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The Influence of Lipolytic Enzymes and Lipid Transfer Proteins on the Fate of Very Low Density Lipoprotein and High Density Lipoprotein Constituents

by

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Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
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I dedicate this work to the memory of my mother and my father. I dedicate it to my dear dad for instilling in me a love of science and a need to know "why". I dedicate it to my precious mom for her abounding love and her unwavering belief in me.

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ABSTRACT

The process of the conversion of triglyceride-rich lipoproteins to low density lipoprotein (LDL) and high density lipoprotein (HDL) is considered central to an understanding of the positive association of intermediate density (IDL) and LDL with the development of atherosclerosis and the apparent protective effect of HDL. The conversion process is thought to involve lipolytic enzymes and the lipid transfer proteins in the vascular compartment.

The purpose of this study was to investigate *in vitro* the role of lipoprotein lipase, hepatic lipase and the lipid transfer proteins in the metabolism of VLDL in the presence of HDL. The results of the *in vitro* incubations were compared to those observed *in vivo* as a result of heparin-induced lipolysis in normolipidemic subjects.

The metabolism of VLDL by lipoprotein lipase resulted in 88-94% hydrolysis of the triglycerides and 30% hydrolysis of the phospholipids of VLDL. The transfer of apo CII and CIII from VLDL to HDL was extensive (88-100%) while the apo E transfer was substantially lower (30%). Unesterified cholesterol and phospholipid were transferred to HDL and in some experiments there was a transfer of cholesteryl ester from VLDL to the HDL region. The transfer of cholesteryl ester was accompanied by an increased transfer of phospholipid from VLDL to the HDL. The metabolized VLDL did not form typical plasma LDL since the particles were considerably larger, less dense, contained a significant amount of apo E and had a very high surface to core ratio.

The addition of hepatic lipase to the lipoprotein lipase incubation had little effect on VLDL conversion to LDL but caused an extensive hydrolysis of HDL triglycerides.

The addition of the lipid transfer proteins to the lipoprotein lipase incubation increased the transfer of unesterified cholesterol, phospholipid and apo E from VLDL to HDL by 1.5 to 2 fold. Net phospholipid transfer activity to HDL was increased while net cholesteryl ester transfer activity from HDL to VLDL was decreased in the presence of lipoprotein lipase. The metabolized VLDL particles were more like plasma LDL than those produced by lipoprotein lipase alone since the particles contained less apo E, had a lower surface to core ratio and approached LDL size.

The *in vivo* studies of heparin-induced lipolysis yielded results that were similar to those observed *in vitro* when the combined effects of lipoprotein lipase, hepatic lipase and the lipid transfer proteins were considered.

It is concluded that lipoprotein lipase and the lipid transfer proteins are the most important factors for the conversion of plasma VLDL to LDL with an associated accumulation of lipid and apoprotein mass in HDL.

LIST OF ABBREVIATIONS

apo:	apoprotein
β-VLDL:	β-migrating very low density lipoprotein
CE:	cholesteryl ester
CE/TG:	molar ratio of cholesteryl ester to triglyceride
CETP:	cholesteryl ester transfer protein
cpm:	counts per minute
d:	density
DEAE:	diethylaminoethyl
DTNB:	5,5-dithiobis 2-nitrobenzoic acid
EDTA:	Ethylenediamine tetraacetate (disodium salt)
ER:	endoplasmic reticulum
HAL:	hepatic lipase
HDL:	high density lipoprotein
IDL:	intermediate density lipoprotein
LDL:	low density lipoprotein
LCAT:	lecithin-cholesterol acyltransferase
LPL:	lipoprotein lipase
LpX:	lipoprotein-X
LTP:	lipid transfer protein preparation containing cholesteryl ester transfer protein and phospholipid transfer protein.
NaN_3:	sodium azide
PAGGE	polyacrylamide gradient gel electrophoresis
PL:	phospholipid
PLTP:	phospholipid transfer protein
rpm:	revolutions per minute

S/C:	molar ratio of surface lipid to core lipid
SD:	standard deviation
S_f^o	Svedberg flotation unit of flotation rate in a medium of d1.063 g/ml at 26°C
TG:	triglyceride
Tris:	tris (hydroxymethyl) aminoethane
Trisil BSA:	N,O-Bis-(trimethylsilyl) acetamide in pyridine
UC:	unesterified cholesterol
UC/PL:	molar ratio of unesterified cholesterol to phospholipid
VLDL:	very low density lipoprotein

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CHAPTER I

INTRODUCTION

An inverse relationship between the concentration of VLDL triglyceride and the concentration of HDL has been observed in individuals with varying degrees of hypertriglyceridemia or in subjects when triglyceride concentrations are changing due to high carbohydrate dietary intake, weight loss, endurance exercise and clofibrate treatment. The change in VLDL triglyceride concentration was usually associated with an opposite change in the HDL_2 subfraction. Due to the gain of mass in HDL during VLDL lipolysis *in vitro*, it has been suggested that the inverse relationship is mainly associated with the catabolism of VLDL. Post-heparin LPL activity has been negatively correlated with coronary heart disease and the composition of HDL in coronary heart disease patients is similar to that observed in normal subjects with low lipoprotein lipase. Subjects with high HDL cholesterol have elevated levels of lipoprotein lipase activity as compared to subjects with low HDL cholesterol. It has been suggested that low HDL may be a marker for inefficient lipolysis of chylomicrons and VLDL which results in poor transfer of lipid mass to HDL and, in the case of VLDL, inefficient conversion of VLDL to LDL. These observations suggest that it is important to understand the role of lipoprotein lipase in lipoprotein metabolism and the influence of other lipolytic enzymes and lipid transfer proteins on this process.

In vitro studies have demonstrated several general effects on the relationship between the catabolism of the VLDL particle and the effect on LDL and HDL mass. The results are consistent in the observation of a loss of mass in the VLDL density range with a gain in the IDL/LDL density range and a concomitant gain of apo CII, CIII, apo E, phospholipid and unesterified cholesterol in the HDL region. The results are inconsistent in terms of the extent of VLDL lipid and apoprotein mass transfer to HDL. The mechanisms that control the transfer of VLDL apoprotein and lipid to HDL have not been well defined. The results are also contradictory with respect to whether cholesteryl ester

is transferred from VLDL to the HDL density region and whether discs or spheres are formed and appear in the HDL region as a result of VLDL lipolysis by lipoprotein lipase. Whether true plasma LDL can be formed from plasma VLDL, with the concomitant loss of apo E and in some instances cholesteryl ester as a result of lipoprotein lipase activity on VLDL has not been demonstrated and there are opposing reports concerning the effect of lipoprotein lipase on the conversion of HDL_3 to HDL_2.

The effect of another extracellular lipase, hepatic lipase, on the modification of the particles produced under the influence of lipoprotein lipase is not clear. Despite the fact that this enzyme is frequently implicated in the conversion of IDL to LDL there is little direct evidence for this role.

The plasma lipid transfer proteins, cholesteryl ester transfer protein and phospholipid transfer protein have been suggested to influence the process of lipolysis but the effect on the lipid and apoprotein redistributions has not been well investigated. It has been suggested from *in vitro* incubation studies that lipolysis of VLDL by lipoprotein lipase increases the activity of the cholesteryl transfer protein resulting in an increased transfer of cholesteryl esters from HDL to VLDL/VLDL remnants. This effect may promote the accumulation of VLDL remnants and IDL which are positively associated with the incidence of atherosclerosis, unless the process also facilitates delivery of these lipoproteins to the liver. Such a role for lipoprotein lipase in the development of atherosclerosis would be contrary to the data indicating an inverse relationship of lipolysis with atherosclerosis. However, many studies have used proportions of lipoproteins, enzymes and transfer proteins that differ greatly from those normally present in the physiological situation. Thus, the present study was undertaken to determine the influence of hepatic lipase and the lipid transfer proteins on the process and products of lipolysis of VLDL by lipoprotein lipase under conditions that approximate those present in the plasma of normolipidemic individuals.

CHAPTER II

REVIEW OF LITERATURE

The purpose of the present investigation is to determine: 1) how the lipolytic enzyme, lipoprotein lipase, affects the formation of high density lipoprotein and low density lipoprotein as a result of the hydrolysis of very low density lipoprotein lipids and 2) how hepatic lipase and the lipid transfer proteins modulate this process. Therefore it is necessary to provide an overview of lipoprotein structure, function and metabolism with an emphasis on the factors effecting HDL and LDL formation.

A. LIPOPROTEIN FUNCTIONS, DEFINITIONS AND COMPOSITION

1. LIPOPROTEIN FUNCTIONS:

Lipoproteins are complexes made up of a neutral lipid core and a surface containing phospholipid, unesterified cholesterol and protein. The general function of lipoproteins is to transport insoluble lipids in the blood from the source of synthesis or absorption to their site of uptake and utilization.

The major classes of lipoproteins are chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). The study of lipoprotein metabolism is of interest due to the suggested involvement of LDL, IDL and in some cases VLDL in the development of the atherosclerotic plaques which lead to the development of coronary heart disease [1-3]. On the other hand, HDL concentrations have been inversely related to the incidence of coronary heart disease [1-4]. In most studies, the relationships of the lipoproteins with the formation or reduction of the atherosclerotic plaque are based mainly on epidemiological data that have correlated the lipoprotein levels with the incidence of coronary heart disease [2,3]. Recently, studies in humans and animals have demonstrated that a reduction in the level of plasma LDL and an increase in HDL can lead to a lower incidence of coronary heart disease [1,5] and regression of the atherosclerotic plaque [6].

The roles of the lipoproteins in the development or prevention of atherosclerosis have not been clearly elucidated. It has been suggested that high LDL and IDL (or certain forms of VLDL and LDL) lead to the development of atherosclerosis by causing the excessive cholesterol deposition observed in atherosclerotic plaques. In the case of HDL, two different mechanisms have been suggested to account for its preventive role in the development of atherosclerosis. The first role is a causative one where HDL acts directly in the removal of cholesterol from the atherosclerotic plaques or inhibits cholesterol deposition in peripheral cells [7]. The other role for HDL is referred to as a "non-causative" one indicating that HDL need not exert a direct effect on the atherosclerotic plaque but that high HDL concentrations reflect activity of an ongoing process that is in itself antiatherogenic [8,9]. An example of a situation where HDL may have a non-causative role would be one where the activity of a particular enzyme or lipid transfer protein reduces or prevents of the formation of "atherogenic" lipoproteins with an accompanying increase in the HDL concentration. The processes involved in the formation and catabolism of the lipoproteins are therefore of interest due to their possible roles in the development or prevention of coronary heart disease.

2. LIPOPROTEIN STRUCTURE:

The general structure of the lipoproteins is that of a spherical particle comprised of a neutral lipid core, containing cholesteryl esters and triglycerides, and a surface made up of phospholipids, unesterified cholesterol and proteins. The proteins that associate with the lipid are referred to as apolipoproteins or apoproteins. Other lipids and proteins may be associated with the lipoprotein particles but are minor components (example: α tocopherol [Vitamin E], β carotene). The surface of lipoproteins contains a small amount of neutral lipid of approximately three mole percent due to the equilibration of neutral lipid between the core and the surface monolayer.

The surface of lipoproteins is considered to be made up of a 20.5 Å monolayer of phospholipid and unesterified cholesterol. The core varies in diameter depending on the

amount of neutral lipid present. The apoproteins contain specific regions referred to as amphipathic alpha helices which associate with the surface lipid. The alpha helical regions contain specific sequences of amino acids resulting in one side of the helix being charged and the opposing side being neutral [10]. The protein can therefore associate with both the phospholipid polar head groups on the outer surface of the particle as well as the apolar regions of the phospholipid fatty acids. The major interaction of the apoproteins with the lipid surface is thought to be through the hydrophobic forces in the apolar regions rather than through electrostatic interaction with the phospholipid head group. The amphipathic nature of the alpha helix of the apoproteins allows for their integration in the surface lipid coat. The interaction of apo B with the lipoprotein lipid is considered to occur via hydrophobic binding domains on the apo B [11] as well as by fatty acylation of apo B through a thioester bond [12].

3. LIPOPROTEIN SEPARATION AND COMPOSITION

The lipoproteins vary in composition depending on the content of lipids and apoproteins. Their separation is dependent on the differing compositions.

Lipoproteins can be isolated according to densities by ultracentrifugation, according to charge by agarose gel electrophoresis or according to size by gel filtration chromatography. Ultracentrifugation is the more common method used to isolate lipoproteins although it is a more vigorous method than gel filtration. Ultracentrifugation isolates the lipoproteins according to size, in most cases, as the particle size of lipoproteins tends to decrease as the density increases. The particle size decreases because there is less neutral lipid core relative to surface lipids and proteins. Since neutral lipids of the core (triglyceride and cholesteryl ester) are less dense than the surface lipids (unesterified cholesterol and phospholipid) and proteins, there is a resultant increase in the density of the particle. The density of a particle is dependent on the percent composition of the various lipid and apoprotein components since the average densities

for the various components are: triglycerides 0.91 g/ml, cholesteryl ester 0.98 g/ml, phospholipid 1.04 g/ml, unesterified cholesterol 1.067 g/ml and protein 1.35 g/ml.

The general characteristics of the major plasma lipoproteins are listed in Table 1 and 2. The lipid and apoprotein composition of the lipoproteins are listed in Table 3. The major lipoprotein classes are chylomicrons, VLDL, IDL, LDL and HDL. The lipoproteins are defined according to their flotation characteristics which do not reflect the origin of the lipoproteins but only their density. Therefore it is possible that a lipoprotein such as a chylomicron which is synthesized in the intestine, after being metabolized in the plasma, may attain the density of a VLDL particle and therefore be isolated in the VLDL fraction. Chylomicrons are triglyceride-rich lipoproteins synthesized in the intestinal epithelium from exogenous lipids. VLDL are triglyceride-rich lipoproteins synthesized in the liver, from endogenous lipids. LDL is the major cholesterol carrier in the plasma transporting 65-70% of the total cholesterol in normolipidemics. LDL is thought to be derived from VLDL, in most cases. HDL is also rich in cholesteryl esters relative to triglycerides and carries 22-30% of the total plasma cholesterol, VLDL transporting the remainder. HDL is synthesized mainly in the liver. Chylomicron remnants and IDL or VLDL remnants are thought to arise from the metabolism of chylomicrons and VLDL, respectively, and are intermediate in their neutral lipid core content between the triglyceride-rich lipoproteins and LDL. There are sub-classifications within lipoprotein categories as outlined in Table 2. For example, VLDL has been separated into $VLDL_1$, $VLDL_2$ and $VLDL_3$. Because early definitions of LDL included IDL, the LDL subcategories are LDL_1(IDL) and LDL_2. Presently LDL refers to LDL_2. HDL has been divided into HDL_2 and HDL_3

The particles range in size from chylomicrons > VLDL > chylomicron remnants, IDL > LDL > HDL. Several ratios have been used to describe the lipoproteins. The surface to core ratio (S/C) reflects the content of phospholipid and unesterified cholesterol relative to cholesteryl ester and triglyceride. The surface to core ratio increases as the particles become smaller due to the fact that the surface area required to

TABLE 1

HUMAN PLASMA LIPOPROTEIN GENERAL CLASSIFICATIONS AND PROPERTIES [1]

Property	Lipoprotein				
	Chylomicrons	VLDL	IDL(LDL1)	LDL(LDL2)	HDL
Hydrated Density:	<.94 g/ml	.94-1.006g/ml	1.006-1.019 g/ml	1.019-1.063 g/ml	1.063-1.21g/ml
Flotation Rate:					
$S°_f$ (d 1.063 g/ml)	>400	20-400	12-20	0-12	——
F° (d 1.21 g/ml)	——	——	——	——	0-9
Electrophoretic Mobility:	origin	pre-beta	beta	beta	alpha
Molecular Weight:	400 x 10^6	5-17 x10^6	3.9-4.8 x 10^6	1.5-2.7 x10^6	.18-.39 x10^6
Particle Diameter:	300-5,000 Å	250-750 Å	220-240	200-225	40-140

[1] Data compiled from references 10, 13 and 14

TABLE 2

HUMAN PLASMA VLDL AND HDL SUBCLASS CLASSIFICATIONS AND PROPERTIES[1]

Property	Lipoprotein Subfraction				
	VLDL1	VLDL2	VLDL3	HDL2	HDL3
Hydrated Density: (g/ml)	___	___	___	1.063-1.125	1.125-1.21
Flotation Rate:				___	___
$S°_f$ (d 1.063 g/ml)	100-400	60-100	20-60		
F° (d 1.21 g/ml)	___	___	___	4-9	0-4
Particle Diameter: (Å)	546 Å	432 Å	377Å	60-140Å	40-100Å

[1] Data compiled from references 10, 13 and 14

TABLE 3

HUMAN PLASMA LIPOPROTEIN LIPID AND APOPROTEIN COMPOSITION

The data in Table 3 are compiled from data listed in references [10,13,14]

The apoprotein compositions are listed only for chylomicrons, VLDL, LDL and HDL.

The molar ratios listed refer to the following:

UC/PL = unesterified cholesterol to phospholipid

CE/TG = cholesteryl ester to triglyceride

S/C = surface lipid to core lipid based on $\frac{\text{(phospholipid + unesterified cholesterol)}}{\text{(cholesteryl ester + triglyceride)}}$

[1]Lipids and apoproteins are expressed in weight percent.

TABLE 3
HUMAN PLASMA LIPOPROTEIN LIPID AND APOPROTEIN COMPOSITION[1]

Component	Chylomicrons	VLDL	VLDL1	VLDL2	VLDL3	IDL	LDL	HDL	HDL2	HDL3
Unesterified Cholesterol:	2	6	5	6	7	9	10	3	4	2
Phospholipid:	7	19	17	19	22	22	20	26	29	22
Cholesteryl Ester	6	12	7	10	18	35	45	18	19	15
Triglyceride:	83	56	65	57	42	19	6	3	3	2
Protein:	2	8	5	7	10	14	20	53	46	61
Percent of Total Protein:										
apo AI	7.4(lymph)	trace					—	67		
apo AII	4.2	trace					—	22		
apo B	22.5	36.9					98	trace		
apo CI	15	3.3					trace	1-3		
apo CII	15	6.7					trace	1-3		
apo CIII	36	39.9					trace	3-5		
apo E		13					trace	+		
UC/PL	.57	.63	.59	.63	.64	.81	1	.24	.28	.18
CE/TG	.095	.28	.14	.23	.56	2.5	10	8	8	10
S/C	.133	.476	.402	.489	.604	.66	.7	1.32	1.46	1.319

cover a sphere increases relative to the core volume as the size of the particle decreases. The cholesterol to phospholipid ratio (UC/PL) is highest in LDL, intermediate in VLDL and lowest in HDL. A lower ratio reflects a more fluid surface and is a characteristic of a lipoprotein category. The cholesteryl ester to triglyceride ratio (CE/TG) reflects the core composition and is lowest for chylomicrons and VLDL as compared to LDL and HDL. These ratios appear to be related to the process of lipoprotein metabolism and can be used when comparing the characteristics of the lipoproteins formed under different metabolic conditions *in vitro* and *in vivo* to those of the plasma lipoproteins.

Other lipoproteins exist which are less frequently present in plasma or comprise a minor component. Beta-VLDL, which is a cholesteryl ester enriched VLDL or chylomicron remnant, is present in certain dyslipidemic subjects [15]. Lp(a) is a LDL-like particle with an apoprotein, designated as apo (a), bound via a disulfide bond to the major apoprotein of LDL, apo B [16]. Lp (a) contains regions that are homologous to parts of the plasminogen molecule [17]. Lp(a) is found in varying concentrations in the plasma and a concentration greater than 300 mg/L is considered pathological. HDL_1, a minor lipoprotein in humans but a major one in rats, has been identified as a large cholesteryl ester-rich and apo E-rich particle [18] that may give rise to the HDL_C which appears with cholesterol feeding in certain animals [19]. VHDL and pre-Beta HDL are extremely small dense HDL particles present in very small amounts in plasma at a density greater than that of HDL (d>1.21g/ml) [20]. HDL discs, comprised of a bilayer of phospholipid, unesterified cholesterol and apoprotein, are considered to be the nascent form of HDL synthesized by the liver and intestine which are rapidly converted to spherical particles in the plasma through the action of the enzyme lecithin-cholesterol acyltransferase (LCAT) [21]. LCAT catalyses the esterification of cholesterol using a phospholipid fatty acid at *sn*-2 position of phosphatidylcholine resulting in the formation of cholesteryl ester and lysophosphatidylcholine. HDL lipids are the favoured substrates. The presence of cholesteryl ester results in the formation of a spherical particle since the

neutral lipid must be shielded in a hydrophobic core from the aqueous environment. HDL discs are rarely present in plasma except in the case of LCAT deficiency [21,22]. Unilamellar vesicles made up of a bilayer of unesterified cholesterol and phospholipid covering a water core have been described in certain *in vitro* studies [23]. Similar particles have been described *in vivo* in subjects undergoing lipid infusions [24,25], in LCAT deficiency [26] or with cholestasis [27] and are referred to as LpX. LpX is a bilayer liposome with a water core containing albumin and is made up mainly of unesterified cholesterol and phospholipid, with a small amount of protein and triglyceride [23,25,28]. LpX can contain the apo C proteins as well as apo E and AI in lesser amounts than the apo C's [25]. The vesicles and LpX exhibit a high ratio of unesterified cholesterol to phospholipid.

4. APOPROTEINS FUNCTIONS AND LIPOPROTEIN ASSOCIATIONS:

Apoproteins are considered to have three functions with respect to their role in lipoprotein metabolism: 1) as ligands for receptor binding; 2) as co-factors for enzymes; 3) as structural proteins. The apoproteins are presently referred to by an alphabetical nomenclature which was initially developed for apo AI of HDL and apo B of LDL according to their alpha and beta electrophoretic mobility, respectively. The other apoproteins were named according to their sequence of discovery. Table 3 lists the apoprotein content of the lipoproteins.

Apo AI is the major apoprotein of HDL comprising 60% of the apoprotein mass. It has a molecular weight of 28,300 and a pI of 5.5-5.6 [29]. Apo AI is synthesized in the liver and intestine [30] and can be found on newly synthesized chylomicrons [31]. It is considered to be the major structural protein of HDL and is an activator of LCAT [32]. It is postulated that apo AI may be involved in reverse cholesterol transport. Reverse cholesterol transport refers to the transport of cholesterol from the peripheral tissues to the liver for excretion. Apo AI may act as a ligand for HDL binding in peripheral cells

[33] and the binding may facilitate the uptake of cellular unesterified cholesterol by HDL [34,35].

Apo AII is the second major protein of HDL making up 20% of the total apoprotein mass. It has a molecular weight of 8,700 a pI of 5.05 [29] and is present as a dimer in humans. Apo AII is synthesized in the liver with little or no apo AII synthesized in the intestine [30]. Apo AII is also present on chylomicrons [29]. Apo AII may act as a structural apoprotein but a definite role for apo AII has not been established.

Apo AIV is a minor component of HDL and chylomicrons. The majority is isolated in the d>1.21 g/ml after ultracentrifugation. Apo AIV has a molecular weight of 46,000 and a pI of 5.5 [29,36]. It is the most hydrophilic of the apoproteins and is usually found unassociated with lipoproteins in the d>1.21 g/ml. A specific role for apo AIV has not been discovered. Apo AIV has been reported to increase the apo CII-stimulated activity of lipoprotein lipase in chylomicrons by aiding in the transfer of apo CII from HDL to chylomicrons [37].

Apo B is the major apoprotein of chylomicrons, chylomicron remnants, VLDL, IDL and LDL. It is one of the largest plasma proteins with an amino acid molecular weight of 512,723 [38]. Analysis of the composition of apo B-containing lipoproteins indicates that there is one apo B molecule per lipoprotein particle [39]. Apo B is referred to as B_{100} and B_{48}, representing the entire apo B molecule or a truncated form representing the N terminal 48% of molecule[40]. Apo B_{48} is the major form synthesized by the intestinal epithelium. B_{48} arises from the same gene as B_{100} but is truncated due to different RNA editing as compared to that of B_{100} [40]. Apo B_{48} is the major form present on chylomicrons and chylomicron remnants in humans. The liver is responsible for synthesizing apo B_{100} in humans, but there is evidence that apo B_{100} may also be synthesized in the human intestine [41]. Apo B is considered to play a structural role as an integral protein. Apo B_{100} is also the ligand for LDL uptake by the LDL receptor, binding via positively charged amino acid residues such as arginine and lysine [11]. The

receptor binding regions of apo B shows homology to that of apo E. B48 is not recognized by the LDL receptor

Apo CI is present in chylomicrons, VLDL and HDL. It has a molecular weight of 6,600 and an isoelectric point of 7.5 [29]. Apo CI has been reported to inhibit the apo E-mediated uptake of VLDL subfractions and IDL by the LDL receptor [42]. Apo CI has been shown to inhibit apo-E mediated β-VLDL uptake by the LDL receptor related protein (LRP)[43].

Apo CII is principally found in chylomicrons, VLDL and HDL. Apo CII has a molecular weight of 8,800 and an isoelectric point of 4.9 [29]. The major role is that of a cofactor for the enzyme lipoprotein lipase which is the major enzyme responsible for the hydrolysis of chylomicrons and VLDL triglycerides *in vitro* [44] and *in vivo* [45]. Apo CII has been reported to inhibit apo E-mediated uptake of VLDL, IDL and β-VLDL [43].

Apo CIII is distributed in lipoproteins in a manner similar to that of apo CII. The molecular weight is 9,200-9,700 [29]. Apo CIII has several isoelectric points of 4.62, 4.82 and 5.02. The three isoforms are referred to as CIII-2, CIII-1 and CIII-0, respectively [46]. The differences in the isoforms are due to different degrees of sialylation of the attached carbohydrate. The role of apo CIII is not clear although it has been reported to prevent the uptake of chylomicrons and VLDL by the liver [47,48] and has been shown to inhibit lipoprotein lipase [49]. It was not found to inhibit the apo-E mediated uptake of β-VLDL by the LRP receptor [43].

Apo E is present in varying amounts in most lipoproteins but its presence is minimal in LDL. It has a molecular weight of 36,000 and has several pI values of 5.64, 5.78, 5.89 and 6.02 [29]. Apo E can bind to the LDL receptor [50]. Apo E mediates the binding of chylomicron remnants, VLDL remnants and β-VLDL in the liver [51-53]. It is considered to be the ligand for the LRP receptor for chylomicrons, β-VLDL and possibly VLDL remnants [53-55]. Apo E has been reported to mediate HDL uptake in the liver [56]. Apo E has three major isoforms designated E2, E3 and E4. The differences are

due to multiple alleles at a single gene locus. Therefore 6 common phenotypes exist, E2/E2, E3/E3, E4/E4, E2/E3, E3/E4 and E2/E4. The E3/E3 phenotype is the most frequent form occurring in normal plasma [57]. The differences in the apo E isoforms are due to the presence of cysteine at positions 112 and 158 of the amino acid sequence for apo E2 and of arginine at position 158 for apo E3 [58]. Apo E4 contains arginine in both positions. The apo E2 isoform is unable to bind to the LDL receptor which is postulated to be due to the absence of arginine at position 158. Arginine 158 is in or near the receptor binding site. The apo E2/E2 phenotype can lead to cholesterol accumulation in the plasma [15] since chylomicron remnants, VLDL, VLDL remnants and β-VLDL are considered to use apo E as a ligand for binding and hence uptake by the liver.

Other minor apoproteins have been identified such as apo D (AIII), F, G and H. A role for these apoproteins has not been established.

B. LIPOPROTEIN SYNTHESIS, CATABOLISM AND INTERCONVERSIONS:

1. CHYLOMICRON METABOLISM:

Chylomicrons are synthesized in the intestine as a vehicle for the transport of exogenous lipids. In the intestine, dietary triglycerides and cholesteryl esters are hydrolysed to fatty acids and monoglycerides, and fatty acids and unesterified cholesterol, respectively, to enable their absorption. After re-esterification of the lipids, chylomicrons are formed with a core made up primarily of triglycerides with a small amount of cholesteryl esters and a surface comprised of phospholipids, unesterified cholesterol, apo B, apo AI and the partial complement of apo E, AIV and the apo C's. Once formed they diffuse into the lymphatic vessels.

Chylomicrons are extremely large relative to other lipoproteins and have a very short plasma half-life. On entering the plasma, chylomicrons partially lose apo AI and AIV and gain their full complement of apo E and the apo C's [31]. Although apo E is considered to be the ligand for chylomicron remnant uptake, intact chylomicrons are not

removed from plasma. The lack of removal may be due to the presence of the apo C's [47,48] or the rapid hydrolysis of the chylomicron. Lipoprotein lipase, on the capillary endothelium, extensively hydrolyses the triglyceride core and some phospholipid surface. There is a concomitant loss of apo CII and CIII, phospholipids and unesterified cholesterol from the surface which are transferred mainly to HDL. Through this process, chylomicron remnants are formed which are rapidly taken up by the liver hepatocytes. The enzyme hepatic lipase has been suggested to play a role in chylomicron remnant uptake and the phospholipase A_1 activity which hydrolyses the fatty acid ester bond of the *sn*-1 position of phospholipids has been implicated [59]. It is speculated that hydrolysis of the phospholipids effects the expression of apo E, favouring its recognition by the hepatocyte receptor.

The type of receptor that is responsible for chylomicron uptake remains controversial. Early reports that the liver expressed a specific chylomicron receptor [52,56], different from the LDL receptor, could not be substantiated [60] and it appeared that the protein isolated was an intracellular one [61]. Some studies demonstrated that the LDL receptor, via apo E binding, was responsible for a majority of the chylomicron uptake [62,63]. Other investigators have argued that a specific receptor, separate from the LDL receptor, must be present since subjects who lack functional LDL receptors do not accumulate chylomicron remnants in their plasma [64,65]. Recently, a membrane protein on the hepatocyte has been hypothesized to be an apo E-specific receptor which mediates the uptake of remnants in the liver [55]. The protein is referred to as the LDL receptor related protein (LRP) since its external region contains four imperfect copies of the external domain of the LDL receptor [66]. LRP has been found in human liver membranes and Hep G2 cells (a human hepatoma cell line) as well as in fibroblasts. The mRNA has been located in a variety of tissues such as lung and brain [66].

2. VLDL METABOLISM:

VLDL is synthesized in the liver hepatocytes from endogenous lipid. Exogenous lipids can be incorporated into VLDL as a result of chylomicron remnant uptake in the liver. The assembly of VLDL in the hepatocyte is a complex process due to the variety of lipids and apoproteins required for the formation of nascent VLDL [67-69]. The events include synthesis of the apoproteins apo B and apo E on the rough endoplasmic reticulum (ER). The majority of the lipids are synthesized on the ER. Phospholipids can be synthesized on the ER and in the Golgi. The following description of the events in the synthesis of VLDL remain controversial. Apo B and possibly apo E combines with lipids at the junction of the rough and the smooth ER. Triglycerides are synthesized on the cytoplasmic side of the ER and migrate to the hydrophobic region in the middle of the membrane bilayer. Apo B may become associated at this stage with the triglyceride as well as associating with the inner membrane. The association of apo B with triglyceride may occur where the rough ER joins the smooth ER because it is the first location that the triglyceride can encounter apo B. Subsequently, by the process of budding of the inner (luminal) side of the membrane, the triglyceride attains its surface coat of phospholipid and unesterified cholesterol and can enter the hydrophilic environment of the lumen of the ER and Golgi. The lipoprotein is transferred to the Golgi for processing of the carbohydrate moieties of the apoprotein and for phospholipid exchange. Secretory vesicles containing the lipoprotein are released from the Golgi, transported to and fuse with the plasma membrane, releasing the nascent lipoprotein into the space of Disse. In the space of Disse, VLDL gains the apo C's from HDL particles [70]. Active synthesis of phosphatidyl choline and the presence of cholesterol has been reported to be required for VLDL synthesis [69]. Apo B is constitutively synthesized and therefore, although its presence is vital for VLDL formation, its formation is not a limiting factor in VLDL synthesis, in the normal situation [68]. If apo B is not incorporated into a lipoprotein it is considered to be degraded intracellularly [68,71]. From observations of the effect of a

sucrose, fatty acids or a cholesterol-enriched diet on the formation of VLDL, the synthesis of lipids in HepG2 cells was not directly coupled to the synthesis and secretion of apoproteins, particularly apo B [72]. Thus the lipid supply appears to be the primary controlling factor in VLDL formation, when apo B synthesis is normal, and triglyceride supply controls the size of the particle secreted.

Once in the plasma, VLDL undergoes extensive modification. Nascent human VLDL is thought to gain cholesteryl esters in plasma through the action of one of the lipid transfer proteins, cholesteryl ester transfer protein (CETP). CETP can mediate the hetero-exchange of triglyceride for cholesteryl esters in plasma [73]. CETP has been reported to mediate the transfer of cholesteryl esters from HDL to VLDL which was accompanied by a transfer of triglyceride from VLDL to HDL [74]. The length of time that VLDL is present in the plasma may influence the degree of cholesteryl ester enrichment. The mechanisms controlling the catabolism of the VLDL are not completely understood but involve the action of lipoprotein lipase. The effect of lipoprotein lipase has been discerned from the results of *in vitro* and *in vivo* studies [23,75-82]. Lipoprotein lipase is more active on larger particles than smaller particles. This may occur because more substrate (triglyceride) is dissolved in the surface of the larger particles due to the lower unesterified cholesterol to phospholipid ratio relative to that of smaller VLDL/IDL particles. Lipoprotein lipase hydrolyses the VLDL triglyceride core and some surface phospholipid with an accompanying transfer of apo CII, apo CIII, apo E, phospholipid and unesterified cholesterol to HDL [23,75]. The loss of apo E is slower than that of apo CII and CIII and therefore apo E remains with the particles longer [78] as they being lipolysed. Several studies of VLDL lipolysis have also reported a gain of VLDL cholesteryl ester by HDL [23,77,82,83]. However, the specific mechanisms controlling the transfer of apo E and lipid mass from VLDL to HDL during lipolysis are not well understood and require further study.

Through the action of lipoprotein lipase as well as by other modifications which are not clear at present, it is thought that plasma VLDL is converted to IDL which is converted to LDL. It has been suggested that hepatic lipase modifies the lipolysed VLDL particles particularly in the IDL density range since subjects with hepatic lipase deficiency have demonstrated elevated levels of IDL (and a reduction of LDL) [45,84,85]. The LDL present is triglyceride rich [84]. The lipid transfer proteins, CETP and phospholipid transfer protein (PLTP) may be involved in the conversion process. Large LDL is present in subjects with CETP deficiency which may indicate a role for CETP in the conversion of large LDL to the size of normal LDL [86]. The activity of CETP [87,88] and PLTP [89] have reported to increase during lipolysis. However, very few studies have been carried out and the effect of the lipid transfer proteins on the formation of LDL and the transfer of lipid and protein to HDL during lipolysis of VLDL by lipoprotein lipase has not been well investigated.

