egg phosphatidylcholine, 7α-hydroxycholesterol in the molar ratio 1:100: 9.09. Microscopic analysis was performed as described in the experimental section. The bar represents 100nm in both micrographs.

Fig 5.13 (a)

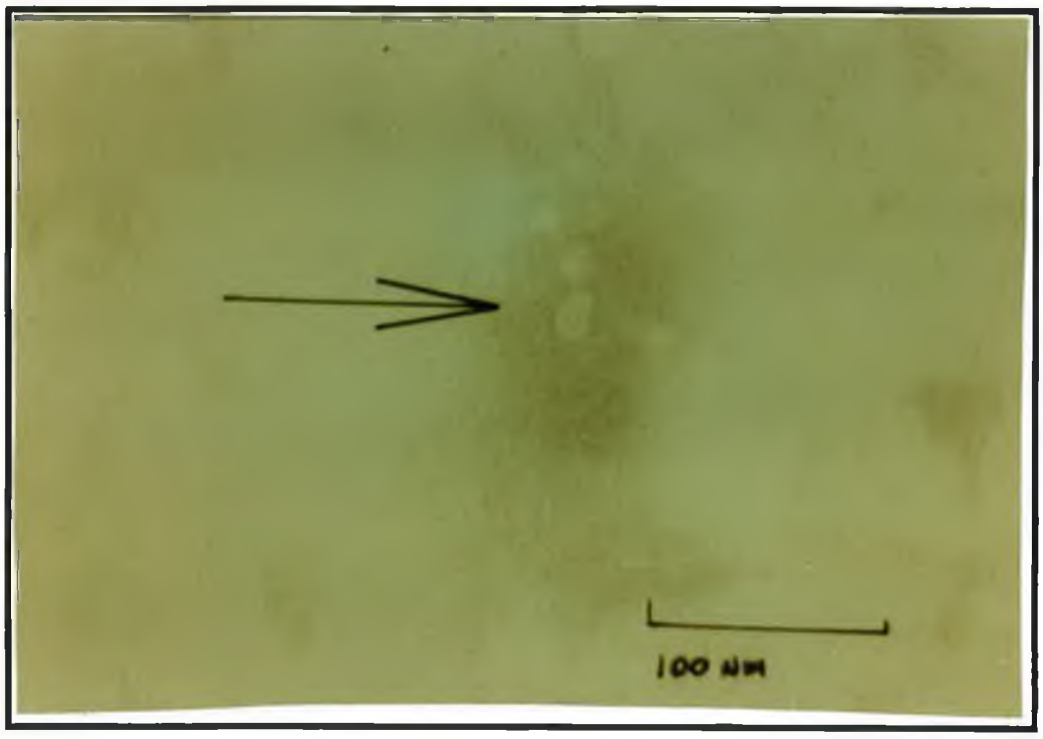


Fig 5.13 (b)

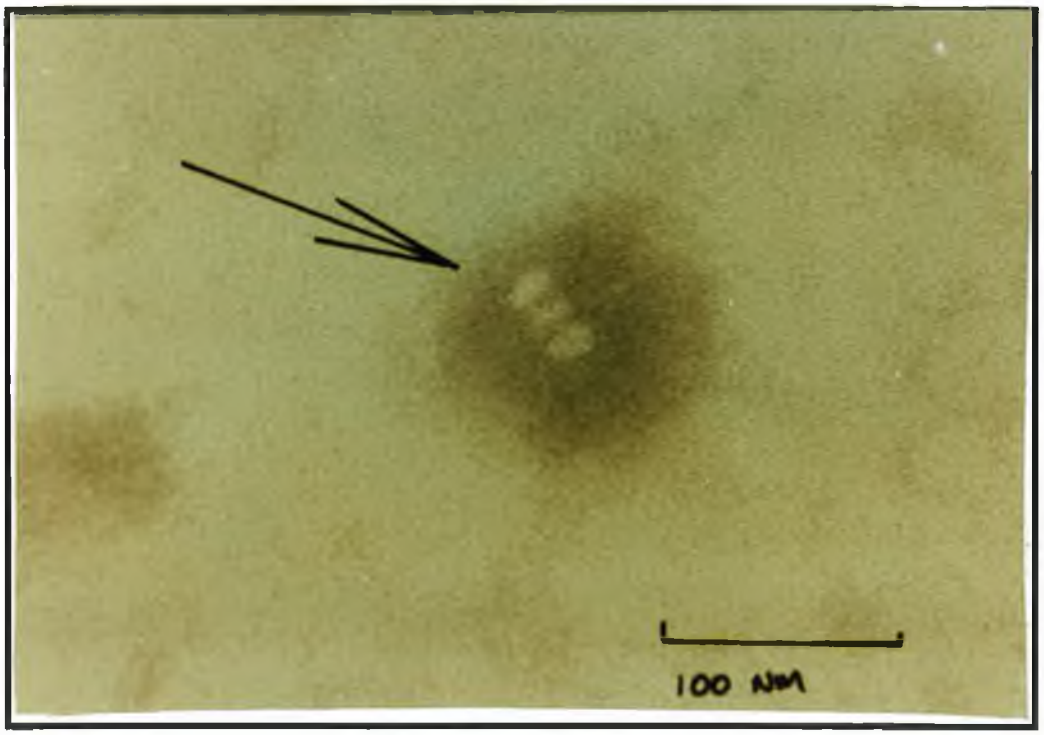


Table 5.1: Partial purification of Lecithin-Cholesterol Acyltransferase from bovine plasma.

	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (units*)	SPECIFIC ACTIVITY	YIELD (%)	PURIFICATION FOLD
BOVINE PLASMA (168ml)	**15854.84**	**9332.4**	**0.588**		
∂ 1.21-1.25 (g/ml)	**336.78**	**6785.8**	**20.15**	**72.7**	**34.27**
$(NH_4)_2SO_4$	**70.57**	**4102.94**	**58.14**	**43.96**	**98.88**
DE-52	**11.06**	**4067.76**	**367.79**	**43.59**	**625.49**

*** One unit of enzyme activity catalysed the esterification of 1nmole unesterified cholesterol per hour at 37°C with bovine apolipoprotein A-I, egg phosphatidylcholine and cholesterol complexes as substrate.**

The final step carried out in the partial purification of the enzyme involved the application of the dialysed ammonium sulphate fraction to an anion exchange column, DE-52. The fraction was dialysed against 10mM Tris-HCl, pH 7.6 containing 5mM EDTA and 50mM NaCl, which was the buffer used for the equilibration of the DE-52 resin. When the dialysed ammonium fraction was applied to the column, approximately 84% of proteins were removed **Fig 5.14** shows the elution profile of this step, indicating the fractions that were to be pooled. Three activity peaks were observed corresponding to elutions at salt concentrations of 110, 135 and 158 mM NaCl. It is apparent that DE-52 chromatography did not change the enzyme yield. All of the enzyme activity that was applied was recovered in 3 peaks of activity in 26 fractions (**Fig 5.14**). The final LCAT preparation, having a specific activity of 368 units/mg was purified approximately 625- fold, with 44% yield.

Aliquots of the various purification stages were analysed by SDS-PAGE under reducing conditions (**Fig 5.15**). Lane 1 (left) indicates the positions of the markers, which range from 32 kDal to 195 kDal. Lane 2 shows the electrophoretic pattern of raw plasma. Lane 3 shows the pattern for the dialysate of the middle fraction obtained from the ultracentrifugation step, while lane 4 shows the ammonium sulphate fraction and lane 5 the concentrated DE-52 fraction. All of these protein samples contained approximately 10µg protein per well, except for the DE-52 fraction which contained approximately 8µg protein. The final lane on the right is that of the DE-52 fraction. Here, four distinct bands and also a fifth weaker band were visible. Reference to the migration of the SDS-7B prestained molecular weight markers (Sigma) indicated the following M_r values: 33.5, 36, 47 and 62.5 kDals.

5.2.3 Kinetic studies of partially purified LCAT

A time course of LCAT activity in the presence of 10nmoles of proteoliposomal [^{14}]C-cholesterol and 40, 75 and 100µl partially purified enzyme is shown in **Fig 5.16/Table 5.2**. With all three enzyme concentrations examined, LCAT activity was linear with repect to time, up to 60 minutes. Approximately 21% cholesterol was esterified in the presence of 40µl enzyme after 60 min incubation. This however increased to 38% and 46% when 75µl and 100µl enzyme were assayed for 60 min respectively. Data for time courses carried out with 40µl, 75µl and 100µl are presented in **Table 5.2**.

It is apparent that enzyme activity was also proportional to enzyme concentration. **Fig 5.17** demonstrates the dependence of enzyme activity on enzyme concentration. Reaction of 100µl enzyme with substrate for 30min resulted in approximately 26% esterification.

Fig 5.14: DE-52 chromatography of butanol-ammonium sulphate fraction. The enzyme fraction was applied to a DE-52 column (2.5x6.0cm), equilibrated with 10mM Tris, 5mM EDTA and 50mM NaCl, pH 7.6. After the sample was applied, the column was eluted with a gradient of 75mM to 200mM NaCl. Flow rate was 1.0ml per minute and 2ml fractions were collected.

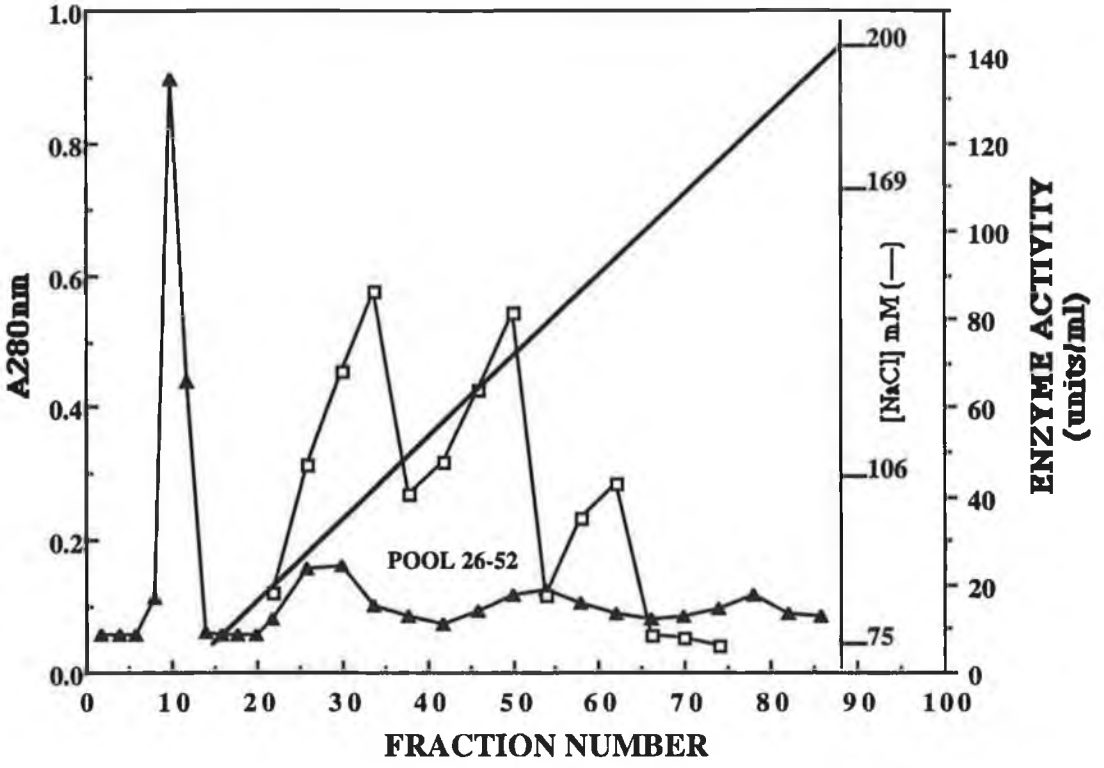


Fig 5.15: 7.5% SDS-PAGE gel demonstrating the various steps in the partial purification of LCAT, from bovine plasma. Molecular weight markers, ranging from 32-195kDal are shown in Lane 1. The various stages of the sample preparation are as described in Chapter 2. The lower gel shows a clearer version of the $(NH_4)_2SO_4$ step.

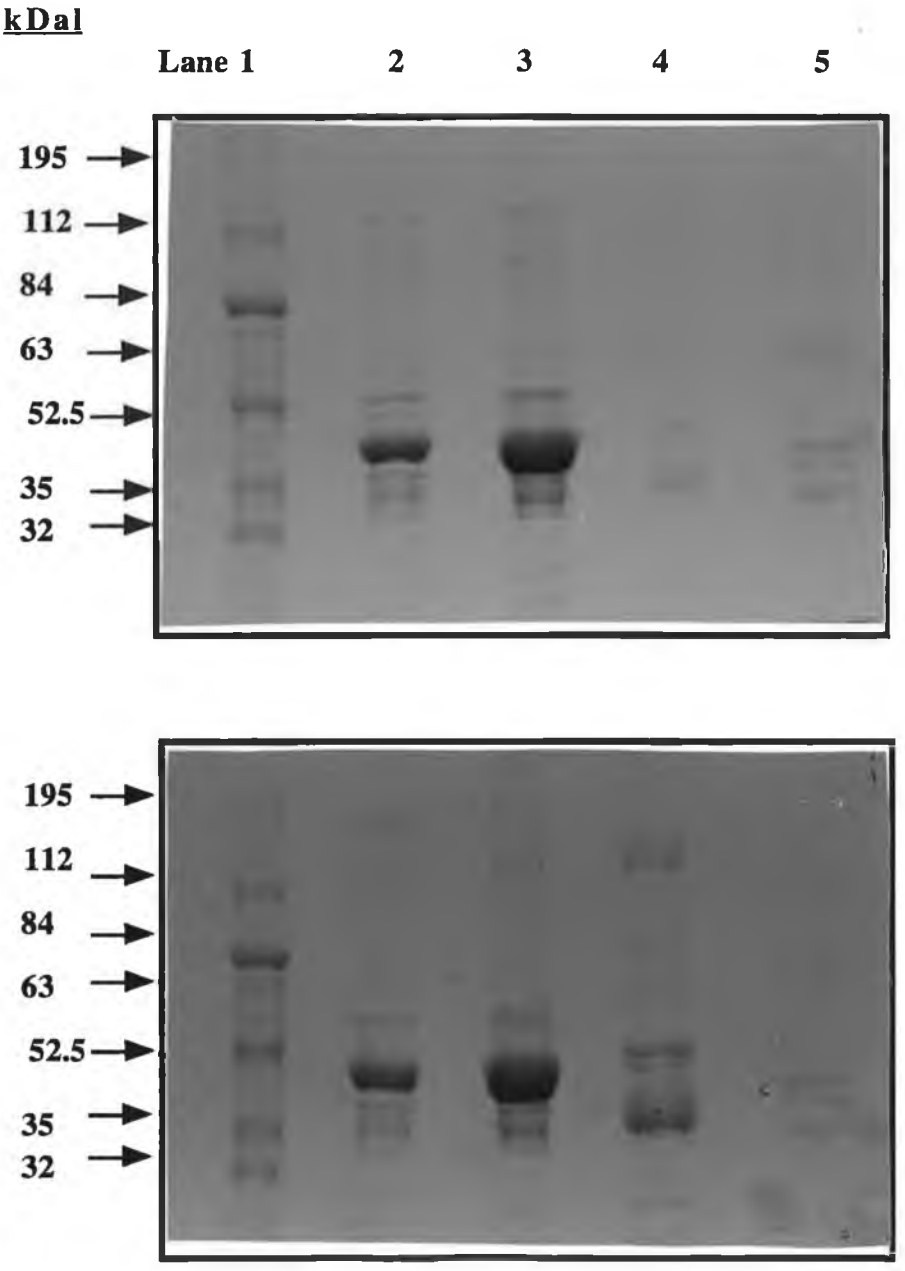


Fig 5.16 / Table 5.2 Time course assay (10-60 min) for varying LCAT concentrations (40-100µl) in the esterification of cholesterol.

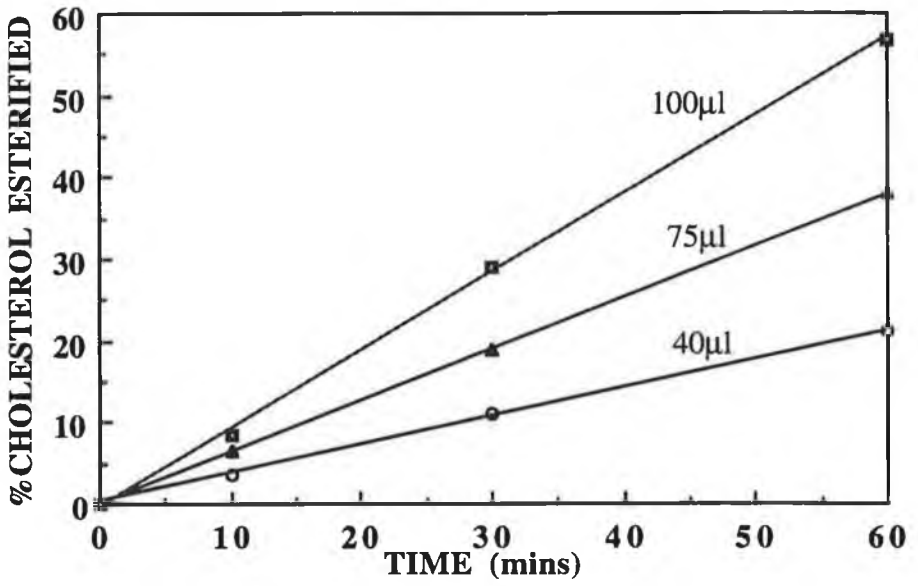
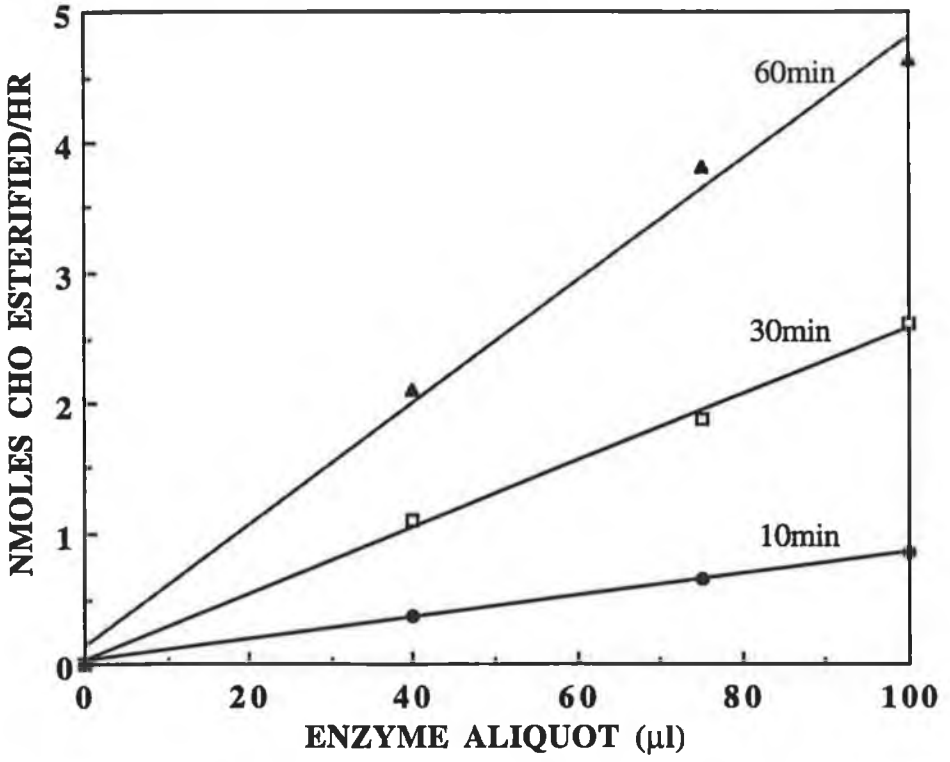


Table 5.2

Enzyme (µl)	Time (min)	nmoles est./hr	%CHO est/hr
40	10	0.369	3.69
	30	1.003	10.03
	60	2.105	21.05
75	10	0.651	6.51
	30	1.879	18.79
	60	3.813	38.13
100	10	0.857	8.57
	30	2.615	26.15
	60	4.624	46.24

Fig 5.17 Plot of enzyme activity as a function of enzyme concentration, at varying times (10-60min). Reaction conditions as described in Chapter 2.