VLDL was considered to be completely metabolized to LDL until metabolic tracer studies demonstrated that this was apparently not always the case [90-92]. From the results of apo B kinetic studies [91,92,93,94], it has been reported that during the process of conversion of VLDL or VLDL subfractions to LDL, the particles may be taken up by the liver or the peripheral tissues at several stages along the metabolic pathway. Furthermore, VLDL subfractions 1, 2 and 3 may be independently produced rather than be interconverted and therefore enter at different points on the metabolic route. A significant input of IDL as well as direct input of LDL has been observed in hypercholesterolemic subjects who demonstrated a 30% hepatic input of apo B from IDL versus 10% in normals [90]. From metabolic studies it has been noted that the uptake versus conversion to LDL varies depending on the size/type of VLDL particles [91]. The apo B label of large VLDL particles ($S^{\circ}f$ 100-400)(VLDL 1) was reported to appear quantitatively in the 12-100 Sf° range (VLDL 2, VLDL 3 and IDL) but little label was observed in the LDL density. The apo B label which appeared in the Sf° 12-100 range

demonstrated a biexponential decay implying that more than one metabolic route was present. At this stage removal of the particle derived from large VLDL was dependent on uptake by receptors requiring apo B arginine residues, indicative of LDL receptor involvement. It was postulated that conversion of the S_f^o 100-400 particles (VLDL 1) to the smaller S_f^o 12-100 particles changed the conformation of the apo B molecule facilitating binding to receptors with a resultant uptake of the smaller particles. It was concluded in this study that the majority of VLDL of the S_f^o 100-400 density range was not converted to LDL. Little conversion to LDL was found in the particles of S_f^o 60-100 (VLDL 2) as well. It was concluded that VLDL of S_f^o 20-60 (VLDL 3) was the major source of plasma LDL. The finding of minimal conversion of VLDL S_f^o60-400 (VLDL 1 and 2) to LDL has recently been confirmed using endogenously labeled rather than exogenously labeled VLDL [93]. The process of VLDL conversion to LDL remains controversial since the reports of the amount of VLDL apo B reaching LDL density vary from 30-90% [91-94]. Reports of the uptake in normolipidemics have varied from 40-50% for VLDL/IDL [92] to 18% [94] of the VLDL apo B. The factors which determine the complete conversion of VLDL to LDL require further study.

The uptake of the VLDL remnant is considered to be due to an increased expression of apo E in the lipolysed particle [95]. The LDL receptor has been implicated since VLDL remnant uptake was found to be under the same control mechanism as LDL uptake but chylomicron uptake was not [52]. The observation of a change in the apo B conformation in LDL as compared to VLDL led to the proposal that the reason that VLDL and VLDL remnants are not taken up via apo B is due to a more cryptic conformation of the binding site which changes as VLDL remnants/IDL are converted to LDL [95]. Lipolysed VLDL particles can be referred to as VLDL remnants (indicating particles that are taken up) and IDL (indicating particles that are converted into LDL). However the terms have been used interchangeably since the density and size of the particles can be similar. Normally, plasma IDL is a "remnant " of VLDL metabolism.

Some investigators suggest that the preference for uptake rather than conversion is a characteristic of the particle type. Apo E-rich and apo E-poor VLDL particles have been described [96]. Preferential uptake of apo E containing particles has been demonstrated in rabbits [97]. A decreased apo E content in small VLDL as compared to large VLDL has been reported in normolipidemic humans [98]. The role of apo E and the mechanisms controlling apo E removal during VLDL metabolism are unclear.

When studying VLDL metabolism and the conversion to LDL, the results may be confusing because the composition and metabolism of VLDL particles isolated at a specific density or of a particular particle size can vary. For example, small nascent VLDL can co-isolate with large lipolysed VLDL remnants but their metabolic endpoints may be completely different. Only the use of true nascent particles would indicate the exact metabolic routes of VLDL. It is possible that VLDL production is a variable process that produces particles of different size or characteristics depending on the amount of triglyceride formed on the ER and the apo E content. The uptake of VLDL as it is being lipolysed in plasma may depend on the balance between the two actions of lipoprotein lipase in that lipoprotein lipase can cause removal of apo E from the VLDL particle consequently reducing its ability to be taken up by the liver. On the other hand, lipoprotein lipase can cause an increase in the expression of apo E epitopes allowing for increased receptor interaction and possibly increased uptake. A role for hepatic lipase in increased apo E expression has also been postulated but a clear mechanism for this role has not been established [99]. In the plasma, the metabolism may vary depending on whether the particle encounters high levels of lipoprotein lipase on the capillary endothelium of the heart or adipose tissue versus being rapidly returned to the liver where hepatic lipase is present. One may speculate that when present in the circulation of the heart or adipose tissue apo E may be transferred from VLDL to HDL but in the liver uptake may be facilitated due to an increased expression of apo E. The role of the lipid transfer proteins must also be considered in the metabolism of VLDL and transfer or

exchange of VLDL mass with HDL. Excessive transfer of triglyceride to HDL (or to existing LDL) in exchange for cholesteryl ester can result in the formation of a cholesteryl ester-rich VLDL [73, 74]. The neutral lipid core of the ensuing particle cannot be reduced by lipolysis as compared to a triglyceride-rich VLDL particle unless there are mechanisms for removal of the cholesteryl ester in plasma. Therefore, conversion of the cholesteryl ester-rich particle to LDL may not occur. Overall, the factors that control the uptake or the mechanism of conversion of VLDL remnants or IDL to LDL are poorly understood at present.

3. IDL METABOLISM:

IDL make up a small fraction of the apo B-containing lipoproteins. IDL particles have been subfractionated into IDL1 and IDL2 [98,100] and it has been suggested that certain fractions give rise to specific fractions of LDL [100]. As IDL becomes more and more dense it becomes enriched in cholesteryl esters, depleted of triglycerides, depleted in apo E and the apo C's relative to apo B due to the continued action of lipoprotein lipase. A role for hepatic lipase in the conversion of IDL to LDL has been postulated from the reports of increased IDL concentrations in hepatic lipase deficiency but the mechanism has not been clearly elucidated. The lipid transfer proteins may also play a role in modifying the particle (see section on lipid transfer proteins).

4. LDL METABOLISM:

LDL is the major transport vehicle for cholesteryl ester in the plasma. LDL can be taken up by peripheral cells as well as hepatocytes via the LDL receptor . Sixty percent of the plasma LDL is considered to be taken up by the LDL receptor with 40% of LDL uptake occurring in the liver [92]. The LDL receptor is a specific saturable receptor found on the membrane of most cells [101]. The receptor binds LDL via apo B but can bind through apo E . The internalization of the receptor-LDL complex with subsequent lysosomal hydrolysis of the cholesteryl esters results in inhibition, via a putative

oxysterol intermediate, of receptor synthesis and cholesterol synthesis. A stimulation of cholesterol esterification by the enzyme acyl cholesterol acyl transferase (ACAT) ensues. Thus, uptake of cholesterol and cholesteryl esters by the LDL receptor is a tightly controlled process which prevents the accumulation of excessive amounts of cholesterol/cholesteryl esters in cells. However, the lack of or dysfunction of the LDL receptors leads to an accumulation of LDL in the plasma that is associated with the development of premature atherosclerosis. The reason that cholesterol deposition occurs in the absence of LDL receptors is thought to be due to uptake via a type of receptor that is unable to regulate the cellular cholesterol content. The receptor has been hypothesized to be the scavenger receptor present on macrophages. Macrophages and smooth muscle cells are considered to be the precursors of lipid-laden foam cells characteristic of the atherosclerotic plaque. Intact LDL is not taken up by the scavenger receptor but modification of LDL (such as peroxidation of the fatty acids or oxidation of apo B), particularly when there is a long plasma half-life, may affect the LDL particle such that it can specifically bind to scavenger receptors. *In vitro*, it has been shown that highly oxidized or acetylated LDL can be taken up by scavenger receptors [102]. Although this is not typical of the physiological situation, there is some evidence that LDL oxidation can occur *in vivo*. The details of the scavenger receptor and oxidized LDL uptake are complex but this mechanism for LDL uptake may play a role in the development or progression of atherosclerotic plaques.

LDL is heterogeneous and various subfractions have been described and related to the development of atherosclerosis or the affinity for the LDL receptor. In general, light and dense LDL subfractions have been described [103-107]. A predominance of large, light LDL has been correlated with HDL cholesterol concentrations [107] while small dense LDL has been found to be higher in subjects with coronary heart disease, hypertriglyceridemia and familial combined hyperlipidemia [90,103,105,107-110]. The cause of the subclass pattern is unresolved. Enrichment with triglyceride in exchange for

cholesteryl esters and subsequent hydrolysis has been suggested to be the cause of the increased concentration of the small dense LDL particles. Large light LDL particles have been observed in hypercholesterolemics [90,107] and postulated to be the result of a decreased conversion of light to heavy LDL. The benefit of having light LDL relative to heavy LDL may depend on the mechanism of the formation of the light particle. Large, light LDL particles have been reported with *in vitro* VLDL lipolysis by lipoprotein lipase [82], in plasma of CETP-deficient patients [86], and following hepatic lipase inhibition [84]. In one study, complex independent routes for the formation of LDL particles have been proposed to explain the observation of four LDL bands on polyacrylamide gradient gels [111]. It was postulated that the subfractions LDL I-IV arise from two different IDL particles, IDL 1 and IDL 2. Small VLDL particles give rise to IDL 1 which are lipolysed to LDL II. IDL 2, whose source is unclear, are cholesteryl ester enriched relative to IDL I and consequently would give rise to the larger LDL I particles. It has been suggested that in hypertriglyceridemics IDL-I and LDL-I particles, which are richer in cholesteryl esters relative to LDL II, would undergo exchange of cholesteryl ester core for triglyceride from large VLDL. With hydrolysis of the triglyceride core, the particles may be converted to the small, dense LDL particles which are observed in hypertriglyceridemics.

Contradictory results have been obtained for the influence of LDL size on receptor uptake of LDL. Relatively more small LDL (200Å) were bound, internalized and degraded than large LDL(270Å) [112]. The interaction of LDL with the LDL receptor was determined in human U-937 monocyte-like cells [113]. The mid- density LDL fraction, which is the LDL subspecies of the highest concentrations in normolipidemic subjects, bound the LDL receptor with a greater affinity and were degraded at a higher rate than the larger or smaller fractions of LDL. Other investigators report a greater uptake of lighter LDL than denser LDL in Hep G2 cells [114]. The difference may be due to the definitions of light (buoyant) and heavy (dense) LDL. Until LDL subfractions are

more consistently defined in terms of density and composition it is difficult to interpret these data.

5. HDL METABOLISM:

HDL was initially defined as a particle that isolates in the density 1.063-1.21 g/ml. Apo AI is the predominant protein present in most particles while the composition of the other apoproteins varies. The major site of synthesis of nascent HDL is considered to be the liver in the form of phospholipid/unesterified cholesterol AI/E/C discs. Recently, from isolation of rat Golgi contents, it was reported that all triglycerides, apo B, apo E, apo AI and apo C's were isolated at a density < 1.010 g/ml. No particles the size of HDL or LDL could be seen in the d<1.010 g/ml fraction by negative stain electron microscopy [115]. The nascent VLDL was highly enriched in phospholipids as compared to plasma VLDL. It was suggested that at a very early stage in plasma pro apo AI, apo E and phospholipid dissociate from the nascent VLDL giving rise to nascent discoidal HDL. Other routes for HDL synthesis have been reported. HDL may be formed in the intestine and enter either the lymph or the plasma [116]. Macrophages also have been reported to synthesize and secrete discs that contain apo E, unesterified cholesterol and phospholipid that can interact with existing HDL [117].

HDL undergoes extensive modification in the plasma. Nascent HDL becomes enriched in cholesteryl ester through the action of LCAT resulting in the conversion of the particle from a disc to a sphere. This process must occur rapidly since HDL, isolated from plasma of human hepatic veins, is predominantly spherical with a few disc shaped particles [21]. The small triglyceride content in HDL is considered to arise by CETP-mediated exchange of HDL cholesteryl esters for VLDL triglyceride since this has been demonstrated in incubations containing VLDL, HDL and CETP [118].

The mechanisms involved in the augmentation of HDL mass are of interest since high HDL concentrations have been found to be inversely related to the incidence of coronary heart disease. Through the action of lipoprotein lipase on chylomicrons and

VLDL, HDL gains unesterified cholesterol, phospholipid, apo CII, apo CIII and apo E. The loss of these components has been suggested to be due to the fact that the surface of the lipolysing particle becomes excessive (redundant) relative to the core. A gain in VLDL cholesteryl ester by HDL has been demonstrated in some studies [23, 78]. The method and factors controlling the extent of transfer of components from VLDL to HDL are unknown. The method of transfer is unclear with respect to whether a new HDL particle is formed or the mass is gained by the existing HDL. In vitro, a HDL-sized spherical particle containing apo E, CII and CIII has been isolated which exchanged apoproteins and lipids when incubated with plasma HDL [119]. Others have hypothesized that the redundant surface of lipolysed triglyceride-rich particles (VLDL and chylomicrons), extrudes as a bilayer [120]. If the apoprotein content is adequate, the redundant surface would separate in the form of a phospholipid /unesterified cholesterol /apoprotein bilayer disc. If there is inadequate protein, a vesicle may form, containing an aqueous core. The particles would be converted to spheres through the action of LCAT and apo AI may be gained from chylomicrons or from existing HDL for stabilization of the particles. *In vitro* hydrolysis of chylomicrons has demonstrated extrusions from the surface of the remnant particles in the presence of low amounts of albumin [121]. Whether this process occurs *in vivo* is unclear.

HDL can be modified by the action of hepatic lipase via the hydrolysis of core triglyceride and surface phospholipid [122,123]. The relative roles of lipoprotein lipase and hepatic lipase in the conversion of HDL_2 to HDL_3 are controversial and require further study.

In general, HDL metabolism has been described as a somewhat circular process in which particles become enlarged by gaining core through the action of LCAT and surface (possibly accompanied by core) from lipolysing triglyceride-rich particles. Large HDL particles can be converted to smaller particles by first gaining triglyceride at the expense of cholesteryl ester from VLDL and chylomicrons through the action of CETP, and

subsequently losing core triglyceride due to hydrolysis by lipases, particularly hepatic lipase [123]. Hepatic lipase may also reduce the surface by phospholipid hydrolysis.

HDL may gain unesterified cholesterol from peripheral cells due to a specific interaction with the cell and the presence of more unesterified cholesterol relative to phospholipid on the cell membrane than on the HDL surface. It is possible that unesterified cholesterol may desorb into the interstitial fluid and therefore interact with HDL [124-126]. However, HDL may facilitate the process of removal of unesterified cholesterol by specifically binding to the cell membrane through apoproteins such as apo AI [127]. Putative HDL receptors have been reported in several cell types [33,35,128-130] allowing for HDL binding without uptake or uptake without degradation via retroendocytosis [35]. Retroendocytosis has been reported in rat macrophages [35], rat liver [131] and in rat intestinal crypt cells [34]. The HDL released from the macrophage was bigger and contained more apo E [35]. The process of cholesterol removal from peripheral cells is referred to as reverse cholesterol transport and has been suggested to be the role that HDL plays in the prevention of atherosclerosis. HDL can cause a reduction of cellular cholesterol *in vivo* [35,129] but whether this process actually occurs *in vivo* is uncertain.

The catabolic route for HDL is considered to be via uptake by the liver but the ligand is uncertain. Apo E-mediated uptake of HDL has been demonstrated in rat [132] and canine hepatocytes [133]. HDL, lacking apo E, was observed to bind to human hepatic membranes [134], rat liver [131] and pig hepatocytes [135]. Uptake of cholesteryl ester was four-fold higher than uptake of the apoproteins using apo E-deficient HDL indicating preferential uptake of lipid in the perfused rat liver [131]. Subsequently, a more dense HDL particle was released. This selective uptake would provide a route for cholesteryl ester removal from plasma in the absence of removal of the HDL particle. Uptake of HDL by other tissues such as the kidney, adrenal and ovary has also been reported. [136].

The HDL particle can transfer some of its components to other lipoproteins which represent an alternate mode of removal of HDL "mass". HDL can lose cholesteryl esters by transfer to VLDL/remnants through the action of CETP. It has been suggested that CETP may increase the rate at which HDL is catabolized since subjects with a genetic absence of the transfer protein demonstrate very high levels of both HDL cholesterol and apo AI [86]. HDL mass can decrease by transfer of apo CII, CIII and apo E to nascent chylomicrons and nascent VLDL. HDL acts as a reservoir of apoproteins supplying the activator of lipoprotein lipase (apo CII) and the ligand for the hepatic uptake of chylomicron and VLDL remnants (apo E) to nascent chylomicrons and VLDL. Possibly a low HDL level does not provide for such a "sink" of apoproteins and therefore these apoproteins are not available in sufficient concentrations required by VLDL and chylomicrons. A lack of apoprotein CII would cause a reduced level of lipolysis while a lack of apo E would reduce the uptake of chylomicron and VLDL remnants. A prolonged plasma half-life of the particles would ensue allowing for enrichment with cholesteryl esters and "oxidative modification" of the remnants. In a study of subjects with coronary artery disease it was found that the subjects demonstrated low levels of apo AI and HDL cholesterol but also low apo CIII and apo E [137] as compared to normolipidemics. This observation may reflect such a process. A slow HDL catabolism may be important in relation to the role of HDL in the prevention of atherosclerosis.

The inverse relationship of HDL with coronary heart disease and the progression of atherosclerosis has been associated with the larger, lighter HDL subfraction, HDL_2. HDL_2 [138], and HDL_{2b} in particular [139], were inversely correlated with coronary heart disease. HDL_2 is variable in plasma whereas HDL_3 tends to be static. The variation in HDL_2 accounts for the differences in total HDL concentrations in most individuals.

C. ENZYMES AND LIPID TRANSFER PROTEINS INVOLVED IN LIPOPROTEIN METABOLISM.

1. LIPOPROTEIN LIPASE:

SYNTHESIS, SECRETION AND UPTAKE:

Lipoprotein lipase mRNA has been found in adipose tissue, muscle, adrenal, lung, brain, macrophages and liver [141]. The major enzyme sources are adipose tissue and cardiac and skeletal muscle. Human and rat adult liver hepatocytes do not produce lipoprotein lipase [142]. However, Intralipid infusions increase lipoprotein lipase activity in the liver on the luminal side of endothelial cells [143]. It is unclear whether the observation is a result of transfer of lipoprotein lipase borne on the Intralipid particle or indicates a function for lipoprotein lipase in the liver. Lipoprotein lipase can enhance the specific binding of chylomicrons to Hep G2 cells [144]. The increased binding is a characteristic of the enzyme protein rather than the process of lipolysis. Facilitated uptake of remnants by hepatocytes may therefore occur through interaction of LPL, which is bound to lipoproteins or emulsions, with the LRP receptor [144].

Lipoprotein lipase is synthesized in the ER as a glycoprotein of molecular weight (MW) 55,500 bearing two N linked oligosaccharide chains of high mannose type. Transportation to the trans Golgi results in the formation of the mature enzyme where the high mannose oligosaccharides are processed to complex oligosaccharides. In the Golgi, the active homodimer is formed [146]. Lipoprotein lipase can bind to specific cell surface heparan sulphate proteoglycan receptors and may be released from the receptor by binding to competing molecules in the extracellular space. The release may prevent the internalization and degradation of lipoprotein lipase in parenchmal cells and allow for lipoprotein lipase to be transferred to and bind endothelial cells. A protein which binds lipoprotein lipase and contains heparan sulphate chains was identified on endothelial cells [147]. The protein was suggested to be the endothelial cell surface lipoprotein lipase receptor. It was proposed that glycosyl phosphatidyl inositol may act as a membrane

anchor for the proteoglycans that bind lipoprotein lipase. Phosphatidyl-inositol specific phospholipase C caused release of lipoprotein lipase from heart cells through release of heparan sulphate proteoglycans which have bound to lipoprotein lipase [148]. A catabolic route for lipoprotein lipase was postulated to occur by detachment from the rat heart endothelium and degradation in the liver rather than by local intracellular degradation [149]. Fatty acids have been shown to cause the detachment of lipoprotein lipase from endothelial cells by a specific interaction that is not ionic nor due to detergent activity [149]. A putative fatty acid binding site has been reported on lipoprotein lipase. The purpose of the release of the enzyme by free fatty acids has been suggested to be the prevention of too high an accumulation of fatty acids in a specific location. The release could serve as a means of allowing lipoprotein lipase to be transported to the liver for degradation. However, the effect of fatty acids on lipoprotein binding remains open to question since albumin is present to bind the released fatty acids and fatty acid uptake by tissues is considered to be rapid.

The mature form of lipoprotein lipase is a N-glycosylated secretory protein with a molecular weight of approximately 60,600 daltons [145]. Lipoprotein lipase is formed in parenchymal cells rather than endothelial cells and is secreted as a homodimer which is the active form [146]. Lipoprotein lipase binds on the luminal side of capillary endothelial cells to glycosaminoglycans containing heparan sulfate via a specific heparin binding site on lipoprotein lipase [141]. In this location on the endothelium, lipoprotein lipase is available for interaction with the plasma lipoproteins. The glycosaminoglycans appears to anchor or stabilize lipoprotein lipase on the endothelium and are not involved in the lipid binding or catalytic activity of the enzyme. Through the interaction of the lipid binding site of lipoprotein lipase with the surface lipid of the lipoprotein, lipoprotein lipase is able to bind and hydrolyse the small amount of triglyceride present on the surface of the lipoprotein (approximately 3 mole percent of the surface). Rapid hydrolysis

of triglyceride is achieved by the continuous replacement of the hydrolyzed surface triglyceride with core triglyceride.

The concentration of lipoprotein lipase present at the capillary endothelium can vary and is subject to hormonal/dietary regulation [141,150]. Tissue-specific differences exist in the hormonal response. For example, starvation decreases adipose tissue lipoprotein lipase but increases cardiac and skeletal muscle lipoprotein lipase. The response appears to be related to the need for fatty acid uptake in the different tissues. It has been suggested that lipoprotein lipase directs triglyceride fatty acid removal from the blood so as to provide for the changing requirement of the different tissues [141]. Several hormones affect lipoprotein lipase concentrations [141,150]. Insulin can increase lipoprotein lipase in adipose tissues and the effect is further enhanced by the addition of dexamethazone. Insulin can decrease the activity in muscle. There is evidence that the effect of insulin may be via stimulation of phospholipase C activity specific to phosphatidyl-inositol in adipose tissue. Catecholamines and glucagon are inhibitory. The opposite effect of the hormones are observed in cardiac muscle.

ACTIVITY AND CHARACTERISTICS:

Lipoprotein lipase (EC 3.1.1.34) hydrolyses the triglycerides of the plasma lipoproteins, particularly those of chylomicrons and VLDL. By hydrolysing the lipoprotein triglycerides, the enzyme supplies tissues with fatty acids to be used for storage in the case of adipose tissue or energy metabolism in the case of muscle tissue. Lipoprotein lipase demonstrates phospholipase A_1 activity but phospholipid hydrolysis occurs at a much slower rate than triglyceride hydrolysis. The phospholipase activity of lipoprotein lipase varies depending on the tissue source in that phospholipid hydrolysis is higher in the lipoprotein lipase purified from bovine skim milk [83] or human milk [140] as compared to the lipoprotein lipase released from rat cardiac endothelium [23,76]. One could speculate that this may be due to small differences in the amino acid sequence

between species or by a difference in post-translational modification in different tissues of the same species.

Lipoprotein lipase is classified as a serine esterase due to the observed inhibition by serine esterase inhibitors such as di-isopropyl fluoro phosphate and phenyl methyl sulphonyl fluoride. The activity is considered to be due to a typical catalytic triad of serine, aspartate and histidine characteristic of serine esterases [149]. Serine 132 is considered to be the catalytic serine comprising part of a putative interfacial lipid binding domain (Gly-X-Ser-X-Gly) shared by hepatic lipase, pancreatic lipase and other serine esterases [151]. Lipoprotein lipase hydrolyses the fatty acids in the sn-1 and sn-3 position of triglycerides, with a preference for the sn-1 position [152]. It has low activity towards monoacylglycerols and requires migration of the fatty acid from the sn-2 position to the sn-1 or 3 position. Lipoprotein lipase requires the presence of apo CII as a co-factor for activity on water-insoluble substrates [44,153]. Lipoprotein lipase is thought to contain a specific binding site for apo CII. Apo CII is thought to act by either aiding in the positioning of the triglyceride ester bond of the triglyceride or positioning of the catalytic site of lipoprotein lipase. Apo CII has a lipid (phospholipid) binding domain, a domain that activates lipoprotein lipase and two lipoprotein lipase binding domains, one possibly binding by hydrophobic interactions and the other by ionic interactions. The importance of the lipid binding domain of apo CII increases as the surface pressure of the lipoprotein increases. Apo CII may enable LPL to act on VLDL which has a higher surface pressure than HDL as compared to the enzyme hepatic lipase which does not demonstrate high triglyceride hydrolysing activity on VLDL but is active on HDL *in vitro* [154]. Albumin is required for full activity of lipoprotein lipase since it acts as an acceptor of the free fatty acids formed during the hydrolysis of the triglycerides (or hydrolysis of phospholipids) [155]. In the absence of albumin the cleaved fatty acid product is removed very slowly from the active site. In the presence of excess albumin, this step is no longer rate limiting [155]. These observations imply that in the *in vivo* situation, where albumin

concentrations in plasma are substantial (4 g/dl), it is unlikely that free fatty acid accumulation frequently occurs since fatty acids can be taken up by the surrounding tissues or by albumin in subjects with normal triglyceride concentrations [156]. Several apoproteins have been tested for their ability to inhibit or stimulate lipoprotein lipase. Apo CIII appears to be a non-competitive inhibitor of triglyceride hydrolysis [49]. Several lipoprotein lipase molecules may bind to one lipoprotein particle at a time resulting in an increased rate of lipolysis. An increase in the number of enzyme molecules bound would account for the substantial increase in triglyceride hydrolysis observed as a result of a heparin infusion which releases the lipase from the endothelium.

Lipoprotein lipase differs in characteristics from hepatic lipase, another lipase that is released as a result of heparin infusion, in that lipoprotein lipase is inhibited to basal levels by 1M NaCl [157] and by protamine while hepatic lipase is activated by high salt and is not significantly inhibited by protamine nor does it require apo CII for maximal activity. Lipoprotein lipase is released from binding to heparin Sepharose at a higher salt concentration (1.2 M versus 0.8M) than hepatic lipase[222]. The difference in inactivation and heparin binding have been suggested to be due to the fact that lipoprotein lipase is active as a homodimer while hepatic lipase is active as a monomer. High salt would therefore have opposing effects on the active form of these two enzymes.

ROLE IN LIPOPROTEIN METABOLISM:

The role of lipoprotein lipase in lipoprotein metabolism has been determined from *in vitro* incubations and *in vivo* heparin infusions .

In vitro lipolysis of VLDL by lipoprotein lipase results in the appearance of most of the lipolysed VLDL mass in the LDL density range. Some mass remains in the VLDL/VLDL remnant/IDL density range. A gain of mass in the HDL density range also occurs. The HDL region has been reported to gain apo CII and CIII, apo E as well as phospholipid and unesterified cholesterol as previously described. A gain in cholesteryl ester in the HDL region has been observed but remains controversial. The results vary in

terms of : 1) the extent of transfer of the lipid apoprotein components to HDL; 2) whether the HDL formed is spherical or discoidal indicating the presence or absence of a neutral lipid core; 3) the effect of lipolysis on the conversion of HDL_3 to HDL_2; 4) whether lipolysis can form LDL similar to that of plasma. The *in vitro* studies differed substantially from each other in the methods or enzyme preparation used and many of the studies employed conditions that would rarely or never be present in the true physiological state. These variations in experimental conditions can account for some of the differences in the results. For example, the enzyme preparations differed in that bovine or human skim milk lipase were used in some experiments while the perfused rat heart or lipoprotein lipase released from the heart after a heparin infusion were used in others. Milk lipase has a higher phospholipase activity [76] compared to rat heart lipoprotein lipase which may affect the resultant products of VLDL lipolysis. *In vitro* incubations were frequently carried out at pH 8.2-8.4 rather than at physiological pH. The different pH may affect the activity of the enzyme (less phospholipid hydrolysis occurs at pH 8.6 than at 7.4) [158] or may affect the association of the apoproteins or the enzyme with the lipoprotein particles. The degree of triglyceride hydrolysis varied from 50% to 99% [78,83]. Some studies [75,78] did not use albumin as a fatty acid acceptor. Insufficient albumin would lead to the accumulation of fatty acids which can inhibit lipolysis [155], dissociate the enzyme from the VLDL, have a detergent effect or cause aggregation of the lipoproteins [75,100]. Other investigators included albumin in the incubation but the amount was frequently inadequate or the albumin preparation may have contained phospholipid and apo AI [76,77,83]. In the majority of studies the ratio of VLDL to HDL mass was excessively high. A high ratio may be representative of that in hypertriglyceridemics but does not represent the ratio in the normolipidemic subjects. The concentration of the lipoproteins in the incubation medium often differed from the concentration present in the physiological situation. Finally the density cuts used to

define HDL varied in that some studies used a "HDL" density cut that overlapped the LDL density range (d>1.04 g/ml)[76,77].

Early studies of VLDL lipolysis included VLDL in the absence of HDL in order to determine what type of particles were isolated in the LDL and HDL density range [75-77,83]. The inclusion of albumin varied. The extent of VLDL triglyceride hydrolysis ranged from 50-97% in these studies. As a consequence, there was great diversity in the reports of the gain by HDL of apo CII, CIII, unesterified cholesterol, phospholipid and in some cases cholesteryl ester. Most of the lipolysed VLDL mass was found in the LDL density range but some mass remained in the VLDL, VLDL remnant and IDL, i.e. d<1.019 gm/ml. The LDL were larger, lighter, had a molecular weight similar to IDL (5 million) and a moderate increase in the surface to core ratio relative to normal plasma LDL [83]. The particles were also enriched in cholesteryl ester relative to apo B. Enrichment in unesterified cholesterol and lipid phosphorus was reported by Alaupovic [158]; they claimed that all of the lipolysed VLDL mass was found in the LDL density range but differed somewhat in composition from normal plasma LDL. Several types of structures were observed with electron microscopy. Dory [75] reported the observation of VLDL remnants as particles smaller than VLDL with irregularities in shape, and some membrane-like borders around partially empty particles. In the LDL density range, spherical particles were observed [82,158] as well as electron-lucent particles considered to be unilamellar liposomes (vesicles) formed from the redundant surface of lipolysing VLDL [82]. Discs were also reported in the LDL density [82]. The HDL region contained discs [76,77,82]. From these observations, the theory for HDL formation from the redundant surface of VLDL during lipolysis was hypothesized with the remaining VLDL particle giving rise to spherical LDL. However, Deckelbaum [83] later reported that the albumin preparation in some of the studies may have contained phospholipid and apo AI which increased the phospholipid content of the incubation by 32%. When an albumin preparation free of apo AI and lipid was substituted, the amount of vesicles in

the LDL range was reduced and the HDL mass was decreased by one-sixth. The presence of phospholipid and AI in albumin appeared to increase the mass gained from VLDL by the HDL region during lipolysis. Deckelbaum et al reported that discs persisted in the HDL region with the purified albumin. The discs were defined as particles of 150-400 Å in diameter and 60-90Å in width. A 90 Å width indicates the presence of two bilayers, i.e. a desiccated unilamellar liposome rather than a disc since the bilayer of a disc would be in the range of 20.5 x 2 = 41 Å. If the dye entered the liposome the width would also appear to be in this range.

More recent studies using the perfused rat heart have reported the presence of spheres in the HDL region as a result of VLDL lipolysis in the absence of HDL. There was a gain of cholesteryl ester and a small amount of triglyceride in the HDL region [23,78]. No disks were observed in the HDL region. The particles were isolated by gel filtration (according to size) rather than by ultracentrifugation (according to density) which may have removed vesicles that would overlap the HDL-LDL density range. When the spherical HDL particles from the perfusate were incubated with a preparation of plasma HDL (lacking apo E), the plasma HDL lacking-apo E lost cholesteryl ester and apo AI and gained phospholipid from the perfusate HDL [119]. The particles remained distinct since the ones which arose from lipolysis maintained the high unesterified cholesterol to phospholipid ratio (0.75) and the larger particle size (139Å) while the plasma HDL maintained an unesterified cholesterol to phospholipid ratio of 0.23 and a size of 121Å. This study also observed vesicles in the LDL region but the mass was greatly reduced when albumin was included in the perfusion. Possibly, if higher amounts of albumin had been used, a further reduction of the vesicles would have occurred. There are other reports of the formation of spherical particles containing cholesteryl ester in the HDL density range with the *in vitro* lipolysis of VLDL [79,159]. Notably, Taskinen did not observe discs in the HDL density range after lipolysis in the presence of HDL. A

gain of cholesteryl ester in the HDL region has been reported in many studies [75,82,83,158] but the effect on the formation of a spherical particle was not rationalized.

When HDL was included with VLDL and lipoprotein lipase in the incubation, a complete conversion of HDL_3 to HDL_2 was reported by Patsch [160] while Taskinen [79] observed a small gain of mass in the HDL_2 region and a small loss from the HDL_3 region without a complete conversion of HDL_3 to HDL_2 . The difference between the two studies is that the plasma was included in the incubation of Patsch but not in the incubation of Taskinen. Plasma contains the lipid transfer proteins and LCAT which may have aided in the conversion of HDL_3 to HDL_2. This suggestion of a role for the lipid transfer proteins and LCAT in HDL conversion is substantiated by the results of another study which determined the effect of VLDL lipolysis on the conversion of HDL_3 to HDL_2 [161]. In this study, only the addition of the lipid transfer proteins with LCAT resulted in a significant conversion of HDL_3 to HDL_2.

In summary, *in vitro* studies have demonstrated that the activity of lipoprotein lipase is associated with the formation of an LDL particle and the augmentation of HDL mass. Although some studies have claimed that VLDL can be completely converted to LDL and HDL_3 can be converted to HDL_2, the majority of the studies indicate that lipoprotein lipase activity does not result in the formation of LDL that is the same as plasma LDL nor can it effect a large shift in the density of the HDL mass. Other factors must be involved in both these conversion processes but few studies have been carried out which address these issues. The factors controlling the degree of augmentation of HDL mass during VLDL lipolysis are unclear. Whether the VLDL mass is transferred directly to the existing HDL or whether a new HDL particle is formed from VLDL which exchanges or donates mass to the existing HDL is not known. If a new HDL particle is formed, it is unclear whether the mass is transferred to the HDL region in the form of a disc (derived from the VLDL surface) or a sphere (derived from both surface and core).

The actions of lipoprotein lipase have been studied *in vivo* by administering an infusion of heparin. Heparin releases lipoprotein lipase from the capillary endothelium with a resultant increase in lipolytic activity. Hepatic lipase is released as well. The *in vivo* studies differ further from the *in vitro* studies in that LCAT and the lipid transfer proteins are present in plasma and lipoprotein uptake and catabolism can be ongoing. Therefore, the effect of lipoprotein lipase on lipoprotein metabolism can not be as easily quantitated in the *in vivo* studies as the *in vitro* ones. Another type of *in vivo* study is that of alimentary lipemia . Alimentary lipemia demonstrates how lipolysis of a fat load affects the plasma lipoprotein concentrations and composition. In such studies, the changes observed in the lipoprotein compositions after several hours reflects the formation of chylomicrons and their lipolysis and metabolism in the plasma.

With heparin infusions in normolipidemics, it was reported that more than 50% apo CII and CIII of VLDL were gained by HDL and there was a loss of mass from Sf° 400-60 and gain in Sf° 60-20, 20-12 and 12-0 after 45 minutes [80]. HDL gained mass in the HDL_2 region and the mass decreased in the dense HDL_3 region. Nestel [81] reported that the apo C's were transferred together to HDL in normolipidemics. Most studies were carried out in hypertriglyceridemics. Forte [162] noted a gain in the HDL_2 region and a loss in the HDL_3 region. At 2.5-10 minutes post-heparin, large flattened particles (vesicles) were observed in the Sf°12-20 region and small (58Å) spherical particles were noted in the HDL_3 region. Both types of particles disappeared after 30-60 minutes and it was suggested that HDL_3 was transformed into HDL_{2a} by incorporation of VLDL constituents. In one subject, used as an example, ten minutes after the infusion there was an increase in protein, phospholipid and unesterified cholesterol mass in IDL, LDL, HDL_2 and HDL_3, the increase being greater in HDL_2 than in HDL_3. Cholesteryl ester increased in IDL, LDL and HDL_2. There was a decrease in cholesteryl ester in HDL_3 which corresponded to the gain in HDL_2. HDL_2 and HDL_3 both lost triglycerides.

With alimentary lipemia, a gain in the light HDL region and a loss in the dense region was observed [163]. It was suggested that the gain observed in phospholipid (up to 18%) with a smaller gain in protein (up to 6%) by HDL resulted in a decreased density of the particles (increase in the flotation rate) of both HDL_2 and HDL_3. Apo AI mass in the plasma increased as did cholesterol.

The effect of lipoprotein lipase on the metabolism of apo E has been investigated. Interest in apo E metabolism arises from its role as a ligand in lipoprotein uptake by the LDL receptor, chylomicron remnant uptake and HDL uptake by the liver [51-53,53-55,132]. Another reason for the interest in the transfer of apo E is that extensive removal of apo E from lipolysing particles is required for the formation of plasma LDL. In one study of lipolysis of VLDL by bovine skim milk lipase, approximately 40-50% of apo E was transferred from VLDL to HDL [164]. This is greater than the 30% transfer reported in another study but only 50% of the VLDL triglyceride had been hydrolysed [78]. Very few studies of VLDL lipolysis by lipoprotein lipase have documented the extent of apo E transfer to HDL from VLDL. The results of the inhibition of lipoprotein lipase or hepatic lipase in post-heparin plasma demonstrated that lipoprotein lipase mediated the loss of apo E from VLDL with a gain or no change in the apo E content of IDL. Inconsistent changes in HDL were reported. Hepatic lipase caused a redistribution of apo E from IDL particles to HDL [165]. In subjects that were either lipoprotein lipase deficient or hepatic lipase deficient, the results of heparin-induced lipolysis indicated that lipoprotein lipase caused a transfer of apo E from the VLDL-sized particles to IDL and HDL-sized particles [166]. Hepatic lipase activity resulted in a transfer of apo E from VLDL to HDL but the apo E content of IDL did not increase. This suggests that transfer of apo E from IDL to HDL had occurred. From the results of these studies, a role for hepatic lipase rather than lipoprotein lipase in the transfer of apo E from IDL to HDL has been suggested. In normal subjects a heparin infusion resulted in a decrease in VLDL apo E and an increase

in HDL with a moderate gain or lack of gain in the IDL region reflecting the activities of both lipoprotein lipase and hepatic lipase.

2. HEPATIC LIPASE:

SYNTHESIS AND SECRETION:

Hepatic lipase (hepatic triacylglycerol lipase) is an enzyme of 65,500 daltons [145]. It is an N-linked glycoprotein, the glycosylation being necessary for secretion but not activity. Hepatic lipase is synthesized by hepatocytes and is located on the capillary endothelium. The majority of hepatic lipase is in the liver but it has been found in the adrenal and ovary where it may play a role in supplying cholesterol for steroidogenesis [167]. Hepatic lipase is also anchored to the endothelium by glycosaminoglycans.

ACTIVITY:

Hepatic lipase has both triglyceride and phospholipid hydrolysing activities *in vitro*. Using artificial emulsions of phospholipid and triglycerides or labeled HDL, hepatic lipase has been found to hydrolyse triglyceride and phosphatidylethanolamine much more effectively than phosphatidylcholine [122,168]. Hepatic lipase has been reported to preferentially hydrolyse the phospholipids of HDL_2 as compared to HDL_3 or LDL [122,123] and hydrolyse the triglycerides of HDL rather than those of VLDL. The specificity of the substrate has been reported to be due to a decrease in hepatic lipase activity as the density of surface packing increases [154], HDL, being much smaller than VLDL or IDL has a greater angle of curvature of the surface and consequently a lower surface packing density.

Hepatic lipase is considered to be a serine esterase. Serine 147 has been implicated since a change of serine 147 to glycine resulted in a loss of activity [169]. Serine 147 may be in the catalytic site but play a role in maintenance of the structural integrity of the active site or be part of the interfacial binding domain. Hepatic lipase has no absolute requirement for a cofactor. Apo E has been reported to stimulate and apo AI,

CII and CIII to inhibit hepatic lipase activity [170]. Apo AII has been reported to have both effects [170]. Hepatic lipase is not as sensitive to diet and hormones as is lipoprotein lipase [122]. Catecholamines [122] can increase the activity while estrogens decrease it [171].

ROLE IN LIPOPROTEIN METABOLISM:

The role of hepatic lipase in lipoprotein metabolism has not been clearly established. Post-heparin plasma hepatic lipase levels have been positively correlated with VLDL mass and negatively correlated with HDL cholesterol. Genetic defects in hepatic lipase have been defined in three kindred. The role of hepatic lipase has been suggested from the alterations in their lipoprotein profiles [45,84,85,172]. Descriptions of the lipoprotein profiles of the three kindred vary. The differences may be due to variations in caloric intake or the presence of another genetic defect causing hyperlipidemia [84,173]. From the observations of the lipoprotein profiles of subjects exhibiting hepatic lipase deficiency, it has been found that there was an accumulation of β-VLDL and IDL. The LDL cholesterol varied in that it was normal or high in one family [45] but LDL was low or absent in the Scandinavian family [85]. HDL cholesterol was elevated. The LDL and HDL particles were enriched in triglycerides. The LDL were large and buoyant [84] and the HDL distribution showed a shift in the distribution with a predominance of the larger, lighter HDL_2 particles. Hypertriglyceridemia and hypercholesterolemia were present in some individuals. Some subjects demonstrated premature coronary heart disease except in the Scandinavian deficiency family [85].

From the lipoprotein profiles and from determination of apo B kinetics in one patient of the Scandinavian family, there was a 50% reduction in the rate of conversion of small VLDL to IDL and the 2.4 fold increase in IDL concentration. From these observations it has been postulated that hepatic lipase is involved in the conversion of small VLDL to IDL but more importantly in the conversion of IDL to LDL [174]. A role

in the transfer of apo E from IDL to HDL particles during VLDL lipolysis has been suggested [164,166].

Some skepticism exists as to effect of hepatic lipase in the interconversion of VLDL-IDL-LDL as it has been reported that hepatic lipase did not play a role in the metabolism of large and small VLDL [175] or in the conversion of VLDL to LDL *in vitro* in one study [174]. In contradiction to these findings, in subjects with lipoprotein lipase deficiency, there is hypertriglyceridemia due to an elevation of chylomicrons but not VLDL[141]. The hypertriglyceridemia can be alleviated by restriction of dietary fat. However, in a subject who demonstrated an autoimmunity to both lipoprotein lipase and hepatic lipase, a low fat diet did not reduce the hypertriglyceridemia [176]. The results suggest that hepatic lipase is involved in the catabolism of endogenous triglyceride-rich lipoproteins. Thus the role of hepatic lipase in VLDL/IDL/LDL metabolism requires further study.

The effect of hepatic lipase on HDL metabolism is postulated to be the conversion of HDL_2 to HDL_3 through hydrolysis of the core triglyceride and surface phospholipid. Incubation of human serum with hepatic lipase resulted in a loss of mass from the HDL_2 density region and a gain in the HDL_3 region, particularly in the dense region of HDL_3 [176]. From *in vitro* studies it appears that for the complete conversion of HDL_2 to HDL_3, the cholesteryl ester transfer protein may be required to first act upon the HDL particles. Through the action of the cholesteryl ester transfer protein, HDL_2 may become enriched with triglycerides from triglyceride-rich particles in exchange for cholesteryl esters. In the presence of hepatic lipase the triglyceride core would be extensively hydrolysed resulting in the formation of small, dense, HDL_3 particles. It has been found that hepatic lipase, in the presence of the cholesteryl ester transfer protein, also stimulated the loss of apo AI from HDL. The loss of apo AI was suggested to increase the catabolism of the particle [177].

The effects of alimentary lipemia and modulation by hepatic lipase on the conversion of HDL_2 to HDL_3 was investigated [163]. HDL_2 was isolated after a fatty meal or after fasting. The HDL_2 isolated after the fatty meal was lighter due to a decreased proportion of protein and an increase in phospholipid as compared to the fasting HDL_2. This effect was presumably due to the transfer of lipids during the lipolysis of chylomicrons (which had formed during fat ingestion). After the ingestion of the fatty meal, the HDL_2 became enriched in triglycerides reflecting the activity of the cholesteryl ester transfer protein. The triglyceride enrichment of HDL_2 was directly related to the magnitude of post-prandial lipemia and inversely with the fasting HDL_2 levels in plasma. The ability of hepatic lipase to convert the triglyceride-enriched HDL_2 to HDL_3 was studied *in vitro*. Substantial hydrolysis of the HDL_2 triglyceride and phospholipid was observed after the lengthy incubation period. The authors reported that subjects with a small extent of triglyceride enrichment of the HDL_2 (<15% of the core neutral lipid) demonstrated very minor shifts to a higher HDL_2 density when this triglyceride enriched-HDL_2 was incubated with hepatic lipase. A shift of the triglyceride-enriched HDL_2 into the HDL_3 density only occurred in subjects with a high amount of triglyceride-enrichment of the HDL_2 (36-53% of the neutral lipid core). If fasting HDL_2 from any of the subjects was incubated with hepatic lipase, the HDL_2 increased in density but in no case was there a shift to a HDL_3 density. These observations indicate a co-operative role for high post-prandial triglycerides, the action of the cholesteryl ester transfer protein and hepatic lipase in the conversion of HDL_2 to HDL_3.

A role for hepatic lipase in chylomicron removal by the liver has been suggested and related to the hydrolysis of the surface phospholipid [178,179]. A resultant change in the conformation of apo E has been postulated [179]. The type of phospholipid hydrolysed may be important since chylomicron remnants and hepatic lipase-treated chylomicrons but not lipoprotein lipase treated chylomicrons were efficiently taken up by the rat liver [59]. The use of phospholipids, isolated from the different types of

chylomicron remnants, in an emulsion demonstrated uptake by the liver similar to the lipoprotein source [59]. Hepatic lipase preferentially hydrolyses phosphatidylethanolamine rather than phosphatidylcholine as compared to lipoprotein lipase which may account for the different effect on uptake. The amount of phospholipid which was lost from the particle was greater with hepatic lipase than with lipoprotein lipase which may also effect the uptake. It has been suggested that in the absence of hepatic lipase, the expression of apo E-binding domains on chylomicron remnants is masked. This is based on the observation that as a result of an injection of an antibody to hepatic lipase in rats, lipoproteins containing apo B48 which may be indicative of an intestinal source accumulated in the LDL density range [180]. It is also possible that apo B_{48}-containing VLDL, synthesized in the rat liver, may be affected by hepatic lipase inhibition. The LDL was larger and demonstrated a higher surface to core ratio than normal LDL which may reflect the activity of lipoprotein lipase alone since the LDL formed *in vitro* with lipoprotein lipase was also large with a high surface to core ratio. VLDL apo E expression differed with hepatic lipase inhibition in that there was less reactivity of apo E with a polyclonal antibody [99].

A role in the uptake of HDL cholesterol by the liver has also been suggested since increased uptake of hepatic-lipase treated HDL by rat hepatoma cells has been observed [181]. The increased uptake has been related to phospholipase A_2 activity of hepatic lipase.

3. LIPID TRANSFER PROTEINS:

The lipid transfer proteins, cholesteryl ester transfer protein and phospholipid transfer protein are thought to play a major role in the redistribution of lipids between lipoproteins. It has been suggested that their activity is interrelated with the effect of hepatic lipase on HDL, as described, as well as with the process of lipolysis of VLDL by lipoprotein lipase.

(a) CHOLESTEROL ESTER TRANSFER PROTEIN:

Cholesterol ester transfer protein (CETP) is an extremely hydrophobic acidic glycoprotein with a molecular weight of 74,000 [182-184] whose activity in the reciprocal transfer of triglyceride for cholesteryl ester was identified by Nichols and Smith [73] and in detail by Morton and Zilversmit [185]. The human gene has been cloned and sequenced [183]. The cDNA predicts a mature protein of 53,108 daltons whose size is thought to increase due to the addition of N-linked oligosaccharides [186] and potentially by a post translational modification. CETP activity is high in rabbits, high to moderate in humans and rhesus monkeys and low or undetectable in rats, mice and pigs based on the measurement of either liver RNA message [187] or on the activity assayed in the plasma [208]. It is important to note that CETP activity is high in animals that demonstrate a high incidence of atherosclerosis and low in those with a low incidence. Normal values for CETP activity range from 69-130 nm CE/ml/hour range [188,189]. CETP deficiency in humans is accompanied by extremely high HDL levels and an absence of coronary heart disease [190-193].

A plasma inhibitor has been identified [194]. The effect of the inhibitor was on the substrate rather than on CETP. The inhibitor is postulated to act by dissociating the lipoprotein / CETP complex [195].

Two mechanisms for the interaction of CETP with the lipoproteins have been proposed. Evidence exists for the formation of a tertiary collision complex of CETP with the two lipoproteins involved in the exchange/transfer process [196]. Others suggest that CETP acts as an intermediate carrier of lipid [197]. The binding of CETP to lipoproteins is reported to be by interaction with a negatively charged molecule. It has been suggested that the phospholipid phosphate groups of the lipoproteins are the primary sites for binding to CETP and the stability of the binding increases as the negative charge on the lipoprotein increases [198].

CETP is synthesized in the liver, adipose tissue, small intestine, adrenals, spleen and macrophages [183]. The majority of CETP in plasma has been reported to be associated with HDL (HDL_3) when isolated by density gradient ultracentrifugation or gel filtration [199]. Most of the CETP was found in the density d>1.25 g/ml when HDL_3 was isolated by a centrifugation of density 1.125-1.210 g/ml with two washes [200]. The binding of CETP to the various lipoproteins has been studied. The binding affinity was similar (and high) for VLDL, LDL and HDL but HDL binding was more stable [195]. The plasma activity appears to be related to the lipid status of the individual since CETP activity is increased with long term cholesterol feeding and post-prandial lipemia [201,202] and CETP activity is elevated in dyslipidemics, chylomicronemics and hypercholesterolemics [203]. The transfer of cholesteryl ester from HDL to VLDL was increased in hypertriglyceridemics but CETP activity was elevated [204] or unchanged [205]. The increase in activity may be due to variations in the lipoprotein substrate of hypertriglyceridemics as compared to normolipidemics. There are inconsistencies in reports of CETP activity in the various hyperlipidemias. The variability may be due to the assay of CETP mass versus assay of activity or the use of exogenous substrate as compared to the use of an endogenous substrate (the subject's lipoproteins) since composition can effect the transfer rate.

CETP has been reported to mediate: 1) the hetero-exchange of cholesteryl ester for triglyceride between lipoproteins, resulting in a net transfer, or 2) the homo-exchange of cholesteryl ester reflecting exchange in the absence of net transfer. CETP exhibits phospholipid transfer activity and has been reported to be responsible for 50% of the phospholipid transfer present in the plasma [199]. The direction of phospholipid transfer is primarily from VLDL to HDL. The direction of cholesteryl ester transfer is controversial as some authors report a net gain of cholesteryl ester by VLDL from HDL and an exchange of cholesteryl ester between HDL and LDL while others report a net transfer to HDL in normal subjects [188,206] which is reversed in hyperlipidemics [206].

The transfer appears to be due to transfer of cholesteryl esters from LDL to HDL more than from VLDL to HDL. HDL gained triglycerides as well which reflects a lack of the hetero-exchange process. These studies differ from ones that report the transfer of cholesteryl ester from HDL to VLDL in that they used normal concentrations of lipoproteins, short incubation times (1 hour versus 18-36 hours) and LDL was present in the incubation [188]. Others have corroborated these results [206] and related the transfer to the HDL triglyceride content. The transfer of cholesteryl ester from LDL to HDL is postulated to occur in the fasting state while a transfer of cholesteryl esters from HDL to apo B containing lipoproteins (chylomicrons and VLDL) may occur with alimentary lipemia, due to a large influx of triglyceride rich chylomicrons [201,202].

Some of the evidence for the direction of transfer has been based on determination of mass transfer. However, the majority of the studies quantitated the activity by measuring the transfer of label. The measurement of movement of label is not a direct reflection of cholesteryl ester mass transfer since CETP stimulates exchange as well as net transfer. Therefore the results can be deceptive. In many of the assays the ratio of VLDL or LDL to HDL was disproportionately high as compared to the ratio normally found in plasma.

It has been reported that the direction of transfer is dependent upon the nature of the donor and acceptor and their relative concentrations [74,207]. Specifically, it has been suggested that the direction of transfer is dependent upon the triglyceride content relative to the cholesteryl ester content. Thus a triglyceride-rich particle such as large VLDL would transfer triglyceride to a cholesteryl ester-rich particle such as HDL (or LDL) while HDL would transfer cholesteryl ester to the VLDL [74,207]. The ratio of triglyceride to cholesteryl ester was reported to be more important in the donor particle as compared to the acceptor particle but the cause is unclear. The difference in triglyceride or cholesteryl ester content of the core would be reflected by the concentration of neutral lipid in the surface of the lipoprotein. There is evidence to

suggest that it is the concentration of neutral lipids on the surface of a lipoprotein that determines the transfer activity [207]. The action of CETP on cholesteryl ester movement between LDL and HDL has been reported to be an exchange of cholesteryl esters by some investigators [208] but a transfer from LDL to HDL by others [188,206]. LDL can have a higher cholesteryl ester to triglyceride ratio than HDL which may account for net transfer to HDL as compared to exchange in some studies.

Other factors such as the amount of free cholesterol present on the surface of the particle are reported to effect the transfer activity . With LDL or VLDL as the lipid donor, an increase in the unesterified cholesterol content inhibited the transfer of cholesteryl ester from the particles in a concentration dependent manner [209]. Triglyceride transfer was not affected. The observations were the reverse for HDL in that increasing the unesterified cholesterol content had little effect on cholesteryl ester transfer but inhibited the transfer of triglyceride. The overall effect on the net mass transfer was that as the amount of unesterified cholesterol in HDL was increased, the transfer of cholesteryl ester to VLDL increased (a 4-fold increase in unesterified cholesterol led to a 3-fold stimulation of cholesteryl ester transfer to VLDL) and VLDL became a more effective donor of triglyceride to HDL. Morton [209] suggests that increasing the unesterified cholesterol on the surface may decrease the solubility of neutral lipid dissolved in the surface as observed in model membranes studies. Since triglycerides are more soluble than cholesteryl esters in the phospholipid monolayer of emulsions, they are less readily displaced by unesterified cholesterol. This would account for the effect on VLDL and LDL. HDL has a relatively small neutral lipid core so the effect may differ. There is a report that the transfer of cholesteryl esters can be inhibited by prolonged incubation with cholesteryl ester analogs without inhibiting triglyceride transfer indicating that there may be separate lipid binding sites [210].

The beneficial or detrimental effect of CETP with respect to the development of atherosclerosis remains controversial. Some authors suggest that CETP plays a beneficial

role in reverse cholesterol transfer by transferring cholesteryl ester from HDL to VLDL [211]. Removal of cholesteryl ester from HDL has been postulated, but not proven, to increase LCAT activity. Increased LCAT activity has been hypothesized to decrease the content of free cholesterol on the HDL surface allowing for increased uptake from the periphery [211]. This would only be the case if HDL gained phospholipids from another lipoprotein or plasma source since LCAT would use one mole of phospholipid per mole of unesterified cholesterol. The VLDL particle would be metabolized to LDL and taken up by LDL receptors in the liver for excretion as bile acids and cholesterol. This pathway provides a route for the transfer of cholesterol from the periphery to the liver for excretion. However, the beneficial effect of transfer to VLDL is questionable as the atherogenic lipoproteins are considered to be those derived from the metabolic cascade of VLDL to LDL. The gain of HDL cholesteryl esters by VLDL would theoretically increase the mass of the atherogenic lipoproteins, thereby potentially increasing the mass available for deposition in the periphery (arterial wall). On the other hand, the lack of transfer of cholesteryl ester to VLDL or the transfer from LDL to HDL would result in most of the plasma cholesteryl ester mass being carried by HDL. It is the HDL cholesterol concentrations rather than that of VLDL, IDL and LDL that have been inversely related to the development of coronary heart disease. Some authors hypothesize that CETP is not atherogenic in the normolipidemic state since CETP transfers cholesteryl esters from LDL to HDL [188,206] for transport by HDL to the liver for excretion. These investigators suggest that the action of CETP would be atherogenic in the hyperlipidemic state where the direction of transfer may change from HDL to VLDL or to IDL.

The effect of CETP on the formation of the plasma lipoproteins has been investigated to some extent. It has been suggested that the cholesteryl ester-rich LDL formed during in *vitro* lipolysis by lipoprotein lipase can be reduced in size by transferring cholesteryl ester to VLDL in exchange for triglyceride. With the ensuing

hydrolysis of the triglyceride gained by LDL, the reduction in core size can result in the formation of a smaller LDL particle [212]. It has been suggested that this mechanism is involved in the formation of plasma LDL as well as the cause of the small LDL particles observed with hypertriglyceridemia.

The effect of CETP on HDL was studied. HDL was incubated with CETP and high concentrations of VLDL or Intralipid [118]. The HDL became larger and less dense (if HDL_3 was the starting HDL), lost cholesteryl ester and unesterified cholesterol and was triglyceride-enriched. A discrete population of very small HDL particles were also observed which were denser than the starting HDL. The effect of CETP on HDL formation during VLDL hydrolysis by lipoprotein lipase has been reported in a few studies. However, the specific effects on lipid gain and apoprotein gain have not been thoroughly investigated. Incubation of purified CETP, bovine skim milk lipoprotein lipase, HDL_3 and a high concentration of VLDL triglyceride for 24 hours (in the presence of low amounts of albumin) resulted in HDL_3 enriched in triglycerides and were lighter. This was only observed if low amounts of albumin were used [161]. In another study, high amounts of VLDL relative to HDL were incubated with HDL_2 or HDL_3 for 18 hours and a d> 1.21 g/ml preparation of plasma or a semi-purified preparation of CETP (which removed LCAT). HDL lost cholesteryl ester and gained triglyceride from VLDL but the phospholipid/protein ratio did not change. If the HDL was re-isolated and incubated (in the absence of VLDL) with lipoprotein lipase or hepatic lipase, the HDL particles became smaller. When this process of triglyceride-enrichment of HDL_2 and hydrolysis of the core by bovine lipoprotein lipase was repeated, the resultant HDL became poorer in cholesterol ester and increased in the percentage composition of triglyceride. The particles became more dense and were isolated in the HDL_3 density range. Therefore, it was hypothesized that this was a mechanism for the formation of HDL_3 from HDL_2. The authors note that VLDL was absent from the incubations and *in vivo* the process may be more a function of hepatic lipase activity than lipoprotein lipase

since lipoprotein lipase is very effective in hydrolysing VLDL triglycerides while hepatic lipase preferentially hydrolyses HDL triglycerides [123].

The interaction of the process of lipolysis by lipoprotein lipase and CETP activity has been investigated [87,88]. The effect of lipolysis on the activity of CETP was reported to be a stimulation of the transfer of cholesteryl ester from HDL to VLDL [87]. The cause of the stimulation was due to the increased binding of the CETP to VLDL [88] and to HDL as well. The increased binding was due to the presence of free fatty acids on the VLDL particle that were released during lipolysis of the triglyceride (or phospholipid) [88]. The fatty acids were thought to act by increasing the negative charge on the surface of the VLDL which increased the binding to CETP . This was determined by the observation that lowering the pH or the inclusion of adequate amounts of albumin abolished the increase in binding and activity [88]. As the pH was reduced from 7.5-6 the effect became more and more reduced and was abolished at pH 6. From these observations it was suggested that the process of lipolysis stimulates the transfer of cholesteryl ester from HDL to VLDL due to the increased binding of CETP by fatty acids present on the lipoproteins. However, the authors pointed out the stimulatory effects were abolished above an albumin/VLDL triglyceride molar ratio of 0.5 which represents the ratio present in plasma. Therefore, the effect may not occur in normolipidemics with normal concentrations of albumin of 4-5 g/dl and normal VLDL triglycerides but may occur in hypertriglyceridemics or with post-prandial lipemia.

Post prandial lipemia (alimentary lipemia) has been reported to increase the transfer of cholesteryl esters from HDL and LDL to triglyceride rich lipoproteins in normolipidemics and from HDL in hypertriglyceridemics [202]. Incubation of alimentary lipemic plasma from normolipidemics demonstrated a 2-3 fold stimulation of transfer of cholesteryl ester from HDL to apo B containing lipoproteins (lipoproteins of $d < 1.063$ g/ml). This transfer activity was very low in the fasting plasma of the subjects who had

low levels of plasma triglycerides [201]. The activity was shown to increase as the amount of triglyceride-rich lipoproteins increased relative to the mass of HDL.

(b) PHOSPHOLIPID TRANSFER PROTEIN

Phospholipid transfer protein (PLTP) is a less well studied protein as compared to CETP. PLTP was initially identified by its ability to transfer phospholipid from unilamellar liposomes (vesicles) to HDL [213]. PLTP is postulated to be responsible for the transfer of phospholipid from VLDL to HDL in plasma. PLTP has an estimated molecular weight of 41,000 [214]. It differs from CETP in that it can be found in the plasma of rat and pig [215] and tends to isolate with HDL_2 particles rather than HDL_3 [215]. There is some controversy concerning the characteristics of the phospholipid transfer activity of PLTP. Tollefson and Albers [199,216] have reported that PLTP is responsible for 50% of the plasma phospholipid transfer activity and CETP is responsible for the other 50%. On the other hand, Tall [214] claims that facilitated phospholipid transfer activity is due to PLTP and can be separated from the facilitated phospholipid exchange activity associated with CETP. These activities which co-isolated with phenyl Sepharose chromatography, were partially separated by carboxymethylcellulose and completely separated on DEAE Sepharose. (The term facilitated transfer was used because phospholipids have been observed to spontaneously transfer from phospholipid vesicles to the HDL density in the absence of HDL. High spontaneous phospholipid transfer was observed by Tall when a $d>1.19$ g/ml preparation was used as a source of phospholipid transfer activity. The reason for the high "spontaneous" transfer is likely due to the presence of very dense HDL in the preparation which would contribute to the phospholipid content of the HDL region).

The different reports of transfer versus exchange activity may be due to the type of assay used to measure the activities. Tollefson and Albers [199,216] measured the mass transfer of phospholipids from VLDL to HDL which is a more physiological assay

than that of Tall which measured the transfer of phospholipid mass from phospholipid vesicles to HDL (facilitated transfer) and the transfer of labeled phospholipid from HDL to LDL (facilitated exchange).

PLTP activity has been reported to be stimulated in the presence of lipolysis of VLDL by bovine skim milk lipoprotein lipase [89]. The transfer of phospholipid from VLDL to HDL was increased as was phospholipid exchange.

In summary, CETP and PLTP activity have been reported to be affected by the process of lipolysis of VLDL by lipoprotein lipase but few in depth studies have been carried out and the conditions used in the previous studies varied greatly from those present *in vivo*.

D. RATIONALE OF THE PRESENT STUDY:

The effect of lipolysis by lipoprotein lipase on the formation and interconversions of lipoproteins is of current interest since active lipolysis by lipoprotein lipase has been recently suggested to be an anti-atherogenic process which, by a poorly defined process, tends to increase HDL mass. However, lipolysis also results in the production of atherogenic IDL and LDL from VLDL. The interplay between the two processes may be critical in the overall atherosclerotic process. Previous studies have shown that VLDL lipolysis by lipoprotein lipase can augment HDL lipid and apolipoprotein mass either through incorporation of components of VLDL into pre-existing HDL or by the formation of distinct HDL-like particles. Studies of the conversion of VLDL to LDL by lipoprotein lipase, *in vitro*, usually indicate that the process is incomplete and yields a product which is larger and less dense than LDL containing more cholesteryl ester and apo E than plasma LDL. Since LPL alone appears to be unable to accomplish the entire process it is possible that other serum factors assist in the process. The objective of the present study was to assess the role of hepatic lipase and the lipid transfer proteins on the

lipoprotein lipase-induced conversion of VLDL to LDL along with the augmentation of HDL mass.

Hepatic lipase was studied because it has been suggested that it is involved in the conversion of IDL to LDL and in the loss of apo E during the interconversion process. Hepatic lipase has also been implicated in the interconversion of HDL_2 to HDL_3. Cholesteryl ester transfer protein and phospholipid transfer protein were studied because they are thought to be important in the transfer of neutral lipids between lipoproteins and phospholipid transfer to HDL. Although these enzymes and activities have been studied extensively in some instances the objective has been to demonstrate a process rather than assess the physiological importance of that process. Thus, in many instances the studies have not used physiological proportions of lipoproteins, enzyme and transfer activities. Adequate albumin concentrations to bind all free fatty acids released during lipolysis have not been used and in most instances the pH used has been at the enzyme optima rather than physiological pH. Thus, in the present study, a major emphasis was placed on using physiological proportions of lipoproteins and conditions in order to obtain a realistic assessment of *in vitro* studies in relation to *in vivo* processes.

The present study will focus on: 1) the extent of transfer of VLDL lipid and apoprotein mass to HDL; 2) the ability of lipoprotein lipase to cause a transfer of cholesteryl esters, in particular, from VLDL to the HDL density range as this transfer is very controversial at present; 3) whether there is evidence to suggest that HDL mass is augmented by a gain in mass by the original HDL or the formation of "new" HDL; 4) whether the HDL mass distribution can be shifted from a higher density to a lower density indicative of conversion of HDL_3 to HDL_2; 5) whether an extensive transfer of apo E to HDL from the lipolysing VLDL can occur as removal of apo E is required for LDL formation; 6) whether lipoprotein lipase in the presence of hepatic lipase or the lipid transfer proteins can cause a complete conversion of VLDL to LDL.

CHAPTER III

MATERIALS AND METHODS

A. METHODS

1. Isolation of Lipoproteins:

Pooled human plasma was obtained from fresh citrated blood for isolation of VLDL, LDL and HDL. All density adjustments were made by the addition of solid potassium bromide (KBr). Centrifugations were carried out at 4°C. Disodium ethylenediaminetetraacetate (Na_2EDTA) and sodium azide (NaN_3) were added to the plasma at a final concentration of 0.01% and 0.02%, respectively. Chylomicrons were removed by overlayering the plasma with distilled water and centrifuging in a SW 27 rotor at 27,000 rpm for one hour. The chylomicrons, which floated in the distilled water layer, were removed by aspiration. VLDL and HDL were isolated from the remaining plasma by sequential ultracentrifugation in a 50.2 Ti rotor. VLDL was isolated by ultracentrifugation for 18 hours at 33,000 rpm at d 1.006 g/ml with one wash at d 1.006 g/ml. HDL was isolated at a d 1.063-1.21 g/ml by ultracentrifugation at 40,000 rpm for 18 hours at d1.063 g/ml and ultracentrifugation of the infranatant at d 1.21 g/ml for 19 hours at 50,000 rpm. A wash of the HDL at d1.063 and 1.21 g/ml was also carried out. The lipoproteins were concentrated by repeated dialysis and treatment with Aquacide II. After a final exhaustive dialysis, 0.01% Na_2EDTA and 0.02% NaN_3 were added and the lipoprotein solution was flushed with nitrogen and stored at 4° C. The dialysis buffer varied. HDL was dialysed against the buffer used for heparin Sepharose chromatography and VLDL was dialysed against the buffer used for the experimental incubations or against the buffer used for labeling.

2. Heparin Sepharose Chromatography:

Heparin Sepharose chromatography was carried out for isolation of HDL, free of apo E, in order to more easily follow the redistribution of apo E from VLDL to HDL as a result of the experimental incubations. Initially a commercial preparation of heparin Sepharose (Pharmacia) was tested for apo E binding but the binding was low relative to the amount of Sepharose used. Therefore, heparin Sepharose was synthesized by a modification of the methods of March [217] and Iverius [218].

(a) Preparation of Heparin Sepharose:

Sepharose CL-6B (Pharmacia) was used for heparin binding by cyanogen bromide activation. The ethanol was removed and 200 ml of the gel were rinsed with distilled water and kept at 4°C. In the fume hood, acetonitrile (30 mls) was chilled to 4°C and added to cyanogen bromide (40 gms) and the solution was stirred until dissolved. The water was removed from the gel and the gel was taken up in 2.0 M Na_2CO_3 (200 mls) and added to a flask containing 2.0 M Na_2C0_3 (200 mls). The gel mixture was stirred rapidly while the cyanogen bromide/acetonitrile solution was quickly added and the mixture was stirred rapidly for 2 minutes. The gel mixture was filtered through a coarse sintered glass funnel and immediately washed with 0.1M $NaHCO_3$ (4 L) pH 9.5, followed by distilled water (4 L) pH 9.5, 0.2 M $NaHCO_3$ (4 L) pH 9.5. All solutions had been previously cooled to 4° C. The gel was dried to a moist cake. Heparin (370 mg) was dissolved in 0.2M $NaHCO_3$ (200 mls) pH 9.5. The gel was added to the heparin solution, mixed and the heparin and Sepharose gel solution was left at 4°C for 20 hours. Glycine (1.0M) (400 ml) was added to the gel solution to bind unreacted sites and the mixture was left at 24°C for 4 hours. The gel was washed with distilled water (8L) pH 7.4, 0.5M NaCl (4 L) pH 7.4 and distilled water (8 L) pH 7.4. The gel was taken up in 0.005M Tris HCl, pH 7.4 containing 0.01% Na_2EDTA and 0.02% NaN_3 and stored.