5.2.4 Reactivity of partially purified LCAT with micellar complexes containing cholesterol and 7α-hydroxycholesterol

Having demonstrated interaction of partially purified bovine LCAT with cholesterol-containing proteoliposomes, its reactivity with 7α-hydroxycholesterol was next examined. However, due to the fact that there there was no commercially available radiolabelled 7α-hydroxycholesterol and also due to difficulties in its synthesis (examined in the discussion later) the method of product analysis was different to that for the radiolabelled cholesterol containing complexes. Thus, the sample preparation and analysis by HPLC, as described in Chapter 2, is employed in this analysis, *i.e.*, incubation, lipid extraction, alkaline hydrolysis, sterol extraction, reaction with cholesterol oxidase and HPLC analysis.

An aliquot (30μl) of the discoidal complexes, containing 10nmoles 7α-hydroxycholesterol was incubated with 100μl aliquot of enzyme. TLC was used to separate and compare the products obtained upon reaction of the enzyme source with the two different substrate complexes. **Fig 5.18** is a diagrammatic representation of the TLC chromatogram showing products of each reaction. Lane 1 contains the products of the reaction with cholesterol complexes, while Lane 2 shows the products of the reaction with 7α-hydroxycholesterol-containing complexes. Two distinctive bands were observed in Lane 1 with Rf values of 0.26 and 93. These correspond with the relative partitioning of the cholesteryl oleate and the free cholesterol standards in Lanes 3 and 4 respectively. A contaminating band corresponding to cholesterol is also observed in Lane 3. Lane 2 shows the presence of two bands, one with an Rf value (0.27) similar to cholesterol , the other with a slightly higher Rf of 0.32, indicating a less polar structure.

It was decided to run duplicates of the described assays- one set which were analysed by TLC- the others run by HPLC, as described in Chapter 2. This was to account for the possibility that the Rf of esterified 7α-hydroxycholesterol may have been higher and that such a band may not have been visible with the iodine staining method of visualisation. **Table 5.3** demonstrates results obtained when the reactivity of LCAT towards 7α-hydroxycholesterol and cholesterol was compared. Both reactions showed the esterification potential of LCAT: 5.6 nmoles cholesterol ester/assay and 3.54 nmoles/assay. Specific activity of LCAT towards cholesterol (354.93nmoles/hr/mg protein) was observed to be 1.6- fold higher than that towards 7α-hydroxycholesterol (224.36nmoles/hr/mg protein). It is apparent from this study that 7α-hydroxycholesterol can, like cholesterol, serve as an acyl acceptor for the transacylation reaction catalysed by LCAT.

Fig 5.18 A schematic representation of a T.L.C. plate indicating positions of products obtained upon LCAT reacting with discoidal complexes containing a) 10 nmoles cholesterol (Lane 1) b) 10nmoles 7α-hydroxycholesterol (Lane 2) as substrate for the enzyme.
Lane 3: Cholesteryl oleate standard. Lane 4: Cholesterol standard.
Mobile phase: Petroleum ether : Diethyl ether : Acetic acid (76 : 20 : 1) (v : v: v)

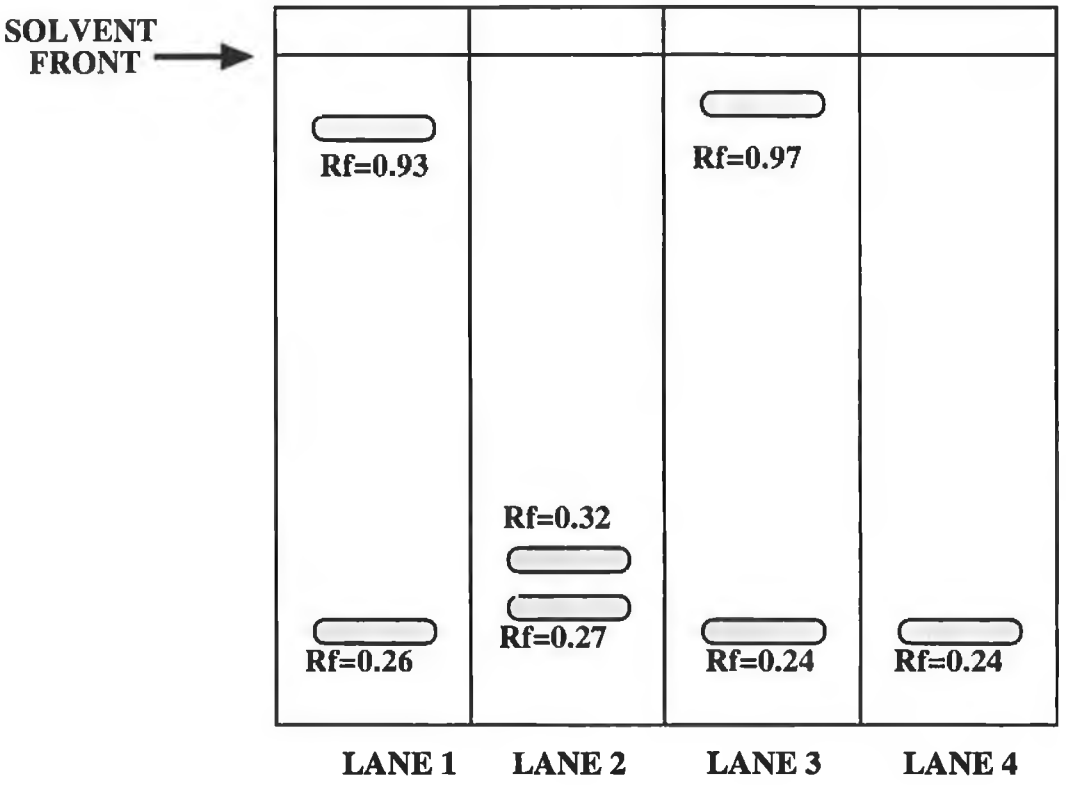


Table 5.3: Typical values of free and esterified cholesterol / 7α-hydroxycholesterol obtained when measuring the activity of LCAT using 10 nmoles cholesterol / 7α-hydroxycholesterol discoidal complexes as substrates. Analysis was carried out by reverse-phase HPLC and values tabulated below are the corresponding concentrations (nmoles) obtained by extrapolation from the standard curves in Chapter 3.

SUBSTRATE FOR LCAT	NMOLES/ ASSAY	FREE (nmol/ hr/ 100µl)	ESTERIFIED (nmol/ hr/ 100µl)	NMOLES ESTERIFIED /HR /MG PROTEIN
CHOLESTEROL	10.00	4.40	5.60	354.93
7a-HYDROXY-CHOLESTEROL	10.00	6.46	3.54	224.36

5.2.5 Reactivity of 7α-hydroxycholesterol with acyl-coenzyme A:cholesterol acyltransferase (ACAT)

The final experiment in this project was to determine if 7α-hydroxycholesterol could serve as acyl acceptor for ACAT, the liver microsomal enzyme that regulates cholesterol esterification. 100µl aliquots of microsomal fraction of bovine liver containing 3.42µg cholesterol, prepared as described in Chapter 2, were treated for varying lengths of time wiith 2-hydroxypropyl-ß-cyclodextrin (final concentration 30mM) to unsaturate or desaturate the enzyme of endogenous cholesterol. **Fig 5.19** shows the extent of cholesterol removal using cyclodextrin after 45 min and after 90 min. Concentration of cholesterol in the microsomal fraction after 45 min was 25.6µg/ml (27.24%) depletion, while after a further 45 min it was reduced to 5.5µg/ml (overall depletion of 84.21%).

An aliquot of this fraction was incubated with varying amounts of 7α-hydroxycholesterol and 25 nmoles of [^{14}C] oleoyl Co A as second substrate and assayed for esterifcation as described in the methods chapter. The products of the reaction of the ACAT source (almost totally depleted of cholesterol) with 24 nmoles of 7α-hydroxycholesterol as substrate, are depicted in the diagrammatic representation of the TLC chromatogram, in **Fig 5.20**. Lane 1 depicts the presence of one band (Rf 0.541) for cholesterol oleate, while Lane 2 has a band at an Rf of 0.113, the location of the cholesterol standard. Lane 3 depicts five bands which were subsequently scraped and analysed by HPLC for cholesterol and 7α-hydroxycholesterol as well as for radioactivity. The bulk of the 7α-hydroxycholesterol was found in the bands with Rf 0.113 and 0.308. The higher Rf of the latter was presumed to indicate the presence of the more non-polar 7α-hydroxycholesterol oleate species. The strongest band, intensity-wise on the TLC plate, was that of Rf 0.233, which on HPLC analysis was shown to contain cholesterol. The proportion of cholesterol contaminating, the Rf 0.113 (0.59% of total cholesterol present) and Rf 0.308 (30% total cholesterol) bands suggest the inability of the cyclodextrin to completely solubilise the cholesterol. Radioactivity analysis of the reaction products indicated a predominantly rich [^{14}]C band at Rf 0.233. This band may be that of unreacted oleoyl CoA.

Fig 5.19 Effect of incubation of 2-HPBCD with bovine microsomal preparation, in the depletion/solubilisation of cholesterol. 0.1ml of microsomal preparation, containing 3.42 µg cholesterol and 150 µg protein was incubated with buffer containing 2-hydroxypropyl-ß-cyclodextrin. Mixture was incubated at 37^{0}C for 45 mins per cycle. After two depletion cycles were carried out, 84.21% cholesterol was solubilised.

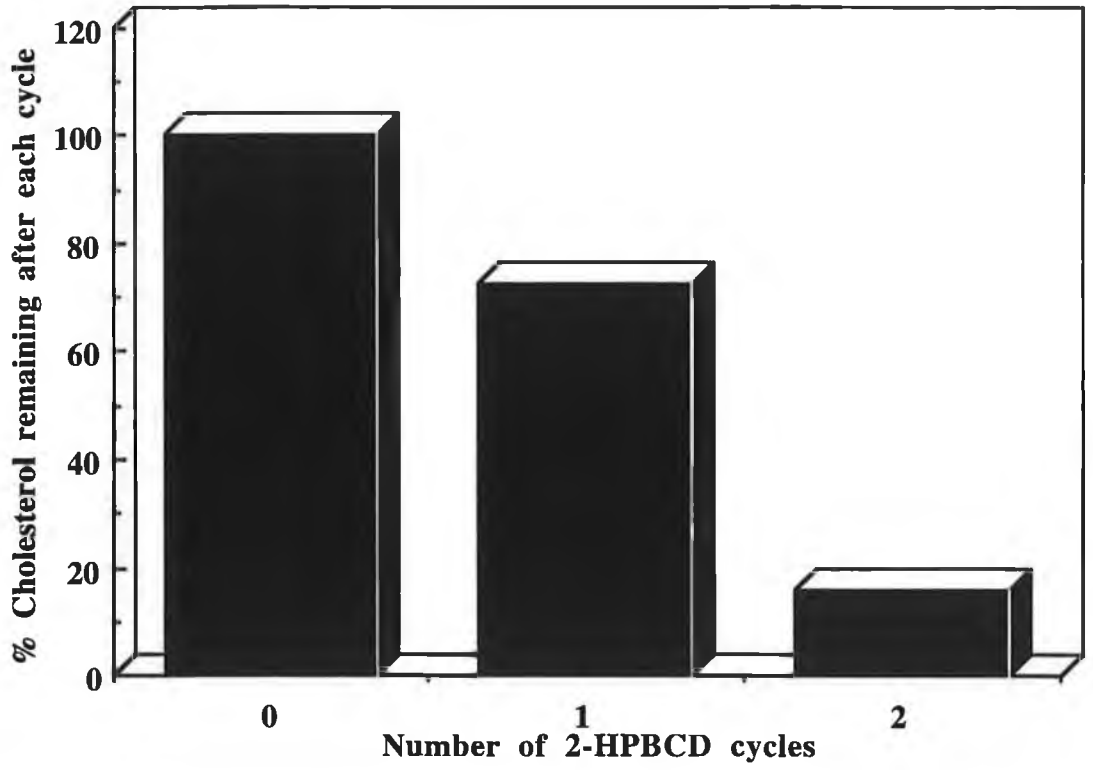


Fig 5.20: Schematic representation of a T.L.C. plate, indicating Rf values of products obtained upon reacting ACAT source with 7α-OHCHO

Lane 1: Cholesteryl oleate. Lane 2: Cholesterol standard.

Lane 3: Sample of ACAT reacted with 7α-OHCHO.

Mobile phase: Hexane : Ethyl acetate (95 : 5) (v : v)

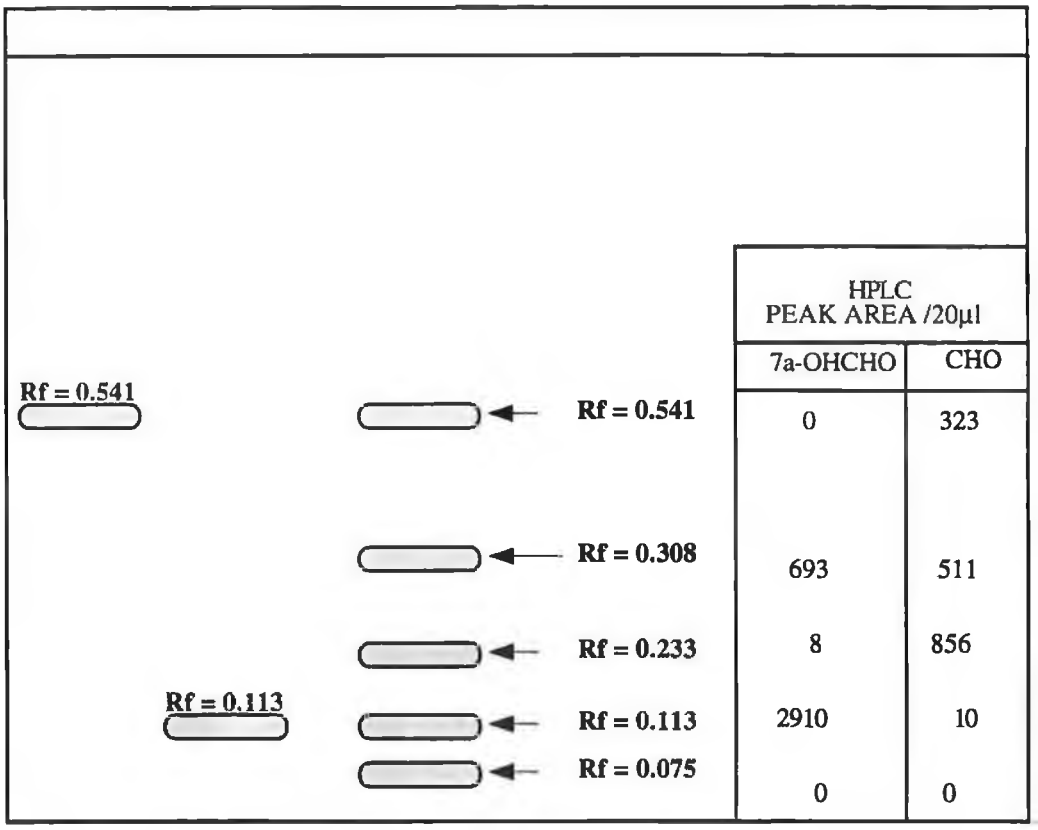
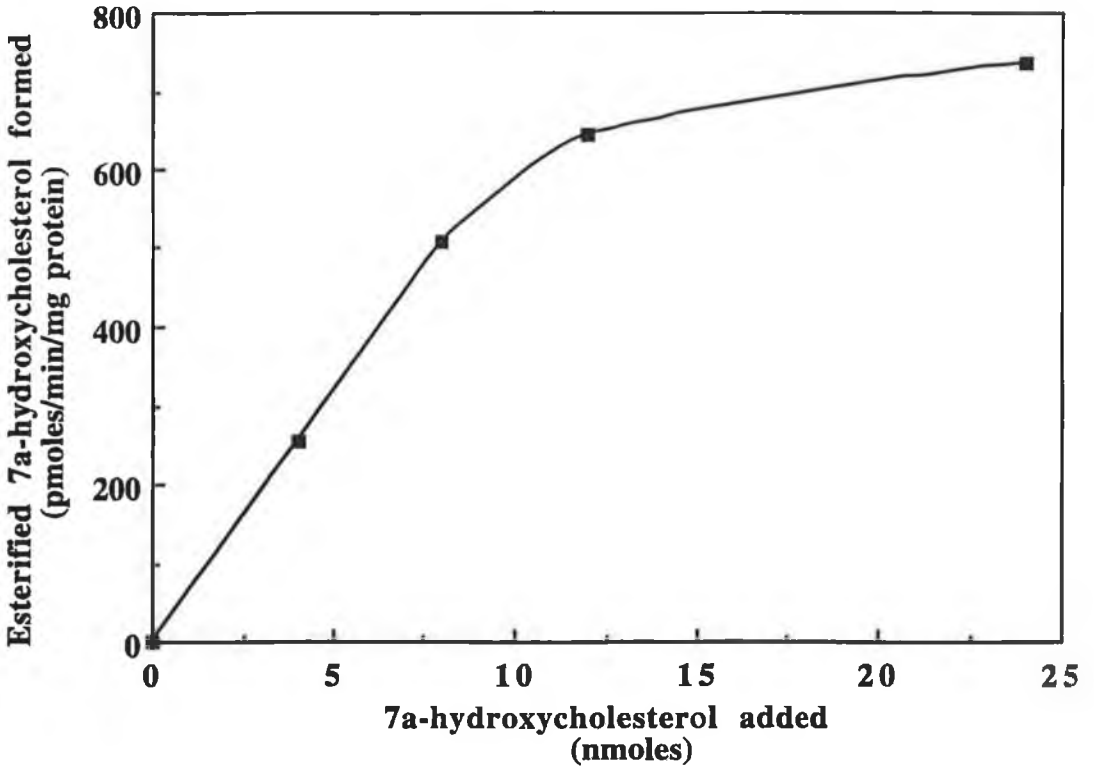


Fig 5.21 shows that ACAT activity was proportional to the concentration of 7α-hydroxycholesterol used as co-substrate. Radioactivity analysis of product bands formed after incubation of 4, 8, 12 and 24 nmoles of 7α-hydroxycholesterol with ACAT source demonstrated a linear increase in the concentration of 7α-hydroxycholesterol oleate formed up to a concentration of approximately 8 nmoles; V_{max} was seen to be approached at a substrate concentration of 24nmoles/assay.

Thus it would appear that ACAT esterified a proportion of the 7α-hydroxycholesterol in this study. Under the given reaction conditions was esterified, the specific activity of bovine hepatic microsomal ACAT, towards 7α-hydroxycholesterol was 736.12 pmoles/min/mg protein. Thus, it is apparent that there was an approximate 5-fold lower esterification of 7α-hydroxycholesterol by ACAT relative to its esterification by LCAT.

Fig 5.21 Plot of varying concentrations of 7α-hydroxycholesterol (4, 8, 12 and 24 nmoles) against the number of pmoles of 7α-hydroxycholesterol esterified/min/mg protein, upon incubation with ACAT source (bovine microsomes depleted of 82% of endogenous cholesterol). Methods as in Chapter 2.



5.3 Discussion

In general, the concentration of cholesteryl esters is greatest in serum and in those tissues (liver, intestine, adrenal and ovary) which play a major role in the anabolism and catabolism of cholesterol (D'Hollander and Chevallier, 1969). These organs (liver, adrenal and the ovary) are also found to have the highest concentration of LDL receptors. Large amounts of cholesteryl ester are known to accumulate in certain tissues as a result of lipid metabolism, such as atherosclerosis. Cholesteryl ester formation may play a role in a number of pathological conditions, most notably coronary heart disease (CHD). This disease involves the accumulation of lipids-particulary free cholesterol and cholesterol ester, in and between the vascular bed (Fielding and Fielding, 1985). The ester form seems to initally accumulate in macrophages which, in turn invade the arterial wall to scavenge lipid and other cellular debris (Gerrity, 1981). The end result is that cells take up large amounts of free cholesterol, due to a poorly regulated receptor which recognises modified LDL, thereby resulting in the increase of cholesteryl ester synthesis by as much as 100-fold (Brown and Goldstein, 1983).

Lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43) is an enzyme synthesised in the liver, secreted into and acts in the plasma, catalyses the synthesis of cholesteryl esters and lysolecithin from lipoprotein lecithin and free cholesterol. The physiological consequences of this reaction are profound and are illustrated by the pathological changes seen in medical cases where there is a familial deficiency of the enzyme (Glomset *et al.*, 1983). In such patients as those that are deficient in the enzyme, there is an elevation in the level of free cholesterol at the expense of esterified cholesterol. Glomset *et al.*, (1983) reported that there is a predeposition towards premature atherosclerosis in these subjects and that there is also an accumulation of free cholesterol and phospholipids in different tissues. Norum *et al.*, (1971) reported that in familial LCAT deficiency, there are very low levels of HDLs present, which are mostly as discoidal nascent particles devoid of cholesteryl esters. Thus, studies on LCAT are deemed to be of importance in the study of lipid metabolism.

The study undertaken here, in Chapter 5, involves measuring the activity of LCAT toward a metabolite of cholesterol- 7α-hydroxycholesterol. Previous work has been carried out on the measurement of LCAT activity toward cholesterol (Fielding and Fielding, 1971, Doi and Nishida, 1981). Due to the heterogenity, both chemically and physically, of native high density lipoprotein particles, it has been deemed necessary to rely on more chemically and physically defined synthetic substrates to investigate the reaction between LCAT and varying substrates.

As referred to in the introduction (Chapter 5), there are different types of synthetic substrates. The main components for most reconstituted substrates are purified apolipoproteins, synthetic or natural phosphatidylcholines and free cholesterol (Jonas, 1986b). The type used in these experiments described here are defined as micellar,

discoidal complexes and were synthesised using the sodium-cholate method as decribed by Jonas (1986a). These complexes, as opposed to vesicles, are reported to be devoid of an internal aqueous compartment (Jonas, 1986b). Jonas (1986b) reported that a noteworthy difference between vesicles and discoidal complexes of similar composition is the discoidal complexes (egg PC complexes) are about 4- fold better substrates for LCAT than the vesicular counterparts. This may be due to the fact that there is a higher content of bound apo A-I in the micellar complexes than in the corresponding vesicles. Thereby, apo A-I may adopt a confirmation that makes it a better activator of the LCAT reaction when present in micellar complexes as opposed to being bound to vesicles. The complexes are also capable of storing large amounts of cholesteryl esters formed: at least 20 mole % of the particle lipid, while the vesicle lipid can store 3 mole % (Matz and Jonas, 1982a).They are also reported to be of similar size and density of HDL (Jonas, 1986a). Such complexes were synthesised using the sodium-cholate dialysis method (Jonas, 1986a). This method allows the incorporation of a large variety of phospholipids into discoidal, HDL-like particles.

Initally, discoidal complexes were synthesised here which contained cholesterol as substrate for the LCAT enzyme. This method was selected to use for such a synthesis, as the kinetic block in the spontaneous reaction of phosphatidylcholine vesicles with apolipoproteins can be bypassed using complexes prepared by the sodium-cholate diaylsis method. This kinetic block occurs as the vesicle bilayer formed in the initial step of synthesis must be disrupted in order to allow the apo A-I to penetrate the structure. Thus it was necessary to work strictly at temperatures close to the T_c (gel to liquid-crystalline phase transition temperature) of the lipid (phosphatidylcholine- 4^0C). Some vesicular lipid preparations, upon exposure to apo A-I at or above the T_c, can be transformed into micellar complexes (Jonas *et al.*, 1980).

The molar ratios chosen to work with, being apo A-I, phosphatidylcholine and cholesterol, (1:100:9.1), were selected for the following reason- reports show that native HDL are the best substrates for LCAT (Glomset, 1972), second to these are small spherical HDL and then larger HDL subclasses. The complexes synthesised here had morphological similarities to native HDL. Highest yields of micellar complexes (Matz and Jonas, 1982b), are obtained at cholesterol contents of less than 20 mol% and at incubation temperatures approaching the transition temperature of the corresponding phosphotidylcholine, where cholesterol mole% gives the number of cholesterol molecules/100 lipid molecules (Phosphatidylcholine + cholesterol).

Little information is available on the difference between the disc and sphere as regards reactivity with LCAT (Jonas, 1991). Native spherical (Marcel *et al.*, 1980) and reconstituted spherical HDL (Jonas *et al.*, 1990) are considerably less reactive with LCAT than the discoidal HDL particles.

Jonas (1991) suggests that as regards the native particles, the changes in apolipoprotein conformation, changes in composition and packing of the surface lipids may result in a decrease in the reactivity with LCAT. Thus by synthesising complexes in the specified molar ratio the resultant complexes would be relatively small in size, thus ressembling native HDLs in some respects.

The complexes synthesised here were analysed by gel filtration, gradient gel electrophoresis and electron microscopy. A combination of these three analytical tools seems to indicate that the complexes containing cholesterol, were approximately 3.71 x 10^5 daltons in molecular weight and with a Stokes radius of 58Å. Further analysis by electron microscopy revealed they measured approximately 10.908nm in diameter. Thus the values obtained for the diameter from the different means of measurement are in agreement. These studies show that the complexes formed were fully associated structures made up of the three components apo A-I, cholesterol and phosphatidylcholine. These measurements would result in the complexes being classified as "small discs", which Jonas (1986b) groups as discs having molecular weights in the range from 10^5 to 4 x 10^5, axial ratios of 2/1 to 3/1, diametres from 100 to 140Å and the thickness of a lipid bilayer. Large discs, on the other hand, have molecular weights ~10^6, axial ratios ~4/1 or larger and diametres around 200Å.

Others obtained complexes measuring 123.4 ± 5.1Å for the apparent vesicle radius, for complexes comprised of apo A-I: lecithin: cholesterol in the molar ratio of 0.8: 250: 12.5 (Chen and Albers, 1982). Complexes with POPC (palmitoyloleoyl phosphatidylcholine), cholesterol and apo A-I in molar ratios of 80:8.1:80, yielded four differently sized products of 77, 86, 96 and 109Å (Jonas *et al.*, 1989). Such discoidal complexes resemble very closely the discoidal nascent HDL in physical and chemical properties. They are remarkably stable entities (Jonas, 1986b). The selected ratios worked with here (1:100:9.1; apo A-I:phosphatidylcholine:cholesterol) were manipulated in terms of the ratio of phosphatidylcholine:apolipoprotein A-I, in order to alter the size of the complex.

The elution profile obtained by passing the complex mixture over the Sepharose CL-4B column indicated that there was a possibility that the preparation was not homogenous, which was later confirmed by running the preparation on a native gradient polyacrylamide gel and also by electron microscopy. The complex peaks obtained are occasionally broad and often the protein and lipid elutions do not coincide exactly, indicating the presence of chemical and size heterogeneity in the complexes (Jonas, 1986a). Contaminants include apolipoprotein D. This glycoprotein is found in the VHDL fraction but is also a consistent constituent of HDL_3. Both HDL_3 and VHDL are good substrates for LCAT (Fielding and Fielding, 1971).

Thus, these methods for synthesising and characterising such described complexes are suitable for the present study. Native HDL particles of humans and animals, which have been isolated between densities of 1.063 and 1.210 g/ml have the characteristic discoidal make-up diametres of about 130 to 240 Å and widths from 40 to 50 Å (Jonas, 1986b). The cholesterol containing complexes described here had diametres of approximately 112 Å, falling in the lower end of the size scale reported for native HDL.

Having synthesised the required complexes, the enzyme lecithin:cholesterol acyltransferase (LCAT) was partially purified prior to reaction with the substrates. The reason for the partial purification was to attempt to remove possible major interferring compounds such as LDLs, which may, with cholesteryl ester acyl transfer protein, further metabolise the product- cholesteryl ester. However, there are still uncertainties of a direct association between LCAT and CETP while it has been shown that both are involved in the metabolism of plasma lipoproteins and act on HDLs. Nishida *et al.*, (1990) found that the lipid transfer activity of CETP was enhanced upon reaction of CETP, LCAT and vesicles of apo A-I and egg-PC or with HDL_3. When discoidal rHDL or HDL_2 were reacted with LCAT, they did not find that this was the case.

Some workers have reacted plasma as a crude LCAT source, with the substrate cholesterol (Chen and Albers, 1982), while others have used a fully purified source (Steinmetz and Utermann, 1985). The various steps in the partial purification resulted in an overall purification fold of 625.49 and a yield of 43.59% (Table 2.1). The first step involved ultracentrifugal centrifugation after adjusting the density to 1.21 g/ml with solid KBr. KBr and NaBr (or a combination of these salts) are routinely used for high-density isolations while NaCl is used for low-density work (Beckman, 1989).

Salt, for adjusting the density of the plasma, may be added as a solution or as a solid. The latter was selected here as minimal dilution of the sample was required. However, if a solution of the salt was to be added, it would generally be added in the ratio of 1 or 2 volumes of salt solution to 1 of plasma (Mills *et al.*, 1984). The method was tedious as the salt was added slowly with continuous stirring. The density of the plasma was checked using a density meter. Although expensive, the instrument has the merit that it can determine the density of a solution (minimum of 2ml) to 1.999 $g.cm^{-3}$ with an accuracy of $\pm 1 \times 10^{-3}$ very rapidly.

The time of centrifugation was 30 hours. As a large proportion of the HDL has an apparent flotation rate of less than 3.5 Svedbergs, a long centrifugation is necessary if a good recovery of this fraction is to be obtained (Mills *et al.,* 1984). This long time for centrifugation also is required if the upper half of the tube is to be adequately cleared of protein (Plasma albumin has a significantly reduced sedimentation rate in the presence of a highly dense and highly viscous solvent). The reason that this fraction was selected to

work with is that LCAT in plasma is mostly associated with high-density lipoproteins ($1.063 < \partial < 1.21$ g/ml) and very high density lipoproteins ($1.21 < \partial < 1.25$ g/ml), (Akanuma and Glomset, 1968; Fielding and Fielding, 1971), thus the region obtained from the ultracentrifugation step ∂ = 1.21-1.25 g/ml (VHDL) contained most of the plasma enzyme activity and only a fraction of the total protein.

The next step resulted in a further increase in purification- the ammonium sulphate precipitation in the presence of butanol. This step has been reported to reduce the amount of contaminating protein in the interfacial precipitate wiithout altering the enzyme yield and the the addition of sucrose also stabilises the enzyme (Doi and Nishida, 1981).

The final step- ion exchange chromatography on DE-52 anion exchange resin resulted in an overall purification of 625.49 fold. The enzyme activity peaks obtained indicate that the protein was eluted off at a salt concentration between 110-158mM. Previous findings for the elution of LCAT by means of a salt gradient include 160mM NaCl (human plasma; Doi and Nishida (1981)), approx.170-183mM NaCl (human plasma; Steinmetz and Utermann, (1985)), 120mM NaCl eluted enzyme which was then subjected to a further gradient of 60-120mM NaCl (Mahadevan and Soloff, 1983) and finally Jauhianen and Dolphin, (1986) found that LCAT eluted from a DEAE column between 130 and 150mM NaCl. Chen and Albers, (1985) purified LCAT from different animal species including pigs, dogs, goats and rabbits. Upon passing the enzyme fraction through DEAE-Sepharose CL-4B, they found the enzyme activity to elute at an NaCl concentration of approx. 120-160mM, while their earlier work on human LCAT (1981) resulted in an enzyme elution at a NaCl concentration of approximately 95-164mM.

Discrepencies in the molecular weight of LCAT are reported. Chung *et al.*, (1979) and Chong *et al.*, (1983) recorded human LCAT to be a 60,000 molecular weight, single polypeptide chain glycoprotein, containing 25% by weight carbohydrate. Albers *et al* ., (1976) characterised it as a protein with an apparent molecular weight of 68,000±1,600 (human source), which was similar to 65,000 to 69,000 by Doi and Nishida (1981). Pig, dog, goat and rabbit LCAT were found to be 66,000:65,000:65,000 and 67,000 respectively by Chen and Albers, (1985). Mahadevan (1983) 66,000±2,000 for human LCAT. Matz and Jonas (1982a) purified human LCAT of M_r = 60,000±4,000.

One of the bands obtained in the present study appears to be of an approximate molecular weight of 62,500 daltons. This band may be that of LCAT. There is also another band corresponding to the molecular weight of 47,000 daltons. This may be a contaminating enzyme- Cholesteryl ester transfer protein (CETP), which has a reported weight of 41,000 (Tall *et al.*, 1983), while Hasler *et al.*, (1988) reported it to be of a 74 Kdal glycoprotein. In addition, free apolipoproteins and serum amyloid may be present as proteins of molecular weights of less than 45 Kdal.

Phospholipid tranfer protein (PTP) is another lipid transfer protein, which is temperature-sensitive with reported apparent molecular weights ranging between 41,000 and 75,000 (Tall *et al.*, (1983), Tollefson *et al.*, (1988) and Jauhiainen *et al.*, (1993)). Interspecies comparisons demonstrated that some species, among them rat and mouse, lack the cholesteryl ester and triglyceride transfer activities but not the facilitated phospholipid transfer activity (Ha and Barter, 1982; Ihm and Harmony, 1980). Both CETP and PTP are also present in the ∂>1.21 g/ml infranatant (Lagrost *et al.*, 1994), whose results showed that LCAT was separated from the two lipid transfer enzymes upon passing the mixture over a coupled Heparin-Ultragel/Blue-Trisacryl column. Groener *et al.*, (1984) detected most of CETP activity in the HDL_3 fraction ($1.125 < \partial <$ 1.210 g/ml).

Such proteins, if actively present in experiments containing varying lipoproteins, could transfer phosphatidylcholine between HDL and LDL (CETP), as CETP is involved in the transport of cholesteryl ester from HDL to VLDL and LDL. Thus, the products of the LCAT reaction, being esterified cholesterol or esterified 7α-hydroxycholesterol, may when within the discoidal complex, react further with CETP to transfer the product to LDL and VLDL. Thus, in the present study the ultracentrifugation step was undertaken in an attempt to eliminate some of the lipoproteins from the study. The DE-52 anion-exchange resin was employed in order to remove further proteins, such as apolipoproteins, as reported by Chen and Albers (1985), who also reported that DEAE-Sepharose chromatography separated LCAT from lipid transfer protein activity, the former being eluted at a salt concentration of approximately 120-160mM, the latter at salt concentrations below 115mM. The band obtained on the gel (**Fig 5.15**) corresponding to approximately 33.5 Kdal may be similar to that (33.0 Kdal) reported by Doi and Nishida (1981) and shown to be a contaminating protein which was removed by passing the fraction over a second DEAE-Sephadex column.

The assay conditions selected to work under seemed to be suitable for the assay of LCAT activity. The reaction time and amount of enzyme used allowed for a linear transformation and approximately 26% esterification of substrate in 30 mins. However, with increasing concentrations of enzyme, the plot became more non-linear.This may be due to the reaction approaching enzyme saturation. The discs synthesised here contained approximately 4.35 mol% cholesterol. Perhaps further esterification may have taken place if there was a higher mol% substrate, as Matz and Jonas (1982a) found that when discoidal complexes of egg PC and apo A-I, containing 20 mol% cholesterol were reacted with LCAT, almost complete transformation (90%) of cholesterol to cholesteryl ester took place. Such micellar complexes consisted of apo A-I:PC:Cholesterol (1:23.8:94.4; Molar ratio). Matz and Jonas (1982a) also reported that beyond 20-30 mol% cholesterol content, the reaction decreased markedly.

The final step resulted in a specific activity of 367.79 units. Reacting this LCAT source with 10nmoles cholesterol under the conditions specified resulted in 5.6nmoles cholesterol being esterified in 30mins. The K_m value for LCAT towards cholesterol or 7α-hydroxycholesterol were not determined due to the expense of synthesising the micellar complexes.

Human plasma LCAT activity (results not tabulated) towards the complexes containing cholesterol as substrate show that an average of 62.83nmoles cholesterol were esterified/hr/ml plasma. Such values are difficult to compare with other work, as LCAT activity may be measured using either an exogenous (proteoliposome substrate) assay or measuring the cholesterol esterification rate where endogenous cholesterol is converted to cholesterol ester. Even within these two divisions, many variations appear. LCAT activity in control human plasma, as measured by the exogenous method, was found to be 95nmoles/hr/ml plasma (Chen and Albers, 1985), 122 nmoles/hr/ml plasma (Franceschini *et al.*, 1990) and 30 nmoles/hr/ml plasma (Funke *et al.*, 1991).

Under the same conditions, 7α-hydroxycholesterol was reacted with the LCAT source, yielding three noteworthy points:

*Firstly, that 3.54 moles of 7α-hydroxycholesterol were esterified by LCAT (after incubation of 10 nmoles/reaction). This is comparable with the 5.60 nmoles of the 10 nmoles cholesterol which were esterified.

*Secondly, the discoidal complexes synthesised by the sodium-cholate method, using the selected molar ratios, were suitable models for such a study.

*Finally, the developed method of analysis described in Chapter 2 proved to a valuble analytical tool, as the 7α-hydroxycholesterol used in these experiments was not radiolabelled, thus a different method for product analysis had to be employed.

The procedure for synthesising radiolabelled 7α-hydroxycholesterol would be difficult as it may not be possible to label commercially available 7α-hydroxycholesterol, because the molecule is labile and could possibly be destroyed by exposure to tritium gas or by chemical reactions to introduce tritium to 3α- or 7ß- positions. Thus, it seems necessary to start with labelled cholesterol, according to Björkhem (1969). This method involves the use of pyridine and benzene and subsequent reduction with sodium borohydride.

In synthesising esterified 7α-hydroxycholesterol, it is necessary to protect, as an ester, the 3ß-hydroxyl group of cholesterol, because introduction of an oxygen molecule at the 7- position with chromium reagent would result in the oxidation of 3ß-hydroxyl group of cholesterol to 3-keto. Such a protecting group may be acetyl, formyl or other higher fatty

acids as stearate, as described by Kishinaka *et al.*, (1992). Sodium borohydride reduces the 7-ketocholesterol-3ß-ester to the esterified form of 7α-hydroxycholesterol. It has been suggested (Kuroki, 1995- personal communication) that reduction with lithium aluminium hydride would, while reducing the 7-keto group, simultaneously release the protecting group to yield mixtures of free 7α-hydroxycholesterol and 7ß-hydroxycholesterol. Difficulties, such as humidity, may be encountered and pose problems in oxidation reactions. Although 7α-hydroxycholesterol may be stable under neutral or alkaline conditions, it may be destroyed to cholesta-5,7-dien-3ß-ol under strongly acidic conditions. For a non-organic chemist, this may become a problem, as the reduction reaction of 7-ketocholesterol-3ß-ester to the corresponding 7-hydroxy esterified epimers- by sodium borohydride, is carried out in the presence of 1M HCl (Kishinaka *et al.*, 1992).

Thus, from these observations, it seems worthwhile to have an assay for measuring the activity of LCAT toward a substrate, without the use of radiolabelled compounds. However, there were some disadvantages to this method. The overall method was time-consuming and tedious. Further product loss would have occured (which was only validated to the HPLC stage) after the initial LCAT reaction, when the reacion was terminated by addition of chloroform/methanol (2/1 v/v) and the method was continued as for the sample preparation for the HPLC method. However, the recovery should not be much less than that obtained for the HPLC only, as only one further step was carried out. Another factor to be considered is that due to lack of discoidal substrate (expense of commercial apolipoprotein A-I), the reaction conditions chosen were those suitable for cholesterol as substrate and were not optimised for 7α-hydroxycholesterol. Thus, this could be viewed as a noteworthy shortcoming of this study.