(b) Isolation of HDL lacking apo E, by Heparin Sepharose Chromatography:

Heparin Sepharose affinity chromatography was carried out using a modified method of Weisgraber and Mahley [219]. A column (30 cm x 3 cm, LKB) of the heparin Sepharose was equilibrated with the starting buffer, 0.005 M Tris HCl, 0.025 M $MnCl_2$, pH 7.4. All steps of the affinity chromatography were carried out at 4° C with degassed buffers. HDL (densities 1.063-1.21 g/ml) which had been dialysed against the starting buffer was applied to the column (24 mg protein in approximately 2-4 mls of the starting buffer) . Immediately prior to the application of HDL solution to the column, solid $MnCl_2$ was added to the HDL solution to give a final concentration of 0.025 M $MnCl_2$. Elution of the HDL lacking apo E was carried out by pumping the starting buffer through the column at a rate of 25 ml/ hour with the aid of a peristaltic pump. After the peak containing HDL, lacking apo E, had eluted, a buffer containing 0.005 M Tris HCl, 1.2 M NaCl, pH 7.4 was pumped through the column at a rate of 100 ml/hour in order to elute the apo E-containing lipoproteins. From the size of the peaks it appeared that the HDL, lacking apo E, made up approximately two thirds of the starting HDL and the HDL, containing apo E, made up one-third of the mass. Each fraction that eluted in the HDL, lacking apo E peak , was tested for the presence of apo E by electroimmunoassay and apo E was found to be absent (the fractions which eluted with 1.2 M NaCl were also tested and apo E reactivity was present). The fractions lacking apo E were combined, dialysed against a 0.05 M Tris HCl buffer, pH 7.4 containing 0.195 M NaCl, 0.01% Na_2EDTA, 0.02% NaN_3, and were concentrated by repeated treatment with Aquacide and dialysis. After a final dialysis, the HDL was stored under N_2 at 4°C. An aliquot of the HDL was tested for LCAT activity. No activity was detected.

3. VLDL Labeling:

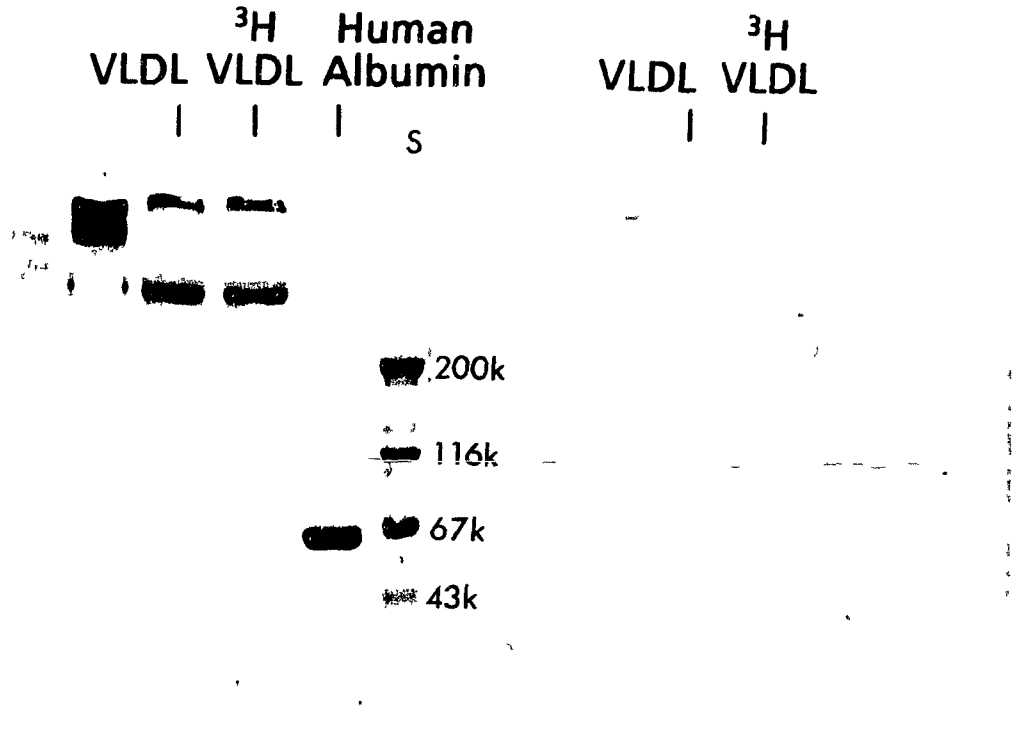
VLDL was labeled with [^{3}H[cholesteryl oleate in experiments B-E. Tritiated cholesteryl oleate was dried, taken up in chloroform and tested for purity by thin layer chromatography using a solvent system of petroleum ether/ether/acetic acid in a ratio of 90/10/1 v/v. Unlabeled cholesteryl oleate and cholesterol were used as carriers. The purity was assessed by measurement of the distribution of radioactivity using a Bioscan Imaging Scanner System 200-IBM (Bioscan Inc., Washington, D.C., U.S.A.) in most cases (initially the purity was assessed by locating the region containing cholesteryl ester and cholesterol by exposure to iodine crystals, scraping of the regions and determining the location of the label by liquid scintillation counting). If the purity was determined to be less than 97%, the [^{3}H] cholesteryl oleate was purified using the same solvent system but the procedure was carried out under nitrogen and in the absence of carriers. Unlabeled cholesterol and cholesteryl oleate were run in a separate lane and sprayed with fluorescein dye to locate the position of cholesteryl oleate. The corresponding region of the plate containing the [^{3}H[cholesteryl oleate was scraped and eluted with ether through a small column of silica gel (Silica Gel 60 GF, EM Science) in the presence of nitrogen, dried and taken up in chloroform. A sample was retested for purity as described above.

VLDL labeling was carried out by modifications of the method of Tollefson and Albers [199]. All components had been previously dialysed against the buffer used in the labeling incubation (0.01M Tris HCl, 0.195 M NaCl, pH 7.4). [^{3}H] Cholesteryl ester (10-20 μCi) (82 Ci/mmol) was dried down in a siliconized glass vial. HDL (2 mg protein) and the incubation buffer were added for a total volume of 2 mls. The preparation was sonicated in an ice water bath at 10% for 4 x 1 minute intervals with a 30 second break using a Microson, Ultrasonic Cell Disruptor, (Heat Systems-Ultrasonics Inc). VLDL (12 mg protein) and a d> 1.21 g/ml plasma preparation (15 mls) were added to the sonicated HDL. The mixture was flushed with nitrogen and incubated for 3-31/2 hours at 37°C in a shaking water bath. The preparation was placed on ice, Na_2EDTA and

NaN_3 were added to achieve a final concentration of 0.01% and 0.02%, respectively . The preparation was overlayered with a d 1.006 g/ml solution containing 0.01% Na_2EDTA and 0.02% NaN_3 and centrifuged at d 1.006 g/ml in a 50.2 Ti rotor for 16 hours at 40,000 rpm.

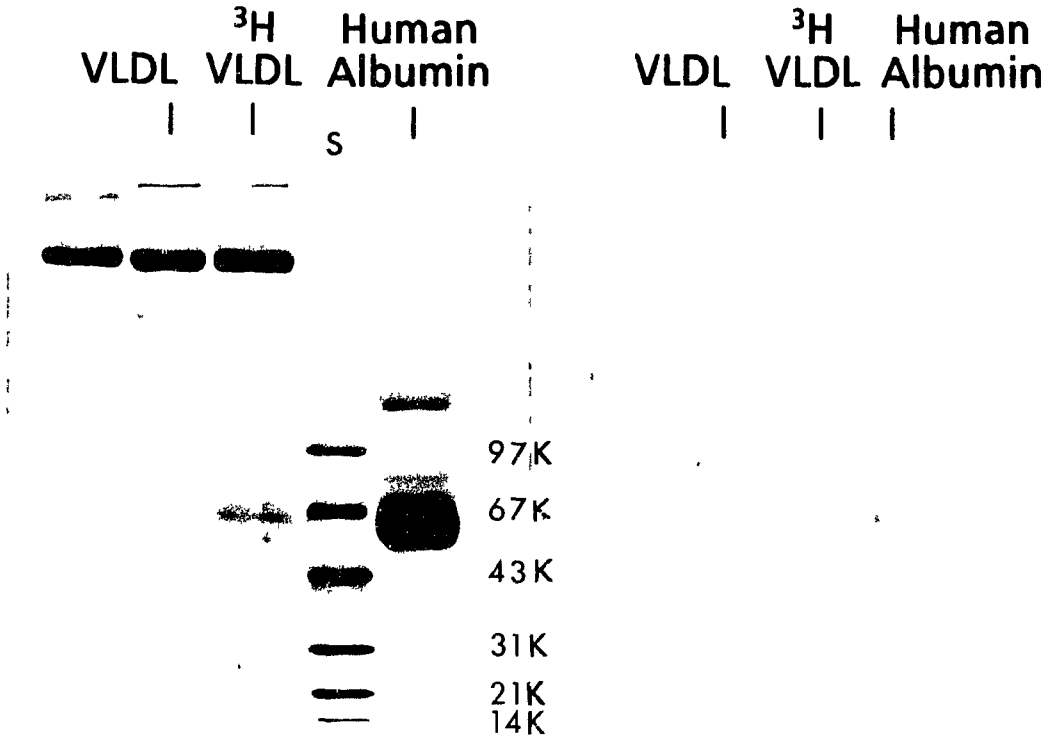
The VLDL was reisolated and the labeling efficiency was determined. In most experiments 71% (range 56-77%) of the HDL [^{3}H] cholesteryl ester had been transferred to the VLDL. The percent lipid composition was determined by gas liquid chromatography to compare the starting VLDL to that of the labeled VLDL to determine if the incubation had increased the cholesteryl ester mass. There was very little difference in the percentage of the total lipid mass made up of cholesteryl esters (example: the maximum difference was + 0.8%, with 15.2% pre-incubation and 16% post-labeling). The gain of cholesteryl esters with labeling was estimated from this change in percentage composition and the gain of cholesteryl esters ranged from of 0.6-5% of the starting VLDL cholesteryl ester mass. For every labeled VLDL preparation, an aliquot was applied to a 4-16% SDS polyacrylamide gradient gel [220] for determination of the protein composition and for immunoblotting [221] (Figures 1 and 2). The only apparent difference in the protein composition was the presence of a band corresponding in molecular weight to albumin in the labeled VLDL sample. The use of an immunoblotting technique with an antibody to human albumin confirmed the identity of the band (Figure 2). The presence of more albumin post-labeling was presumably due to the fact that the labeled VLDL did not undergo a washing step in the centrifugation procedure as did the starting VLDL. The SDS gel was used for immunoblotting against a polyclonal antibody to apo B to determine if the incubation procedure had caused any degradation of the apo B. No degradation products were present (Figure 1).

FIGURE 1: SDS GEL AND ANTI-APO B IMMUNOBLOT OF ^{3}H VLDL



The original VLDL and VLDL labeled with ^{3}H cholesteryl esters were applied to an SDS gel (on left). An anti-apo B immunoblot was prepared from the gel (on right), and demonstrated that apo B in the labeled VLDL remained intact after labeling, since breakdown products were not apparent on the gel.

FIGURE 2: SDS GEL AND ANTI-ALBUMIN IMMUNOBLOT OF ^{3}H VLDL



The original VLDL and VLDL labeled with ^{3}H cholesteryl esters were applied to an SDS gel (on left). An anti-albumin immunoblot was prepared from the gel (on right), and indicated that the lower band which appeared in the labeled VLDL corresponded to human albumin.

4. Hepatic Lipase Isolation and Assay:

(a) Hepatic Lipase Isolation:

Hepatic lipase was semi-purified from human post-heparin plasma according to the method of Boberg [222] with some modifications. All steps in the isolation procedure were carried out on ice or at 4° C. A subject was infused with 20 units of heparin per kilogram body weight and a post heparin blood sample was obtained. The plasma was removed and 50 mls were mixed with 50 mls of 0.45M NaCL, 5% glycerol, 0.005 M Na-barbital buffer, pH 7.4 and filtered through a coarse sintered glass funnel. This mixture was applied to a column (21x1.5 cm, Pharmacia) of heparin Sepharose (Pharmacia) which had been equilibrated with a 0.3M NaCl, 0.005M Na-barbital buffer, pH 7.4. All solutions were applied with the aid of a peristaltic pump at a rate of 25 ml/hr. Two hundred milliliters of the 0.3M NaCl equilibration buffer were pumped through the column. A large peak was observed. When the absorbance returned to baseline, 0.8 M NaCl, 0.005 M Na-barbital buffer, pH 7.4 was applied and a small peak was observed. The fractions which corresponded to the small peak were combined and 120 mg of bovine serum albumin were added and mixed gently. The mixture was concentrated and dialysed against a 0.05 M Tris HCl buffer, pH 7.4 and concentrated and dialysed against a 0.195 M NaCl, 0.1M Tris HCl buffer, pH 7.4, flushed with nitrogen and frozen at -70°C. Several aliquots (200µl) were frozen separately for the hepatic lipase activity assay.

(b) Hepatic Lipase Activity Assay:

A frozen aliquot was thawed in ice water and treated the same way as that of the preparation used in the experimental incubation in terms of buffer used for dialysis and length of time of dialysis in order to mimic the experimental conditions. The assay was carried out according the method of Krauss [223]. A triolein emulsion was prepared by mixing 100 mg triolein (Sigma) with 2 ml of a solution of [^{14}C] triolein (2µCi) in

chloroform. The [^{14}C] triolein (104.9 mCi/mmol) had previously been tested for purity by thin layer chromatography and found to be 98.7% pure. The chloroform was evaporated under nitrogen and bovine serum albumin (200 mgs), 1% Triton X-100 (0.6 ml) and 0.15M NaCl, 0.2M Tris HCl buffer (11 mls) pH 8.2 were added. The emulsion was formed by sonicating the mixture immersed in ice water using a Branson Sonifier-Cell Disruptor (Model W185, Heat Systems- Ultrasonics Inc) at an output of 4 for 10 X 1 minute periods with 30 second rest intervals. The emulsion remained on ice and was used within one-half hour. The assay mixture contained 10-100 µl of the hepatic lipase preparation or 10-100 µl of the dialysis buffer as a control, and was made up to a total volume of 1 ml with the emulsion. All assays were carried out in duplicate. The assay mixture was vortexed and incubated in a shaking water bath at 37°C for 45-60 minutes. The reaction was stopped with the addition of 8 ml of a chloroform/ methanol/ heptane solution (5 / 5.6 /4). One milliliter of 0.1M NaOH was added and the mixture was vortexed and centrifuged (I.E.C. PR-6,000) at 2,000 rpm for 45 minutes. The total volume of the top layer was recorded and 1 ml was placed in a scintillation vial, 10 ml of Aquasol were added and the radioactivity was determined in a Beckman LS 7000 liquid scintillation counter. Total free fatty acid released was calculated after correction for the control value. The final activities for experiments A, B and C were 6.0, 13.6 and 12.4 µmoles free fatty acid released/ml/hr, respectively.

Inhibition by protamine sulphate (0.75 gm/ml) was tested in one assay as a means of verifying that the lipolytic activity was due to hepatic lipase and not lipoprotein lipase since hepatic lipase is much more resistant than lipoprotein lipase to protamine inhibition. The inhibition was 10-15% which is in the range reported for a preparation of hepatic lipase derived from a perfusion of rat livers with heparin which presumably contained only hepatic lipase and not lipoprotein lipase [223]. The difference in the results between using an enzyme preparation that had been dialysed at pH 7.4 versus pH 8.2 was assessed and the activity at pH 7.4 was 94% of that at pH 8.2. Therefore the activity measured may

be a slight underestimate as the enzyme preparations used in the assays had all been dialysed at pH 7.4 rather than at pH 8.2.

5. Lipid Transfer Protein Isolation and Assay:

(a) Lipid Transfer Protein Isolation:

A semi-purified preparation of the lipid transfer proteins, CETP and PLTP, was prepared according to the method of Tollefson and Albers [199]. In brief, fresh plasma (450 ml/preparation or, if 2 preparations were required per experiment, 900 ml) containing 0.01% Na_2EDTA and 0.02% NaN_3 was adjusted to density 1.25 g/ml with solid KBr and centrifuged in a 50.2 T1 rotor at 43,000 rpm for 26 hours at 4°C. The contents of the top half of the tube were removed after tube slicing and mixed with a solution of d 1.19 g/ml (d 1.006 g/ml NaCl adjusted to d 1.19 g/ml with KBr) in a ratio of 3/2 (resulting in a density of d 1.21 g/ml) and centrifuged in a 50.2 Ti rotor at 43, 000 rpm for 48 hours at 4°C. The contents of the top and bottom 1/3 of the tube were discarded and the middle 1/3 (clear zone) was collected. The middle fraction was applied directly to a phenyl Sepharose column (35 X 1.5 cm, Pharmacia) which had previously been equilibrated with a 2.0 M NaCl, 0.01 M Tris HCl buffer, pH 7.4. All chromatography was carried out at 4°C at a flow rate of 60-70 ml/hr. Approximately 1,000 mls of equilibration buffer were pumped through the column. The column was then washed with 1000 ml of a 0.15M NaCl, 0.01M Tris HCl buffer, pH 7.4. The lipid transfer protein preparation was eluted from the column with distilled water, dialysed against 0.01M Tris HCl, pH 7.4 containing 0.01% Na_2EDTA, concentrated and dialysed and concentrated two more times with a final dialysis in 0.01% Na_2EDTA, pH 7.4. The lipid transfer protein preparation was flushed with nitrogen and frozen at -70°C. Several small aliquots were frozen separately for assays of transfer activities. For experiment B, the clear middle zone from 60 ml of plasma was used directly in the experimental incubation without isolation by phenyl Sepharose chromatography.

(b) Lipid Transfer Assays:

i) Cholesteryl Ester Transfer Assay:

The assay for cholesteryl ester transfer activity reflecting the activity of the cholesteryl ester transfer protein (CETP) was based on the method of Tall [214]. The facilitated exchange of cholesteryl oleate between HDL and LDL was assayed by determining the transfer rate of [^{3}H] cholesteryl oleate from HDL to LDL.

Labeling of HDL:

The HDL was labeled by the method of Groener et al [189]. HDL (d1.11-1.19 g/ml) was dialysed overnight in a 0.15 M NaCL, 0.01% Na_2EDTA, 0.01% NaN_3, 0.01M Tris HCL buffer, pH 7.4. In a siliconized glass vial, 5 µCi of [^{3}H] cholesteryl oleate dissolved in chloroform, 1 µmole egg phosphatidylcholine, 20 nmol butylated hydroxytoluene were mixed and evaporated under nitrogen. Two milliliters of 0.01% Na_2EDTA, 0.05M Tris HCl buffer, pH 7.4 were added and the tube was flushed with nitrogen. The mixture was sonicated under nitrogen for 5 X 2 min. with a 30 second break at 50% power using a Microson, Ultrasonic Cell Disruptor,(Heat Systems-Ultrasonics Inc). The sonicated lipid was added to the HDL (8 µmol total cholesterol, 13 mg protein) and a d 1.21 g/ml plasma preparation (5 mls)(dialysed against the incubation buffer) and then 0.01M 5,5-dithiobis 2-nitrobenzoic acid (DTNB) (6 ml) and 100 g/ml Na_2EDTA (80 µl) were added subsequently. The mixture was flushed with nitrogen and incubated for 24 hours at 37°C in a shaking water bath. The HDL (d 1.11-1.19 g/ml) was re-isolated with one wash at d 1.11 g/ml, dialysed against the CETP assay buffer, flushed with nitrogen and kept at 4°C.

Assay:

LDL (d1.02-1.05 g/ml) was dialysed against the assay buffer (0.01% NaN_3, 0.1% Na_2EDTA, 0.15 Tris-phosphate buffer, pH 8). The lipid transfer protein preparation was treated in a similar way as the preparation used in the experimental incubation by thawing a frozen aliquot of the lipid transfer protein in ice water and dialysing the aliquot against

the buffer used in the experimental incubations (0.195 M NaCl, 0.1M Tris HCl, pH 7.4) for the same length of time the lipid transfer protein preparation to be used in the experimental incubation was to be dialysed. The preparation was diluted to the same concentration as that used in the experimental incubation.

The incubations contained LDL (0.1 mg protein), [^{3}H] HDL (0.1 mg protein) with 40-80 µl of the lipid transfer protein preparation and the volume was made up to a total of 4 ml with the assay buffer. As a control, the assay buffer was used in place of the lipid transfer protein preparation. All analyses were carried out in duplicate. The mixture was mixed, flushed with nitrogen and incubated in a shaking water bath at 37°C for 2 hours. At the end of 2 hours the incubation was placed on ice and 3.8 mls were adjusted to d1.063 g/ml, overlayered with d 1.063 g/ml solution and centrifuged in a 50.3 rotor for 14 hours at 50,000 rpm at 4°C. The top 1.5 ml, middle 1.4 ml and bottom 2.9 ml were separated and 1 ml of each were mixed with Aquasol and the radioactivity was determined by liquid scintillation counting (Beckman, LS 7000). The facilitated cholesteryl ester exchange was determined by calculating the transfer of label from HDL to LDL in the presence of the lipid transfer protein preparation minus the transfer observed in the absence of the preparation. The cholesteryl ester (CE) transfer activity of the lipid transfer proteins used in experiments C, D and E were 81 nmoles CE/ml/hr, 99 nmoles CE/ml/hr and 91 nmoles CE/ml/hr. In one assay the effect of the addition of 1.4 mM DTNB and 4.0 mM DTNB (either preincubated for 30 minutes with the lipid transfer protein preparation or added directly to the assay) were assayed in order to determine if the DTNB which was included in the experimental incubations affected the cholesteryl ester transfer activity. The activity was reduced an average of 9.8 % with the addition of DTNB after a 2 hour incubation. There was no difference if the sample was pre-incubated with DTNB or the DTNB was added at the beginning of the incubation. There was no difference in the inhibition using a concentration of 1.4 mM as compared to 4.0 mM DTNB.

ii) Phospholipid Transfer Activity :

The measurement of phospholipid transfer reflects the activity of the phospholipid transfer protein (PLTP) and CETP since the transfer activity in plasma is thought to be equally divided between these two transfer proteins according to Tollefson and Albers [199,216]. The assays were based on the methods of Tall [214].

Labeling of Vesicles with Phosphatidylcholine:

Vesicles were labeled with phosphatidylcholine by a modified method of Tall [214]. ^{14}C Phosphatidylcholine (153 mCi/mmol) which had been tested for purity and found to be 98.8% pure was mixed with egg phosphatidylcholine dissolved in chloroform in the ratio of 43,000 dpm/mg for a total of 172,000 cpm [^{14}C] phosphatidylcholine in 4 mg egg phosphatidylcholine. The chloroform was evaporated under nitrogen and 4 mls of the assay buffer (0.1% Na_2EDTA, 0.01% NaN_3, 0.15 M Tris-phosphate, pH 8) were added and the mixture was sonicated under nitrogen in ice water for 5 X 1 minute at 10% power with a 30 second break using a Microson, Ultrasonic Cell Disruptor (Heat Systems-Ultrasonics Inc). The vesicles were used immediately. This assay was used for experiment B and C. To facilitate the measurement of phospholipid transfer activity, for experiment C, D and E, the HDL was labeled by the addition of 2 μCi of [^{14}C] phosphatidylcholine to the vesicle preparation used for the cholesteryl ester transfer assay and re-isolated (d 1.11-1.19 g/ml) and the transfer of label from HDL to LDL was assayed. Both methods of assaying phospholipid transfer/exchange were carried out for the lipid transfer preparation of experiment C so that a comparison of the activities could be made.

Assay:

The lipid transfer protein preparation was diluted and dialysed against the buffer used in the experimental incubations (0.195 M NaCl, 0.1M Tris HCl, pH 7.4) so that treatment of the lipid transfer protein preparation mimicked that of the preparation used

in the experimental incubations. For measurement of facilitated phospholipid transfer, 250 µl or less of the lipid transfer protein preparation, 0.15 mg HDL protein and 0.5 mg of phosphatidylcholine vesicles were mixed in that order and the assay buffer (0.1% Na_2EDTA, 0.01% NaN_3, 0.15 M Tris-phosphate, pH 8) was added to result in a total volume of 4 mls. For the controls, phosphatidylcholine vesicles were mixed with HDL and assay buffer (no lipid transfer preparation) or phosphatidylcholine vesicles were mixed with the assay buffer and the lipid transfer protein preparation (no HDL). For the assay measuring the exchange of label from HDL to LDL, the control incubation contained labeled HDL and LDL with the substitution of buffer for the lipid transfer protein preparation. All assays were carried out in duplicate. When transfer of [^{14}C] phosphatidylcholine from HDL to LDL was determined in experiments C, D and E, the incubation contained [^{14}C] HDL and LDL in the absence or presence of the lipid transfer protein preparation using the same concentrations and conditions described for the cholesteryl ester transfer assay in the previous section. The incubation mixture was vortexed for 15 seconds, flushed with nitrogen and incubated in a shaking water bath at 37°C for 2 hours. The incubation mixture was placed on ice and 3.8 ml were adjusted by the addition of solid KBr to a density of d 1.063 g/ml, overlayered with a solution of d 1.063 g/ml and centrifuged for 14 hours at 50,000 rpm in a 50.3 rotor. The top (1.5 ml), middle (1.4) mls and bottom (2.9 mls) were counted in Aquasol in a Beckman LS 7000 liquid scintillation counter. The radioactivity in the bottom 2.8 ml was used as a measure of the transfer of label from the vesicles to HDL and the radioactivity in the top 1.5 ml was used as a measure of transfer of label from HDL to LDL. The amount of facilitated phospholipid transfer added to the experimental incubations using the vesicle -to-HDL assay for experiments B and C were 26.4 and 514 nmoles/ml/hr. The spontaneous transfer (transfer in the absence of HDL) was high in experiment B which may be due to the amount of the lipid transfer protein preparation used in the assay (250 µl of a d 1.21-1.25 g/ml preparation in experiment B versus 6 µl of a preparation eluted from phenyl

Sepharose in experiment C) which was also noted by Tall using a d> 1.21 preparation [214]. Using the [^{14}C] HDL-to-LDL assay for experiments C, D and E, the phospholipid exchange activity added to the experimental incubations were 45.4, 69.3 and 66 nmoles/ml/hr, respectively. The value of 45.4 nmoles/ml/hr of exchange activity as compared to 514 nmoles/ml/hr of facilitated transfer activity in experiment C, approximately a 10 fold difference in the two types of activities, is similar to that reported by Tall [214].

6. Lecithin-Cholesterol Acyltransferase Assay:

The activity of the enzyme lecithin-cholesterol acyltransferase (LCAT) was assayed since the lipid transfer protein preparation was not purified to the stage where these activities were separated. The experimental incubations that included the lipid transfer protein preparation contained 1.4 mM DTNB in order to inhibit LCAT. The effectiveness of DTNB inhibition was assayed by determining LCAT activity in the experimental incubation. An aliquot was removed at the start of the experimental incubation (0 time) and 15 minutes into the incubation and the LCAT activity of the sample was assayed according to the method of Jauhiainen and Dolphin [224]. One hundred microliters of a lecithin, cholesterol, apo AI proteoliposome substrate (250:12.5:0.8 mole/mole) which was labeled with [^{3}H] cholesterol was pre-incubated with 200 µl assay buffer (0.14M NaCl, 0.001M Na_2EDTA, 0.01M Tris HCl, pH 7.4) and 125 µl of 2% bovine serum albumin at 37°C for 20 minutes. Mercaptoethanol was not included in the assay as the effect of DTNB would be reversed. An aliquot of the experimental incubation (20-50 µl) was added, the volume was made up to 500 µl with the assay buffer, the mixture was mildly vortexed, flushed with nitrogen and incubated in a shaking water bath at 37°C for 30 minutes. The control contained 50 µl of assay buffer in place of the experimental incubation sample. The tubes were placed on ice, 8 ml of a chloroform/methanol (2:1) solution, cold cholesteryl ester as a marker and 2 ml of 0.58% NaCl were added. The mixture was centrifuged, the protein interface layer was removed,

the upper phase was aspirated and discarded. Two mls of a methanol/0.58% NaCl/chloroform solution (48/47/3 v/v) was added, the mixture was shaken and centrifuged and the upper phase was discarded. The lower phase was dried down under nitrogen, dissolved in chloroform and the lipids were separated by thin layer chromatography in a solvent system of n-heptane/isopropyl ether/glacial acetic acid/methanol (60:40:4:2 v/v). The location of cholesterol and cholesteryl ester was established using iodine crystals, the region was scraped and the radioactivity measured in Readi-gel using a Beckman LS 7000 scintillation counter. From the results of the LCAT assay, the maximum amount of cholesteryl ester formed by LCAT in the incubations for experiment D and E were 31.3 and 52.9 μg cholesteryl ester per incubation (12.7 mls) over 2 hours when measured at 0 time, respectively. When measured after incubating for 15 minutes, the maximum amount of cholesteryl ester formed in experiments D and E was 18.3 and 31.4 μg per incubation (12.7 ml) over 2 hours, respectively.

7. Lipoprotein Lipase Isolation:

Lipoprotein lipase was freshly isolated on the day of the experiment from rat hearts. Male, Long Evans rats (350-450 gms) were fasted overnight. The rats were anaesthetized with sodium pentobarbital (65-80 mg/animal). The hearts were removed while still beating, cannulated through the aorta and rinsed with 20 ml of 0.195 M NaCl, 0.005 M phosphate buffer, pH 7.4 (kept on ice). A solution of heparin containing 11.3 units of heparin/ml in 0.195 M NaCl, 0.005 M phosphate buffer, pH 7.4 was infused with one pass through the heart using an infusion pump (model 355, Sage Instruments) set at 80% X 1 which was approximately 30 ml/minute. Usually 15 mls of the same heparin solution were infused through 32 hearts if four experimental incubations were required in order to release enough lipoprotein lipase to achieve the extensive triglyceride hydrolysis required. It had previously been established in experiments not reported in this thesis that 8 hearts were required per incubation in order to consistently achieve 90-95% VLDL

triglyceride hydrolysis in 2 hours. The heparin solution containing the released lipoprotein lipase was kept on ice when not being infused. The solution was centrifuged at 2,000 rpm, for 25 minutes at 4°C to remove any red blood cells that might have been present and the lipoprotein lipase solution was used immediately.

8. *In Vitro* Experimental Incubations:

All work was carried out at 4 °C or kept on ice. Since the lipoprotein products of the incubations were to be isolated by density gradient ultracentrifugation, it was imperative that the salt content was carefully controlled and was identical for each incubation in order to achieve the same final density in all of the incubations. Consequently exhaustive dialysis against the incubation buffer, 0.195 M NaCl, 0.1M Tris HCl, pH 7.4, was required for any component which was to be added to the incubations.

The incubations were prepared in the following manner. The lipoprotein lipase solution was added to each incubation tube. For the control incubation and the incubation containing the lipid transfer protein in the absence of lipoprotein lipase activity, the lipoprotein lipase solution was heated to 70°C for 20 minutes and placed on ice. Since the lipoprotein lipase solution contained 0.195 M NaCl, 0.005 M phosphate buffer, an appropriate volume of 0.195 M NaCl, 1.0 M Tris-HCl was added to the lipoprotein lipase solution in order to achieve a final concentration of 0.1 M Tris-HCl . Bovine serum albumin was slowly added and mixed (660 mg of albumin per incubation giving a final concentration of 4.7%). Since the hepatic lipase preparation contained 120 mg of albumin, 540 m were added to the hepatic lipase incubation. VLDL (2 mg protein) and HDL (10 mg protein) were then added and gently mixed. The lipid transfer protein preparation or the hepatic lipase preparation was added and an equal amount of the incubation buffer was added to the incubations which did not contain these preparations. A small volume (200 µl) of a DTNB solution was added to the incubations that contained the lipid transfer protein preparation to achieve a final concentration of 1.4 mM and 200 ul of the experimental buffer were added to those that did not contain the preparation.

The DTNB solution, which was used within several hours of its preparation, was prepared by dissolving 0.194 gm DTNB in 5 mls of a 2% $KHCO_3$ solution. The total volume of the incubations was made up to 14 ml with the incubation buffer. The incubations were gently but thoroughly mixed, flushed with nitrogen and placed in a shaking water bath at 37°C for 2 hours. The incubations were immediately placed in ice water and 13 mls were adjusted to d 1.04 g/ml with the addition of 0.578 gms of KBr. The density gradient was formed in a 40 ml Quick Seal tube (polyallomer, 25 X 89 mm, Beckman) whose volume had been verified before use. The density gradient was set up by pipetting 12.7 mls of the incubation solution, which had been set to d 1.04 g/ml, into a 60 ml syringe case. The syringe case had a 18 gauge, 1.5 ' needle attached and the needle had polyethylene tubing on the end of it. The tip of the polyethylene tubing rested on the bottom of the Quick Seal tube. The incubation mixture was underlayed by pipetting with a burette 12.7 ml of a d 1.25 g/ml solution into the 60 ml syringe case. The d 1.25 g/ml solution was made by adjusting a d 1.006 g/ml (0.195 M) NaCl solution containing 0.01% Na_2EDTA and 0.02% NaN_3 to d 1.25 g/ml with solid KBr. The incubation solution was then overlayered with 14.6 mls of the d 1.006 g/ml solution. The tubes were centrifuged for 24 hours at 45,000 rpm in a 50. 2 Ti rotor at 4°C. The gradients were collected as described in section 10 below.

9. *In Vivo* Heparin Infusions:

Pre- and post-heparin samples were obtained from 2 normolipidemic males, subjects A and B. Informed consent was obtained. The subjects were being studied at the University of Western Ontario Health Science Center. The subjects were of normal body weight with low-normal plasma lipids (see figures 3 and 4 for plasma lipid values). The mean values for total plasma cholesterol and triglycerides were 150 mg/dl and 40 mg/dl. The details of the plasma lipids are listed in the Results section in the legends of figures 3 and 4. The subjects were required to follow a balanced weight-maintaining diet for the 2 week period prior to the study. After taking a pre-heparin blood sample, the subjects

received a priming dose of 4,000 IU/hr over the first one-half hour. Heparin was then infused at a rate of 2,000 IU/hr for the remaining study period. The post-heparin sample was obtained after 1.5 hours for subject A and 1 hour for subject B. Amino phenylboronic acid (4 mM) was added to the blood to inhibit lipoprotein lipase, hepatic lipase and LCAT. The samples were shipped to Halifax on wet ice. The plasma from each sample was removed and immediately subjected to density gradient ultracentrifugation. The gradients were collected as described in section 10 below.