Another enzyme which is to be considered in the possible esterification of 7α-hydroxycholesterol is acyl-coenzyme A:cholesterol acyltransferase (ACAT). This is an intrinsic endoplasmic reticulum protein, which although it has been solubilised (Suckling *et al.*, 1982), it has not been purified (Erickson *et al.*, 1994). This enzyme may be assayed, as in earlier assays, using exogenous labelled cholesterol as substrate. However, an associated draw-back to such an assay would be the determination of the extent of exogenous cholesterol mixing with endogenous microsomal cholesterol. The most widely used ACAT assay involves a [^{14}C]-labelled fatty acyl coenzyme A as substrate.

[^{14}C]-labelled oleoyl CoA was employed in this study. This is the preferred substrate, as palmitoyl-CoA is particularly sensitive to degradation by a second microsomal enzyme- acyl-CoA hydrolase. This enzyme competes with ACAT for oleoyl CoA

substrate (Billheimer *et al.*, 1981). Oleoyl CoA itself is a detergent which can inhibit enzyme activity (Billheimer, 1985). The use of essentially fatty-acid free bovine serum albumin, as was used in this study, prevents the detergent effect of oleoyl CoA disrupting the membrane.

As cholesterol is a well defined and studied substrate of ACAT, it was decided to attempt to deplete it from the microsomal preparation in order to study the reaction between ACAT and 7α-hydroxycholesterol (Einarsson, 1995- personal comunication). This was carried out by means of 2-hydroxypropyl-ß-cyclodextrin (2-HPBCD), which is a cyclic heptamer of glucose capable of solubilising steroids into aqueous solutions through the formation of inclusion complexes. In such complexes, the steroid molecule is partially contained in the nonpolar cavity of the macrocycle formed by the glucose residues of ß-cyclodextrin (Gerloczy *et al.*, 1994). Results by these workers conclude that hydroxypropyl ß-cyclodextrin was clearly the most efficient solubiliser of cholesterol, with respect to other derivatives of ß-cyclodextrin. Such complexes may be used therapeutically, in the depletion of cholesterol, as they also bind bile acids, thus preventing their reabsorption, similar to cholestyramine, but the former being better tolerated than the latter.

Martin *et al.*, (1993) reported the successful use of this dextrin in the depletion of cholesterol from hamster liver microsomes (81%) while examining the activity of cholesterol 7α-hydroxylase. The activity of the enzyme (microsomal) was found to be unchanged despite the removal of its endogenous substrate. The concentration of 2-HPBCD used here was in accordance with Martin *et al.*, (1993) and also De Caprio *et al.*, (1992) reported that the solubility of cholesterol in 2-HPBCD was 15.5 µmol/ml of 0.9% NaCl containing 45% 2-HPBCD, which would accommodate the concentration of cholesterol in the microsomal preparation. It was successfully used as it depleted more than 83% of microsomal cholesterol. 7α-hydroxycholesterol was added to the reaction mixture, suspended in Triton WR-1339. This detergent was selected as reports have indicated that even under optimal conditions, the increase in ACAT activity obtained by suspending cholesterol in acetone was much less than that obtained by dispersing cholesterol in Triton WR-1339 (Billheimer *et al.*, 1981). Organic solvents such as acetone containg sterol substrate would precipitate out the sterol upon addition to an aqueous reaction mixture and the solvent itself may inhibit ACAT. Erickson *et al.*, (1980) and Egan, (1976) reported that such non-ionic detergents as Triton X-100 can disrupt the membrane and inhibit ACAT, as their hydrophile-lipophile balance value is between 12 and 15.

7α-hydroxycholesterol was seen to be esterified by ACAT under the given reaction conditions. The range of 7α-hydroxycholesterol to be added to the assay mix posed

another problem. So, it was roughly calculated from work carried out by Sahlin *et al.*, (1994) whereby they added exogenous cholesterol in the concentration of 50nmol/100μg microsomal protein. This is approximately 9- to 10- times the endogenous amount. Thus, as the endogenous amount of 7α-hydroxycholesterol was found to be approximately 4 nmoles/mg microsomal protein, then it was decided that at least a ten-fold substrate concentration should be added to the assay. It is apparent from **Fig 5.21** that incubation with increasing amounts of substrate that was a linear increase in the amount esterified between 4 and 8 nmoles substrate added. However, this linearity seemed to diminish with increasing concentration of 7α-hydroxycholesterol, perhaps indicating saturation of the enzyme (approachingVmax at 24nmoles substrate). The amount that was esterified was in the pmole/min/mg protein range, when nmoles of substrate were added to each assay.

Once again, the sample preparation and reverse-phase HPLC analysis described in Chapter 3 proved useful in the identification and quantitation of 7α-hydroxycholesterol present. Having separated out the products of the reaction by TLC, each band was analysed separately for the presence of 7α-hydroxycholesterol. 7α-hydroxycholesterol, being more polar than cholesterol, has a slightly lower Rf value, using a relatively non-polar mobile phase. Hutton *et al.*, (1966) found that mitochondria, in the absence of NAD^+ (which is required in the conversion of 7α-hydroxycholesterol to 7α-hydroxy-4-cholesten-3-one) when incubated with 7α-hydroxycholesterol formed the more polar product cholesten-5-en-3ß,7α,26-triol. This was also formed upon incubation of 7α-hydroxycholesterol and the supernatant fraction of rat liver. This triol, being more polar than 7α-hydroxycholesterol (Danielsson, 1961) may be the band corresponding to the lowest Rf value of 0.075 (**Fig 5.20**). Other bands may be attributed to unreacted oleoyl CoA. The Rf values of esterified 7α-hydroxycholesterol (0.31) and that of free cholesterol (0.113) on the TLC plate of ACAT results, differ slightly from the values for the esterified 7α-hydroxycholesterol obtained upon reaction of the substrate with the LCAT source (0.32; **Fig 5.18**) while that of free cholesterol was approximately 0.24. Two points may be referred to in explaining the slight difference in Rf values in the two above cases. (1): The mobile phase for LCAT being petroleum ether:diethyl ether:acetic acid(76:20:1) (v/v/v) which is a slightly more polar mobile phase than that used in the ACAT assay: hexane:ethyl acetate (95:5) (v/v). Thus, 7α-hydroxycholesterol being relatively more polar than free cholesterol will migrate further with a more polar mobile phase. Also, cholesterol migrated further in this mobile phase relative to the hexane:ethyl acetate mobile phase. Kuroki, S. (personal communication) suggested that the Rf value of esterified 7α-hydroxycholesterol is expected to be very close to that of free

cholesterol, as both compounds have one hydroxyl group. The hydroxyl group ranks highly in the order of interactivity of functional groups: $RCO_2H>RCONH_2>ROH>RNH_2>$ $RCO_2CH_3>RH\text{-}(CH_3)_2>ROCH_3>RH$. This proved a very useful tool of analysis, as 7α-hydroxycholesterol-3ß-oleate was not available commercially and its synthesis would have been difficult to do as it is a long and tedious procedure. It involves the esterification of cholesterol and the subsequent oxidation with chromium trioxide/methylene chloride to yield the esterified 7-keto form. Further reactions are carried out and contaminating 7ß-hydroxycholesterol-3ß-oleate is removed. By using the preparative extraction and HPLC procedures, the esterified 7α-hydroxycholesterol was hydrolysed and subsequently measured after enzymatic conversion to 7α-hydroxy-4-cholesten-3-one.

Another advantage of the sample preparation and reverse-phase HPLC method of analysis is that the enzyme for conversion of products to enone derivatives detectable by HPLC at 240nm, being cholesterol oxidase, is specific for the 3ß-position of cholesterol and seems to require a double bond in the Δ^5- or Δ^4- positions (Richmond, 1973). It does not catalyse the oxidation of hydroxyl groups at position C-7α and C-7ß (Ikawa *et al.*, 1979) Thus, if 7α-hydroxycholesterol is esterified at position 3 of the steroid nucleus, then in order to analyse it by HPLC, the acyl group at position 3 is hydrolysed and the resulting hydroxyl group is converted to an enone derivative by cholesterol oxidase. Therefore the free and esterified 7α-hydroxycholesterol can be identified by means of the characteristic retention time of 7α–hydroxy-4-cholesten-3-one. This also shows that the esterification which occured was at position 3 of the steroid nucleus. This would be in accord with Oda *et al.*, (1990), who recently reported that approximately 75% of serum 7α-hydroxycholesterol was esterified with fatty acid(s) at the 3ß-position.

Due to the expense of radiolabelled oleoyl CoA, there were experiments which would have been beneficial and worthwhile carrying out, but, unfortunately were not. These include the following:

* Measurement of the activity of ACAT toward cholesterol in the bovine microsomal preparation, in order to compare it with that obtained for 7α-hydroxycholesterol.
* The activity of ACAT should, ideally, be measured after cholesterol depletion has occured, in order to determine if such treatment has harmed the enzyme in any way. (As referred to above- Martin *et al.*, (1993), found that the activity of cholesterol 7α-hydroxylase was unaffected by the depletion of cholesterol).
* The assay should be standardised for 7α-hydroxycholesterol. Such parameters as the

variations in the concentration of oleoyl CoA, microsomal protein and time should be investigated.

* Studies involving competition/inhibition between 7α-hydroxycholesterol and cholesterol for ACAT should be carried out. Thus, the addition of varying concentrations of 7α-hydroxycholesterol, in the presence of both endogenous and exogenous cholesterol should be carried out.

* Possible product inhibition studies.

* Two neutral cholesteryl ester hydrolases have been reported in the liver, a more quantitatively prominent one, whose activity was detected in the soluble fraction, having a pH optimum of 6.5-7.5. A second activity has been reported, which accounts for approximately 11-32% of the cholesteryl esterase activity of the homogenate- this was in the microsomal fraction and had a pH optimum of 6.1 (Deykin and Goodman, 1962). Whether the activity of the latter may affect the products of the ACAT reaction should be examined.

* Addition of ACAT inhibitors to the above assay should be considered in order to determine if they have any effect, direct or indirect, on the esterification of 7α-hydroxycholesterol.

However, this part of the project was only a preliminary study on the possibilty that ACAT may esterify 7α-hydroxycholesterol. As the results indicate 7α-hydroxycholesterol which was esterified by ACAT. However, in relation to the amount of 7α-hydroxycholesterol which was esterified by LCAT, the amount esterified by ACAT was quite small. The overall significance of these results will be discussed in the overall discussion and conclusion.

CHAPTER 6

OVERALL DISCUSSION

The work reported in this thesis has shown the presence of a considerable amount of esterified 7α-hydroxycholesterol in the systemic circulation of both man and rat. In order to assess the meaning and validity of this work it is necessary to address two fundamental items; the security of the identifications proposed and the potential of artificial formation of 7α-hydroxycholesterol. With respect to the first matter, the identification of 7α-hydroxy-4-cholesten-3-one having an intense absorption at 240nm by the specific action of cholesterol oxidase on 7α-hydroxycholesterol appears to be very sound. Cholesterol oxidase, being an enzyme that modifies sterols into conjugated enones will oxidise 3ß hydroxyl group of cholesterol or related sterols to a keto group and isomerise the Δ5-bond to yield a conjugated double bond which acts as a chromopheric group (α,ß unsaturated ketone; $E=16000M^{-1}cm^{-1}$). The method has the advantage of allowing separation and quantitation of cholesterol oxidase products by a highly sensitive HPLC. Identities assigned solely by chromatography are always potentially less secure than those made by brute isolation of crystalline material by classical methods. Nonetheless, the validation of the method wherein serum samples which were spiked with known amounts of 7α-hydroxycholesterol showed almost 80% recovery and the demonstration of acceptable low coefficients of variation for both inter- and intra-assays puts the method of identification of 7α-hydroxycholesterol reported here on a very secure basis.

The second issue of importance is the potential artificial origin of 7α-hydroxycholesteryl ester, which may be an esterified cholesterol oxidation product (i.e. 7α-hydroxycholesterol) , oxidized cholesteryl ester or both. If the origin is that of esterification of an oxidised cholesterol derivative such as 7α-hydroxycholesterol, then it must have occurred as a result of a rapid *post mortem* transesterification of traces of 7α-hydroxycholesterol in the presence of massive amounts of cholesterol and cholesterol esters. Alternatively, the requisite 7α-hydroxycholesterol must be present *in vivo* where enzymic esterification occurs. The latter situation is almost certainly the case for the esterified 7α-hydroxycholesterol as analysis was carried out in the presence of BHT and was not accompanied by the appearance of the two foremost major autooxidative products of cholesterol- 7ß-hydroxycholesterol or 7-ketocholesterol. Though 7α-hydroxycholesterol is a quantitatively minor cholesterol autooxidation product, and though esters of 7α-hydroxycholesterol are formed from the autooxidation of cholesteryl esters, such air oxidations of cholesteryl esters require considerably more exposure in air to radiation or heat than for cholesterol and much more than allowed in the present work. As shown in **Table 4.5**, the concentration of serum cholesterol was approximately

22000-fold that of 7α-hydroxycholesterol in humans (approximately 6000-fold in rats) and minimal autooxidation of cholesterol during the sample preparation might result in an appreciable increase of 7α-hydroxycholesterol. In the present study, however, concentration of total (free and hydrolysed) 7α-hydroxycholesterol increased rapidly during alkaline hydrolysis. The levels of total 7α-hydroxycholesterol after alkaline hydrolysis, being similar to those after enzymatic hydrolysis, suggest that there is a form of 7α-hydroxycholesterol in human and rat serum which is readily converted to quantifiable free 7α-hydroxycholesterol.

The hypercholesterolaemic patients in this study had significantly higher levels of total 7α-hydroxycholesterol than control normolipidaemic subjects. Approximately 78-84% of serum 7α-hydroxycholesterol was esterified with fatty acids at the 3ß position in both groups. Despite this, the ratio of total 7α-hydroxycholesterol to total cholesterol remained unchanged, suggesting cholesterol-bearing lipoproteins as a possible transport medium for 7α-hydroxycholesterol in plasma. Higher levels of 7α-hydroxycholesterol were found in rat serum relative to human serum, probably reflecting a much greater synthesis of this metabolite in the rat than in the liver. If one considers 7α-hydroxycholesterol as the major oxidative product of cholesterol in the bile acid biosynthetic pathway and that the rat , unlike the human has no storage facility for bile acids it is not surprising that such a species difference would exist. A major finding in this study was however the significantly higher level of total 7α-hydroxycholesterol in serum of cholestyramine-treated rats relative to control or diabetic rats. This has previously been demonstrated in patients with primary hypercholesterolaemia on bile acid sequestrant therapy (Van Doormaal *et al.*, (1989) and Bascoul *et al.*, 1990). Furthermore, there was a significant correlation between hepatic levels of 7α-hydroxycholesterol and serum levels of 7α-hydroxycholesterol, indicating that measurement of this metabolite in serum may be a good means of confirming high levels of bile acid synthesis occurring in liver which otherwise is difficult to do, being dependent on surgical liver biopsy for measurement of hepatic cholesterol 7α-hydroxylase. There was no correlation between sterol levels in liver and serum within each group however. Whether this was due to the limited number of animals within the groups or to other factors is not known. Theoretically, the level of 7α-hydroxycholesterol in serum should be dependent upon several factors other than cholesterol 7α-hydroxylase activity, e.g. rate of diffusion/ secretion of the sterol into blood and rate of metabolism / elimination. These factors may vary between animals and contribute to the poor correlation between sterol levels and hepatic levels observed within

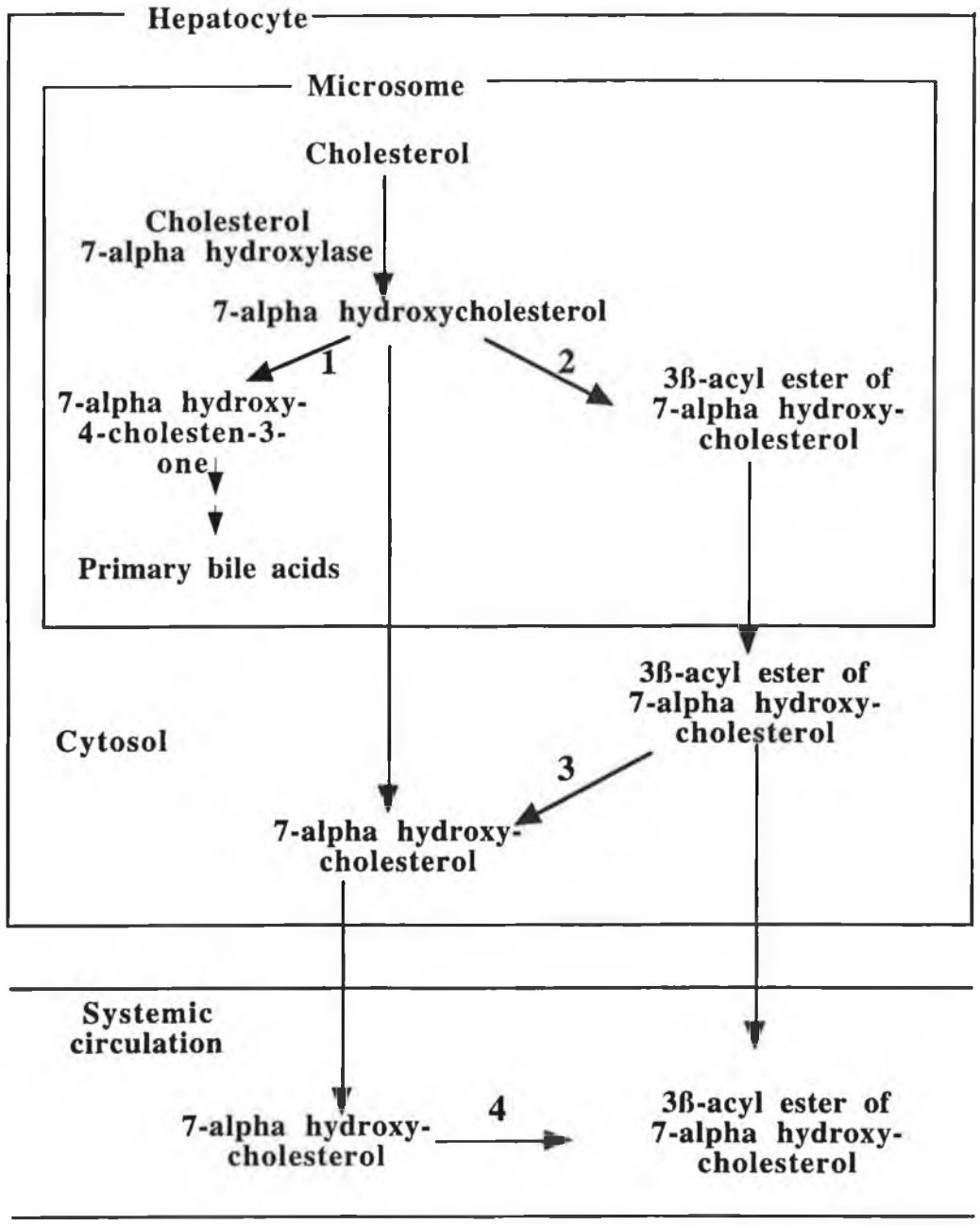
the three rat groups studied. As mentioned in the introduction to this thesis, only one other report on levels of serum 7α-hydroxycholesterol has been reported but in portal blood (Fukushima *et al.*, 1995). Such measurements by gas liquid chromatography were found to be circa 800-1000 pmoles/ml which were significantly higher than those reported in this study (247pmoles/ml serum). Despite the obvious greater concentration of bile acids (and presumably bile acid precursors) in the enterohepatic circulation, it is apparent that quantifiable levels of 7α-hydroxycholesterol do leak into the systemic circulation and that levels mirror levels in liver, potentially its tissue of origin.

A second major outcome of the work reported in this thesis was that 7α-hydroxycholesterol can be esterified by plasma LCAT and to a lesser extent by the liver enzyme, ACAT. Various studies have been carried out on the possible esterification of oxysterols by ACAT but to date it appears that no corresponding study has been published on the plasma esterifying enzyme, LCAT. The findings as described in Chapter 5 may provide answers to some of the questions posed by Oda *et al.*, (1990) which are schematically represented in **Fig 6.1**.