10. Collection of the Density Gradient:

The centrifuge was stopped without the use of a break. The tubes were carefully removed and were placed in ice water. All steps in the collection of the gradient were carried out with the least amount of movement of the tube so as to avoid any disturbance of the gradient. The tube was placed in a 60 ml syringe, the plunger tightly inserted and the syringe was clamped to a retort stand with the base of the plunger firmly pressed against the base of the retort stand. The syringe had previously had about 5 mm of the top removed so the top of the tube could protrude. The tip of the tube was cut off with scissors and .5 ml of the first fraction was aspirated with a pasteur pipette. The top of the tube was sliced off with a razor blade. The remaining 1.5 mls of fraction 1 were collected by careful aspiration from the edges. The next 2 ml fraction was then aspirated and represents fraction 2 of the gradient. The side of the tube was carefully pushed out and the remaining gradient was overlayered to the top of the tube with distilled water. The tube was removed from the syringe and silicon vacuum grease (Beckman) was placed around the top of the tube. The tube was inserted in another 60 ml syringe, the plunger was tightly inserted, the syringe was clamped to the retort stand with the bottom of the plunger placed firmly against the base of the retort stand so that a seal of silicone grease was obvious between the top of the tube and the inner top of the syringe . The syringe in this case has had a hole bored in the side at the level corresponding to the bottom of the tube for insertion of a needle at a consistent position through the wall of the syringe and

into the bottom of the tube. Tygon plastic tubing was attached to the top of the syringe. A solution of d 1.35 g/ml, prepared by the addition of solid KBr to d 1.006 g/ml NaCl, was pumped through a 19 gauge, 1 inch needle that had been inserted through the hole in the syringe into the base of the tube. The gradient was pumped out through the tubing at the top of the syringe by displacement using an infusion pump (model 355, Sage Instruments) set at 386 X 10. The 2 ml fractions were collected manually in 15 ml sterile graduated conical tubes (Polystyrene,17 X 120 mm style, Falcon). The tube volume corresponding to the 2 mls graduation mark had previously been verified as being accurate using a 2 ml volumetric pipette. This verification of the volume had been carried out repeatedly and the tubes had consistently registered 2 ml accurately. The first 4 mls (2 fractions) which were pumped out contained mainly the distilled water overlayer and were considered to be part of fraction 2 of the gradient. The following fractions, numbered 3-20 were then collected sequentially.

11. Analysis of the Fractions of the Density Gradient:

Aliquots were taken of each fraction of the gradient and frozen at - 20° C to be used for lipid analysis by gas liquid chromatography and for apoprotein analysis by rocket electroimmuno assay. Aliquots were also taken for determination of radioactivity by liquid scintillation counting and analysis of particle size by polyacrylamide gradient gel electrophoresis. These aliquots were kept at 4°C and analysed either immediately or within several days.

(a) Gas Liquid Chromatography:

Each fraction of the gradient as well as the VLDL and HDL used in the incubation were analysed by gas liquid chromatography in order to obtain the total lipid profile of each fraction of the gradient or of the lipoprotein. Gas liquid chromatography was performed as described by Kuksis [225]. Phospholipase C (1 ml of a solution containing 0.125 mg Phospholipase C/ml in 0.035 M Tris HCl buffer, pH 7.3) and 1.0% $CaCl_2$ (1 ml) were added to the sample in a glass screw cap centrifugation tube. The mixture was

overlayered with 1 ml of diethyl ether and incubated with vigorous shaking in a Buchler Evapomix for 2 hours at 30°C. The reaction was stopped with 5 drops of a 0.1N HCL solution and an appropriate amount of internal standard (tricaprin) in chloroform was added. The lipids were extracted by adding 2.5 ml of methanol and 2.5 ml (total volume) of chloroform and centrifuged. The chloroform layer was removed and passed through a pasteur pipette containing sodium sulphate as a drying agent. The chloroform was evaporated to dryness under nitrogen and the lipid residue was taken up in 100 µl of Trisil BSA (Pierce) for trimethylsilylation of hydroxyl residues forming trimethylsilyl ethers. The lipids were analysed by an automated Hewlett Packard 5840A gas chromatograph using nickel columns (1/8 X 20 in) packed with 3% OV-1 or 3% SP-2100 on 100/120 Supelcoport. Nitrogen was used as the carrier gas. The temperature program ranged from 170°C to 350°C. Response factors were calculated from a run of lipid standards containing the internal standard tricaprin, cholesterol, cholesteryl oleate, cholesteryl palmitate, tripalmatin and triolein. Phospholipid response factors were assumed to be 1. It had previously been established that there was no significant accumulation of diglycerides as a result of triglyceride hydrolysis by lipoprotein lipase [23]. The diglyceride and ceramide mass, as detected by gas chromatography, represented the phospholipid mass of the sample. Any lysophospholipids that may have been present in the sample as a result of phospholipid hydrolysis would have been detected as monoglycerides by this method of lipid analysis. Particle diameters were calculated from the lipid profile as described by Kuksis [226] and Shen [227].

(b) Electroimmunoassay:

Rocket electroimmuno assay, based on the method of Laurell [228] with some modifications, was carried out for the analysis of apo AI, B, CII, CIII and apo E content of the gradient fractions and of the HDL and VLDL used in the incubations. Triton X 100

(0.1%) was added to all samples beforehand in an attempt to equalize the epitope expression in the pre- and post-incubation samples or pre- and post-heparin samples.

For apo AI, CII, CIII and E, a 1.3 % agarose gel (Seakem LE) containing 2.0% polyethylene glycol was prepared in the appropriate running buffer. The gel used for apo B was a 2.0% agarose gel containing 1.0% polyethylene glycol. The running buffers varied but were all pH 8.6. All buffers except that used for apo B contained 0.01% Triton. The buffers used were the following: Tris/Tricene (0.08 M/0.024M) buffer for apo AI; Tris/Tricine containing 1.0 mM Na_2EDTA for apo B; Tris/Tricine containing 2.5 mM calcium lactate for apo CIII; 0.06 M barbital buffer for apo E; 0.06M barbital buffer containing 2.5 mM calcium lactate for apo CII. The antibodies for apo B, CII, CIII and apo E had been previously prepared in the laboratory and were reported to have no immunoreactivity towards the other apoproteins [78, unpublished data]. The apoprotein content as determined by electroimmunoassay was within 5-6% of that determined by isolation of the individual apoproteins and determination of the protein content by the Lowry method. The antibody for apo AI was a commercial preparation (Boehringer Mannheim) and reported to be monospecific. Standards for the apo B, CII, CIII and apo E had previously been prepared and validated in the laboratory. A preparation of HDL was standardized against a prepared commercial standard (Boehringer Mannheim) for the apo AI electroimmuno assays.

All gels were run for 18 hours at 2.5 V/cm at 4°C. The plates were removed and wrapped with a wet filter paper (Whatman #1), covered with a weighted layer of paper toweling and left for 15 minutes. The paper toweling was replaced with fresh toweling and the plates were left for 15 minutes. The plates were dried in a 80°C oven. The gels were stained in a solution of Coomassie Brillant Blue R-250 (0.63g/250 ml destaining solution). The gels were destained in a solution of water/methanol/acetic acid (5:5:1 v/v) and air dried.

For most rockets, the area (height x width at half the height) was used to determine the apoprotein concentration. However, for apo E, for the fractions from the HDL region of the gradient (fractions 8-20) only the height was used as the shapes of the rockets varied widely, particularly those containing hepatic lipase in the incubations. Using this method of determining apo E concentrations resulted in more consistent recoveries for all of the incubations. The lipid transfer protein incubations demonstrated double peaks in the dense fractions of the gradient. It was determined that the lower peak contained apo E by adding a sample containing apo E to the fraction and determining which of the two peaks became enlarged. The cause of the large peak was not clear. Several proteins were tested for reactivity to the apo E antibody such as human albumin, apo AI, apo AIV, apo CIII, apo CII and the lipid transfer protein preparation but the results were inconclusive.

(c) Measurement of [^{3}H]Cholesteryl Ester Radioactivity:

A 250 µl aliquot was placed in 10 ml of Aquasol and counted in a Beckman LS 7000 scintillation counter. Aliquots containing DTNB were titrated to a clear solution before the addition of Aquasol by the addition of a few drops of 30% hydrogen peroxide in order to avoid quenching by DTNB.

(d) Polyacrylamide Gradient Gel Electrophoresis (PAGGE):

Polyacrylamide gradient gel electrophoresis was carried out by the method of Nichols [229]. For VLDL or fractions in the VLDL/LDL density range (fraction 1-7), 2-16 % gels were used (Pharmacia). For HDL or fractions in the HDL density range (fractions 8-20), 4-30% gels were used (Pharmacia). The samples were prepared by adding 1µl bromphenol blue to 100 ul of sample. Sucrose was added to achieve a final concentration of 25 percent. The high molecular weight standards ranged from 1.3 million to 67,000 and also contained bromphenol blue and sucrose. The gels were electrophoresed in a GE 2/4 electrophoresis apparatus (Pharmacia) in a circulating

Tris/Boric acid buffer (0.09M/0.08M), pH 8.35, containing 3.0 mM Na_2EDTA (0.1%) and 3.0 mM NaN_3 (0.02%). The gels were equilibrated by a prerun for 15 minutes at 125 V constant voltage. The samples were applied (10-35 µl per lane) and pre-electrophoresed at 70 V constant voltage for 20 minutes. The electrophoresis was then carried for 24 hours at 125 V constant voltage at approximately 15°C. The gels were fixed in a solution of 10% sulfosalicylic acid for 1 hour, stained with 0.1% Comassie Brilliant Blue R-250 in 7.0% acetic acid for 3 hours and destained in 5.0% acetic acid. To determine if vesicles were present in the VLDL/LDL fractions, 12 mg of cellobiose were added to a second 100 µl of the fraction in experiment C in order to dehydrate the water core and therefore disrupt any vesicles present [230].

12. Determination of the Salt Density Distribution of the Gradient:

In order to obtain an estimate of the density profile of the gradient, a gradient was set up in the same manner as described for the experimental incubations but contained only the salt background of the gradient. Briefly, 12.7 ml of d 1.04 g/ml solution prepared by the addition of solid KBr to a solution of d 1.006 g/ml NaCl was pipetted into a 40 ml Quick Seal Tube as previously described. The d 1.04 g/ml solution was underlayered by 12.7 ml of a d 1.25 g/ml solution prepared by the addition of solid KBr to a solution of d 1.006 g/ml NaCl . The d 1.04 g/ml solution was then overlayered with 14.6 ml of a density 1.006 g/ml solution of NaCl. The tubes were centrifuged and the gradient collected by the same method previously described. The density distribution of the gradient was determined using 5 density gradients, with 3 gradients being centrifuged at one time and 2 at another time. The density of each fraction was determined at 20° C by determining the refractive index and converting the value for KBr to the corresponding density. The difference in the refractive index between NaCl and KBr at d 1.006 g/ml was subtracted to account for the background d 1.006 g/ml of NaCl in each fraction.

13. Other Methods:

Protein was estimated by the method of Lowry [231] with the addition of 10.0% sodium dodecyl sulphate to solubilize the apoproteins.

For electron microscopy, aliquots of fractions 8-12 of experiment E of the lipoprotein lipase incubation, which had never been frozen, were pooled and dialysed in 0.005 M NH_4HCO_3 buffer, pH 7.4 to remove the salt. The procedure used for visualization of the lipoproteins was that of negative staining. A small amount of the sample was applied to a copper grid. The grid had previously been covered with a film of formvar which was subsequently coated with carbon before the application of the sample. After several minutes the excess sample was removed by absorption with a small piece of filter paper held at the edge of the grid. Staining was carried out with 2.0% aqueous uranyl acetate for 5 minutes. The grids were examined under the electron microscope (Philips).

B. MATERIALS

Sepharose 4B and 6B, heparin Sepharose CL- 6B, phenyl Sepharose CL- 4B, the 2-16%, 4-30 % polyacrylamide gradient gels and the high molecular weight standards used for polyacrylamide gradient gel electrophoresis were obtained from Pharmacia (Canada) Ltd., Dorval, Que.

Heparin (Grade 1, from porcine intestinal mucosa, 162 units/mg, Lot #66F-0727) for preparation of heparin Sepharose and release of lipoprotein lipase from the rat hearts was obtained from Sigma Chemical Company, St. Louis, Mo. Protamine sulphate (Grade 1, from Salmon), 5,5-dithio-bis-2-nitrobenzoic acid, egg phosphatidyl choline(L-α-phosphatidyl choline from fresh chicken egg yolk, type XI-E) and triolein (for the hepatic lipase assay) were also purchased from Sigma Chemical Company, St. Louis, Mo. as was Comassie Brilliant Blue R-250 used for the staining of gels.

Cyanogen bromide, Reagent Grade, was purchased from Fisher Scientific Company, Dartmouth, N.S.

[1, 2, 6, 7, -^{3}H (N)] Cholesteryl oleate, [carboxyl- ^{14}C] triolein, l-α-dipalmitoyl-phosphatidyl [methyl -^{14}C] choline and [1. 2- ^{3}H] cholesterol were obtained from New England Nuclear/Dupont Canada, Montreal, Que., as was Aquasol and Readi-gel for liquid scintillation counting.

Long Evans rats were supplied by Canadian Breeding Farms, St. Constant, Quebec. Citrated blood was generously provided by the Nova Scotia Red Cross Transfusion Unit. Sodium pentobarbital (Somnotol) was obtained from M.T.C. Pharmaceuticals, Hamilton, Ont.

Bovine serum albumin (essentially fatty acid free, from fraction V, #A6003) which was found to contain no detectable apo AI or phospholipid (lower limit of apo AI electroimmuno assay would result in <0.08 μg apo AI/mg albumin) used in the incubations and several of the assays was purchased from Sigma Chemical Company, St. Louis, Mo.

Phospholipase C (from Clostridium perfringens) was from Sigma Chemical Company, St. Louis, Mo. Lipid standards for gas liquid chromatography were obtained from the following companies: tricaprin from Terochem, Rexdale, Ont.; cholesteryl palmitate and cholesteryl oleate from Sigma Chemical Company, St. Louis, Mo; cholesterol, tripalmatin and triolein from Applied Science Division supplied by Terochem, Rexdale, Ont. Tri-Sil BSA was purchased from Pierce Chemical Company, Rockford, Ill. The column packing material used for gas chromatography, 3% OV-1 on 100/120 Supelcoport and 3% SP-2100 on 100/120 Supelcoport, was obtained from Supelco Canada Ltd, Oakville, Ont.

D-(+) cellobiose was purchased from the Eastman Kodak Company, Rochester, N.Y.

SeaKem agarose (LE) for rocket electroimmunoassay was from Mandel Scientific Company, Guelph, Ont.

The apo AI antibody and the commercial standard for apo AI was purchased from Boehringer Mannheim, Dorval, Que. Triton X-100 was purchased from Rohm and Haas, Philadelphia, PA.

Aquacide II (carboxymethylcellulose) was from Calbiochem, c/o Terochem, Mississauga, Ont.

Dialysis tubing (molecular weight cut off of 3,500, 8,000 and 12,000) was purchased from Spectrum Medical Industries Inc., Los Angeles. Tygon flexible plastic tubing was obtained from Fisher Scientific Company, Dartmouth, N.S.

Other chemicals or reagents were purchased from Sigma Chemical Company, St. Louis, MO, Fischer Scientific or BDH Chemicals, Dartmouth, N.S.

CHAPTER IV

RESULTS

The results are reported in three sections. Section A describes the development and characteristics of the density gradient that was used for the isolation of the lipoproteins in the *in vitro* and in *vivo* experiments. Section B contains the results of the *in vitro* studies. This section reports the changes in VLDL and HDL when incubated together in the presence of lipoprotein lipase as well as the effects on the lipoproteins following the addition of hepatic lipase or the lipid transfer proteins to the lipoprotein lipase incubation. Section C describes the results of the *in vivo* studies. This section describes the effects of heparin-induced lipolysis on the plasma lipoprotein profiles of two normolipidemic subjects.

A) DEVELOPMENT OF A SIMPLE DENSITY GRADIENT ULTRACENTRIFUGATION TECHNIQUE FOR THE ISOLATION OF LIPOPROTEINS:

The single spin density gradient ultracentrifugation technique was developed based on several density gradient methods [232-234] in order to observe the complete distribution of the lipoproteins. Density gradient ultracentrifugation was selected as a method to determine the lipoprotein distributions in order to avoid the use of the arbitrary (fixed) boundaries of sequential ultracentrifugation since lipoprotein distributions comprise a continuum of particles whose densities may overlap and whose distributions may vary depending on the sample and the metabolic conditions to which the lipoproteins are exposed. Sequential ultracentrifugation is suitable for the isolation of a particular class of plasma lipoproteins. However, this method does not demonstrate small changes within a lipoprotein class which may be important in determining how the different experimental conditions affected the lipoprotein distributions in the present study. Sequential ultracentrifugation also requires more manipulation of the sample due

to the repeated centrifugation steps, higher salt concentrations and a greater total number of g hours with the longer total centrifugation time. All these factors may effect the integrity of the lipoproteins. As an example, to isolate VLDL, LDL+IDL and HDL sequentially a total of 8,576,191 x g hours are required (this does not include washings or isolation of subfractions) whereas the density gradient technique requires 4,419,888 x g hours. A major concern in the use of ultracentrifugation, as a method of isolating plasma lipoproteins, is the possible dissociation of apoproteins from the lipoprotein particles due to the shear forces and high salt concentrations. The apoproteins which have been reported to be affected by ultracentrifugation are apo AI [235-237] and apo E [238-240] but the conditions causing dissociation differed. Apo AI dissociation was increased with repeated ultracentrifugations but decreased with high salt concentrations while apo E was dissociated with high sheer stress and high salt concentrations. When gel filtration was used to avoid apoprotein dissociation, it was found that the VLDL and LDL regions contained an average of 27% (11-54%) of the plasma apo E while the HDL region contained an average of 73% (46-89%) [241]. However, when sequential ultracentrifugation was used, it was found that the distribution of apo E was 40% (36-56%) in the VLDL/LDL region, 19% (11-33%) in the HDL region and 27-45% in the d>1.21 g/ml, indicating that some dissociation of apo E into the lipoprotein deficient region may have occurred.

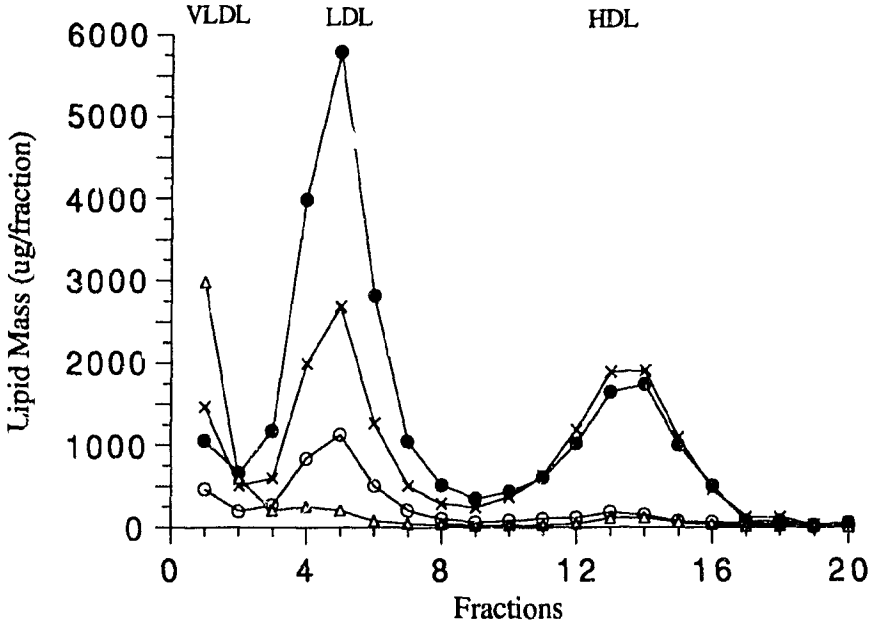
A comparison of the isolation of plasma lipoproteins by sequential ultracentrifugation and a single spin density gradient ultracentrifugation technique noted no differences for the apo B distribution while the density gradient technique reduced apo AI dissociation from HDL (30% versus 41% in the d>1.21 g/ml for sequential ultracentrifugation). However, the effect on the apo E distribution was not reported. When this gradient was used during the development of density gradient for the present study, seventy percent of the apo E was found in the most dense region of the gradient, corresponding to average densities of 1.161-1.282 g/ml (data not shown). There was

essentially no apo E in the fractions that contained most of the HDL lipid mass. When the plasma was adjusted to a lower salt concentration (d 1.04 gm/ml rather than d 1.25 gm/ml), the distribution of apo E normalized with 23-30% in VLDL-LDL, 62-69% in HDL fractions and 8% in fractions in the very dense region of the gradient. The complete distribution for apo E on the gradient is provided for two subjects in the *in vivo* section of this thesis. The distributions again show little apo E mass (10% and 11%) in the very dense fractions with 58% and 69% of the total plasma apo E being located in the HDL region.

Four examples of the lipid distributions of normal plasma samples, isolated using the density gradient ultracentrifugation technique, are outlined in Figures 3-7 in order to demonstrate some characteristics of the distribution. The density gradient was designed for maximum resolution of the HDL region while still allowing for the separation of VLDL and LDL. The HDL region was emphasized since it was the region under investigation with respect to the effect of VLDL lipolysis on the HDL lipid and apoprotein distribution.

VLDL isolated in fraction 1 with a small amount of carry over into fraction 2, particularly if the plasma VLDL concentration was high. In one subject (Figure 5), when an increased concentration of IDL was present, it appeared in fraction 2. As can be observed from the lipid compositions and distributions (Figures 3-6), there was little extension of the LDL peak into fractions 1 and 2. The LDL distributed largely in fraction 3-7 with the peak varying between fractions 5 and 6 depending on whether the individual possessed the majority of LDL as a "light LDL" or "heavy LDL". HDL distributed between fractions 8-18. HDL did not usually demonstrate separate peaks for HDL_2 and HDL_3. As determined by the measurement of the density of the fractions using potassium bromide alone to form the gradient (Figure 7), the density distributions of HDL_2 included fractions 8-14 and HDL_3 included fractions 14 to mid-19. The HDL

FIGURE 3: LIPID DISTRIBUTION OF PLASMA LIPOPROTEINS ISOLATED BY DENSITY GRADIENT ULTRACENTRIFUGATION - SUBJECT A



A fasting sample of plasma was subjected to density gradient ultracentrifugation. Each fraction was analysed for lipid mass by gas liquid chromatography.

The lipids are represented as unesterified cholesterol: O
phospholipid: X
cholesteryl ester: ●
triglyceride: Δ

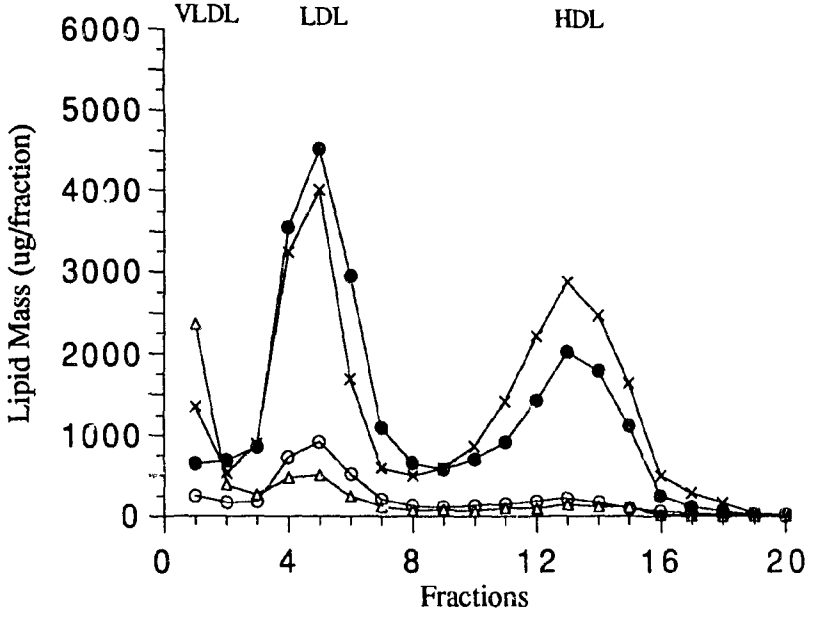
The location of VLDL, LDL and HDL peaks are indicated.

The subject had the following plasma lipid values:

Total cholesterol: 152.5 mg/dl
VLDL cholesterol: 9.9 mg/dl*
LDL cholesterol: 96.5 mg/dl
HDL cholesterol: 46.1 mg/dl*
Total triglycerides: 38.5 mg/dl
Total phospholipids: 137 mg/dl

*These values were calculated by summing fraction 1-2 for VLDL and 8-20 for HDL.

FIGURE 4: LIPID DISTRIBUTION OF PLASMA LIPOPROTEINS ISOLATED BY DENSITY GRADIENT ULTRACENTRIFUGATION - SUBJECT B



A fasting sample of plasma was subjected to density gradient ultracentrifugation. Each fraction was analysed for lipid mass by gas liquid chromatography.

The lipids are represented as unesterified cholesterol: O
phospholipid: X
cholesteryl ester: ●
triglyceride: Δ

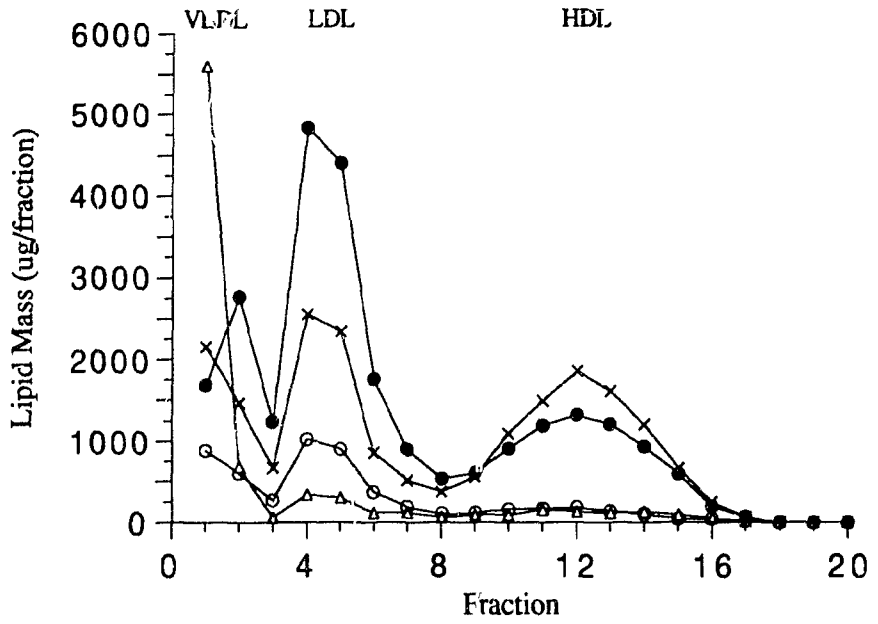
The location of VLDL, LDL and HDL peaks are indicated.

The subject had the following plasma lipid values:

Total cholesterol: 146.9 mg/dl
VLDL cholesterol: 9.6 mg/dl*
LDL cholesterol: 81.3 mg/dl
HDL cholesterol: 56 mg/dl*
Total triglycerides: 40.6 mg/dl
Total phospholipids: 203.8 mg/dl

*These values were calculated by summing fraction 1-2 for VLDL and 8-20 for HDL.

FIGURE 5: LIPID DISTRIBUTION OF PLASMA LIPOPROTEINS ISOLATED BY DENSITY GRADIENT ULTRACENTRIFUGATION - SUBJECTC



A fasting sample of plasma was subjected to density gradient ultracentrifugation. Each fraction was analysed for lipid mass by gas liquid chromatography.

The lipids are represented as unesterified cholesterol: O
phospholipid: X
cholesteryl ester: ●
triglyceride: Δ

The location of VLDL, LDL and HDL peaks are indicated.

The subject had the following plasma lipid values:

Total cholesterol: 164 mg/dl
VLDL cholesterol: 32 mg/dl *
LDL cholesterol: 89.3 mg/dl
HDL cholesterol: 42.7 mg/dl*
Total triglycerides: 64.3 mg/dl
Total phospholipids: 155 mg/dl

*These values were calculated by summing fraction 1-2 for VLDL and 8-20 for HDL.

This subject demonstrated a peak in fraction 2 which may be due to IDL

FIGURE 6

DUPLICATE SAMPLES OF THE LIPID DISTRIBUTIONS OF THE PLASMA LIPOPROTEINS OF SUBJECT D ISOLATED BY DENSITY GRADIENT ULTRACENTRIFUGATION.

Duplicate samples of fasting plasma were subjected to density gradient ultracentrifugation. A duplicate sample for fraction 16 was absent in the lower graph.

Each fraction was analysed for lipid mass by gas liquid chromatography.

The lipids are represented as unesterified cholesterol: O
phospholipid: X
cholesteryl ester: ●
triglyceride: Δ
The location of VLDL, LDL and HDL peaks are indicated.

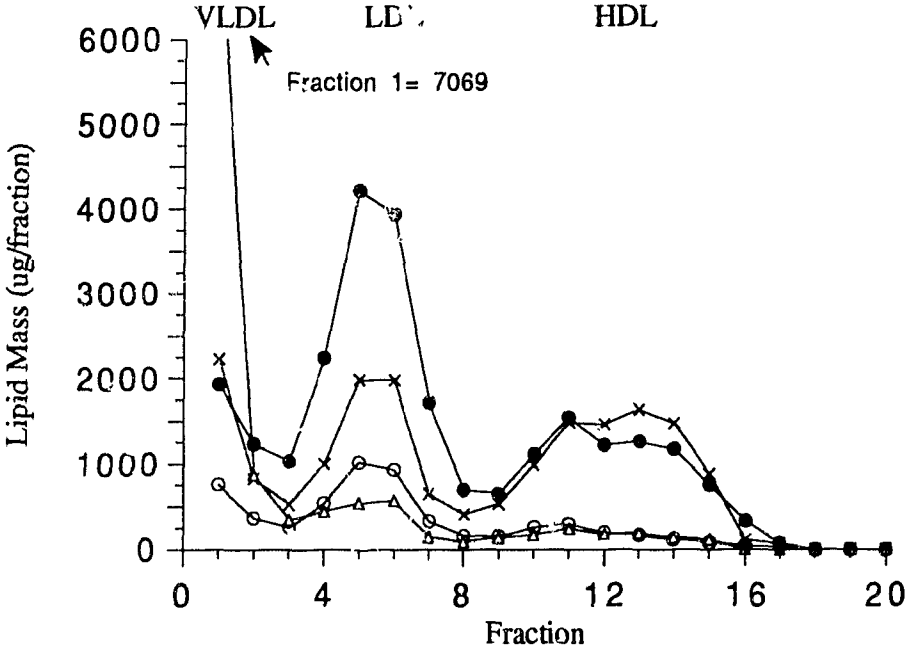
In order to maintain the same range for the lipid mass of 0-6000 µg as in figures 1-3 , the value for triglycerides in fraction 1 did not appear on the graph but is indicated.

The subject had the following plasma lipid values:

Total cholesterol: 164.3 mg/dl
VLDL cholesterol: 24 mg/dl *
LDL cholesterol: 91 mg/dl
HDL cholesterol: 49.3 mg/dl*
Total triglycerides: 89.2 mg/dl
Total phospholipids: 144 mg/dl

*These values were calculated by summing fraction 1-2 for VLDL and 8-20 for HDL.

Figure 6: Duplicate Samples of the Lipid Distributions of the Plasma Lipoproteins of Subject D Isolated by Density Gradient Ultracentrifugation.



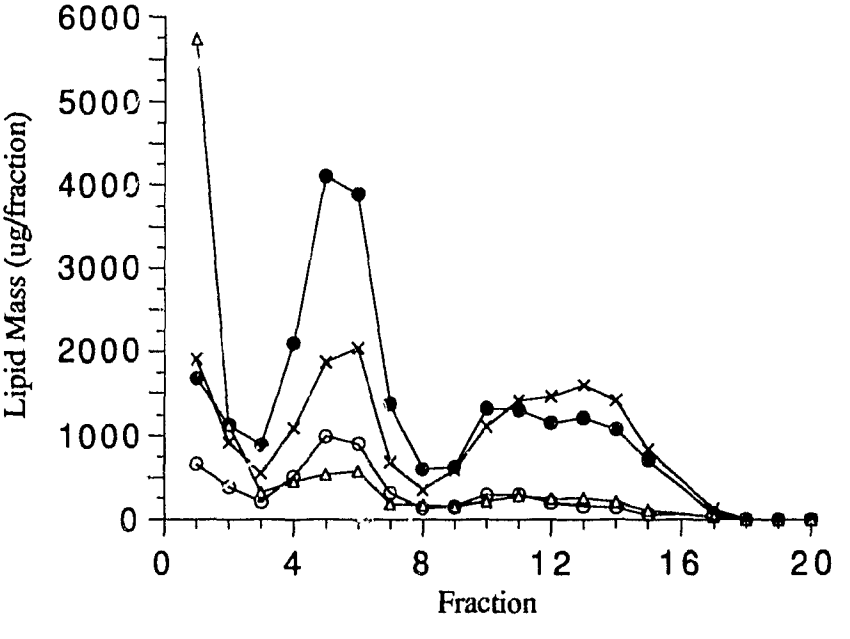
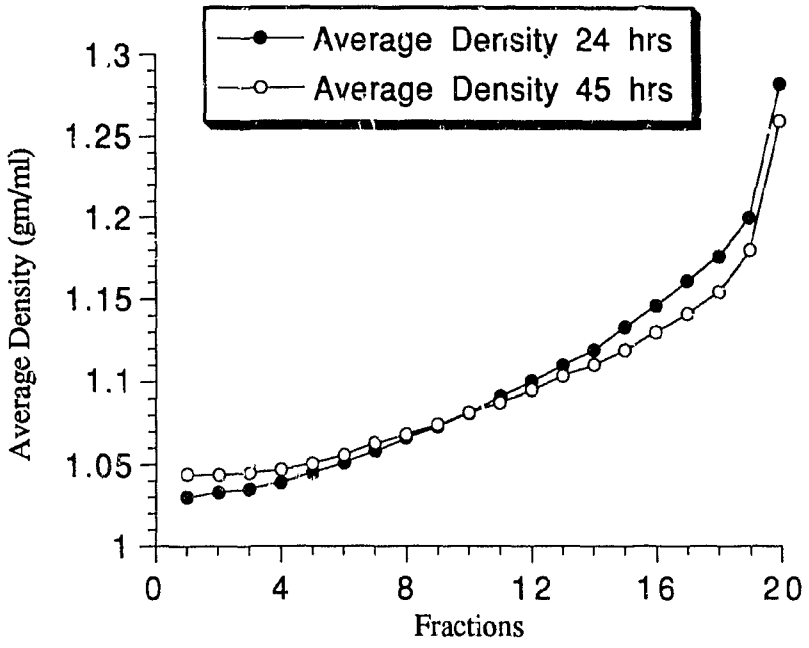


FIGURE 7: DENSITY PROFILE OF THE DENSITY GRADIENT CENTRIFUGED FOR 24 AND 45 HOURS.