1) Does a fraction of 7α-hydroxycholesterol synthesised in the liver microsomes escape the bile acid biosynthetic route? **2)** Is it esterified by ACAT in the liver microsomes? **3)** Is it secreted into the systemic circulation as the esterified form or is it hydrolysed by a cholesterol esterase in liver cytosol? **4)** Is it not esterified in the liver but rather secreted as the free form into the circulation where it is then esterified by LCAT? or **5)** maybe a fraction is esterified by both enzymes?

This study suggests that a possible metabolism of 7α-hydroxycholesterol in hepatocytes and in serum would be that in view of the small proportion of 7α-hydroxycholesterol in serum (pmoles/ml) relative to rat liver (nmoles/ml), that most of the hepatic 7α-hydroxycholesterol is metabolised to bile acids in the liver and that only a small amount of it escapes into the systemic circulation either as free or esterified 7α-hydroxycholesterol. Of the 7α-hydroxycholesterol which enters the circulation a substantial amount may be esterified by LCAT.

Fig 6.1 Schematic representation of the possible metablism of 7α-hydroxycholesterol in hepatocyte and serum. 1= 3ß-hydroxysteroid dehydrogenase; 2= ACAT; 3= Cholesterol esterase and 4= LCAT. Adapted from Oda *et al.*, (1990).



The significance of this finding relates to the transport and further metabolism of 7α-hydroxycholesterol *in vivo*. When this oxysterol was presented to LCAT in complexes which resembled HDL's, esterification resulted; this proposes further areas of research such as reacting the esterified 7α-hydroxycholesterol with other lipoproteins and proteins / enzymes which are involved in the metabolism and exchange reactions of lipoproteins and tracing their pathway of transport and delivery. That transport and metabolism of 7α-hydroxycholesterol would be similar to that of cholesterol may be inferred from a recent study published by Kishinaka *et al.*, (1992) where it was observed that intravenously administered esterified 7α-hydroxycholesterol was taken up by the liver and metabolised via the normal bile acid biosynthetic pathway. Thus 7α-hydroxycholesteryl ester may be delivered to liver in the form of HDL particles for elimination by the body in the form of bile acids, as in reverse cholesterol transport . As to its mode of entry into the systemic circulation, it is probable that it may be either secreted from the liver in the form of nascent HDL's or more likely as part of VLDL's which undergo further metabolism by lipoprotein lipase. It is worth mentioning that possible equilibration of 7α-hydroxycholesterol from liver with 7α-hydroxycholesterol from other non-hepatic sources such as heart, lung and kidney (Chiang *et al.*, 1990) may occur in the circulation. Reverse transport of 7α-hydroxycholesteryl ester by HDL's may account for the rapid hepatic uptake of this sterol as reported by Kishinaka *et al.*, (1990).

The question still remains as to whether 7α-hydroxycholesterol exerts a regulatory role in cholesterol metabolising pathways or if it is an atherogenic oxysterol. However the latter is probably unlikely as it has a very short half life (about 6hours) when injected as 7α-hydroxycholesterol-stearate and its hepatic uptake to primary bile acids is very efficient -at least in hamsters (S. Kuroki , Kyushu University, Fukuoka, Japan, 1995-personal communication). Last month Doerner *et al.*, (1995) reported that 7α-hydroxycholesterol in concentrations of 50μM failed to restore cholesterol 7α-hydroxylase mRNA levels in squalestatin (a specific squalene synthase inhibitor which results in depriving the cell of endogenously synthesised sterols)-treated rat hepatocyte cultures. They deduced that cholesterol rather than oxysterols regulate cholesterol 7α-hydroxylase expression. Whether 7α-hydroxycholesterol might affect transcription of other genes containing sterol regulatory elements is not yet known.

In vitro studies carried out over two decades ago showed that 7α-hydroxycholesterol, 7ß-hydroxycholesterol and 7-ketocholesterol when incubated at concentrations of 5μM or

more with primary cultured mouse liver cells or L cells (a subline of NCTC clone 929 mouse fibroblasts) markedly inhibited both sterol synthesis and the activity of HMG Co A reductase. Another report found 7α-hydroxycholesterol to be potent in the inhibition of cholesterol uptake into aortic smooth muscle cell cultures by up to 51% at concentrations of 3μg/ml (7.45 nmoles/ml) (Peng *et al.*, 1985). These oxysterol studies in cultured cells involved the addition of sterols in organic media such as ethanol. However Goldstein and Brown in 1990 reported that ethanol allows the sterols to enter the cytosol, thereby bypassing the requirement for receptors or lysosomes. The accumulation of such polar sterols in the cytosolic compartment may thus account for their enhanced potency. Thus future work to be considered in this field of research should involve presentation of such sterols to liver cells as components of synthetic complexes which resemble lipoproteins. This would facilitate a study of how oxysterols are taken up by liver cells and metabolised- whether by means of a receptor-mediated pathway or whether they directly enter the cytosolic or microsomal fractions. The inclusion of serum, serum lipids or serum lipoproteins in media is a serious limitation of many *in vitro* studies (Smith and Johnson, 1989). Such factors may influence the proliferation, growth and functioning of the cell. In addition, the presence of undetected levels of oxysterols may also influence the nature of the work being carried out.

Identification of oxysterol- binding proteins that regulate oxysterol trafficking between membrane compartments is another area for future research.

The significance if any of the presence of fatty acyl esters of other oxysterols is a third area for future research. The sterol esters may be simply esterified for further metabolic disposition in the same fashion as is cholesterol. The work described in Chapter 5 on investigating the reactivity of 7α-hydroxycholesterol with LCAT may be adapted for studying interaction of other oxysterols in discoidal complexes with serum LCAT. Cholestane-3ß,5α,6ß-triol, for example is reported to be a toxic metabolite of cholesterol. However, the cytotoxic properties of the oxidised sterols suggest that esterification may be a detoxification mode. The esters may be metabolised nonetheless to yield the active free sterols. More speculatively, the hypothesis of Kandutsch *et al.*, (1978), regarding the possible endogenous regulation of *de novo* sterol synthesis by these oxidised cholesterol derivatives is served by findings of these physioiolgically active sterols in liver (potentially a tissue of origin), plasma (potentially a transport medium) and aorta (potentially a target tissue).

Thus to conclude, the aims of this study being to measure and investigate the biological significance and route of metabolism of 7α-hydroxycholesterol in serum were achieved. The work yielded a suitable method for analysing serum 7α-hydroxycholesterol in human and rat serum as well as in *in vitro* systems comprising reconstituted lipoprotein

complexes. The method allowed for a correlation between levels of serum and hepatic 7α-hydroxycholesterol to be investigated and indicated that this may be of significant use in diagnosing metabolic disorders of bile acid metabolism. Finally, the resulting esterification of this oxysterol by plasma LCAT (involving the synthesis of complexes which resembled HDL's) and also to a lesser extent by hepatic ACAT allows for further studies on the metabolism of 7α-hydroxycholesterol and also of other oxysterols currently implicated in atherosclerosis.

CHAPTER 7

BIBLIOGRAPHY

Albers, J.J., Cabana, V.G. and Dee Bardon Stahl, Y. (1976) Purification and characterization of human plasma lecithin:cholesterol acyltransferase. Biochemistry 15: 1084-1087.

Albers, J.J., Chen, C-H. and Adolphson, J.L. (1981) Lecithin:cholesterol acyltransferase (LCAT) mass; its relationship to LCAT activity and cholesterol esterification rate. J.Lipid Res. 22: 1206-1213

Albrink, M.J., Krauss, R.M. and Lindgren, F.T. (1980) Intercorrelations among plasma high density lipoproteins, obesity and triglycerides in a normal population. Lipids 15: 668-676

Anaki, Y. and Yamasaki, K. (1970) *In vitro* conversion of 7α-hydroxycholesterol to some natural C24-bile acids with special reference to chenodeoxycholic acid biogenesis. J.Biochem. 68: 341-346

Anderson, K., Kok, E. and Javitt, N.B. (1972) Bile acid synthesis in man: metabolism of 7α-hydroxycholesterol-^{14}C and 26-hydroxycholesterol-^{3}H. J.Clin.Invest. 51: 112-117

Angelin, B., Hershon, K.S. and Brunzell, J.D. (1987) Bile acid metabolism in hereditary forms of hypertriglyceridemia: evidence for an increased synthesis rate in monogenic familial hyperglyceridemia. Proc.Natl.Acad.Sci 84: 5434-5438

Ansari, G.A.S. and Smith, L.L.(1979) High performance liquid chromatography of cholesterol autooxidation products. J.Chromatogr. 175: 307-315

Assmann, G., Fredrickson, D.S., Sloan, H.R., Fales, H.M. and Highet, R.J. (1975) Accumulation of oxygenated steryl esters in Wolman's disease. J.Lipid Res. 16: 28-38

Atkinson, D., Smith, H.M., Dickson, J. and Austin, J.P. (1976) Interaction of apoprotein from porcine high density lipoprotein with dimyristoyl lecithin I. Structure of complexes. Eur.J.Biochem. 64: 541-547

Axelson, M., Björkhem, I., Reihnér, E. and Einarsson, K. (1991) The plasma level of 7α-hydroxy-4-cholesten-3-one reflects the activity of hepatic cholesterol 7α-hydroxylase. FEBS Lett. 284: 216-218.

Axelson, M. and Sjövall, J.(1990) Potential bile acid precursors in plasma- possible indicators of biosynthetic pathways to cholic acid and chenodeoxycholic acids in man. J.Steroid Biochem. 36: 631-640

Balasubramaniam, S., Mitropoulos, K.A. and Myant, N.B. (1972) Rhythmic

changes in the activity of cholesterol 7α-hydroxylase in the livers of fed and fasted rats. *In* Bile acids in human diseases, II. Bile acid meeting, Freiburg i. Br., June 30-July 1, 1972. Back, P. and Gerok, W. eds., F.K.Schattauer Verlag, Stuttgart, 97-102.

Balasubramaniam, S., Mitropoulos, K.A. and Myant, N.B. (1972) Rhythmic changes in the activity of cholesterol 7α-hydroxylase in the livers of fed and fasted rats. *In* Bile acids in human diseases, II. Bile acid meeting, Freiburg i. Br., June 30-July 1, 1972. P.Back and Gerok, W. eds., F.K.Schattauer Verlag, Stuttgart, 97-102.

Balasubramaniam, S., Mitropoulos, K.A. and Myant, N.B. (1973) Evidence for the compartmentation of cholesterol in rat-liver microsomes. Eur.J.Biochem 34: 77-83

Balasubramaniam, S., Mitropoulos, K.A. and Myant, N.B. (1975) The substrate for cholesterol 7α-hydroxylase. Biochimica et Biophysica Acta 398: 172-177

Balasubramaniam, S., Mitropoulos, K.A., Myant, N.B., Mancini, M. and Postiglione, A. (1979) Acyl-coenzyme A-cholesterol acyltransferase activity in human liver. Clin.Sci. 56: 373-375

Bascoul, J., Desplan, D., Domergue, N. and Crastes de Paulet, A. (1988) *in* Biological activities of oxygenated sterols (Beck, J.P. and Crastes de Paulet, A., eds), pp 41-46, Colloque INSERM, V166.

Bascoul, J., Gozé, C., Domergue, N. and Crastes de Paulet, A. (1990) Serum level of 7α-hydroxycholesterol in hypercholesterolemic patients treated with cholestyramine. Biochim.Biophys.Acta 1044: 357-360

Bates, S.R., Jett, C.M. and Miller, J.E. (1983) Prevention of the hyperlipidemic serum or LDL-induced cellular cholesterol ester accumulation by 22-hydroxycholesterol and its analogue. Biochim.Biophy.Acta. 753: 281-293

Batzri, S. and Korn, E.D. (1973) Single bilayer liposomes prepared without sonication. Biochim.Biophys.Acta. 298: 1015-1019

Bennett Clark, S. and Tercyak, A.M. (1984) Reduced cholesterol transmucosal transport in rats with inhibited mucosal acyl CoA:cholesterol acyltransferase and normal pancreatic function. J.Lipid Res. 25: 148-159

Berge, R.K. (1979) Purification and characterisation of a long-chain acyl-Co A hydrolase from rat liver microsomes. Biochim.Biophy.Acta. 574: 321-333

Bergman, M., Gidez, L.I. and Eder, H.A. (1986) High density lipoprotein

subclasses in diabetes. Am.J.Med. 81: 488-492

Bergström, S, and Samuelsson, B. (1961) The autooxidation of cholesterol. *in* Autooxidation and Autooxidants. Vol I. Lundberg, W.O. (ed.) John Wiley and Sons, New York. 233-248.

Bergström, S and Wintersteiner, O. (1942) J.Biol.Chem. 145: 309.

Bernheimer, A.W., Robinson, W.G., Linder, R., Mullins, D., Yip, Y.K., Cooper, N.S., Seidman, I. and Uwajima, T. (1987) Toxicity of enzymatically-oxidized low-density lipoprotein. Biochem.Biophys.Res.Commun. 148: 260-266

Bidlingmeyer, B.A., Del Rios, J.K. and Korpé, J. (1982) Separation of organic amine compounds on silica gel with reversed-phase eluents. Anal. Chem 54: 442-447.

Billheimer, J.T. (1985) Cholesterol acyltransferase *in* Methods in enzymology 111: 286-293. (Law, J.H. and Rilling, H.C. eds.,) Academic Press, Inc., Orlando, Florida, USA

Billheimer, J.T. and Gillies, P.J. (1990) Intracellular cholesterol esterification *in* Advances in cholesterol research. Esfahani, M. and Swaney, J.B. (eds.,). Telford Press, Caldwell, N.J. USA. p7-45

Billheimer, J.T., Tavani, D. and Nes, W.R. (1981) Effect of dispersion of cholesterol in Triton WR-1339 on acyl CoA:cholesterol acyltransferase in rat liver microsomes. Anal. Biochem., 111: 331-335

Bischoff, F. (1969) Carcinogeneic effects of steroids. Adv. Lipid Res. 7: 165-244

Björkhem, I. (1969) On the mechanism of the enzymatic conversion of cholest-5-ene-3ß,7α-diol into 7α-hydroxycholest-4-en-3-one. Eur. J.Biochem 8: 337-344

Björkhem, I. (1985) *in* Sterols and Bile acids (Danielsson, H. and Sjövall, J. eds) Elsevier, Amsterdam. pp231-278.

Björkhem, I., Andersson, O., Diczfalusy, U., Sevastik, B., Xiu, R-J., Duan, C. and Lund, E. (1994) Atherosclerosis and sterol 27-hydroxylase: Evidence for a role of this enzyme in elimination of cholesterol from human macrophages. Proc.Natl.Acad.Sci.USA 91: 8592-8596

Björkhem, I. and Danielsson, H. (1975) 7α-hydroxylation of exogenous and endogenous cholesterol in rat liver microsomes Eur.J.Biochem 53: 63-70

Bjorkhem, I. and Kallner, A. (1976) Hepatic 7α-hydroxylation of cholesterol in ascorbate-deficient and ascorbate-supplimented guines pigs. J.Lipid Res. 17: 360-365.

Björkhem, I., Nyberg, B. and Einarsson, K. (1992) 7α-hydroxylation of 27-hydroxycholesterol in human liver microsomes. Biochim.Biophys.Acta. 1128: 73-76

Björkhem, I., Oftebro, H., Skrede, S., and Pederson, J.I. (1981) Assay of intermediates in bile-acid biosynthesis using isotope dilution-mass spectrometry-hepatic levels in the normal state and in cerebrotendinous xanthomatosis. J.Lipid Res 22: 191-200

Björkhem, I., Reihnér, E., Angelin, B., Ewerth, S., Åkerlund, J-E. and Einarsson, K. (1987) On the possible use of the serum level of 7α-hydroxycholesterol as a marker for increased activity of the cholesterol 7α-hydroxylase in humans. J.Lipid Res. 28: 889-894

Blomhoff, J.P. (1974) *In vitro* determination of lecithin:cholesterol acyltransferase in plasma. Scand.J.Clin.Lab.Invest. 33: 35-43

Bollag, D.M. and Edelstein, S.J. (1991) *in* Protein Methods. Wiley-Liss, Inc. New York. pp143-160.

Bösinger, S., Luf, W. and Brandl, E. (1993) 'Oxysterols': Their occurrence and biological effects. Int.Dairy Journal 3: 1-33

Bosisio, E., Galli, G. and Galli Kienle, M. (1983) Influence of species and sex on cholesterol 7α-hydroxylase activity in experimental animals. Eur. J. Biochem. 136: 167.

Boyd, G.S., McNamara, B., Suckling, K.E. and Tocher, D.R. (1983) Cholesterol metabolism in the adrenal cortex. J.Steroid Biochem. 19: 1017-1027

Brady, D.R. and Gaylor, J.L.(1971) Enzymic formation of esters of methyl sterol precursors of choleserol. J.Lipid Res. 12: 270

Brecher, P. and Chan, C.T. (1980) Properties of acyl-CoA:cholesterol O-acyltransferase in aortic microsomes from atherosclerotic rabbits. Biochim.Biophys.Acta 617: 458-471

Brooks, C.J.W., Harland, W.A and Steel, G. (1966) Squalene, 26-hydroxycholesterol and 7-ketocholesterol in human atheromatous plaques. Biochim.Biophys.Acta. 125: 620-622

Brown, M.S., Dana, S.E. and Goldstein, J.L. (1975) Cholesterol ester formation in cultured human fibroblasts- stimulation by oxygenated sterols. J.Biol.Chem. 250: 4025-4027

Brown, M.S. and Goldstein, J.L. (1983) Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. Ann. Rev Biochem 52: 223-261

Brown, M.S. and Goldstein, J.L.(1984) How LDL receptors influence cholesterol and atherosclerosis. Scientific American 251: 58-66

Brown, M.S. and Goldstein, J.L. (1986) A receptor-mediated pathway for cholesterol homeostasis. Science 232: 34-47

Burrier, R.E., Deren, S., McGregor, D.G., Hoos, L.M., Smith, A.A. and Davis, H.R. Jr. Demonstration of a direct effect on hepatic acyl CoA:cholesterol acyltransferase (ACAT) activity by an orally administered enzyme inhibitior in the hamster. Biochem.Pharmacol. 47: 1545-1551

Cadenhead, D.A., Kellner, B.M.J. and Balthasar, D.M. (1982) Cholesterol oxidation and the behaviour of 5α-hydroperoxy-cholesterol at the air/water interface. Chem.Phys.Lipids 31: 87-96

Cali, J.J. and Russell, D.W. (1991) Characterisation of human sterol 27-hydroxylase- a mitochondrial cytochrome P-450 that catalyses multiple oxidation reactions in bile- acid biosynthesis.J.Biol. Chem 266: 7774-7778

Carlile, S.I. and Lacko, A.G. (1981) Strain differences in the age-related changes of rat lipoprotein metabolism. Comp.Biochem.Physiol. 70: 754-758

Ceryak, S., Bouscarel, B. and Fromm, H. (1993) Comparative binding of bile acids to serum lipoproteins and albumin. J.Lipid Res 34: 1661-1674

Chen, C.-H. and Albers, J.J. (1981) Further characterization of purified lecithin:cholesterol acyltransferase from human plasma. Biomedical Medicine. 25: 215-226

Chen, C-H., and Albers, J.J. (1982) Characterisation of proteoliposomes containing apoprotein A-I: a new substrate for the measurement of lecithin:cholesterol acyltransferase activity. J.Lipid Res. 23: 680-691

Chen, C.-H. and Albers, J.J. (1985) A rapid large-scale procedure for purification of lecithin-choleterol acyltransferase from human and animal plasma. Biochim.Biophys.Acta 834: 188-195

Chen, C.H. and Albers, J.J. (1986) Stimulation of lecithin:cholesterol acyltransferase activity by apolipoprotein A-II in the presence of apolipoprotein A-I. Eur.J.Biochem. 155: 589-594

Chen, Y-DI., Jeng, C-Y, and Reaven, G.M. (1987) HDL metabolism in diabetes. Diabetes Metab Rev. 3: 653-668

Chiang, J.Y.L., Miller, W.F. and Lin, G-M. (1990) Regulation of cholesterol 7α-hydroxylase in the liver. J.Biol.Chem 265: 3889-3897

Chong, K-S., Jahani, M., Hara, S. and Lacko, A.G. (1983) Characterization of lecithin:cholesterol from human plasma. 3. Chemical properties of the enzyme. Can. J. Biochem. Cell Biol 61: 875-881

Christians, U. and Sewing, K.-FR. (1989) Whole blood sample clean-up for chromatographic analysis *in* Selective sample handling and detection in high-performance liquid chromatography. Zech, K. and Frei, R.W. (eds.,). Elsevier Science Publishers B.V. Amsterdam

Chung, J., Abano, D.A., Fless, G.M. and Scanu, A.M. (1979) Isolation, properties and mechanism of *in vitro* action of lecithin:cholesterol acyltransferase from human plasma. J.Biol.Chem. 254: 7456-7464

Cighetti, S., Bosiso, E., Galli, G. and Galli-Kienle, M.G. (1983) The effect of cholestyramine on liver HMG-CoA reductase and cholesterol 7α-hydoxylase in various laboratory animals. Life Sci. 33: 2483-2488

Clayton, P.T., Leonard, J.V., Lawson, A.M., Setchell, K.D., Anderson, S., Egestad, B. and Sjövall, J. (1987) Familial giant cell hepatitis associated with synthesis of 3 beta, 7 alpha-dihydroxy- and 3 beta,7 alpha, 12 alpha-trihydroxy-5-cholenoic acids. J.Clin.Invest. 79: 1031-1038

Crastes de Paulet, A., Astruc, M.-E. and Bascoul, J. 1988. Biologie des lipides chez l'homme (Douste-Biazy, L. and Mendy, F. eds.), CNRS-CNERNA Editions Techniques et Documentation (Lavoisier), Paris. pp 154-174.

Csallany, A.S., Kindom, S.E., Addis, P.B. and Lee, J.H. (1989) HPLC method for quantitation of cholesterol and four of its major oxidation products in muscle and liver tissues. Lipids 24: 645-651

Dadgar, D. and Smyth, M.R. (1987) Validation of chromatographic methods of analysis of drugs in biological samples. Tr AC 5: 115-117

Danielsson, H., Einarsson, K. and Johansson, G. (1967) Eur.J.Biochem 2: 44-49

Danielsson, H. and Sjövall, J. (1975) Bile acid metabolism. Ann. Rev. Biochem. 44: 233-253

Davis, R.A., Musso, C.A., Malone-McNeal, Lattier, G.R., Hyde, P.M., Archambault-Schexnayder, J. and Straka, M. (1988) Examination of bile acid negative feedback regulation in rats. J.Lipid Res. 29: 202-211

De Caprio, J., Yun, J. and Javitt, N.B. (1992) Bile acid and sterol solubilization in 2-hydroxypropyl-ß-cyclodextrin. J.Lipid Res 33: 441-443

DeLamatre, J.G. and Roheim, P.S. (1981) Effect of cholesterol feeding on apoB and apoE concentration and distributions in euthyroid and hypothyroid rats. J.Lipid Res 22: 297-306

Devery, R.A.M., O'Meara, N., Collins, P.B., Johnson, A.H., Scott, L. and Tomkin, G.H. (1987) A comparative study of the rate-limiting enzymes of cholesterol synthesis, esterification and catabolism in the alloxan-induced diabetic rat and rabbit. Comp.Biochem.Phys. 88**B**: 547-550.

Devery, R.A.M., O'Meara, N., Collins, P.B., Johnson, A.H., Scott, L. and Tomkin, G.H. (1987) A comparative study of the rate-limiting enzymes of cholesterol synthesis, esterification and catabolism in the rat and rabbit. Comp.Biochem.Physiol. 87**B**: 679-702

Deykin, D. and Goodman, D.S. (1962) The hydrolysis of long-chain fatty acid esters of cholesterol with rat liver enzymes. J.Biol.Chem. 237: 3649-3656

D'Hollander, F. and Chevallier, F. (1969) Estimation qualitative et quantitive des sterols libres et esterifies du rat in toto et de ses tissus ou organes. Biochim. Biophys. Acta 176: 146-162

Dobiasova, M. (1983) Lecithin-cholesterol acyltransferase and the regulation of endogenous cholesterol transport. Adv.Lipid Res. 20: 107-194

Doerner, K.C., Gurley, E.C., Vlahcevic, Z.R. and Hylemon, P.B. (1995) Regulation of cholesterol 7α-hydroxylase expression by sterols in primary rat hepatocyte cultures. J.Lipid Res. 36: 1168-1177

Doi, Y. and Nishida, T. (1981) Lecithin-cholesterol acyltransferase from human plasma *in* Methods in enzymology 71: 753-767

Doolittle, G.M. and Chang, T-Y. (1982) Acyl-CoA:cholesterol acyltransferase in Chinese hamster ovary cells. Enzyme activity determined after reconstitution in

phospholipid/cholesterol liposomes. Biochim.Biophys.Acta. 713: 529-537

Dormandy, T.L. (1969) Biological rancidification. The Lancet ii (Sept. 27): 684-688

Drevon, C.A., Weinstein, D.B. and Steinberg, D. (1980) Regulation of cholesterol esterification and biosynthesis in monolayer-cultures of normal adult-rat hepatocytes. J.Biol.Chem. 255: 9128-9137

Dueland, S., Trawick, J.D., Nenseter, M.S., MacPhee, A.A. and Davis, R. A. (1992) Expression of 7α-hydroxylase in non-hepatic cells results in liver phenotypic resistance of the low density lipoprotein receptor to cholesterol repression. J.Biol.Chem. 267: 22695-22698

Egan, R.W., Jones, M.A. and Lehninger, A.L. (1976) Hydrophile-lipophile balance and critical micelle concentration as key factors influencing surfactant disruption of mitochondrial membranes. J.Biol.Chem. 251: 4442-4447

Einarsson, K., Angelin, B., Ewerth, S., Nilsell, K. and Bjorkhem, I. (1986) Bile acid synthesis in man: assay of hepatic microsomal cholesterol 7α-hydroxylase by isotope dilution-mass spectrometry. J.Lipid Res. 27: 82-88.

Einarsson, K., Benthin, L., Ewerth, S., Hellers, G., Stahlberg, D. and Angeliin, B. (1989) Studies on acyl-coenzyme A: cholesterol acyltransferase activity in human liver microsomes. J.Lipid Res. 30: 739-746

Einarsson, K., Ericsson, S., Ewerth, S., Reihnér, E., Rudling, M., Stahlberg, D. and Angelin, B. (1990) Bile acid sequestrants: mechanisms of action on bile acid and cholesterol metabolism Eur.J.Clin.Pharm 40[Suppl 1]: S53-S58

Eisenberg, S. (1984) High density lipoprotein metabolism. J.Lipid Res 25: 1017-1058

Eisenberg, S. and Levy, R.I. (1975) Lipoprotein metabolism. Adv.Lipid Res.13: 1

Emanuel, H.A., Hassel, C.A., Addis, P.B., Bergmann, S.D. and Zavoral, J.H. (1991) Plasma cholesterol oxidation products (oxysterols) in human subjects fed a meal rich in oxysterols. J.Food Sci. 56: 843-847

Ericksson, S. (1957) Proc.Soc.Exp.Biol.Med. 94: 518-582

Erickson, S.K., Lear, S.R. and McCreery, M.J. (1994) Functional sizes of hepatic enzymes of cholesteryl ester metabolism determined by radiation inactivation. J.Lipid Res 35: 763-769

Erickson, S.K., Shrewsbury, M.A., Brooks, C. and Meyer, D.J. (1980) Rat liver acyl-coenzyme A:cholesterol acyltransferase: its regulation *in vivo* and some of its properties *in vitro*. J.Lipid Res 21: 930

Esterbauer, H., Jürgens, G., Quehenberger, O. and Koller, E. (1987) Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. J.Lipid Res. 28: 495-509

Faggiotto, A., Ross, R. and Harker, L. (1984) Studies of hypercholesterolemia in the nonhuman primate: I. Changes that lead to fatty streak formation. Arteriosclerosis 4: 323-341

Fantappie, S., Catapano, A., Cancellieri, M., Fascoli, L., DeFabiani, E., Bertoli, M. and Bosisio, E. (1992) Plasma lipoproteins and cholestereol metabolism in Yoshida rats- an animal model of spontaneous hyperlipemia. Life Sci. (England) 50: (24), 1913-1924.

Farese, G. and Mager, M. (1970) Protein-free filtrates obtained by membrane ultrafiltration. Clin.Chem. 16: 280-281

Field, F.J., Erickson, S.K. and Cooper, A.D. (1982) **(b)** Intestinal acyl-coenzyme A:cholesterol acyltransferase (ACAT). Regulation *in vivo* and *in vitro*. Gastroenterology 83: 873-880.

Field, F.J. and Mathur, S.N. (1983) Regulation of acyl CoA:cholesterol acyltransferase by 25-hydroxycholesterol in rabbit intestinal microsomes and absorptive cells. J.Lipid Res. 24: 1049-1059

Field, F.J. and Salome, R.G. (1982) **(a)** Effect of dietary fat saturation, cholesterol and cholestyramine on acylcoenzyme A:cholesterol acyltransferase activity in rabbit intestinal microsomes. Biochim.Biophys.Acta. 712: 557-570

Fielding, C.J. (1990) Lecithin:cholesterol acyltransferase *in* Advances in cholesterol research. Esfahani, M. and Swaney, J.B. (eds.,) The Telford Press, Caldwell, N.J. USA. pp 271-314.

Fielding, C.J. and Fielding, P.E. (1971) Purification and substrate specificity of Lecithin-cholesterol acyltransferase from human plasma. FEBS Letts 15: 355-358

Fielding, C.J. and Fielding, P.E. (1985) Metabolism of cholesterol and lipoproteins *in* Biochemistry of lipids and membranes. Vance, D.E. and Vance, J.E. (eds.,). The Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA,USA pp.405-474

Fielding, C.J. and Fielding, P.E. (1995) Molecular physiology of reverse cholesterol transport. J.Lipid Res. 36: 211-228

Finocchiaro, E.T. and Richardson, T. (1983) Sterol oxides in foodstuffs: A review. J.of Food Protection 46: 917-925

Flanagan, R.J. and Jane, I. (1985) High performance liquid chromatographic analysis of basic drugs on silica columns using non-aqueous ionic eluents. I. Factors influencing retention, peak shape and detection response. J. Chromatogr. 323: 173-189

Flegg, M.M. (1973) An investigation into the determination of serum cholesterol by an enzymatic method. Ann.Clin.Biochem 10: 79-84

Folch, J., Lees, M. and Stanley, G.H.S. (1957) A simple method for isolation and purification of total lipides from animal tissues J. Biol. Chem. 226: 497-509.

Fong, L.G., Parthasarathy, S., Witztum, J.L. and Steinberg, D. (1987) Nonenzymatic oxidative cleavage of peptide bonds in apoprotein B-100. J.Lipid Res. 28: 1466-1477

Franceschini, G., Baio, M., Calabresi, L., Sirtori, C.R. and Cheung, M.C. (1990) Apolipoprotein Aimilano partial lecithin cholesterol acyltransferase deficiency due to low levels of a functional enzyme. Biochim.Biophy.Acta 1043: 1-6

Fry, R.P., Mayes, P.A., Suckling, K.E. and Botham, K.M. (1990) The effect of chylomicron remnants on bile acid synthesis in cultured rat hepatocytes. Biochim.Biophys.Acta. 1042: 413-416

Fukushima, K., Ichimiya, H., Higashijima, H., Yamashita, H., Kuroki, S., Chijiiwa, K. and Tanaka, M. (1995) Regulation of bile acid synthesis in the rat: relationship between hepatic cholesterol 7α-hydroxylase activity and portal bile acids. J.Lipid Res. 36: 315-321

Funke, H., Von Eckardstein, A., Pritchard, P.H., Albers, J.J., Kastelein, J.J.P., Droste, C.C. and Assmann, G. (1991) A molecular defect causing fish eye disease-an amino acid exchange in lecithin-cholesterol acyltransferase (LCAT) leads to selective loss of alpha-LCAT activity. Proc.Natl.Acad.Sci.USA 88: 4855-4859

Gerloczy, A., Hoshino, T. and Pitha, J. (1994) Safety of oral cyclodextrins: Effects of hydroxypropyl cyclodextrins, cyclodextrin sulfates and catinic cyclodextrins on steroid balance in rats. J.Pharmaceutical Sciences 83: 193-196

Gerrity, R.G. (1981) The role of the monocyte in atherogensis. I Transaction of blood-borne monocytes into foam cells in fatty lesions. Amer J.Pathol 103: 191-200

Gielen, J., VanCantfort, J., Robaye, B. and Renson, J. (1969) Rythme circadien de

la cholestérol-7α-hydroxylase chez la rat. C.R.Acad.Sci.Paris 269: 731-732

Gillett, M.P.T. and Owen, J.S. (1992) Cholesterol esterifying enzymes-lecithin:cholesterol acyltransferase (LCAT) and acylcoenzyme A:cholesterol acyltransferase (ACAT) *in* Lipoprotein analysis- A practical approach. Converse, C.A. and Skinner, E.R. (eds.,) IRL Press, Oxford University Press, New York. pp187-201

Glick, J.M. (1990) Intracellular cholesteryl ester hydrolysis and clearance *in* Advances in cholesterol research. Esfahani, M. and Ewaney, J.B. (eds) Telford Press, West Caldwell, N.J. pp 171-200

Glomset, J.A. (1964) The plasma lecithin:cholesterol acyltransferase reaction. J.Lipid Res. 9: 155-167

Glomset, J.A. (1972) *in* "Blood lipids and lipoproteins: Quantitation, Composition and Metabolism" G.J.Nelson, (ed.), Wiley (Interscience), New York. p.745.

Glomset, J.A. and Norum, K.R. (1973) The metabolic role of lecithin:cholesterol acyltransferase: perspectives from pathology. Adv Lipid Res 1: 1-65

Glomset, J.A., Norum, K.R. and Gjone, E. (1983) *in* The metabolic basis of inherited disease. Stanbury, J.B., Wyngaarden, J.B. and Fredrickson, D.S. (eds.), McGraw-Hill, New York. pp.643-654

Glomset, J.A. and Wright, J.L. (1964) Some properties of a cholesterol esterifying enzyme in human plasma. Biochim.Biophy.Acta. 89: 266-276

Goldstein, J.L. and Brown, M.S. (1990) Regulation of the mevalonate pathway. Nature 343: 425-430

Goldstein, J.L., Ho, Y.K., Basu, S.K. and Brown, M.S. (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc.Natl.Acad.Sci.USA 76: 333-337

Goodman, D.S., Deykin, D. and Shiratori, T. (1964) J.Biol.Chem. 230: 1325

Gotto, A.M., Pownall, H.J. and Havel, R.J. (1986) Introduction to the plasma lipoproteins *in* Methods in Enzymology 128: Segrest, J.P. and Albers, J.J.(eds.). Academic Press, Inc., Orlando, USA. pp 3-41

Groener, J.E.M., Van Rozen, A.J. and Erkelens, D.W. (1984) Cholesteryl ester transfer activity. 50: 261-271

Grummer, R.R. and Carroll, D.J. (1988) A review of lipoprotein cholesterol metabolism: importance to ovarian function. J.Anim.Sci. 66: 3160-3173

Grundy, S.M.(1988) HMG-CoA reductase inhibitors for treatment of hypercholesterolemia. New England Journal of Medicine 319: 24-33

Grundy, S.M., Ahrens, E.H.Jr., Miettinen, T.A. (1965) Quantitative isolation and gas-liquid chromatographic analysis of fecal bile acid. J.Lipid Res. 6: 397-410.

Grundy, S.M., Aherns, E.H, Jr. and Salen, G. (1971) Interruption of the enterohepatic circulation of bile acids in man; comparative effects of cholestyramine and ileal exclusion on cholesterol metabolism. J.Lab.Clin.Med 78: 94-121

Ha, Y.C., and Barter, P.J. (1982) Differences in plasma cholesteryl ester transfer activity in sixteen vertebrate species. Comp.Biochem.Physiol. 71: 265-269

Halliwell, B. and Gutteridge, J.M.C. (1989) Lipid peroxidation: a radical chain reaction *in* Free radicals in biology and medicine. Halliwell, B. and Gutteridge, J.M.C., (eds.,) 2nd Ed., Clarendon Press, Walton St., Oxford. pp 188-268

Hames, J.M. (1984) *in* Methods in Molecular Biology. Volume 1: Proteins. J.M.Walker, (ed.). Humana Press, Clifton, New Jersey. pp 57-62

Hames, B.D. (1981) *in* Gel Electrophoresis of Proteins: A Practical Approach. pp71-77

Harper's review of Biochemistry (1982) Martin, D.W., Mayes, P.A. and Rodwell, V.W. Lange Medical Publishers, California. p531.

Hasler, C.B., Tall, A.R., Swenson, T.L., Weech, P.K., Marcel,Y.L. and Milne, R.W. (1988) Monoclonal antibodies to the M_r 74,000 cholesteryl ester transfer protein neutralize all the cholesteryl ester and triglyceride transfer activities in human plasma. J. Biol. Chem. 263: 5020-5023

Heftmann, E. and Hunter, I.R.(1979) High-pressure liquid chromatography of steroids. J.Chromatog 165: 283.

Heuman, D.M., Vlahcevic, Z.R., Bailey, M.L. and Hylemon, P.B. (1988) Regulation of bile acid synthesis. II. Effect of bile acid feeding on enzymes regulating hepatic cholesterol and bile acid synthesis in the rat. Hepatology 8: 892-897

Higley, N.A., Beery, J.T., Taylor, S.L., Porter, J.W., Dziuba, J.A. and Lalich, J.J. (1986) Comparative atherogenic effects of cholesterol and cholesterol oxides. Atherosclerosis 62: 91-104

Hill, M.J., Crowther, J.S., Drasar, B.S., Hawksworth, G., Aries, V. and Williams, R.E.O. (1971) Bacteria and aetiology of cancer of large bowel. Lancet 1: 95-100

Hobbs, H.H., Russell, D.W., Brown, M.S. and Goldstein, J.L. (1990) The LDL receptor locus in familial hypercholesterolemia. Annu.Rev.Genet. 24: 133-170

Hodis, H.N., Kramsch, D.M., Avogaro, P., Bittolo-Bon, G., Cazzolato, G., Hwang, J., Peterson, H. and Sevanian, A. (1994) Biochemical and cytotoxic characteristics of an *in vivo* circulating oxidized low density lipoprotein (LDL-). J.Lipid Res. 35: 669-677

Hofmann, A.F. Hofmann, N.E. (1974) Measurement of bile acid kinetics by isotope dilution in man. Gastroenterology 67: 314-323.

Hsu, R.C., Kanofsky, J.R. and Yachnin, S. (1980) The formation of echinocytes by the insertion of oxygenated sterol compounds into red-cell membranes.Blood 56: 109-117.

Hutton, H.R.B. and Boyd, G.S. (1966) The metabolism of cholesten-5-en-3ß-7α-diol by rat fractions. Biochim.Biophys.Acta. 116: 336-361

Hylemon, P.B., Studer, E.J., Pandak, W.M., Heuman, D.M., Vlahcevic, Z, R. and Chiang, J.Y.L. (1989) Simultaneous measurement of cholesterol 7α-hydroxylase activity by reverse-phase high performance liquid chromatography using both endogenous and exogenous [4-14C]cholesterol as substrate. Analytical Biochemistry 182: 212-216.

Ihm, J. and Harmony, J.A.K. (1980) Simultaneous transfer of cholesteryl ester and phospholipid by protein(s) isolated from human lipoprotein-free plasma. Biochem.Biophys.Res.Commun. 93: 1114-1120

Ikawa, S., Takita, M. and Ogura, M. (1979) Steroids as substrates for cholesterol:oxygen oxidoreductase, with special reference to 3ß-hydroxy bile acids. J.Biochem (Tokyo) 85: 1447-1452

Imai, H., Werthessen, N.T., Subramanyam, V., Le Quesne, P.W., Soloway, A.H. and Kanisawa, M. (1980) Angiotoxicity of oxygenated sterols and possible precursors. Science 207: 651-653

Ireland, J.T., Thomson, W.S.T. and Williamson, J. (1980) Lipid metabolism and diabetes *in* Diabetes today. Ireland, J.T., Thomson, W.S.T. and Williamson, J. (eds.,) HM + M Publishers, Aylesbury, U.K. pp 76-83.