A NaCL solution of density 1.006 g/ml was adjusted to densities 1.04 g/ml and 1.25 g/ml with solid NaBr. The density gradient was prepared using the three solutions as described in the methods.

The gradients were collected after centrifugation for 24 and 45 hours and the density of each fraction was obtained by refractometry. The densities of five gradients were averaged to obtain the 24 hour value and the densities of two gradients were averaged for the 45 hour values.

peak was typically in fraction 12-13. There was little lipid mass in the HDL_3 density range of d 1.161- 1.21 g/ml (fraction 17- mid-19).

In order to determine the overall reproducibility of the gradient and the consistency of the location of the LDL or HDL peak, two plasma samples were centrifuged in duplicate and the lipid mass of each fraction of the gradient was determined. Figure 6 demonstrates the lipid distribution of one of the duplicate samples. No significant variation in the LDL or HDL peak was observed and the distribution of the lipids were consistent. The average variation for unesterified cholesterol, cholesteryl esters and phospholipid was 5% which was in the range of accuracy of the gas chromatograph. There was a greater variability for triglycerides (an average of 16%) which was largely due to variability in fraction 1. The higher variability in triglycerides as compared to the other lipids is due to the high concentration of VLDL triglycerides in this fraction which can affect the accuracy of the analysis due to the greater dilution required for analysis.

It was not directly determined whether LDL and HDL had attained their equilibrium density which would require determining the fractions in which previously prepared lipoproteins of known density would isolate. However, the LDL mass was found in fractions 3-7 (d 1.034-1.062 g/ml) with the majority of mass in 4-6 (d 1.037-d 1.055 g/ml) and the HDL mass isolated in fractions 8-16 (d 1.062-1.153 g/ml) with the majority of the mass in fractions 10-15 (d 1.077-1.139 g/ml). These density distributions correspond to the densities identified for plasma LDL and HDL mass when isolated using an equilibrium density gradient technique [234]. The conditions that the lipoproteins were subjected to during ultracentrifugation of the gradient exceeded those required for the isolation of HDL at d 1.21 g/ml. Since the isolation of the other lipoproteins requires less vigorous conditions for isolation, it suggests that the lipoproteins were exposed to the necessary g force for an adequate period of time to achieve their equilibrium density. In

order to determine if the lipoprotein would move any further on the gradient in the event that they had not previously reached their equilibrium density, the gradient was centrifuged for 45 hours rather than 24 hours. The longer ultracentrifugation time caused LDL to move higher up in the tube as compared to 24 hours while the plasma proteins were more concentrated at the bottom of the tube. However, this shift was due to a change in the actual salt gradient (Figure 7) rather than lack of equilibration of the lipoproteins after 24 hours. The change in the gradient was likely due to diffusion of the salt with the prolonged centrifugation time. It should be noted, however that the density of a lipoprotein in a particular fraction may differ slightly from the density determined using the NaCl/KBr gradient even if the lipoproteins had reached their equilibrium density. This is due to the fact that the lipoprotein fractions were collected at 4°C while NaCl/KBr fractions, although collected at 4°C, were measured by refractometry at 20°C for estimation of the density. The thermal expansivity of NaCl/KBr going from 4° to 20°C may differ from that of the lipoprotein particles and thus their densities may differ slightly. Thus one should use the density of a given fraction as an operational reference (an approximate density) rather than as a precise value.

B) *IN VITRO* EXPERIMENTS:

The *in vitro* experiments made up the majority of the study. The conditions were designed to test how lipoprotein lipase affected the HDL mass and the formation of LDL during lipolysis of VLDL and to determine the effect of the addition of hepatic lipase or the lipid transfer proteins to the lipoprotein lipase incubation. All incubations contained VLDL and HDL. Four incubation conditions were used : 1) a control incubation which contained heat denatured lipoprotein lipase; 2) a LPL incubation which contained active lipoprotein lipase; 3) a LPL+HAL incubation which included hepatic lipase with the active lipoprotein lipase; 4) a LPL+LTP incubation which included the lipid transfer protein preparation with the active lipoprotein lipase; 5) a LTP incubation which included

the lipid transfer protein preparation with the heat denatured lipoprotein lipase. Five experiments are shown and are referred to as experiments A, B, C, D and E. The number of experiments for the various conditions are: control= 5, LPL= 4, LPL+HAL= 3, LPL+LTP= 4, LTP= 2 (Table 4). The complete lipid and apoprotein data for the five experiments are listed in the Appendix A. To simplify the description of the net shifts in mass, the VLDL/LDL region was designated as fractions 1-7 and the HDL region as fractions 8-20. Part of fraction 19 and all of fraction 20 were of a greater density than plasma HDL (d> 1.21 g/ml).

The results are reported for the *in vitro* incubations by describing the following: a) the effect on HDL mass of the addition of lipoprotein lipase compared to the control incubation; b) the effect on HDL mass of the addition of hepatic lipase or the lipid transfer proteins to the lipoprotein lipase incubation as compared to the lipoprotein lipase incubation alone; c) the effect of lipoprotein lipase on LDL formation and the effect of the addition of hepatic lipase or the lipid transfer protein preparation to the lipoprotein lipase incubation as compared to that of the lipoprotein lipase incubation alone. The effect of the lipid transfer protein preparation in the absence of active lipoprotein lipase was also reported as it served as a control for the incubation of the lipid transfer protein preparation in the presence of active lipoprotein lipase.

1) COMPOSITION OF VLDL AND HDL: (TABLES 5 AND 6)

The composition of the VLDL and HDL used in the five experiments are listed in Tables 5 and 6, respectively. The VLDL was similar to plasma VLDL III in terms of lipid content and particle size. VLDL III is poorest in triglyceride content as compared to VLDL I and II and represents the majority of the plasma VLDL in normolipidemics. The HDL used in the incubation was one which lacked apo E since we planned to study in detail the loss of apo E from VLDL and gain by HDL. The absence of apo E was verified by an anti-apo E electroimmunoassay. The use of HDL without apo E facilitated the measurement and distribution of the apo E transferred to the HDL region. If the total

TABLE 4

OUTLINE OF *IN VITRO* EXPERIMENTS

	Experiment				
Incubation	**A**	**B**	**C**	**D**	**E**
Control	+	+	+	+	+
LPL	+	+	+(63%) [1]	+	+
LPL+HAL	+	+	+	-	-
LPL+LTP	-	+	+	+	+
LTP	-	-	-	+	+

The conditions included in each of the five experiments are indicated by a + sign.

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein which was carried out for 2 hours at 37°C

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+HAL indicates the addition of hepatic lipase to the LPL incubation.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.
LTP indicates the addition of the lipid transfer protein preparation to the control incubation.

[1] The LPL incubation attained 63% VLDL triglyceride hydrolysis rather than the average of 90% in the other incubations with active lipolysis in experiment C.

TABLE 5

COMPOSITION OF VLDL USED IN THE INCUBATIONS

Component	Experiment A	B	C	D	E
	Weight %				
Unesterified Cholesterol	7.1	5.2	6.4	2.7	5.2
Cholesteryl Esters	18.2	12.7	11.6	15.1	16.7
Phospholipid	21.4	20.2	22.1	27.5	20.6
Triglyceride	39.7	46.6	47.1	43.4	45.9
Protein	13.5	15.2	12.8	10.6	11.6
Particle Diameter (Å)	277	317	284	263	326
UC/PL	.670	.510	.375	.270	.510
S/C	.616	.530	.614	.610	.500
CE/TG	.600	.360	.321	.460	.480

Particle diameter was estimated from the lipid data, based on a calculation relating core volume to surface area as outlined in appendix B[226, 277].

UC/PL= the unesterified cholesterol to phospholipid molar ratio.

S/C= the molar ratio of surface lipids (cholesterol and phospholipid) to core lipids (cholesteryl ester and triglycerides).

CL/TG= the cholesteryl ester to triglyceride molar ratio.

TABLE 6

COMPOSITION OF HDL USED IN THE INCUBATIONS

Components	Experiment A	B	C	D	E
			Weight %		
Unesterified Cholesterol	1.7	2.0	2.4	1.6	1.8
Cholesteryl Esters	16.5	16.1	14.3	18.5	20.5
Phospholipid	20.0	18.8	27.4	29.8	28.3
Triglyceride	3.1	2.6	4.3	5.0	5.1
Protein	58.6	60.5	51.6	45.1	44.3
Particle Diameter (Å)	143	120	112	126	137
UC/PL	.173	.211	.172	.11	.13
S/C	1.040	1.059	1.526	1.245	1.097
CE/TG	6.88	8.20	4.35	4.9	5.24

Particle diameter was estimated from the lipid data, based on a calculation relating core volume to surface area as outlined in appendix B[226, 277].

UC/PL= the unesterified cholesterol to phospholipid molar ratio.

S/C= the molar ratio of the surface lipids (cholesterol and phospholipid) to core lipids (cholesteryl ester and triglyceride).

CE/TG= the cholesteryl ester to triglyceride molar ratio.

plasma HDL had been used, the transfer of apo E would be difficult to measure since HDL contains approximately 65% of the total plasma apo E and a relatively small gain would be much less apparent. The HDL lacking apo E represents approximately two-thirds of the plasma HDL as determined by comparing the size of the of HDL lacking apo E peak to that of the HDL containing apo E peak which eluted from the heparin Sepharose column. The HDL lacking apo E may contain less HDL_2 than plasma HDL since HDL containing apo E preferentially isolates in the HDL_2 density range [166].

2) VLDL TRIGLYCERIDE HYDROLYSIS: (TABLE 7)

The percentage of VLDL triglyceride hydrolysis (lipolysis) for each condition of the experiments is listed in Table 7. Lipolysis was very effective since 88-93% of the VLDL triglycerides were hydrolysed. The percentage of triglyceride hydrolysis was calculated by comparing the amount of triglycerides lost from fractions 1-7 in the 4 experimental incubations to the amount of triglyceride present in these fractions in the control incubation. Fractions 3-7, which are the fractions where plasma LDL normally isolates, were included because with active lipolysis the VLDL mass was found to distribute down the gradient into fractions 3, 4 and 5. Little mass reached fraction 6 and 7 after lipolysis but these were included because they were in the LDL density range.

3) VLDL PHOSPHOLIPID HYDROLYSIS: (TABLE 8)

Hydrolysis of the VLDL phospholipid occurred which was indicated by a net loss of phospholipid in the total gradient. The percentage of phospholipid hydrolysis for the experimental conditions is listed in Table 8. When the percentage hydrolysis was calculated, the net loss of phospholipid from the total gradient (fractions 1-20) was divided by the phospholipid mass in fractions 1-7 of the control incubation. The net loss from the total gradient was used rather than the loss from the fraction in the VLDL/LDL region (1-7) in calculating the percentage hydrolysis. The loss of phospholipid from the total gradient was used since the decrease in fractions 1-7 was partially due to the transfer

TABLE 7

PERCENTAGE OF VLDL TRIGLYCERIDE HYDROLYSIS[1]

Incubations	Experiment A	B	C	D	E
LPL	91.4%	88.9%	63.1%	92.7%	93.0%
LPL+HAL	91.0%	87.5%	87.6%	——	——
LPL+LTP	——	89%	88.2%	89.4%	85%[2]
LTP	——	——	——	8.7%[2]	0%[2]

The conditions of the various experiments are outlined in Table 1

1 The percentage of triglyceride hydrolysis was calculated in the following manner:

$$1 - \frac{\text{(sum of fraction 1-7 in the experimental incubation)}}{\text{(sum of fractions 1-7 in the control incubation)}} \times 100$$

2 Due to the transfer of triglyceride from the VLDL-LDL region to the HDL region in these incubations, the values were adjusted by including the amount of triglyceride transferred to HDL in the triglyceride mass remaining in fractions 1-7.

TABLE 8

PERCENTAGE OF VLDL PHOSPHOLIPID HYDROLYSIS

	Experiment				
Incubations	**A**	**B**	**C**	**D**	**E**
LPL	27.2%	38.8%	(6.8%)	24.7%	31.7%
LPL+HAL	24.3%	47.9%[1]	31.7%	——	——
LPL+LTP	——	35.6%	22.5%	20.6%[2]	18.2%
LTP	——	——	——	0%[2]	0%

The conditions of the various experiments are outlined in Table 1.

The percentage of phospholipid hydrolysis was calculated in the following manner:

$$\frac{\text{differences between experimental and control incubations of fractions 1-20}}{\text{sum of fractions 1-7 in the control incubation}} \times 100$$

This estimate of VLDL phospholipid hydrolysis assumes that the hydrolysis was taking place in VLDL rather than being transferred to HDL and then being hydrolysed. This is based on the observation that there was a large net loss from the VLDL-LDL region with a net gain in the HDL region.

1 The estimate of VLDL phospholipid hydrolysis of the LPL+HAL incubation is an overestimate since there was a net loss of phospholipid from the HDL region as well as from the VLDL region. The loss from the HDL region is indicative of HDL phospholipid hydrolysis.

2 The lipid transfer protein preparation contained phospholipid. The amount of phospholipid in the preparation was subtracted from the net loss in calculating the percentage phospholipid hydrolysis.

of the VLDL phospholipid mass to HDL. When referring to VLDL phospholipid hydrolysis, it was assumed that the phospholipid was hydrolysed in VLDL and not after being transferred to HDL. The average percentage hydrolysis in the LPL incubations was 30.6%. The addition of hepatic lipase did not affect the extent of hydrolysis of VLDL phospholipid. The estimate of phospholipid hydrolysis in the LPL+HAL incubation was complicated by the fact that hydrolysis of HDL phospholipids also occurred. The LPL+LTP incubation demonstrated 24.2% hydrolysis on average. The LTP incubation did not demonstrate loss of phospholipid from the VLDL-LDL region.

4) CHANGES IN THE CONTROL INCUBATION:

In order to determine if the control incubation demonstrated any changes or shifts in the distribution of lipid or apoproteins between the VLDL/LDL and the HDL region as a result of incubating VLDL with HDL for 2 hours at 37°C, the percentage composition of fraction 1 (containing most of the VLDL mass) was compared to that of the starting VLDL (data not shown). Few differences were noted. For example, the lipid composition of the starting VLDL of experiment E was 5.9% unesterified cholesterol, 18.9% phospholipid, 23.3% cholesteryl ester and 51.9% triglyceride. The corresponding values for fraction 1 of the control incubation were 6.3%, 16.7%, 24.7% and 52.3%, respectively. When the mass in fractions 1-7 for the VLDL component were compared to the apoprotein content in the starting VLDL, shifts in apo CIII or CII mass appeared to be minimal. Unlike the other lipid and apoprotein components, transfer of apo E was measurable since the starting HDL did not contain apo E. Transfer of apo E from VLDL to HDL was small, averaging 8.4% (2.3%, 4.9%, 8.9%, 16.5% and 9.6% for experiments A to E, respectively). Overall, the control incubation, when compared to the starting VLDL or HDL, did not demonstrate any major changes in lipid or apoprotein content in any of the experiments.

5) CHANGES IN THE EXPERIMENTAL INCUBATIONS:

a) EFFECT OF LIPOPROTEIN LIPASE ON HDL MASS: (TABLE 9, FIGURES 8-21)

The effect of lipoprotein lipase on the redistribution of mass from VLDL to the HDL region was determined by comparing the mass in the HDL region of the lipoprotein lipase incubation to that of the control incubation. The results of the average lipid and apoprotein mass in the HDL region of the gradient for the lipoprotein lipase incubations (experiment A, B, D and E) and the control incubations are listed in Table 9. The significance was determined using a paired t test.

The inclusion of lipoprotein lipase in the incubation resulted in a consistent gain by HDL of unesterified cholesterol and phospholipid, (mean gain = + 110 μg and +285 μg, respectively) as compared to the control incubation. Both increases in lipid mass were significantly different from the control incubation. There was a greater gain of phospholipid in experiments D and E as compared to A and B.

The effect of lipoprotein lipase on the change in cholesteryl ester mass was highly variable due to a substantial gain observed in experiments D and E but a small loss in experiments A and B (Table 9).The experiments that demonstrated the gain in cholesteryl esters in the HDL region were the same ones that demonstrated an increased transfer of phospholipid from VLDL to HDL (experiments D and E). The reason for this apparent segregation of the experiments into two groups, A and B versus D and E, was not obvious when comparing the percentage of triglyceride or phospholipid hydrolysis of the VLDL used in the four experiments. There was no noticeable difference in the composition of the VLDL used in experiments A and B as compared to D and E. It was only upon inspection of the HDL composition that a difference was noted. The HDL used in the experiments which demonstrated a gain in cholesteryl ester and a greater gain in phospholipid had a markedly lower unesterified cholesteryl ester ratio (UC/PL) (Table 6)

TABLE 9

LIPID AND APOPROTEIN MASS IN THE HDL REGION
OF THE LIPOPROTEIN LIPASE INCUBATIONS AND CONTROL INCUBATIONS

Component	Control (n=4)		LPL (n=4)		
	Mean	SD	Mean	SD	Mean Change
			μg		
Unesterified Cholesterol:	313	± 45	423	± 57	+110 ***
Phospholipid:	3205	± 634	3490	± 694	+285 **
Cholesteryl Esters:	2471	± 392	2581	± 570	+110 n.s.
Triglycerides:	555	± 135	518	± 177	-37 n.s.
Apo CII:	61	± 17	258	± 62	+197 ***
Apo CIII:	215	± 120	640	± 141	+424 ***
Apo E:	32	± 25	107	± 22	+75 ***

*** = $p \leq .005$, ** = $p \leq .01$, n.s. = not significant

Results are the mean ± standard deviation of 4 experiments (Experiment **A, B, D, E**)

A paired t-test was used comparing the values of the LPL incubation to the control incubation

LPL = lipoprotein lipase incubation

than the HDL of the experiments that did not demonstrate these changes. The UC/PL ratio of experiments D and E were 0.11 and 0.13 while the ratios of experiments A and B were 0.17 and 0.21. Unesterified cholesterol is considered to impart rigidity (or order) to a phospholipid monolayer or bilayer at 37°C. Therefore a lower UC/PL ratio reflects the presence of a more fluid surface in the HDL used in experiments D and E as compared to experiments A and B. A more fluid surface in the HDL may facilitate the transfer and incorporation of mass from VLDL to the original HDL or stabilize the formation of a "new" HDL by the donation of lipid or apoprotein mass to the mass transferred from VLDL. The reason for the difference in the UC/PL ratio is not clear as 3-5 different units of blood were used for the preparation of the HDL for each experiment. Due to the high variability in the cholesteryl ester gain by HDL in the four experiments, the average gain of cholesteryl ester (+110 μg) did not reach significance. Apo CII increased an average of 197 μg, apo CIII gained 424 μg and apo E increased 75 μg.

The increases in apo CII, CIII and apo E mass in the HDL region were all significantly different from the control (Table 9). The triglycerides mass in the HDL region did not differ from the control (-37 μg) (Table 9).

The overall effect of lipoprotein lipase on the lipid and apoprotein distributions in the HDL region was a slight shift in the mass to the lighter region of the gradient as compared to the control distribution (Figures 8, 10, 12, 16, 18 and 20). The shift was due to a gain in mass on the light side of the control peak mass and a decrease in the dense region in the experiments that did not gain cholesteryl ester (experiments A and B). The distribution tended to differ in the experiments that gained cholesteryl ester in that there was a gain of HDL mass in the light region without a decrease in the dense region (Figures 9,11,13, 17,19 and 21). A change in the location of the peak fraction as compared to the control incubation was not observed in the majority of the cases in any of the experiments (Figures 8-20).

b) THE EFFECT ON HDL MASS OF THE ADDITION OF HEPATIC LIPASE AND LIPID TRANSFER PROTEINS TO THE LIPOPROTEIN LIPASE INCUBATION.

To determine whether further transfer of VLDL components to HDL could be achieved during VLDL lipolysis by lipoprotein lipase, the role of hepatic lipase and the lipid transfer proteins was investigated. The conditions were the same as those used in the in the lipoprotein lipase incubation.

The amount of hepatic lipase activity added to the incubation was in the low-normal range of that reported for human plasma (6, 13.6 and 12.4 μ moles free fatty acid released/ml/hr for experiments A, B and C, respectively). The amount of cholesteryl ester transfer activity and phospholipid transfer activity added to the incubation was in the average range reported for human plasma. Cholesteryl ester transfer activity was 81, 99 and 91 nmoles cholesteryl ester transferred/ml/hr for experiments C, D and E. Phospholipid transfer activity was 26.4 and 45.4 nmoles phospholipid transferred/ml/hr for experiments B and C, respectively, as determined by the method which measures the transfer of labeled cholesteryl ester from HDL to LDL. As described in the methods, the transfer was substantially higher when assayed by determining the transfer of labeled phospholipid from vesicles to HDL.

The shifts in the distribution of mass in the HDL region are compared to the control distribution for each lipid and apoprotein of the LPL, LPL+HAL, LPL+LTP and LTP incubations (Figures 8-21). The shifts in HDL mass are of interest since the direction and magnitude of the shift may aid in the understanding of how HDL is affected by the various incubations and how mass is transferred from VLDL to HDL. For example, if there was a gain of mass in the light region of the gradient with a corresponding decrease in the dense region, it suggests that the VLDL mass was transferred to the original HDL particles. The particles became lighter because they gained more lipid relative to protein from VLDL. If there was a gain in the light region only without a corresponding decrease in the dense, it suggests that the VLDL mass was either transferred as a separate particle

which appeared in the HDL density which may exchange mass with the existing HDL or that the HDL in the light region preferentially gained the mass transferred from VLDL.

The results of two typical experiments that together contained all of the experimental conditions were used to demonstrate the changes in the density gradient profiles since not all of the experimental conditions were included in each experiment. The data from the 5 experiments were reported in terms of the gains and losses observed in the HDL region. The gains (or losses) in the HDL region were referred to rather than the losses (or gains) in the VLDL/LDL region because the change in the HDL mass was the focus of this section.

The data of the LPL incubation of experiment C are referred to in some cases in the text. However, the percentage of VLDL triglyceride hydrolysis of the LPL incubation was 63% rather than the 88% observed in the LPL+HAL and LPL+LTP incubations of experiment C. The data were not included when averages were calculated since the reduced amount of lipolysis was accompanied by a decrease in the amount of apoprotein CII and CIII mass transferred from VLDL to HDL (73.5 % and 68.1 %) as compared to the transfer observed for the LPL+HAL incubation (91.3 % and 100%) and the LPL+LTP incubation (90.9 and 96.7%). When listed in tables, the data for the LPL incubation of experiment C are included in parentheses to indicate that this incubation was dissimilar to the others.

In experiments involving the lipid transfer protein preparation, the preparation appeared to be free of lipid but apo AI was present in varying amounts in the lipid transfer protein preparations of experiments C, D and E as determined by comparing the recovery of apo AI in the LPL+LTP incubation and LTP incubation to that of the control incubation. Apo AI did not appear to be present in the lipid transfer protein preparation of experiment B as determined by recoveries. The lipid transfer protein preparation of experiment D contained a considerable amount of apo AI as well as some unesterified cholesterol, phospholipid, cholesteryl ester and apo CIII that had co-isolated. The

presence of excess lipid and apoproteins was also determined by inspection of the recoveries of lipid and apoproteins in the LPL+LTP and LTP incubations as compared to the control incubation of experiment D. The amount of lipid and apoprotein of the LPL+LTP and LTP incubation which was in excess relative to the mass in the control incubation of experiment D was assumed to indicate the amount of lipid and apoprotein present in the lipid transfer protein preparation and not due to a difference in recoveries. It was necessary to use the recoveries from the sum of the gradient fractions to determine the amount of excess lipid and apoprotein since it was not possible to obtain an accurate estimate from the lipid transfer preparation due to the small amount available for assay. In calculating the transfer of lipid or apo CIII, the amount present in the lipid transfer protein preparation was subtracted from the amount in the HDL region in experiment D. The data from experiment D were included in the averages because the amount of lipid or apoprotein transferred from VLDL to HDL did not appear to be affected by the presence of excess lipid and apo AI nor was the transfer directly related to the mass of apo AI present when comparing the results of experiment D with those of B, C and E. All data for the experiments are included in appendix A. The phospholipid and cholesteryl ester distributions of experiment D are shown in appendix C.

It was necessary to report the results of two experiments in order to include all of the experimental conditions. Therefore, two figures are included for each lipid or apoprotein distribution of experiment B and E. Experiments B and E were similar in that they both contained a control incubation, a LPL incubation and a LPL+LTP incubation. They differed in that experiment B included a LPL+HAL incubation while experiment E included a LTP incubation.

i) LIPID DISTRIBUTION.

For the lipid data, in order to demonstrate the gains and losses across the gradient, each figure contains an a) and b) section. Section a) depicts the density gradient profile of the absolute mass of the lipid in each fraction of the gradient. Section b) is a differential

plot reflecting changes in mass across the gradient of the experimental incubations when compared to the control incubation (example: change in mass of fraction 1 of the LPL incubation = [mass of fraction 1 of the LPL incubation - mass of fraction 1 in the control incubation]).

Summaries of the net gain or loss of the surface lipids (Table 10) and core lipids (Table 12) in the HDL regions of the gradient are listed. The data for the net gains or losses in the HDL region are averaged for the 5 controls, 4 LPL, 3 LPL+HAL, 4 LPL+LTP and 2 LTP incubations. To determine if the inclusion of hepatic lipase or the lipid transfer protein preparation had an effect on the results of the LPL incubation, an unpaired two sample t test was performed comparing the change in the HDL region of the LPL incubation to that of the LPL+HAL and to that of the LPL+LTP incubation. To compare the results of the LPL+LTP and the LTP incubation, an unpaired two sample t test was also performed.

For reference only, the mass in the VLDL/LDL region (fractions 1-7), the HDL region (fractions 1-20) and the total mass of each incubation of the experiments are listed in Tables 11 for the surface lipids (unesterified cholesterol and phospholipid) and in Table 13 for the core lipids (cholesteryl ester and triglyceride). The values in parentheses indicate how the mass differed from the corresponding mass of the control incubation.

UNESTERIFIED CHOLESTEROL: (Table 10 and 11, Figures 8 and 9)

The gain of unesterified cholesterol by HDL, as observed in the incubation containing lipoprotein lipase, was not affected by the addition of hepatic lipase to the incubation (Table 10). The average gain was +104 μg in the LPL+HAL experiments as compared to +109 μg in the LPL incubation.

TABLE 10

NET GAINS OR LOSSES OF SURFACE LIPIDS IN THE HDL REGIONS

The table contains the values for the net changes in surface lipid mass in the HDL region relative to that of the control incubation.

An unpaired t test was performed.

[1] Significance compared to the LPL incubation.

[2] Significance compared to the LPL+LTP incubation .

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein which was carried out for 2 hours at 37°C

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+HAL indicates the addition of hepatic lipase to the LPL incubation.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.
LTP indicates the addition of the lipid transfer protein preparation to the control incubation.

TABLE 10

NET GAINS OR LOSSES OF SURFACE LIPIDS IN THE HDL REGIONS

Incubations	Experiment A	B	C	D	E	Mean Change	SD
Unesterified Cholesterol			µg				
LPL	+106	+77	(+135)	+114	+139	+109	± 26
LPL+HAL	+92	+73	+146	——	——	+104	± 38 n.s.
LPL+LTP	——	+173	+208	+193	+227	+200	± 23 *** [1]
LTP	——	——	——	+34	+78	+56	± 31** [2]
Phospholipid							
LPL	+192	+186	(+271)	+377	+386	+285	± 111
LPL+HAL	+183	-167	+59	——	——	+25	± 177 * [1]
LPL+LTP	——	+419	+476	+682	+885	+615	± 212 * [1]
LTP	——	——	——	0	-68	-34	± 48** [2]

*** = p ≤ .005, ** = p ≤ .01, * = p≤ .05, n.s. = not significant

TABLE 11

THE DISTRIBUTION OF SURFACE LIPIDS
IN THE VLDL/LDL AND HDL REGIONS AND TOTAL MASS OF THE GRADIENT

All values are expressed in ug.

The conditions of the various experiments are outlined in Table 1.

V/L refers to the VLDL/LDL region, fractions 1-7

HDL region includes fractions 8-20

Total refers to the sum of the mass in fractions 1-20.

The values in brackets indicate how the value differed from the control value.

A net gain or loss in the VLDL/LDL or HDL regions can indicate either transfer or a difference in recovery of the experimental incubation from the control incubation.

A net gain or loss in the Total reflects a difference in recovery of the experimental incubation from the control incubation.

In the case of phospholipid, a loss of phospholipid can indicate hydrolysis as well as a difference in recovery from the control incubation in the VLDL/LDL or HDL region or in the Total.

[1] The LPL incubation of experiment C only attained 65% of VLDL triglyceride hydrolysis.

[2] The LPL+LTP incubation of Experiment D had an excess of + 171 µg of unesterified cholesterol. The LTP incubation had an excess of +185 µg cholesteryl ester and +498 µg of phospholipid. It was assumed that this represented mass in the lipid transfer protein preparation. A net excess of phospholipid in the LPL+LTP incubation was probably masked by hydrolysis of VLDL phospholipid in the presence of LPL.

TABLE 11
THE DISTRIBUTION OF SURFACE LIPIDS
IN THE VLDL/LDL AND HDL REGIONS AND TOTAL MASS OF THE GRADIENT

Incubation		Experiment A	B	C	D	E
Unesterified Cholesterol				μg		
Control	V/L	684	709	658	593	754
	HDL	259	329	397	307	361
	Total	943	1038	1055	900	1115
LPL	V/L	698 (+14)	547 (-162)	556 (-102)	489(-104)	616(-138)
	HDL	365 (+106)	406 (+77)	532 (+135)	421(+114)	500(+139)
	Total	1063(+120)	953 (-85)	1088(+33)[1]	910(+10)	1116(+1)
LPL+HAL	V/L	741 (+57)	596 (-113)	584 (-74)	—	—
	HDL	351 (+92)	402 (+73)	543 (+146)	—	—
	Total	1092(+149)	998 (-40)	1127(+72)	—	—
LPL+LTP	V/L	—	481 (-228)	509 (-149)	400(-193)	548(-206)
	HDL	—	502 (+173)	605 (+208)	671(+364)	588(+227)
	Total	—	983 (-55)	1114(+59)	1071(+171)[2]	1136(+21)
LTP		—	—	—	559(-34)	696(-58)
		—	—	—	526(+219)	439(+78)
		—	—	—	1085(+185)[2]	1135(+20)
Phospholipid						
Control	V/L	2316	2617	2524	2494	3266
	HDL	2789	3115	3958	2789	4127
	Total	5105	5732	6482	5283	7393
LPL	V/L	1493 (-823)	1415 (-1202)	2082 (-442)	1502(-992)	1846(-1420)
	HDL	2981 (+192)	3301 (+186)	4229 (+271)	3166(+377)	4513(+386)
	Total	4474 (-631)	4716 (-1016)	6311 (-171)[1]	4668(-615)	6359(-1034
LPL+HAL	V/L	1570 (-746)	1531 (-1086)	1665 (-859)	—	—
	HDL	2972 (+183)	2948 (-167)	4017 (+59)	—	—
	Total	4542 (-563)	4479 (-1253)	5683 (-800)	—	—
LPL+LTP	V/L	—	1266 (-1351)	1479(-1045)	1298(-1196)	1785(-1481)
	HDL	—	3534 (+419)	4434(+476)	3969(+1180)	5012(+885)
	Total	—	4800 (-932)	5913(-569)	5267(-16)[2]	6797(-596)
LTP	V/L	—	—	—	2525 (+31)	3249 (-17)
	HDL	—	—	—	3256 (+467)	4059 (-68)
	Total	—	—	—	5781(+498)[2]	7308 (-85)

FIGURE 8

UNESTERIFIED CHOLESTEROL DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE EXPERIMENT **B** INCUBATION

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein which was carried out for 2 hours at 37°C

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+HAL indicates the addition of hepatic lipase to the LPL incubation.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.

The conditions of the incubations are represented as:

control: ○
LPL: ●
LPL+HAL: ×
LPL+LTP: ▲

Figure a shows the unesterified cholesterol mass distribution in the fractions isolated by density gradient ultracentrifugation.

Figure b shows the change in unesterified cholesterol mass in the fractions of the experimental incubations relative to the control. The value was calculated by subtracting the mass of a given fraction of the control incubation from the mass of the corresponding fraction of the experimental incubation.