Jauhiainen, M. and Dolphin, P.J. (1986) Human plasma lecithin-cholesterol

acyltransferase. J.Biol.Chem. 261: 7032-7043

Jauhiainen, M., Metso, J., Pahlman, R., Blomqvist, S., Van Toll, A. and Ehnolm, C. (1993) Human plasma phospholipid transfer protein causes high density lipoprotein converesion. J.Biol.Chem 268: 4032-4036

Javitt, N.B. (1994) Bile acid synthesis from cholesterol: regulatory and auxiliary pathways. The FASEB Journal 8: 1308-1311

Javitt, N.B., Kok, E., Cohen, B. and Burstein, S. (1982) Cerebrotendinous xanthomatosis- reduced serum 26-hydroxycholesterol. J.Lipid Res. 23: 627-630

Jelinek, D.F., Andersson, S., Slaughter, C.A. and Russell, D.W. (1990) Cloning and regulation of cholesterol 7α-hydroxylase, the rate limiting enzyme in bile acid biosynthesis. J.Biol.Chem. 265: 8190-8197

Jiao, S., Matsuzawa, Y., Matsubara, K., Kihara, S., Nakamura, T., Tokunaga, K., Kubo, M. and Tarui, S. (1988) Incresaed activity of intestinal acyl-CoA:cholesterol acyltransferase in rats with streptozotocin-induced diabetes and restoration by insulin supplementation. Diabetes 37: 342-346

Jonas, A. (1986a) Reconstitution of high-density lipoproteins *in* Methods in enzymology 128: 553-582. Segrest, J.P. and Albers, J.J. (eds.,). Academic Press, Florida, USA

Jonas, A. (1986b) Synthetic substrates of lecithin:cholesterol acyltransferase (Minireview) J.Lipid Res 27: 689-698

Jonas, A. (1991) Lecithin-cholesterol acyltransferase in the metabolism of high-density lipoproteins. Biochim. Biophys. Acta 1084: 205-220

Jonas, A., Covinsky, K.E. and Sweeny, S.A. (1985) Effects of amino group modification in discoidal apolipoprotein A-I egg phosphatidylcholine-cholesterol complexes on their reactions with lecithin:cholesterol acyltransferase. Biochemistry 24: 3508-3513

Jonas, A., Drengler, S.M. and Patterson, B.W. (1980) Two types of complexes formed by the interaction of apolipoprotein A-I with vesicles of L-alpha-dimyristoyl phosphatidylcholine. J.Biol.Chem 255: 2183-2189

Jonas, A., Hefele Wald, J.H., Harms Toohill, K.L., Krul, E.S. and Kézdy, H.E. (1990) Apolipoprotein A-I structure and lipid properties in homogenous reconstituted spherical and discoidal high density lipoproteins. J.Biol.Chem 265: 22123-22129

Jonas, A., Kézdy, K.E. and Hefele Wald, J. (1989) Defined apolipoprotein A-I

conformations in reconstituted high density lipoprotein discs. J.Biol Chem 264: 4818-4824

Jonas, A. and Matz, C.E. (1982) Reaction of human lecithin:cholesterol acyltransferase with micellar substrates is independent of the phase state of the lipid. Biochemistry 21: 6867-6872

Jonas, A. and McHugh, H.T. (1983) Reaction of lecithin:cholesterol acyltransferase with micellar complexes of apoprotein A-I and phosphatidylcholine, containing various amounts of cholesterol. J.Biol.Chem. 258: 10335-10340

Jonas, A. and McHugh, H.T. (1984) Reaction of lecithin:cholesterol acyltransferase with micellar substrates: effect of particle size. Biochim.Biophys.Acta. 794: 361-372

Kandutsch, A.A. and Chen, H.W. (1973) Inhibition of sterol synthesis in cultured mouse cells by 7α-hydroxycholesterol, 7ß-hydroxycholesterol and 7-ketocholesterol. J.Biol.Chem. 248: 8408-8417

Kandutsch, A. A., Chen, H.W. and Heiniger, H.J. (1978) Biological activity of some oxygenated sterols. Science 201: 498-501

Kandutsch, A.A., Heiniger, H.J. and Chen, H.W. (1977). Effects of 25-hydroxycholesterol and 7-ketocholesterol, inhibitors of sterol-synthesis, administered orally to mice. Biochimica. Biophysica Acta 486: 260-272

Kaneda, T., Nakajima, A., Kobayashi, T. and Yoneyama, S. (1980) Quantitative analysis of cholesterol in foods by gas-liquid chromatography. J. Nutr. Sci. Vitaminol. 26: 497-505.

Kasama, T., Byun, D-S. and Seyama, Y. (1987) Quantitative analysis of sterols in serum by high-performance liquid chromatography- Application to the biochemical diagnosis of cerebrotendinous xanthomatosis. J.Chrom. 400: 241-246

Katayama, K. and Yamasaki, K. (1968) The enzymatic 7α-hydroxylation of cholesterol and its esters by rat liver homogenate. Yonago Acta Medica.12: 103-109

Kelly, M.J. and Roberts, S.M. (1995) Combating cholesterol. Nature 373: 192-193

Kern, F. Jr. (1991) Normal plasma cholesterol in an 88-year-old man who eats 25 eggs a day. N.Engl.J.Med 324: 896-899

Khachadurian, A.K. (1990) Role of cholesterol in atherosclerosis *in* Advances in cholesterol research. pp 385-420. Esfahani, M. and Swaney, J.B. (eds) Telford Press, New Jersey, 1990.

Khachadurian, A.K., Fung, C.H., Van Es, T. and Davis, F.F. (1981) Polyethylated cholesterol derviatives- organic synthesis, cellular uptake and effect on lipid metabolism in cultured skin fibroblasts. Biochim. Biophys. Acta. 665: 434-441.

Kim, S.K. and Nawar, W.W. (1993) Parameters influencing cholesterol oxidation. Lipids 28: 917-922

Kishinaka, M., Kosahara, K., Okamoto, S., Oda, H., Ichimiya, H., Chijiiwa, K. and Kuroki, S. (1992) Metabolism of intravenously administered 7α-hydroxycholesterol-3ß-stearate in the hamster. Biochim.Biophys.Acta. 1165: 222-228

Kitabatake, K., Piran, U., Kamio, Y., Doi, Y. and Nishida, T. (1979) Purification of human plasma lecithin:cholesterol acyltransferase and its specificity towards the acyl acceptor. Biochim.Biophy.Acta. 573: 145-154

Knox, J.H. (1978) Adsorption chromatography *in* High-performance liquid chromatography. Knox, J.H. (ed.,). Edinburgh University Press, Edinburgh. Chapter 3 (p20-33).

Kogaire, M., Gomez, G., Uchida, T., Ishizuka, J., Greeley, G.H.Jr. and Thompson, J.C. (1992) Chronic effect of oral cholestyramine, a bile acid sequestrant, and exogenous cholecystokinin on insulin release in rats. Pancreas 7: 15-20

Koopman, B.J., Van der Molen, J.C. and Wolthers, B.G.(1987) Determination of some hydroxycholesterols in human serum samples. J.Chromatogr. 416: 1-13

Korahani, V., Bascoul, J. and Crastes de Paulet, A. (1982) Autoxidation of fatty acid esters in solid state and aqueous dispersions. Lipids 17: 703-708

Kraemer, F.B. (1986) Insulin deficiency alters cellular cholesterol metabolism in murine macrophages. Diabetes 35: 764-770

Kudchodkar, B.J., Choi Lee, M-J., Lee, S-M., DiMarco, N.M. and Lacko, A.G. (1988) Effect of dietary protein on cholesterol homeostasis in diabetic rats. J.Lipid Res 29: 1272-1287

Kudo, K., Emmons, G.T., Casserly, E.W., Via, D.P., Smith, L.C., St. Pyrek, J. and Schroepfer, G.J. Jr. (1989) Inhibitors of sterol synthesis. Chromatography of acetae derivatives of oxygenated sterols. J.Lipid Res. 30: 1097-1111.

Kumar, N. andSinghal, O.P. (1991) Cholesterol oxides and atherosclerosis: A review. J.Sci.Food.Agric. 55: 497-510

Lagrost, L., Athias, A., Gambert, P. and Lallemant, C. (1994) Comparative study of

phospholipid transfer activities mediated by cholesteryl ester transfer protein and phospholipid transfer protein. J.Lipid Res. 35: 825-835

Lau, B.W.C. and Klevay, L.M.(1981) Plasma lecithin:cholesterol acyltransferase in copper-deficient rats. J.Nutr.111: 1698-1703

Law, B., Gill, R. and Moffat, A.C. (1984) High-performance liquid chromatography retention data for 84 basic drugs of forensic interest on a silica column using an aqueous methanol eluent. J. Chromatogr., 301: 165.

Lee, K.M. and Biellmann,J.F.(1988) Cholesterol conversion to cholestenone by cholesterol oxidase in polyphasic systems: Extension to the selective oxidation of 7ß-hydroxycholesterol. Tetrahedron 44: 1135-1139

Lehninger, A.L. (1982) Vitamins and trace elements in the function of enzymes *in* Principles of biochemistry. Anderson, S. and Fox, J. (eds.,). Worth publishers, New York, USA. pp249-276

Lichtenstein, A. and Brecher, P. (1983) Esterification of cholesterol and 25-hydroxycholesterol by rat liver micrsomes. Biochim.Biophys.Acta. 751: 340-348

Lim, C.K. (1991) Liquid chromatography in biomedical analysis:Basic approach *in* Liquid chromatography in biomedical analysis. Hanai, T. (ed.,). Elsevier Science Publishers B.V., The Netherlands. p 1-20

Lin, Y.Y. and Smith, L.L. (1974) Sterol metabolism. XXVIII. Biosynthesis and accumulation of cholest-5-ene-3ß,24-diol (cerebrosterol) in developing rat brain. Biochim.Biophys.Acta. 348: 189-196

Lindstedt, S. (1957) The turnover of cholic acid in man: Bile acid and steroids 51. Acta Physiol Scand 40: 1-9.

Liu, K., Ramjiawan, B., Kutryk, M.J.B. and Pierce, G.N. (1991) Effects of oxidative modification of cholesterol in isolated low density lipoproteins on cultured smooth muscle cells. Mol., and Cell., Biochem. 108: 49-56

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J.Biol.Chem. 193: 265-275.

Luskey, K.L., Faust, J.R., Chin, D.J., Brown, M.S. and Goldstein, J.L. (1983) Amplification of the gene for 3-hydroxy-3-methylglutaryl coenzyme A reductase, but not for the 53-kDa protein, in UT-1 cells. J.Biol.Chem. 258: 8462-8469

Maechler, P., Wollheim, C.B., Bentzen, C.L. and Niesor, E. (1992) Role of intestinal acyl-CoA:cholesterol acyltransferase activity in the hyperresponse of

diabetic rats to dietary cholesterol. J.Lipid Res 33: 1475-1484
Maerker, G. (1987) Cholesterol autoxidation- Current status. J.Amer.Oil.Chem.Soc. 64: 388-392

Mahadevan, V. and Soloff, L. (1983) A method for isolating human plasma lecithin:cholesterol acyltransferase without using anti-apolipoprotein D and it's characterization. Biochim.Biophys.Acta 752: 89-97

Mahley, R.W. and Holcombe, K.S. (1977) Alterations of plasma lipoproteins and apoproteins following cholesterol feeding in the rat. J.Lipid Res.18: 314-324.

Maor, I. and Aviram, M. (1994) Oxidized low density lipoprotein leads to macrophage accumulation of unesterified cholesterol as a result of lysosomal trapping of the lipoprotein hydrolysed cholesteryl ester. J.Lipid Res. 35: 803-819

Marcel, Y.L., Vezina, C., Emond, D. and Suzue, G. (1980) Heterogeneity of human high-density lipoprotein- presence of lipoproteind without their rates as substrates for lecithin-cholesterol acyltransferase reaction. Proc. Natl. Acad. Sci. USA 77: 2969-2976

Martin, K.O., Budai, K. and Javitt, N.B. (1993) Cholesterol and 27-hydroxycholesterol 7a-hydroxylation: evidence for two different enzymes. J. Lipid Res. 34: 581-588.

Mason, H.S.(1965) Oxidases. Annu. Rev. Biochem 34: 595-634.

Mattock, M.B., Fuller, J.H., Maude, P.S. and Keen, H. (1979) Lipoproteins and plasma cholesterol esterification in normal and diabetic subjects. Atherosclerosis 34: 437-449

Matz, C.E. and Jonas, A. (1982) Micellar complexes of human apolipoprotein A-I with phosphatidylcholines and cholesterol prepared from cholate-lipid dispersions. J.Biol.Chem. 257: 4535-4540

Matz, C.E. and Jonas, A. (1982) Reaction of human lecithin cholesterol acyltransferase with synthetic micellar complexes of apolipoprotein A-I, phosphatidylcholine and cholesterol. J.Biol Chem 257: 4541-4546

Mayer, D. (1972). Some of the new aspects on the regulation of cholesterol 7α-hydroxylase activity. *In* Bile acids in human diseases, II. Bile acid meeting, Freiburg i. Br., June 30-July 1, 1972. P.Back and Gerok, W. eds., F.K.Schattauer Verlag, Stuttgart, 103-109.

Menkes, J.H.. Schimschock, J.R. and Swanson, P.D. (1968) Arch. Neurol. 19: 47-53

Meyer, V.R. Reversed-phase HPLC *in* Practical high-performance liquid chromatography. Meyer, V.R. (ed.,)Wiley, Verlag (1988) Chapter 10. pp132.

Milander, M.M. and Kuhn, M. (1992) Lipid-lowering drugs. AACN Clinical Issues 3: 494-506

Miller, N.E. (1990) Disorders of HDL metabolism: clues to the pathophysiology of reverse cholesterol transport *in* Disorders of HDL, Proceedings of the International symposium held at the Karolinska Hospital, Stockholm, 7-8 June 1990. Carlson, L.A. (ed.,). Smith-Gordon, London.p 1-6

Mills, G.L., Lane, P.A. and Weech, P.K. (1984) Isolation and purification of plasma lipoproteins *in* A Guidebook to Lipoprotein Technique. Burdon, R.H. and van Knippenberg, P.H. (eds.).pp 18-116. Elsevier Science Publishers B.V.Amsterdam, The Netherlands.

Misra, D.P., Staddon, G., Powell, N., Misra, J. and Crook, D. (1974) Lecithin-cholesterol acyltransferase activity in Diabetes mellitus and the effect of insulin on these cases. Clin Chimica Acta 56: 83-89

Morel, D.W. and Chisolm, G.M. (1989) Antioxidant treatment of diabetic rats inhibits lipoprotein oxidation and cytotoxicity. J.Lipid Res. 30: 1827-1834

Morel, D.W., DiCorleto, P.E. and Chislom, G.M. (1984) Endothelial and smooth muscle cells alter low density lipoprotein *in vitro* by free radical oxidation. Arteriosclerosis 4: 357-364

Morin, C.R., Edralin, G.G. and Woo, J.M. (1974) Esterification of cholesterol by subcellular fractions from swine arteries and inhibition by amphipathic and polyanionic compounds. Atherosclerosis 20: 27-39

Mosbach, E.H. (1972) Hepatic synthesis of bile acids- Biochemical steps and mechanisms of rate control. Arch. Intern. Med. 130: 478

Myant, N.B. and Mitropoulos, K.A. (1977) Cholesterol 7α-hydroxylase. J. Lipid Res. 18: 135-153

Nagasaki, T. and Akanuma, Y. (1977) A new colorimetric method for the determinatin of plasma lecithin:cholesterol acyltransferase activity. Clin.Chim.Acta. 75: 371-375

Ness, G.C., Pendleton, L.C., Li, Y.C. and Chiang, J.Y.L. (1990) Effect of thyroid hormone on hepatic cholesterol 7 alpha hydroxylase, LDL receptor, HMG CoA reductase, farnesyl pyrophosphate synthetase and apolipoprotein A-I mRNA levels in hypophysectomized rats. Biochem.Biophys.Res.Commun. 172: 1150-1156

Nichols, A.V., Krauss, R.M. and Musliner, T.A. (1986) Nondenaturing polyacrylamide gradient gel electrophoresis *in* Methods in enzymology 128: 417-431. Segrest, J.P. and Albers, J.J. (eds.,) Academic Press, Inc. USA.

Nikkilä, E.A.(1984) HDL in relation to the metabolism of triglyceride-rich lipoproteins *in* Miller, N.E. and Miller, G.J. (eds) Clinical and Metabolic Aspectsd of High-Density Lipoproteins. Elsevier Science Publishers B.V. Amsterdam. pp 217-245.

Nimmannit, S. and Porter, J.W. (1980) Cholesterol 7α-hydroxylase: Solubilization and determination of enzyme activity. Arch. Biochem. 201: 533-543.

Nishida, H.I., Kato, H. and Nishida, T. (1990) Affinty of lipid transfer protein for lipid and lipoprotein particles as influenced by lecitihin-cholesterol acyltransferase. J.Biol.Chem. 265: 4876-4883

Nishigaki, I., Hagihara, M., Tsunekawa, H.T., Maseki, M. and Yagi, K. (1981) Lipid peroxide levels of serum lipoprotein fractions of diabetic patients. Biochem.Med. 25: 373-378

Nissen, H.P. and Kreysel, H.W. (1987) The use of HPLC for the determination of lipids in biological materials. Chromatographia 30: 686-690

Norum, K.R., Glomset, T.A., Nichols, A.V. and Forte, T (1971) Plasma lipoproteins in familial lecithin:cholesterol acyltransferase deficiency: physical and chemical studies of low and high density lipoproteins. J.Clin Invest 50: 1131-1141

Nourooz-Zadeh, J. (1990) Determination of the autoxidation products from free or total cholesterol: a new multistep enrichment methodology including the enzymatic release of esterified cholesterol. J.Agric.Food Chem. 30: 1667-1673

Oda, H., Yamashita, H., Kosahara, K., Kuroki, S. and Nakayama, F. (1990) Esterified and total 7a-hydroxycholesterol in human serum as an indicator for hepatic bile acid synthesis. J.Lipid Res 31: 2209-2218.

Oftebro, H., Björkhem, I., Skrede, S., Schreiner, A. and Pedersen, J.I. (1980) Cebrotendinous xanthomatosis- a defect in mitochondrial 26-hydroxylation required for normal biosynthesis of cholic acid. J.Clin.Invest. 65: 1418-1430

Ogishima, T. and Okuda, K. (1986) An improved method for assay of cholesterol-7-α-hydroxylase activity. Analytical Biochem. 158: 228-232

Ógren, L., Csiky, I., Risinger, L., Nilsson, L.G. and Johansson, G. (1980) A post column enzyme reactor for detection of oxidized cholesterol in HPLC separations.

Anal. Chim. Acta, 117: 71

Ogura, M. and Yamasaki, K. (1970) Metabolism of 3ß-stearyl-7α-hydroxycholesterol in rats. J.Biochem. 67: 643-653

O'Meara, N., O'Donnell, L., Owens, D., Collins, P., Johnson, A. and Tomkin, G. (1986) The influence of diabetic control on serum lipoproteins on in rabbits. Clin.Sci. 71: 61

Owens, D., Stinson, J., Collins, P., Johnson, A. and Tomkin, G.H. (1991) Improvement in the regulation of cholesterologenesis in diabetes: the effect of reduction in serum cholesterol by simvastatin. Diabetic Medicine 8: 151-156

Parish, E.J., Nanduri, V.B.B., Kohl, H.H. and Taylor, F.R. (1986) Oxysterols: chemical synthesis, biosynthesis and biological activities. Lipids 21: 27-30

Peng, S-K., Morin, R.J., Tham, P. and Taylor, C.B. (1985) Effects of oxygenated derivatives of cholesterol on cholesterol uptake by cultured aortic smooth muscle cells. Artery 13: 144-164

Peng, S-K. and Taylor, C.B. (1984) Cholesterol autoxidation, health and arteriosclerosis. World Rev.Nutr.Diet 44: 117-154

Peng, S.K., Taylor, C.B., Hill, J.C. and Morin, R.J. (1985) Cholesterol oxidation derivatives and arterial endothelial damage. Atherosclerosis 54: 121-133

Petersson, L. and Eriksson, C.G. (1994) Reversed-phase high-performance liquid chromatographic determination of 7α-hydroxy-4-cholesten-3-one in human serum. J.Chrom. 657: 31-36

Philips, N.R., Havel, R.J. and Kane, J.P. (1981) Levels and interrelationships of serum and lipoprotein cholesterol and triglycerides: Association with adiposity and the consumption of ethanol, tobacco and beverages containing caffeine. Arteriosclerosis 1: 13-24

Piran U. and Nishida, T. (1979) Utilisation of various sterols by lecithin-cholesterol acyltransferase as acyl acceptor. Lipids 14: 478-482

Proudlock, J.W. and Day, A.J. (1972) Cholesterol-esterifying enzymes of atherosclerotic rabbit intima. Biochim.Biophys.Acta. 260: 716-723

Reddy, B.S., Martin, C.W. and Wynder, E.L. (1977) Fecal bile acids and cholesterol metabolites of patients with ulcerative colitis, a high-risk group for development of colon cancer. Cancer Res. 39: 1697-1701

Reichl, D. and Miller, N.E. (1989) Pathophysiology of reverse cholesterol transport-Insights from inherited disorders of lipoprotein metabolism. Arterosclerosis 9: 785-797

Reichl, D. and Miller, N.E. (1992) Pathophysiology of reverse cholesterol transport. Arteriosclerosis 9: 785-797.

Russell, D.W. (1992) Cholesterol biosynthesis and metabolism. Cardiovascular drugs and therapy 6: 103-110

Reihnér, E., Björkhem, I., Angelin, B., Ewerth, S. and Einarsson, K. (1989) Bile acid synthesis in humans: regulation of hepatic microsomal cholesterol 7α-hydroxylase activity. Gastroenterology 97: 1498-1505.

Reinhart, M.P., Billheimer, J.T., Faust, J.R. and Gaylor, J.L. (1987) Subcellular localization of the enzymes of cholesterol biosynthesis and metabolism in rat liver. J.Biol.Chem. 262: 9649-9655

Richmond, W. (1973) Preparation and properties of a Cholesterol oxidase from *Nocardia* sp. and its application to the enzymatic assay of total cholesterol in serum. Clin.Chem. 19: 1350-1356

Rosenfeld, M.E. (1991) Antioxidants: Potential inhibitiors of the multiple atherogenic effects of oxidized lipoproteins. An international conference on Lipid lowering agents: A critical review documentation. September 1991, London.

Ross, R. and Glomset, J.A. (1973) Atherosclerosis and the arterial smooth muscle cell. Science. 180: 1332-1339

Russell, P.T., Scott, J.C. and VanBruggen, J.T. (1962). Effects of dietary fat on cholesterol metabolism in the diabetic rat. J.Nutr. 76: 460-466.

Russell, D.W. and Setchell, K.D.R. (1992) Bile acid biosynthesis. Biochemistry 31: 4737-4749

Sahlin, S., Granström, L., Gustafsson, U., Ståhlberg, D., Backman, L. and Einarsson, K. (1994) Hepatic esterification rate of cholesterol and biliary lipids in human obesity. J.Lipid Res 35: 484-490

Sakamoto, A. (1968) The effects of bile salts and thyroid hormone on cholesterol esterase and esterifying enzyme in rat liver. Yonago Acta Medica. 12: 81-91

Salen, G., Meriwether, T.W. and Nicolau, G. (1975) Chenodeoxycholic acid inhibits increased cholesterol and cholestanol synthesis in patients with cercbrotendinous xanthomatosis. Biochem. Med. 14: 57-74

Salen, G., Nicolau, G., Shefer, S. and Mosbach, E.H. (1975) Hepatic cholesterol metabolism in patients with gallstones. Gastroenterology 69: 676-684

Salen, G., Shefer, S. and Berginer, V.D. (1983) *in* The metabolic basis of inherited disease. J.B.Wijngaarden, D.S. Fredrickson, J.L. Goldstein and M.S. Brown (eds). Macmillan, New York, 5th ed., p173.

Sanghvi, A., Grassi, E., Bartman, C., Lester, R., Galli Kienle, M. and Galli, G.(1981) Measurement of cholesterol 7α-hydroxylase activity by selected ion monitoring. J. Lipid Res. 22: 720-724

Scallen, T.J. and Sanghvi, A. (1983) Regulation of 3 key enzymes in cholesterol metabolism by phosphorylation dephosphorylation. Proc.Natl.Acad.Sci 80: 2477-2480.

Schaeffer, E.J., Anderson, D.W., Brewer, H.B. Jr. *et al.*, (1978) Plasma triglycerides in regulation of HDL cholesterol levels. Lancet 2: 391-393

Schernthaner, G., Kostner, G.M., Dieplinger, H., Prager, R and Mühlhauser, I. (1983) Apolipoproteins (A-I, A-II, B), Lp(a) Lipoprotein and Lecithin:cholesterol acyltransferase activity in diabetes mellitus. Atherosclerosis 49: 277-293

Schwartz, C.C., Berman, M., Halloran, L.G., Swell, L. and Vlahcevic, Z.R. (1982). "Cholesterol disposal in man: Special role of HDL free cholesterol," *in* Lipoprotein kinetics and modelling, Berman, M., Grundy, S.M. and Howard, B.V. (eds.,) 337-349. New York Press.

Setagouchi, T., Salen, G., Tint, G.S. and Mosbach, E.H. (1974) A biochemical abnormality in cerebrotendinous xanthomatosis. Impairmant of bile acid biosynthesis associated with incomplete degradation of the cholesterol side chain. J. Clin. Invest. 53: 1393-1401

Setchell, K.D.R., Suchy, F.J., Welsh, M.B., Zimmer-Nechemias, L., Heubi, J. and Balistreri, W.R. (1988) Delta 4-3-oxosteroid 5 beta-reductase deficiency described in identical twins with neonatal hepatitis. A new inborn error in bile acid synthesis. J.Clin.Invest. 82: 2148-2157

Sevanian, A. and Peterson, A.R. (1984) Cholesterol epoxide is a direct acting mutagen. Proc. Natl. Acad. Sci. USA. 81: 4198-4202

Shefer, S., Hauser, S., Bekersky, I. and Mosbach, E.H. (1970) Biochemical site of regulation of bile-acid biosynthesis in rat. J.Lipid Res. 11: 404-411

Shefer, S., Nguyen, L.B., Salen, G., Ness, G.C., Tint, G.S., Batta, A.K., Hauser, S. and Rani, I. (1991) Regulation of cholesterol 7alpha-hydroxylase by by hepatic 7

alpha-hydroxylated bile acid flux and newly synthesized cholesterol supply. J.Biol.Chem. 266: 2693-2696

Shepherd, J. and Packard, C.J. (1992) Atherosclerosis in perspective: the pathophysiology of human cholesterol metabolism *in* Human nutrition- A continuing debate. Eastwood, M., Edwards, C. and Parry, D. (eds.,).Chapman & Hall, London. 33-50

Slotte, J.P. and Grönberg, L. (1990) Oxidation of cholesterol in low density and high density lipoproteins by cholesterol oxidase. J.Lipid Res. 31: 2235-2242

Small, D.M. (1988) Progression and regression of atherosclerotic lesions: insights from lipid physical biochemistry. Arteriosclerosis 8: 103-129

Smith, L.L. (1981) Cholesterol autoxidation. Plenum Press, New York.

Smith, L.L. (1987) Cholesterol autoxidation 1981-1986. Chemistry and physics of lipids 44: 87-125

Smith, A.G. and Brooks, C.J.W (1976) Cholesterol oxidases- properties and applications. J.Steroid Biochem 7: 705-713

Smith, J.L., DeJersey, J., Pillay, S.P. and Hardie, I.R. (1986) Hepatic acyl-CoA:cholesterol acyltransferase. Development of a standard assay and determination in patients with cholesterol gallstones. Clin.Chim.Acta 158: 271-282

Smith, L.L. and Johnson, B.H. (1989) Biological activities of oxysterols. Free radical biology & medicine 7: 285-332

Smith, L.L., Teng, J.I., Lin, Y.Y., Seitz, P.K. and McGehee, M.F. (1981) Sterol metabolism-XLVII. Oxidised cholesterol esters in human tissues. J.Steroid Biochemistry 14: 889-900

Smith, L.L. and Van Lier, J.E. (1970) Sterol metabolism. Part 9. 26-hydroxycholesterol levels in the human aorta. Atherosclerosis 12: 1-14

Snyder L.L., *Principles of Adsorption Chromatography* , Dekker, New York, 1968.

Song, W., Pierce, W.M., Russell, A.P. and Redinger, N. (1991) Characteristics of cholesterol 7α-hydroxylase and 7α-hydroxycholesterol hydroxylase activities of rodent liver. Biochem Pharm 41: 1439-1447.

Stange, E.F., Schneider, A., Preclik, G. and Ditschuneit, H. (1981) Bile acid-induced interconversion of 3-hydroxy-3-methylglutaryl coenzyme A reductase incultured intestine. Biochim.Biophys.Acta. 666: 291-293

Stange, E.F., Suckling, K.E. and Dietschy, J.M. (1983) Synthesis and coenzyme A-dependent esterification of cholesterol in rat intestinal epithelium. J.Biol.Chem. 258: 12868-12875

Steinberg, D., Parthasarathy, S., Carew, T.E. and Witztum, J.L. (1989) Beyond disease- Modifications of low-density lipoprotein that increase its atherogenicity. New Eng.J.Med. 320: 915-924

Steinmetz, A., Hocke, G., Saïle, R., Puchois, P. and Fruchart, J.C. (1989) Influence of serum amyloid A on choleserol esterification in human plsama. Biochim.Biophy.Acta. 1006: 173-178

Steinmetz, A., Kaffarnik, H. and Utermann, G. (1985) Activation of phosphatidylcholine-sterol acyltransferase by human apolipoprotein E isoforms. Eur. J. Biochem 152: 747-751

Steinmetz, A. and Utermann, G. (1985) Activation of Lecithin:Cholesterol Actltransferase by Human Apolipoprotein A-IV. J.Biol.Chem. 260: 2258-2264

Stokke, K.J. and Norum, K.R. (1977) Determination of lecithin:cholesterol acyltransferase in human blood plasma. Scand.J.Clin.Lab.Invest. 27: 21-27

Straka, M.S., Junker, L.H., Zacarro, L., Zgg, D.L., Dueland, S., Everson, G.T. and Davis, R.A. (1990) Substrate stmulation of 7α-hydroxylase, an nzyme located in the choleseterol-poor endoplasmic reticulum. J.Biol.Chem. 265: 7145-7149.

Stravitz, R.T., Vlahcevic, Z.R., Gurley, E.C. and Hylemon, P.B. (1995) Repression of cholesterol 7α-hydroxylase transcription by bile acids is mediated through protein kinase C in primary cultures of rat hepatocytes. J.Lipid Res. 36: 1359-1369.

Streuli, R.A. and Chung, J. (1979) Inhibition of human lymphocyte E-rosette formation by oxygenated sterols. J.Immunology 123: 2897-2902

Streuli, R.A., Chung, J., Scanu, A.M. and Yachnin, S. (1981) Serum lipoproteins modulate oxygenated sterol insertion into human red cell membranes. Science 212: 1294-1296

Subbaiah, P.V., Liu, M. and Paltauf, F. (1994) Role of sn-2 acyl group of phosphatidylcholine in determining the positional specificity of lecithin-cholesterol acyltransferase. Biochemistry 33: 13259-13266

Suckling, K.E., Boyd, G.S. and Smellie, C.G. (1982) Properties of a solubilized and reconstituted preparation of acyl-CoA:cholesterol acyltransferase from rat liver.

Biochim.Biophys.Acta 710: 154-163

Suckling, K.E. and Stange, E.F. (1985) Role of acyl-CoA:cholesterol acyltransferase in cellular cholesterol metabolism. J.Lipid Res 26: 647-671

Suckling, K.E., Stange, E.F. and Dietschy, J.M. (1983) *In vivo* modulation of rat liver acyl-coenzyme A:cholesterol acyltransferase by phosphorylation and substrate supply. FEBS Lett. 158: 29-32

Sundseth, S.S. and Waxman, D.J. (1990) Hepatic P-450 Cholesterol 7α-hydroxylase. Regulation *in vivo* at the protein and mRNA level in response to mevalonate, diurnal rhythm and bile acid fedback. J.Biol.Chem. 265: 15090-15095

Swaney, J.B. (1980) Properties of lipid apolipoprotein association products-complexes of dimyristoyl phosphatidylcholine and human apo A-I. J.Biol.Chem. 255: 877-881

Szanto, A., Ruys, J. and Balasubramaniam, S. (1994) Coordinate diurnal regulation of hepatic acyl-coenzyme A:cholesterol acyltransferase and cellular levels of esterified cholesterol. Biochim.Biophys.Acta. 1214: 39-42

Tall, A.R. (1986) Plasma lipid transfer proteins. J.Lipid Res 27: 361-367

Tall, A. R., Abreu, E. and Shuman, J. (1983) Separation of a plasma phospholipid transfer protein from cholesterol ester/phospholipid exchange protein. J. Biol. Chem 258: 2174-2180

Tang, P.M. and Chiang, J.Y.L. (1986) Modulation of reconstituted cholesterol 7 alpha-hydroxylase by phosphatase and protein kinase. Biochem.Biophys.Res.Commun. 134: 797-802

Tavani, D.M., Nes, W.R. and Billheimer, J.T. (1982) The sterol substrate specificity of acyl CoA:cholesterol acyltransferase from rat liver. J.Lipid Res. 23: 774-781

Taylor, F.R., Saucier, S.E., Shown, E.P., Parish, E.J. and Kandutsch, A.A. (1984) Correlation between oxysterol binding to a cytosolic binding protein and potency in the repression of hydroxymethylglutaryl coenzyme A reductase. J.Biol.Chem. 259: 21382-12387

Tikkanen, M.J. (1989) Intravascular lipoprotein remodelling: lipolytic enzymes *in* Fruchart, J.C. and Shepherd, J. (eds), Human plasma lipoproteins, pp 143-151. Berlin: Walter de Gruyter.

Tikkanen, M.J. (1990) HMG CoA reductase inhibitors: Comparative clinical pharmacology and indications . Balliere's Clinical Endocrinology and Metabolism

4:719-741

Tilvis, R.S. and Miettinen, T.A. (1980) A lack of esterification of lanosterol and other methyl sterols *in vitro* Scand.J.Lab.Invest. 40: 671-674

Tollefson, J.H., Ravnik, S. and Albers, J.J. (1988) Isolation and characterization of a phospholipid transfer protein (LTP-II) from human plasma. J.Lipid Res 29: 1593-1602

Tomkin, G.H. and Owens, D. (1991) Abnormalities of cholesterol metabolism in diabetes. Proceedings of the Nutrition Society 50: 583-589

Turley, S.D. and Dietschy, J.M. (1982) *in* The Liver: Biology and Pathobiology (Arias, I., Poper, H., Schachter, D. and Shafritz, D.A., Eds.) Raven Press, New York. pp467-492.

Uihlein, M. (1986) *in* Practice of high performance liquid chromatography-Applications, equipment and quantitative analysis. Engelhardt, H. Ed. Springer-Verlag, 1986. pp161.

Van Doormaal, J.J., Smit, N., Koopman, B.J., Van Der Molen, J.C., Wolthers, B.G. and Doorenbos, H. (1989) Hydroxycholesterols in serum from hypercholesterolaemic patients with and without bile acid sequestrant therapy. Clin.Chim.Acta. 181: 273-280

Van Lier, J.E. and Smith, L.L. (1967) Sterol metabolism. I. 26-hydroxycholesterol in the human aorta. Biochemistry 6: 3269-3278

Vlahcevic, Z.R., Bell, C.C., Buhac, I. *et al.*, (1970) Diminished bile acid pool size in patients with gallstones. Gastroenterology 59: 165-173

Weight, M.J., Coetzee, H.S., Smuts, C.M., Marais, M.P., Maritz, J.S., Hough, F.S., Benadé, A.J.S. and Taljaard, J.J.F. (1993) Lecithin:cholesterol acyltransferase activity and high-density lipoprotein subfraction composition in type 1 diabetic patients with improving metabolic control. Acta Diabetol. 30: 159-165

Willard H.H, Merritt, Jr.L.L, J.A. Dean, and F.A. Settle, Jr. (1988) *Instrumental Methods of Analysis*, (7th ed.) Chapter 20.

Wilson, K. and Goulding, K.H. (1986) Chromatographic techniques *in* A biologist's guide to principles and techniques of practical biochemistry (3rd ed.) Hodder and Stoughton, Great Britain. Chapter 20.

Wong, R.K.L. and Bruggen, J.T. (1960) Lipid metabolism in the diabetic rat. I. Acteate metabolism and lipid synthesis *in vivo*. J.Biol.Chem 235: 26.

Yokoyama, S., Fukushima, D., Kupferberg, J.P., Kézdy, F.J. and Kaiser, E.T. (1980) The mechanism of activation of lecithin:cholesterol acyltransferase by apolipoprotein A-I and an amphiphilic peptide. J.Biol.Chem. 255: 7333-7339

Yoshida, T., Honda, A., Tanaka, N., Matsuzaki, Y., He, B.and Osuga, T. (1993) Simultaneous determination of mevalonate and 7α-hydroxycholesterol in human plasma by gas chromatography-mass spectrometry as indices of cholesterol and bile acid biosynthesis. J.Chromatogr. 613: 185-193.

Young, N.L., Lopez, D.R., McNamara, D.J. and Benavides, B. (1985) Evaluation of the contribution of dietary cholesterol to hypercholesterolemia in diabetic rats and of sitosterol as a recovery standard for cholesterol absorption. J.Lipid Res 26: 62-69

Young, N.L., Saudek, C.D. Walters, L. and Chang, V. (1982) Preventing hyperphagia normalizes 3-hydroxy-3-methylglutaryl-CoA reductase activity in small intestine and liver of rats. J.Lipid Res 23: 831-838.

Zhang, H., Basra, H.J.K. and Steinbrecher, U.P. (1990) Effects of oxidatively modified LDL on cholesterol esterification in cultured macrophages. J.Lipid Res. 31: 1362-1369

APPENDIX

Abstracts/posters:

Dowling, L. and Devery, R.
"Use of HPLC for the determination of serum 7α-hydroxycholesterol in cholestyramine-treated rats"
Biochemical Society Colloquium at Belfast, September 1993

Dowling, L. and Devery, R.
"Proteoliposomal studies with partially purified lecithin:cholesterol acyltransferase"
The Diabetes and Atherosclerosis meeting at Velen, Germany, September 1994

Dowling, L. Quinn, J. and Devery, R.
"The use of HPLC for quantitation of 7α-hydroxycholesterol in rat liver and serum: The effects of diabetes and cholestyramine"
Association of Clinical Biochemists in Ireland, Dublin, October 1994

Oral communications:

"The measurement, esterification and biological significance of serum 7α-hydroxycholesterol"
Biochemical Society Colloquium at U.C.C. September 1994

"Proteoliposomal studies with partially purified lecithin:cholesterol acyltransferase"
The Diabetes and Atherosclerosis meeting at Velen, Germany, September 1994

Publication:

Dowling, L. and Devery, R.
"Use of HPLC for the determination of serum 7α-hydroxycholesterol in cholestyramine-treated rats" Biochem.Soc.Trans., (1995) 23(2) 362S

Publications in preparation:

"Validation of preparative and chromatographic procedures for the measurement of serum 7α-hydroxycholesterol"

"Relationship between serum 7α-hydroxycholesterol and liver 7α-hydroxycholesterol in diabetic and cholestyramine-treated rats"

"Preparation and characterisation of discoidal complexes containing cholesterol and 7α-hydroxycholesterol for sterol studies"

"Reactivity of discoidal complexes containing 7α-hydroxycholesterol with bovine LCAT"