FIGURE 8: UNESTERIFIED CHOLESTEROL DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE EXPERIMENT B INCUBATION

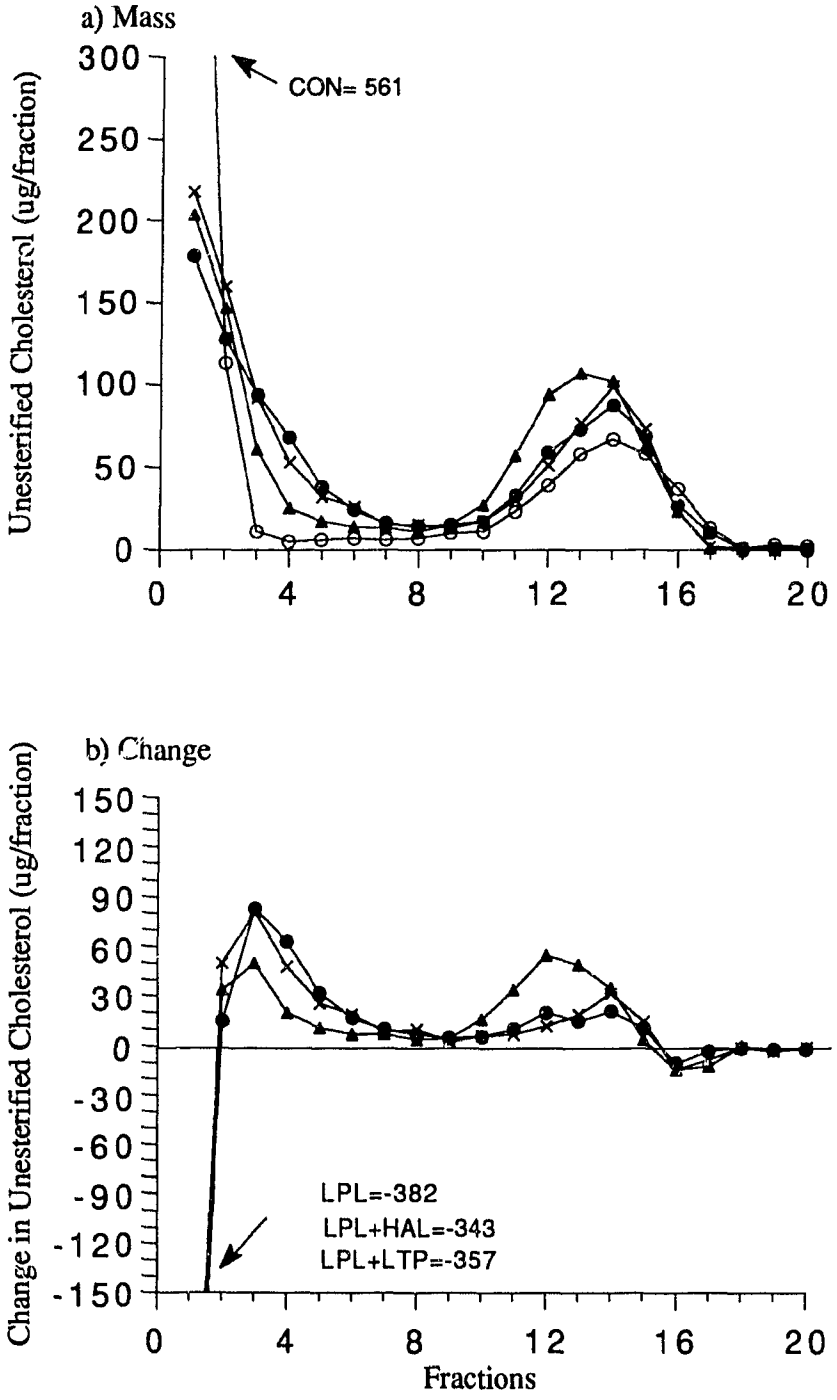


FIGURE 9

UNESTERIFIED CHOLESTEROL DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENTE

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein which was carried out for 2 hours at 37°C

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.
LTP indicates the addition of the lipid transfer protein preparation to the control incubation.

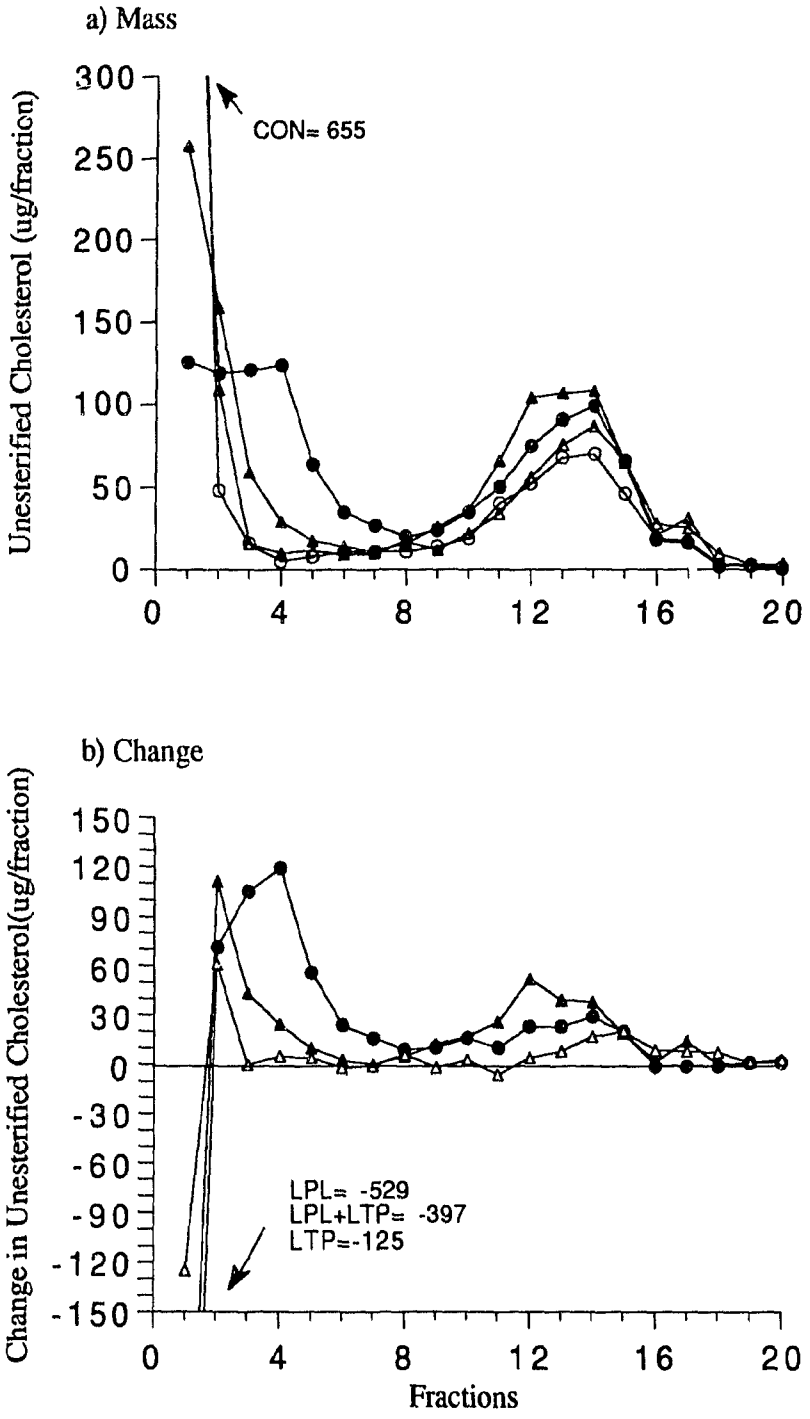
The conditions of the incubations are represented as:

control:	O
LPL:	●
LPL+LTP:	▲
LTP:	Δ

Figure a shows the unesterified cholesterol mass distribution in the fractions isolated by density gradient ultracentrifugation.

Figure b shows the change in unesterified cholesterol mass in the fractions of the experimental incubations relative to the control. The value was calculated by subtracting the mass of a given fraction of the control incubation from the mass of the corresponding fraction of the experimental incubation.

FIGURE 9: UNESTERIFIED CHOLESTEROL DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT E



The addition of LTP to the LPL incubation increased the amount of unesterified cholesterol transferred to HDL (Table 10). The amount was approximately double that transferred in the LPL incubation (an average of +200 μg in the LPL+LTP experiments) and this increased amount of transfer was significant ($p \leq .005$). The LTP incubation demonstrated an average gain of 56 μg which was significantly lower than that observed in the LPL+LTP incubation (Table 10).

The gain of unesterified cholesterol by HDL in the LPL incubation was on the light side (less dense) of the control peak with a smaller decrease in the dense region but the peak did not shift in position on the gradient in the experiments that did not gain cholesteryl ester as previously described.(Figure 8). In the experiments that gained cholesteryl ester, the HDL gained unesterified cholesterol in the light region of the control peak but a decrease in unesterified cholesterol mass was not observed in the dense region of HDL in most cases. A shift in the peak did not occur. The LPL+HAL incubation distributed in a manner similar to that of the LPL incubation although there was a tendency for the mass to maintain the position of the control incubation on the gradient more so than that observed with LPL (Figure 8).

The LPL+LTP incubation gained unesterified cholesterol mass on the light side of the control peak as was observed with the LPL incubation but a shift of the peak to a lighter density was also observed (Figure 8 and 9). The gain in the LTP incubation was on the dense side of the control HDL peak (Figure 9).

PHOSPHOLIPID: (Tables 10 and 11, Figures 10 and 11)

As previously reported, the LPL incubation resulted in a net gain of phospholipid in the HDL region in all LPL experiments (Table 10). The amount transferred differed between experiments as previously described in that it was greater in experiments D and E as compared to experiments A and B. The addition of hepatic lipase to the LPL incubation resulted in a significantly lower gain relative to the LPL incubation. The

FIGURE 10

PHOSPHOLIPID DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT B

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein which was carried out for 2 hours at 37°C

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+HAL indicates the addition of hepatic lipase to the LPL incubation.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.

The conditions of the incubations are represented as:

control: O
LPL: ●
LPL+HAL: ×
LPL+LTP: ▲

Figure a shows the phospholipid mass distribution in the fractions isolated by density gradient ultracentrifugation.

Figure b shows the change in phospholipid mass in the fractions of the experimental incubations relative to the control. The value was calculated by subtracting the mass of a given fraction of the control incubation from the mass of the corresponding fraction of the experimental incubation.

FIGURE 10: PHOSPHOLIPID DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT B

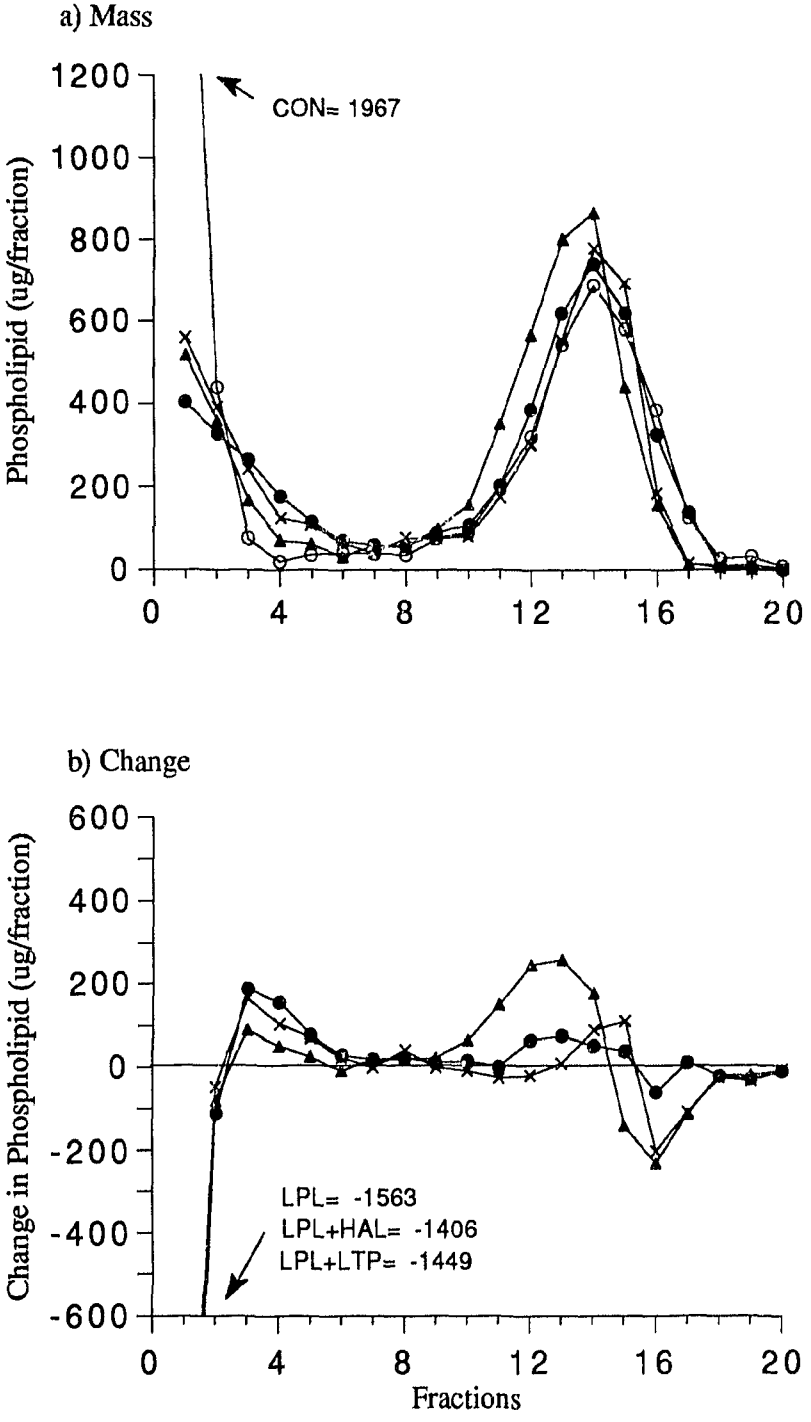


FIGURE 11

PHOSPHOLIPID DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT **E**

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein which was carried out for 2 hours at 37°C

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.
LTP indicates the addition of the lipid transfer protein preparation to the control incubation.

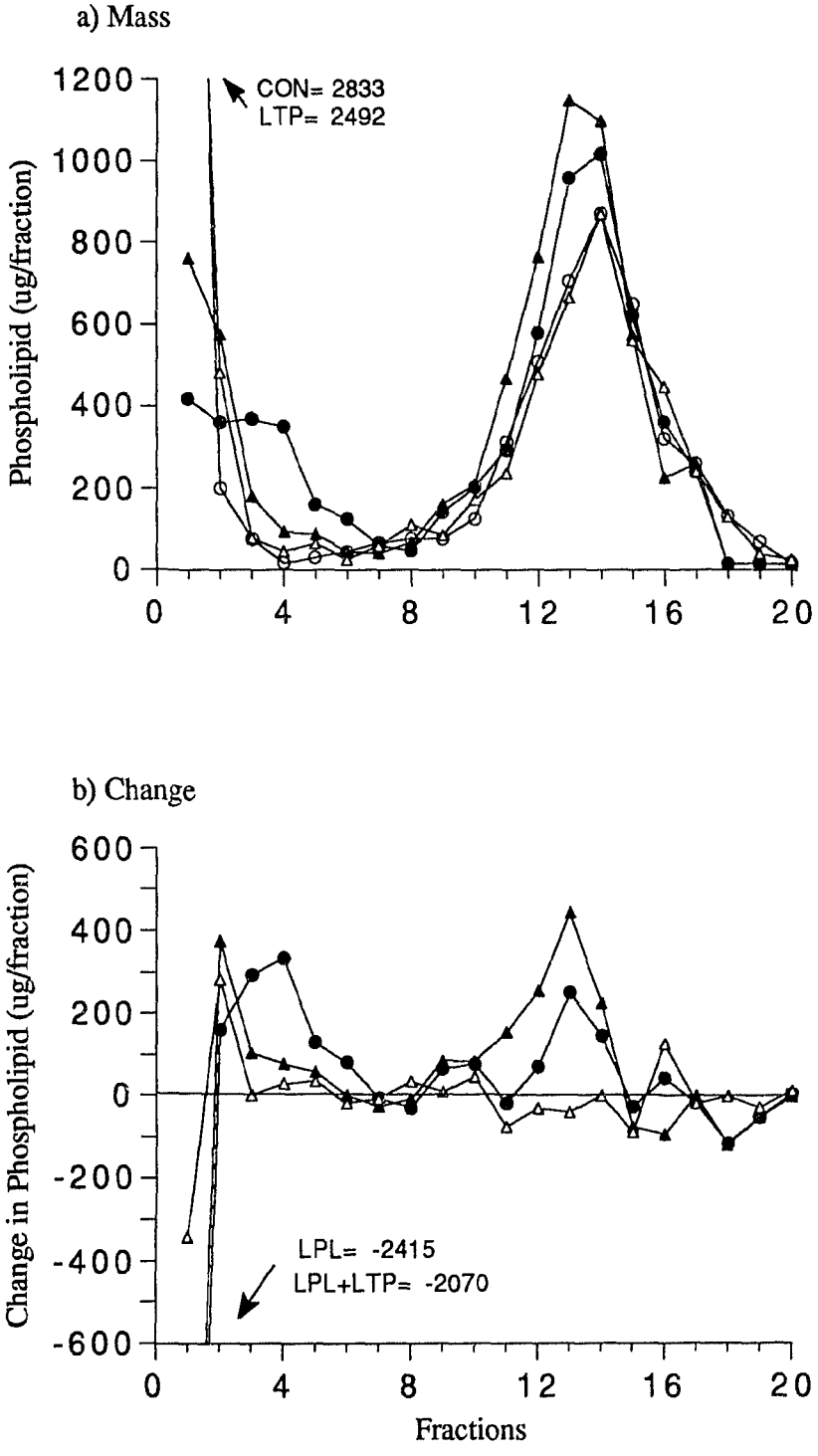
The conditions of the incubations are represented as:

control: ○
LPL: ●
LPL+LTP: ▲
LTP: △

Figure a shows the phospholipid mass distribution in the fractions isolated by density gradient ultracentrifugation.

Figure b shows the change in phospholipid mass in the fractions of the experimental incubations relative to the control. The value was calculated by subtracting the mass of a given fraction of the control incubation from the mass of the corresponding fraction of the experimental incubation.

FIGURE 11: PHOSPHOLIPID DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT E



LPL+HAL incubation showed a gain of phospholipid by HDL in experiment A similar to that observed with LPL alone. In experiment C, the amount gained by HDL was smaller than that of the LPL incubation (which in this experiment only attained 63% triglyceride hydrolysis). A net loss from the HDL region was observed in experiment B. From these results, it appears that some phospholipid hydrolysis had occurred in experiments B and C when hepatic lipase was included which was not observed in the LPL incubation. The amount of hydrolysis was difficult to estimate because HDL phospholipid hydrolysis by hepatic lipase may be masked by a gain due to lipoprotein lipase. If, in experiment B, it is assumed that in the LPL+HAL incubation lipoprotein lipase had stimulated transfer of phospholipid to HDL (+186 μg) but HDL phospholipid was hydrolysed by hepatic lipase (net loss = -167 μg) the total amount hydrolysed(186 μg +167 μg = -353 μg) would be 11% of the control HDL phospholipid mass. In experiment C the corresponding value (271 μg -59 μg = -212 μg) would be 5% of the control. The lack of phospholipid hydrolysis of experiment A may be due to the lower activity of the hepatic lipase preparation used in this experiment as compared to that of experiments B and C.

The addition of LTP to the LPL incubation resulted in a significant stimulation of phospholipid transfer which was twice the amount transferred in the LPL incubation (Table 10). The doubling of the amount of phospholipid transferred to HDL in the LPL+LTP incubation compared to the LPL incubation was maintained when the amount of phospholipid transferred in the LPL incubation increased in experiments D and E. The LTP incubation did not demonstrate transfer of VLDL phospholipid to HDL (Table 10).

The gain in HDL phospholipid in the LPL incubation was on the light side of the control peak (Figure 10 and 11). There was a small decrease in the dense region of HDL in all experiments. An actual shift in the HDL peak did not occur. The phospholipid gain in the LPL+HAL incubation centered on the control HDL peak rather than tending to the lighter side as in the LPL incubation (Figure 10). The loss occurred on the dense side of

control peak, in the very dense HDL fractions (fractions 16 -18). The combination of these effects tended to result in a narrowing of the HDL peak on the density gradient.

The gain in phospholipid in the LPL+LTP incubation was located on the light side of the control HDL peak as was observed in the LPL incubation (Figure 10 and 11). The distribution of the LPL+LTP incubation differed from the distribution of the LPL incubation as the peak shifted to a lighter fraction and a substantial decrease in phospholipid mass was observed in the dense fractions of the gradient. The decrease in phospholipid observed in fractions 15-18 in the various LPL+LTP experiments represents a shift in mass from the denser fractions into the lighter fractions. The phospholipid distribution of the LTP incubation was similar to the control HDL (Figure 11).

CHOLESTERYL ESTERS: (Tables 12 and 13, Figures 12 - 15)

The transfer of cholesteryl ester mass from VLDL to the HDL region was highly variable in the LPL incubation as described in the previous section (Table 12). Experiments A and B lost -59 µg and -51 µg in the HDL region while experiments D and E gained +121 µg and +430 µg. The addition of hepatic lipase had no significant effect when compared to that of LPL (mean change= -58 µg) (Table 12). LPL+HAL was not one of the conditions tested in the experiments D and E where a substantial gain of cholesteryl ester in the HDL region was observed in the LPL incubation.

The LPL+LTP incubation was not significantly different from the LPL incubation (mean change= +35 µg) (Table 12). There is evidence to suggest that there was a small amount of exchange of cholesteryl ester from HDL for triglyceride from VLDL in the LPL+LTP incubation in experiment D and E. For example in experiment E, which demonstrated a good recovery of cholesteryl ester in all incubations, there was a gain of cholesteryl ester in the HDL region of +430 µg in the LPL incubation, +177 µg in the LPL+LTP incubation but a loss of -421 µg in the LTP incubation. The triglycerides in the HDL region increased by +33 µg in the LPL incubation, by +283 µg in the LPL+LTP incubation and by +525µg in the LTP incubation. The VLDL/LDL region showed a

TABLE 12

NET GAINS OR LOSSES OF CORE LIPIDS IN THE HDL REGIONS

The table contains the values for the net changes in core lipid mass in the HDL region relative to that of the control incubation.

An unpaired t test was performed.

[1] Significance compared to the LPL incubation.

[2] Significance compared to the LPL+LTP incubation .

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein which was carried out for 2 hours at 37°C

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+HAL indicates the addition of hepatic lipase to the LPL incubation.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.
LTP indicates the addition of the lipid transfer protein preparation to the control incubation.

TABLE 12
NET GAINS OR LOSSES OF CORE LIPIDS IN THE HDL REGIONS

Incubation	Experiment A	B	C	D	E	Mean Change	SD
Cholesteryl Esters			µg				
LPL	-59	-51	(-81)	+121	+430	+110	±229
LPL+HAL	-150	-7	-18	——	——	-58	±80 n.s.[1]
LPL+LTP	——	+48	-54	-32	+177	+35	±105 n.s.[1]
LTP	——	——	——	-286	-421	-354	± 96 ** [2]
Triglyceride							
LPL	-83	-43	(+45)	-55	+33	-37	±50
LPL+HAL	-339	-313	-396	——	——	-349	±43 *** [1]
LPL+LTP	——	-21	-9	+1	+283	+63	±147 n.s. [1]
LTP	——	——	——	+217	+525	+371	±218 n.s. [2]

*** = $p \leq .005$, ** = $p \leq .01$, n.s. = not significant

TABLE 13

DISTRIBUTION OF CORE LIPIDS
IN THE VLDL/LDL AND HDL REGIONS AND TOTAL
MASS OF THE GRADIENT

All values are expressed in µg.

The conditions of the various experiments are outlined in Table 1.

The V/L refers to the VLDL/LDL region which includes fractions 1-7

The HDL region includes fractions 8-20

Total refers to the sum of the mass in fractions 1-20.

The values in brackets indicate how the value differed from the control value.

A net gain or loss in the VLDL/LDL or HDL regions can indicate either transfer or a difference in recovery of the experimental incubation from the control incubation.

A net gain or loss in the Total reflects a difference in recovery of the experimental incubation from the control incubation.

A net gain or loss in cholesteryl ester reflects a difference in recovery from the control incubation.

A net loss of triglyceride indicates hydrolysis as well as a difference in recovery from the control incubation.

[1] The LPL incubation of experiment C only attained 65% of VLDL triglyceride hydrolysis.

[2]The LTP incubation had an excess of +88 µg cholesteryl ester. It was assumed that +88 µg represented mass in the lipid transfer protein preparation.

TABLE 13
DISTRIBUTION OF CORE LIPIDS IN THE VLDL/LDL AND HDL REGIONS AND TOTAL MASS OF THE GRADIENT

Conditions		Experiment A	B	C	D	E
Cholesteryl Ester				µg		
Control	V/L	1939	1541	1517	1698	2096
	HDL	2046	2669	2402	2256	2913
	Total	3985	4210	3919	3954	5009
LPL	V/L	1987 (+48)	1220 (-321)	1406 (-111)	1209 (-489)	1586 (-510)
	HDL	1987 (-59)	2618 (-51)	2321 (-81)	2377 (+121)	3343 (+430
	Total	3974 (-11)	3838 (-372)	3727 (-192)[1]	3586 (-368)	4929 (-80)
LPL+HAL	V/L	2009 (+70)	1309 (-232)	1408 (-109)	—	—
	HDL	1896 (-150)	2662 (-7)	2384 (-18)	—	—
	Total	3905 (-80)	3971 (-239)	3792 (-127)	—	—
LPL+LTP	V/L	—	1285 (-256)	1451 (-66)	1336 (-362)	1995 (-101)
	HDL	—	2717 (+48)	2348 (-54)	2312 (+56)	3090 (+177
	Total	—	4002 (-208)	3799 (-120)	3648(-306)[2]	5085 (+76)
LTP		—	—	—	1984 (+286)	2554(+458
		—	—	—	2058 (-198)	2492(-421)
		—	—	—	4042(+88)[2]	5046(+37)
Triglyceride						
Control	V/L	4350	3946	4827	5423	6054
	HDL	472	428	699	593	728
	Total	4822	4374	5526	6016	6782
LPL	V/L	373 (-3977)	437 (-3509)	1782 (-3045)	396 (-5027)	427(-5627
	HDL	389 (-83)	385 (-43)	744 (+45)	538 (-55)	761 (+33)
	Total	762 (-4060)	822 (-3552)	2526(-3000)[1]	934 (-5082)	1188(-559
LPL+HAL	V/L	392 (-3957)	494 (-3452)	598 (-4229)	—	—
	HDL	133 (-339)	115 (-313)	303 (-396)	—	—
	Total	526 (-4296)	609 (-3765)	901 (-4625)	—	—
LPL+LTP	V/L	—	434 (-3512)	568 (-4259)	573(-4850)	630(-5424
	HDL	—	407 (-21)	690 (-9)	594(+1)	1011(+283)
	Total	—	841 (-3533)	1258 (-4268)	1167(-4849)	1641(-5141
LTP	V/L	—	—	—	4735 (-688)	5512(-542)
	HDL	—	—	—	810(+217)	1286(+525
	Total	—	—	—	5545 (-471)	6798(+16)

FIGURE 12

CHOLESTERYL ESTER DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT **B**

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein and was carried out for 2 hours at 37°C

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+HAL indicates the addition of hepatic lipase to the LPL incubation.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.

The conditions of the incubations are represented as:

control: O
LPL: ●
LPL+HAL: X
LPL+LTP: ▲

Figure a shows the cholesteryl ester mass distribution in the fractions isolated by density gradient ultracentrifugation.

Figure b shows the change in phospholipid mass in the fractions of the experimental incubations relative to the control. The value was calculated by subtracting the mass of a given fraction of the control incubation from the mass of the corresponding fraction of the experimental incubation.

FIGURE 12: CHOLESTERYL ESTER DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT**B**

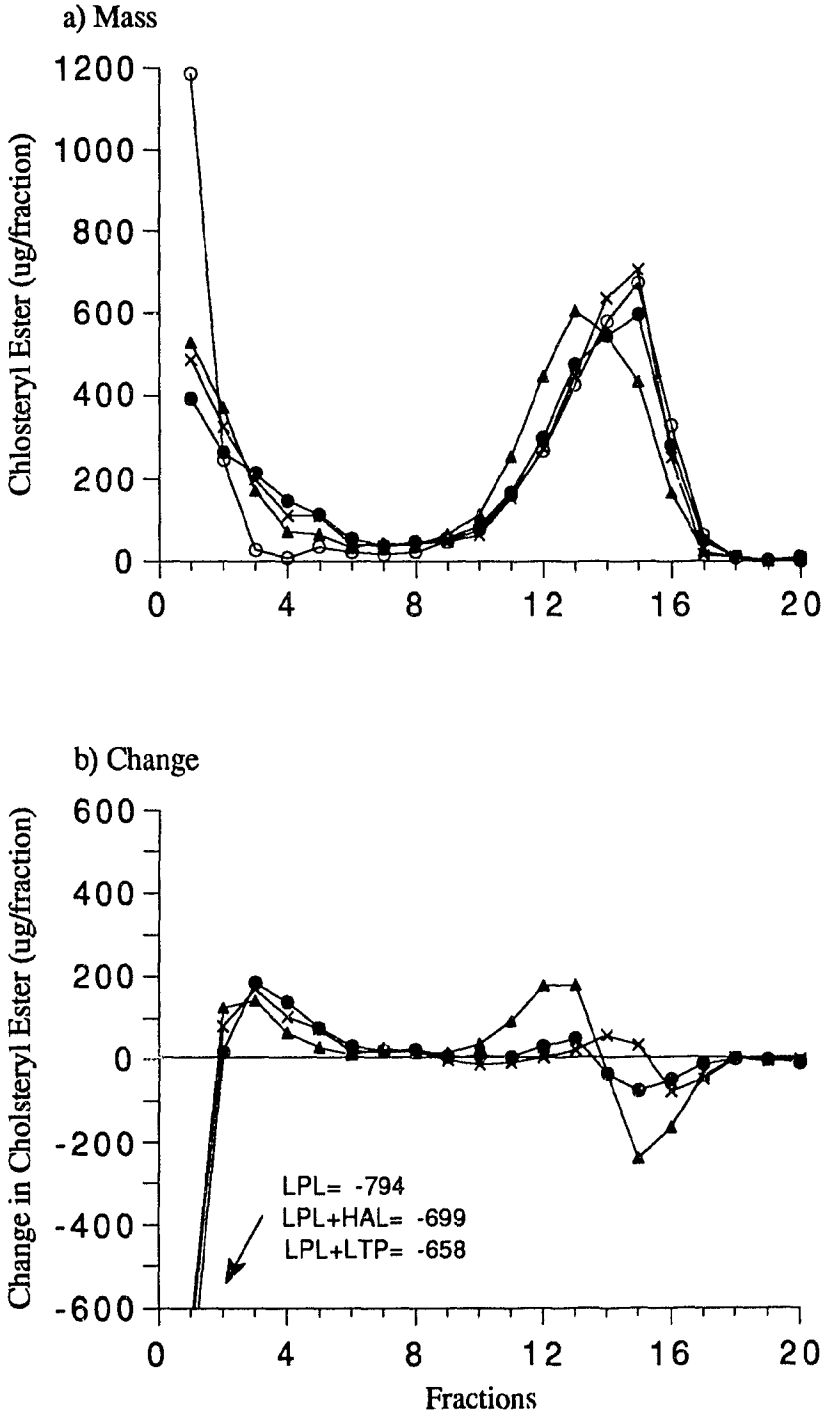


FIGURE 13

CHOLESTERYL ESTER DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT E

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein which was carried out for 2 hours at 37°C

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.
LTP indicates the addition of the lipid transfer protein preparation to the control incubation.

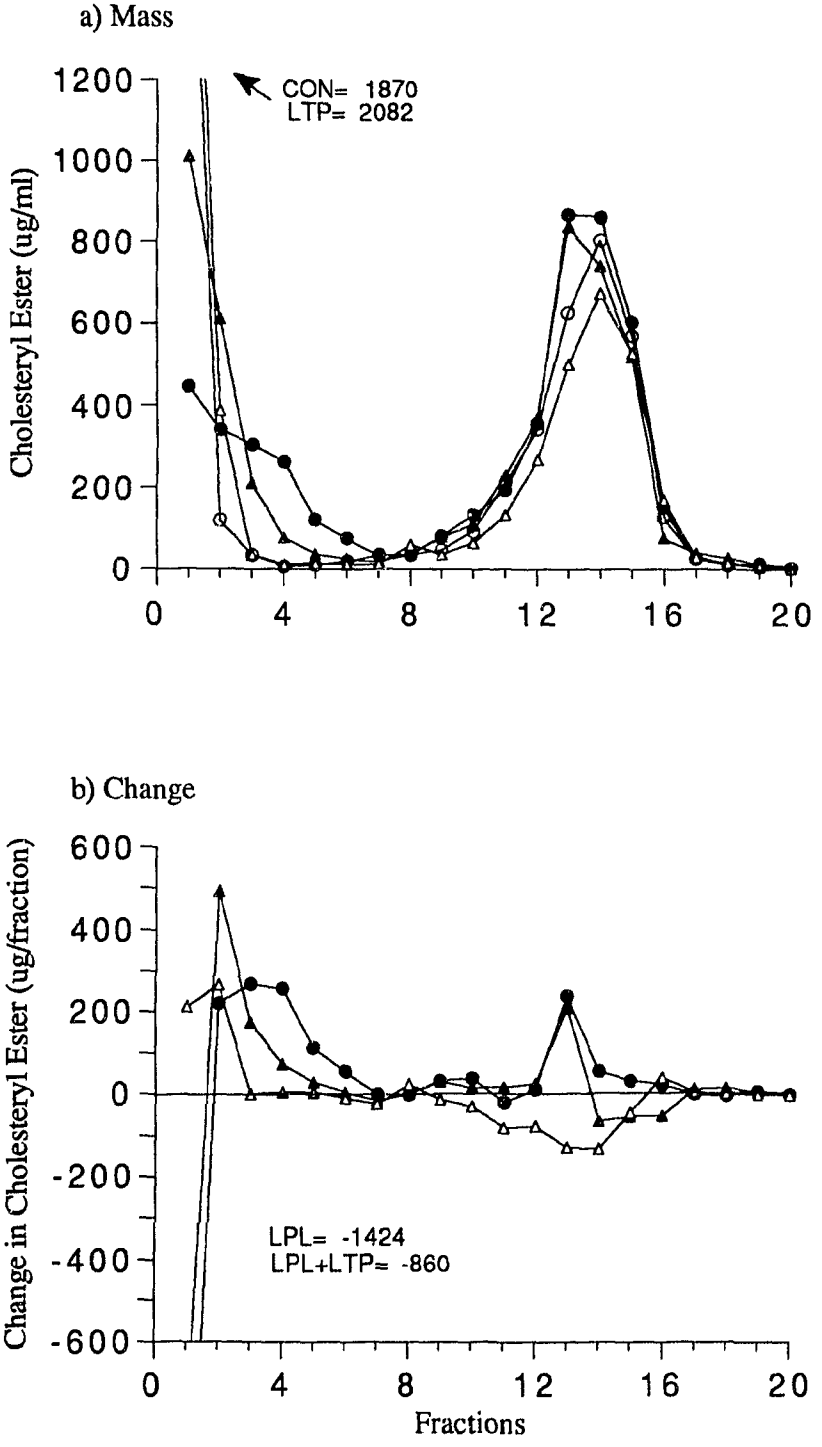
The conditions of the incubations are represented as:

control: ○
LPL: ●
LPL+LTP: ▲
LTP: △

Figure a shows the cholesteryl ester mass distribution in the fractions isolated by density gradient ultracentrifugation.

Figure b shows the change in cholesteryl ester mass in the fractions of the experimental incubations relative to the control. The value was calculated by subtracting the mass of a given fraction of the control incubation from the mass of the corresponding fraction of the experimental incubation.

FIGURE 13: CHOLESTERYL ESTER DISTRIBUTION IN THE LIPOPROTEIN FRACTION FOR THE INCUBATIONS OF EXPERIMENT E



loss of cholesteryl ester of -510 μg in the LPL incubation, -100 μg in the LPL+LTP incubation but a gain of 458 μg in the LTP incubation. A change in triglycerides in the VLDL/LDL region due to factors other than triglyceride hydrolysis could not be established for the LPL and LPL+LTP incubation but the triglycerides in the VLDL/LDL region decreased in the LTP incubation (-542 μg) and corresponded to the gain of triglycerides observed in the HDL region. These differences in cholesteryl ester movement between VLDL and HDL indicate that there was substantial transfer of cholesteryl esters from the HDL region to the VLDL/LDL region in the LTP incubation. However this transfer was reversed in the LPL incubations. The net gain of cholesteryl ester by HDL in the LPL+LTP incubation indicates: 1) that the transfer of cholesteryl ester to the VLDL/LDL regions observed with LTP alone was reduced in the presence of lipoprotein lipase; and 2) that lipoprotein lipase was still effective in causing a transfer to the HDL region although the amount transferred was less than that observed with lipoprotein lipase alone.

The cholesteryl ester distribution in the HDL region of the LPL incubation was similar to that of the control in experiments where there was little change in the cholesteryl ester mass in the HDL region (experiment A and B) (Figures 12 and 13). There was slightly more cholesteryl ester mass on the light side of the control peak with a small loss in the dense region. The gain of cholesteryl ester on the light side of the control peak was more apparent in experiments D and E which demonstrated a substantial gain in cholesteryl esters in the LPL incubation (Figure 13). A loss of cholesteryl ester mass in the dense region of the control peak was not observed in this case. The distribution of cholesteryl esters of the LPL+HAL incubation was similar to the LPL incubation but the peak tended to be narrower (Figure 12).

The cholesteryl ester mass in the LPL+LTP incubations increased in the light regions of the HDL gradient but decreased in the dense regions relative to the control distribution (Figure 12 and 13). This shift in the cholesteryl ester mass of the LPL+LTP

incubation was similar to that observed for the surface lipid, unesterified cholesterol and phospholipids, irrespective of whether cholesteryl ester was gained or lost in the HDL region of the LPL+LTP incubation. The gain of cholesteryl ester in the light region with a loss in the dense region is indicative of a shift in the original HDL mass.

In order to further define the extent of accumulation of cholesteryl ester in the HDL region in the LPL incubations, VLDL labeled with [^{3}H] cholesteryl ester was used in experiments B-E. Figures 14 and 15 contain the distributions of the [^{3}H] cholesteryl ester of experiment B and E, respectively. Each figure contains 3 sections which show: a) the distribution of the radioactive counts; b) the percentage distribution of the counts; c) the specific activity for each fraction. The results indicate that a very small amount of label appears in the HDL region in the control incubation. There was a small increase of label as compared to the control (4.74% versus 4.04% in experiment B and 7.25% versus 5.7% in experiment C) when there was no net gain of cholesteryl ester mass measured in the HDL region in the LPL incubation. The increases in label would be equivalent to a gain of 11 and 23 μg of VLDL cholesteryl ester in the HDL region. The gain of label by HDL relative to the control was greater in experiments D and E which demonstrated a substantial gain in mass in the HDL region as compared to experiments A and B which did not record a gain in cholesteryl ester mass (experiment D gained 8.3% versus 4.7% in the control and experiment E gained 7.6% versus 2.7% in the control). The gain in label did not precisely parallel the gain in mass. There was a marginal change in the label in the LPL+HAL incubations (3.84% in experiment B and 8.38% in experiment C) equivalent to a loss of 3 μg and a gain of 40 μg in the HDL region, respectively. The use of the label suggests that there was a trend of cholesteryl ester gain in the HDL region which was not observed when mass was determined in some experiments.

In the LTP incubations (experiments D and E), there was a substantial amount of label that appeared in the HDL region (Figure 15) despite the fact that there was a marked decrease in cholesteryl ester mass in the HDL region and a corresponding gain by the

FIGURE 14

[^{3}H] CHOLESTERYL ESTER DISTRIBUTION AMONG THE LIPOPROTEINS OF THE INCUBATIONS OF EXPERIMENT B

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein and was carried out for 2 hours at 37°C. VLDL was labeled with [^{3}H] cholesteryl esters and the distribution of the label was determined.

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+HAL indicates the addition of hepatic lipase to the LPL incubation.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.

The conditions of the incubations are represented as:

control: O
LPL: ●
LPL+HAL: ×
LPL+LTP: ▲

The figures shows the [^{3}H] cholesteryl ester mass distribution in the fractions isolated by density gradient ultracentrifugation.
The distributions are expressed as counts per minute (cpm), percentage of the total cpm and the specific activity per fraction.

Specific activity was expressed as the cpm/μg cholesteryl ester for each fraction.

FIGURE 14: [^{3}H]CHOLESTERYL ESTER DISTRIBUTION AMONG THE LIPOPROTEINS OF THE INCUBATION OF EXPERIMENT **B**

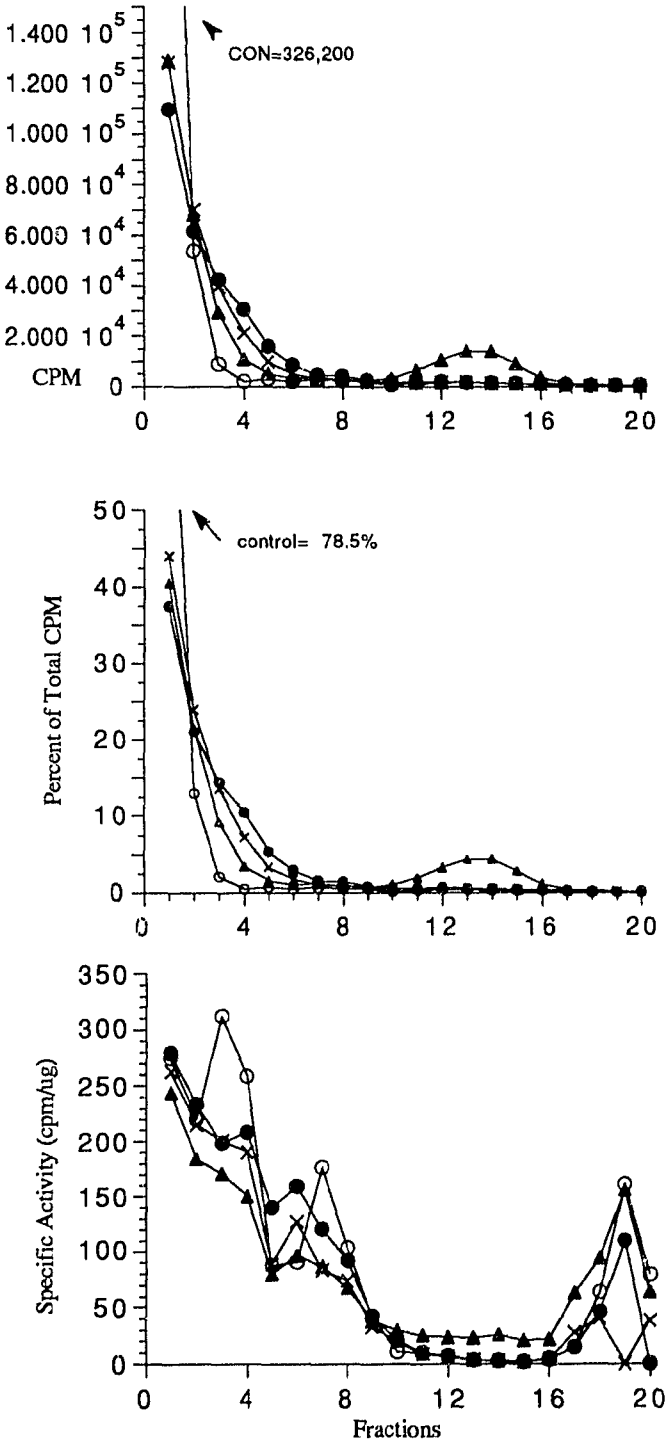


FIGURE 15

[^{3}H] CHOLESTERYL ESTER DISTRIBUTION AMONG THE LIPOPROTEINS OF THE INCUBATIONS OF EXPERIMENT E

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein and was carried out for 2 hours at 37°C. VLDL was labeled with [^{3}H]cholesteryl esters and the distribution of the label was determined

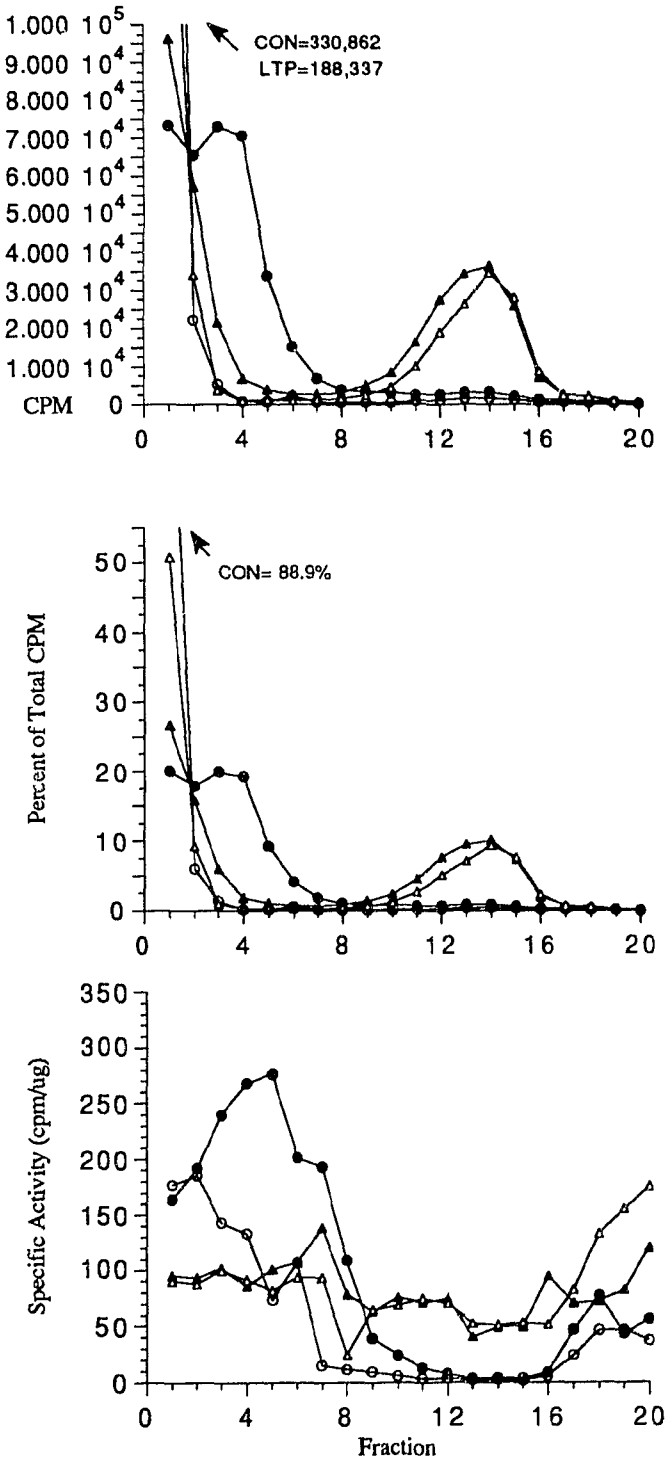
Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.
LTP indicates the addition of the lipid transfer protein preparation to the control incubation.

The conditions of the incubations are represented as:

control: O
LPL: ●
LPL+LTP: ▲
LTP: Δ

The figures shows the [^{3}H]cholesteryl ester mass distribution in the fractions isolated by density gradient ultracentrifugation.
The distributions are expressed as counts per minute (cpm), percentage of the total cpm and the specific activity per fraction.
Specific activity was expressed as the cpm/μg cholesteryl ester for each fraction.

FIGURE 15: [^{3}H]CHOLESTERYL ESTER DISTRIBUTION AMONG THE LIPOPROTEINS OF THE INCUBATIONS OF EXPERIMENT E



VLDL/LDL region (Table 12 and Figure 13). The presence of label in the HDL region in the LTP incubation indicates that there was an exchange process occurring between VLDL and HDL cholesteryl esters which allowed for equilibration of the label. The decrease in cholesteryl ester mass in HDL and gain by VLDL reflects the net transfer process. When lipoprotein lipase was included in the incubation (LPL+LTP incubation), more label was observed in the HDL region than with the lipid transfer protein alone (LTP incubation). The greater amount of label in the HDL region for the LPL+LTP incubation as compared to the LTP incubation is indicative of a greater cholesteryl ester mass of VLDL in the HDL region since the label appeared to be equilibrated (Figure 15).

The explanation for the difference between the LPL+LTP and the LTP incubation is that there are two processes that are active in the LPL+LTP incubation. With lipoprotein lipase alone (LPL incubation) there was a gain of mass in the HDL region (+430 μg) while the lipid transfer protein preparation alone (LTP incubation) caused a transfer of cholesteryl ester mass from HDL to VLDL(-421 μg) (Table 12). When these conditions were combined, the lipid transfer protein caused some transfer of cholesteryl ester from HDL to VLDL but the transfer was significantly reduced as the particles were lipolysed and the effect of lipoprotein lipase on the transfer of cholesteryl ester from VLDL to HDL was observed. The two opposing processes resulted in a net gain of +177 μg of cholesteryl ester by the HDL region in the LPL+LTP incubation rather than a gain of +430 μg in the LPL incubation or a loss of -421 μg in the LTP incubation.

TRIGLYCERIDES: (Tables 12, 13 and 14, Figures 16 and 17)

Lipoprotein lipase had little effect on HDL triglycerides as described in the previous section. Hepatic lipase exhibited its most remarkable effect in the LPL+HAL incubations where an extensive hydrolysis of HDL triglycerides was observed (Table 12). The decrease in triglycerides in the LPL+HAL incubation was highly significant relative to the LPL incubation (Table 12). Based on the recoveries in the HDL region in the LPL+HAL incubation, the percentage of hydrolysis was 71.8%, 73.1% and 56.7% for

TABLE 14

HYDROLYSIS OF HDL TRIGLYCERIDES IN THE LPL+HAL INCUBATION

Fraction	Experiment A			B			C		
Fraction #	Control µg	LPL+HAL µg	% Loss	Control µg	LPL+HAL µg	% Loss	Control µg	LPL+HAL µg	% Loss
8	18	1	94	5	1	80	11	1	90.9
9	9	5	10.5	35	0	100	21	13	38.1
10	3	3	0	16	2	87.5	43	20	53.5
11	31	23	25.8	45	10	77.8	30	29	3.3
12	46	15	67.4	50	17	66	115	25	78.3
13	114	31	72.8	58	13	77.6	139	47	66.2
14	113	25	77.9	78	18	76.9	161	108	32.9
15	83	20	75.9	96	25	74	113	45	60.2
16	35	0	100	27	15	44	24	2	91.7
17	16	0	100	10	9	10	33	3	90.9
18	2	6		5	5		9	10	
19	2	3		3	0		0	0	
20	0	1		0	0		0	0	

The conditions of the various experiments are outlined in Table 1.

The percentage hydrolysis was calculated from the loss in a given fraction of the LPL+HAL incubation as compared to that of control incubation, divided by the mass in the control incubation of that fraction.

The percentage hydrolysis was not calculated for fractions 18, 19 and 20 due to the small mass of triglycerides in these fractions.

FIGURE 16

TRIGLYCERIDE DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT **B**

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein and was carried out for 2 hours at 37°C

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+HAL indicates the addition of hepatic lipase to the LPL incubation.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.

The conditions of the incubations are represented as:

control: O
LPL: ●
LPL+HAL: ×
LPL+LTP: ▲

Figure a shows the triglyceride mass distribution in the fractions isolated by density gradient ultracentrifugation.

Figure b shows the change in triglyceride mass in the fractions of the experimental incubations relative to the control. The value was calculated by subtracting the mass of a given fraction of the control incubation from the mass of the corresponding fraction of the experimental incubation.

FIGURE 16: TRIGLYCERIDE DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT B

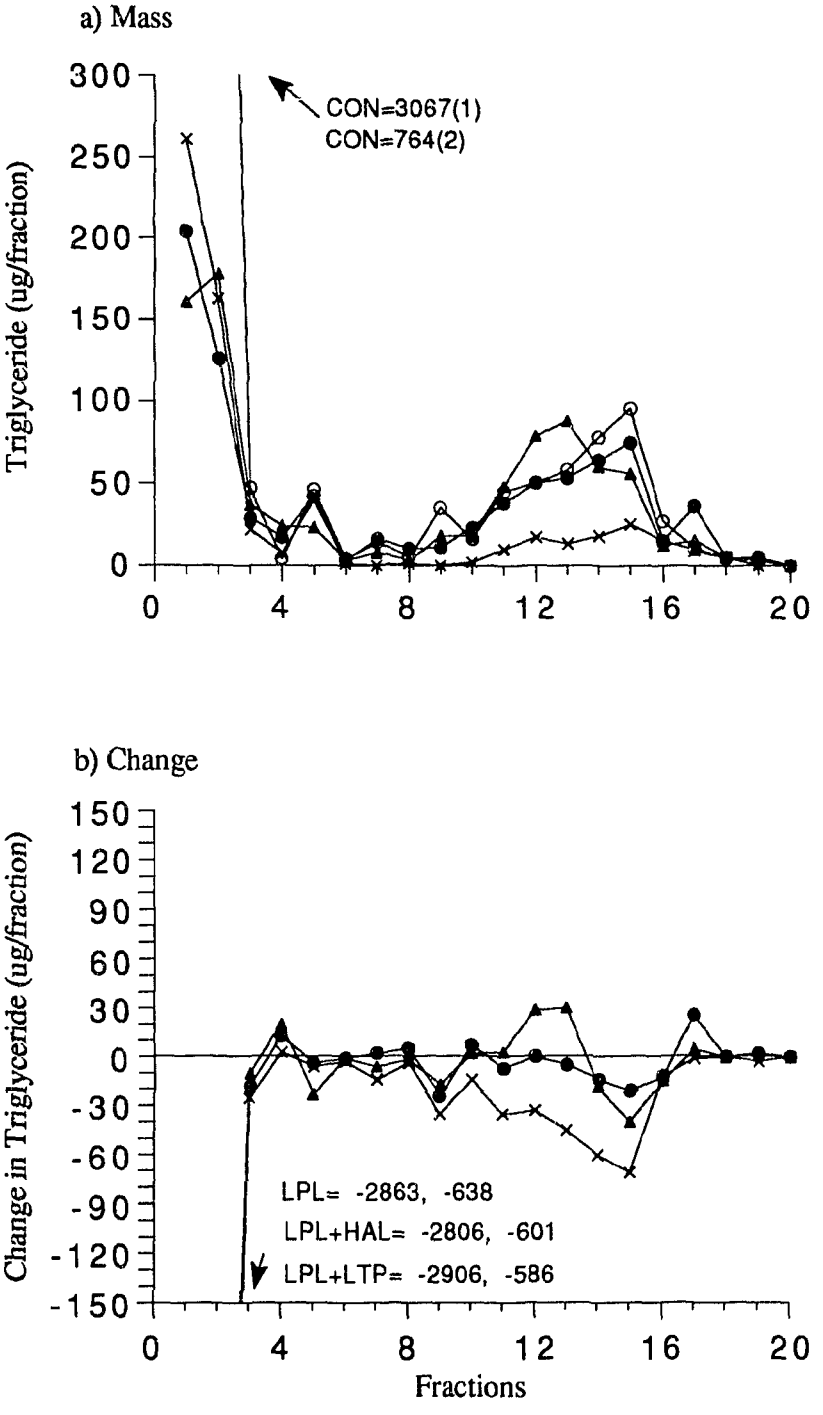


FIGURE 17

TRIGLYCERIDE DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT E

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein which was carried out for 2 hours at 37°C

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.
LTP indicates the addition of the lipid transfer protein preparation to the control incubation.

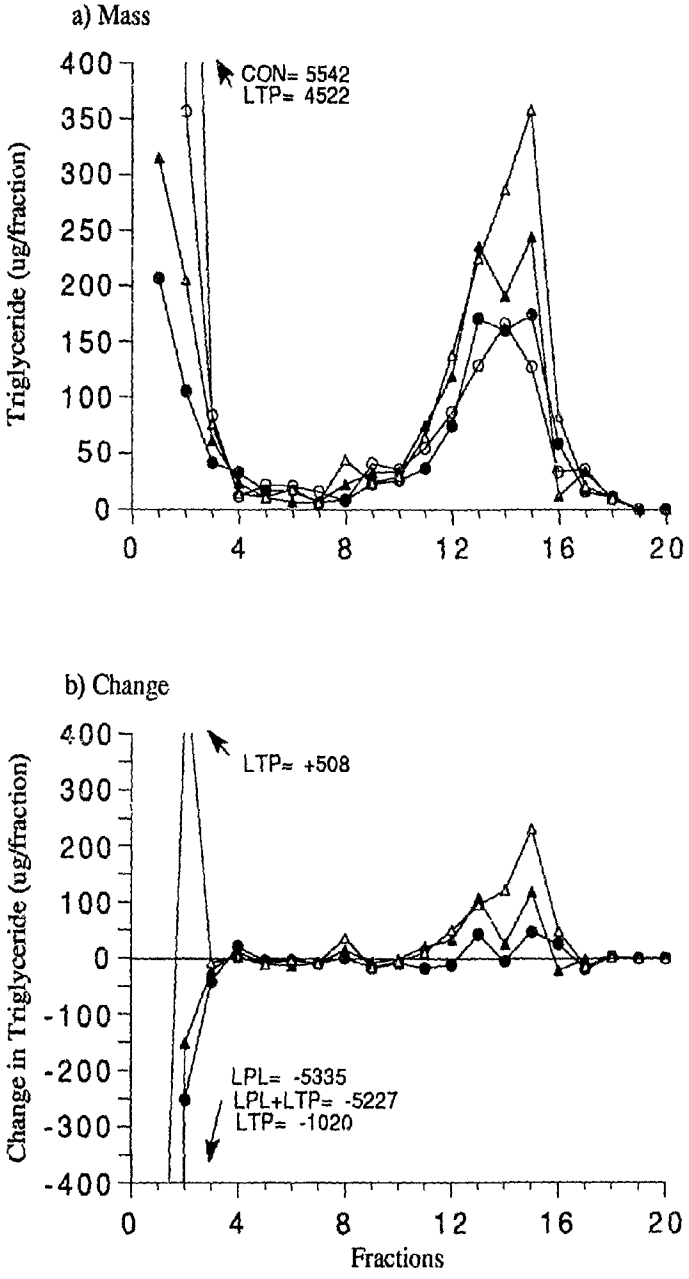
The conditions of the incubations are represented as:

control:	O
LPL:	●
LPL+LTP:	▲
LTP:	Δ

Figure a shows the triglyceride mass distribution in the fractions isolated by density gradient ultracentrifugation .

Figure b shows the change in triglyceride mass in the fractions of the experimental incubations relative to the control. The value was calculated by subtracting the mass of a given fraction of the control incubation from the mass of the corresponding fraction of the experimental incubation.

FIGURE 17: TRIGLYCERIDE DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT E



experiments A, B and C, respectively. The triglyceride hydrolysis occurred throughout the HDL region in the LPL+HAL incubation. On inspection of the percentage hydrolysis of the control triglyceride for each HDL fraction of the LPL+HAL incubations (Table 14, Figure 16), no clear trends could be observed in terms of a preferential region for hydrolysis. Experiment A and C tended to have the greatest percentage hydrolysis on the dense side of the peak while the opposite appeared to occur in experiment B.

The mean gain of triglyceride (+63 μg) in HDL for the LPL+LTP incubation was not significant relative to the LPL incubation (Table 12). The LTP incubation demonstrated a large gain of triglyceride by HDL in both experiments (+217 μg and +525 μg). Due to the high variability in the data, the gain was not was significantly different from the LPL+LTP incubation. The gain of triglyceride and a comparable loss of cholesteryl esters from the HDL region in the LTP incubation is indicative of the hetero exchange/transfer activity of CETP (Table 12, Figures 13 and 17).

ii) APOPROTEIN DISTRIBUTIONS:

Table 15 contains the net gains of apo CII, CIII and apo E in the HDL regions. The data for the net gains or losses in the HDL region are averaged for the 5 controls, 4 LPL, 3 LPL+HAL, 4 LPL+LTP and 2 LTP incubations. To determine if the inclusion of hepatic lipase or the lipid transfer protein preparation had any effect on the results of the LPL incubation, a unpaired t test was performed comparing the change in the LPL incubation to that of the LPL+HAL and comparing the change in the LPL to that of the LPL+LTP. An unpaired t test was performed to compare the change of the LPL+LTP incubations to that of the LTP incubation.

For reference only, the mass in the VLDL/LDL region (fractions 1-7), the HDL region (fractions 1-20) and the total mass of each incubation are listed for all five experiments for apo CII and apo CIII in Table 16 and for apo E, AI and Apo B in Table 17. The values in parentheses indicate how the mass differed from the corresponding mass of the control incubation.

TABLE 15

NET GAINS OR LOSSES OF APO CII, CIII AND APO E IN THE HDL REGIONS

The table contains the values for the net changes in apo CII, CIII and apo E in the HDL region relative to that of the control incubation.

An unpaired t test was performed.

[1] Significance compared to the LPL incubation.

[2] Significance compared to the LPL+LTP incubation .

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein which was carried out for 2 hours at 37°C

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+HAL indicates the addition of hepatic lipase to the LPL incubation.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.
LTP indicates the addition of the lipid transfer protein preparation to the control incubation.

TABLE 15

NET GAINS OR LOSSES IN THE HDL REGIONS
APO CII, CIII AND E

Incubations	Experiment A	B	C	D	E	Mean Change	SD
Apo CII			μg				
LPL	+134	+173	(+128)	+237	+233	+194	± 50
LPL+HAL	+134	+194	+177	——	——	+168	± 31 n.s. [1]
LPL+LTP	——	+247	+170	+241	+245	+226	± 37 n.s. [1]
LTP	——	——	——	+7	+21	+ 14	± 10 *** [2]
Apo CIII							
LPL	+560	+284	(+169)	+401	+452	+424	± 115
LPL+HAL	+644	+306	+345	——	——	+432	± 185 n.s. [1]
LPL+LTP	——	+343	+383	+504	+474	+426	± 76 n.s. [1]
LTP	——	——	——	-38	-18	-28	± 14 *** [2]
Apo E							
LPL	+90	+83	(+36)	+74	+52	+75	± 17
LPL+HAL	+111	+142	+66	——	——	+106	± 38 n.s. [1]
LPL+LTP	——	+131	+90	+120	+117	+115	±17 ** [1]
LTP	——	——	——	+39	+35	+37	± 3 * [2]

*** p≤ .005, ** = p≤ .01, * = p ≤ .05, n.s. = not significant

TABLE 16

DISTRIBUTION OF APO CII AND CIII IN THE VLDL/LDL AND HDL REGIONS AND THE TOTAL MASS OF THE GRADIENT

All values are expressed in μg.

The conditions of the various experiments are outlined in Table 1.

The V/L refers to the VLDL/LDL region, fractions 1-7.

The HDL region includes fractions 8-20.

Total refers to the sum of the mass in fractions 1-20.

The values in brackets indicate how the value differed from the control value.

A net gain or loss in the VLDL/LDL or HDL regions can indicate either transfer or a difference in recovery of the experimental incubation from the control incubation.

A net gain or loss in the Total reflects a difference in recovery of the experimental incubation from the control incubation.

[1] The LPL incubation of experiment C only attained 65% of VLDL triglyceride hydrolysis.

[2] The LPL+LTP incubation had an excess of + 397 μg of apo CIII. The LTP incubation had an excess of +220 μg of apo CIII. It was assumed +220 represented mass in the lipid transfer protein preparation.

TABLE 16
DISTRIBUTION OF APO CII AND CIII IN THE VLDL/LDL AND HDL REGIONS AND THE TOTAL MASS OF THE GRADIENT

Incubations		Experiment A	B	C	D	E
Apo CII				µg		
Control	V/L	148	249	275	318	270
	HDL	37	73	87	64	72
	Total	185	322	362	382	342
LPL	V/L	16 (-132)	20 (-229)	73 (-202)	10 (-308)	17 (-253)
	HDL	171 (+134)	256 (+173)	215 (+128)	301 (+237)	305 (+233)
	Total	187 (+2)	276 (-46)	288 (-74)[1]	311 (-71)	322 (-20)
LPL+HAL	V/L	16 (-132)	19 (-230)	24 (-251)	—	—
	HDL	171 (+134)	267 (+194)	264 (+177)	—	—
	Total	187 (+2)	286 (-36)	288 (-74)	—	—
LPL+LTP	V/L	—	17 (-232)	25 (-250)	48 (-270)	41 (-229)
	HDL	—	320 (+247)	257 (+170)	305 (+241)	317 (+245)
	Total	—	337 (+15)	282 (-80)	353 (-29)[2]	358 (+16)
LTP		—	—	—	313 (-5)	254 (-16)
		—	—	—	71 (+7)	93 (+21)
		—	—	—	384 (+2)[2]	347 (+5)
Apo CIII						
Control	V/L	605	367	338	408	529
	HDL	71	165	191	298	328
	Total	676	532	529	706	857
LPL	V/L	13 (-592)	2 (-365)	108 (-230)	0 (-408)	11 (-518)
	HDL	631 (+560)	449 (+284)	360 (+169)	699 (+401)	780 (452)
	Total	644 (-32)	451 (-81)	468 (-61)[1]	699 (-7)	791 (-66)
LPL+HAL	V/L	13 (-592)	2 (-365)	0 (-338)	—	—
	HDL	715 (+644)	471 (+306)	536 (+345)	—	—
	Total	728 (+52)	473 (- 59)	536 (+7)	—	—
LPL+LTP	V/L	—	2 (-365)	11 (-327)	81 (-327)	78 (451)
	HDL	—	508 (+343)	574 (+383)	1022 (+724)	802 (+474)
	Total	—	510 (-22)	585 (+56)	1103 (+397)[2]	880 (+23)
LTP	V/L	—	—	—	446 (+ 38)	540 (+11)
	HDL	—	—	—	480 (+182)	310 (-18)
	Total	—	—	—	926 (+220)[2]	850 (-7)

TABLE 17

THE DISTRIBUTION OF APO E AND APO AI
IN THE VLDL/LDL AND HDL REGIONS AND TOTAL MASS OF THE GRADIENT

All values are expressed in μg.

The conditions of the various experiments are outlined in Table 1.

The V/L refers to the VLDL/LDL region, fractions 1-7.

The HDL region includes fractions 8-20.

Total refers to the sum of the mass in fractions 1-20.

The values in brackets indicate how the value differed from the control value.

A net gain or loss in the VLDL/LDL or HDL regions can indicate either transfer or a difference in recovery of the experimental incubation from the control incubation.

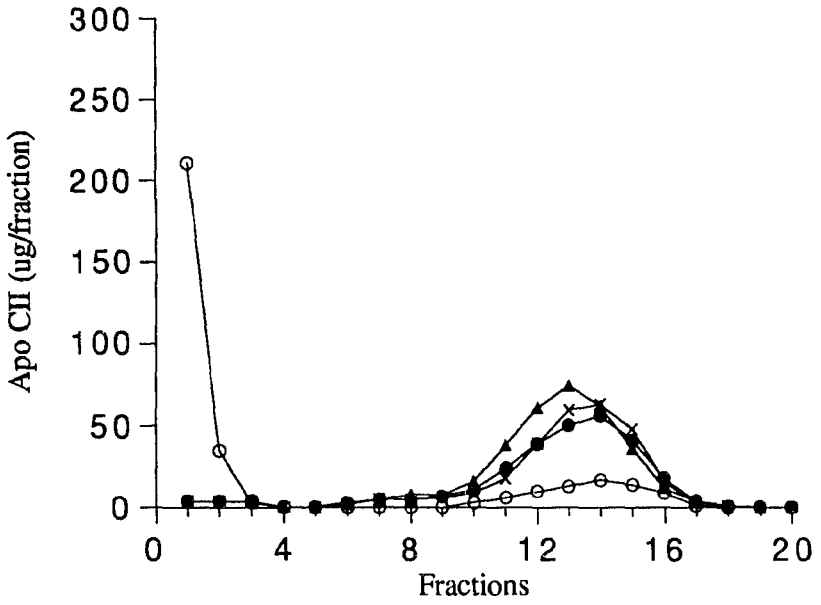
A net gain or loss in the Total reflects a difference in recovery of the experimental incubation from the control incubation except for the incubations containing the lipid transfer protein preparation which contained apo AI in some experiments.

[1] The LPL incubation of experiment C only attained 65% of VLDL triglyceride hydrolysis.

TABLE 17
THE DISTRIBUTION OF APO E AND APO AI IN THE VLDL/LDL AND HDL REGIONS AND TOTAL MASS OF THE GRADIENT

Incubations		Experiment A	B	C	D	E
				µg		
Apo E						
Control	V/L	420	292	336	335	358
	HDL	10	15	33	65	38
	Total	430	307	369	400	396
LPL	V/L	317 (-103)	208 (-84)	263 (-73)	275 (-60)	299 (-59)
	HDL	100 (+90)	98 (+83)	69 (+36)	139 (+74)	90 (+52)
	Total	417 (-13)	306 (-1)	332 (-37)[1]	414 (+14)	389 (-7)
LPL+HAL	V/L	335 (-85)	211 (-81)	292 (-44)	—	—
	HDL	121 (+111)	157 (+142)	99 (+66)	—	—
	Total	456 (+26)	368 (+61)	391 (+22)	—	—
LPL+LTP	V/L	—	151 (-141)	233 (-103)	218 (-117)	235 (-123)
	HDL	—	146 (+131)	123 (+90)	185 (+120)	155 (+117)
	Total	—	297 (-10)	356 (-13)	403 (+3)	390 (-6)
LTP		—	—	—	314 (-21)	339 (-19)
		—	—	—	104 (+39)	72 (+35)
		—	—	—	418 (+18)	411 (+15)
Apo AI						
Control	V/L	94	113	187	302	238
	HDL	6437	7633	6917	7437	7964
	Total	6531	7746	7104	7739	8202
LPL	V/L	89	100	82	214	96
	HDL	6459	6980	6426	6730	6808
	Total	6548 (+17)	7080 (-666)	6508 (-596)[1]	6944 (-795)	6904(-1298
LPL+HAL	V/L	90	74	52	—	—
	HDL	6381	7092	6654	—	—
	Total	6471 (-60)	7166 (-580)	6706 (-398)	—	—
LPL+LTP	V/L	—	106	71	177	108
	HDL	—	7666	8822	20969	14084
	Total	—	7772 (+26)	8893(+1789)	21146 (+13407)[2]	14192 (+5990)
LTP	V/L	—	—	—	305	245
	HDL	—	—	—	20504	14734
	Total	—	—	—	20809 (+13070)[2]	14979 (+6777)

FIGURE 18: APO CII DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT B



The figure shows the apo CII mass distribution in the fractions isolated by density gradient ultracentrifugation.

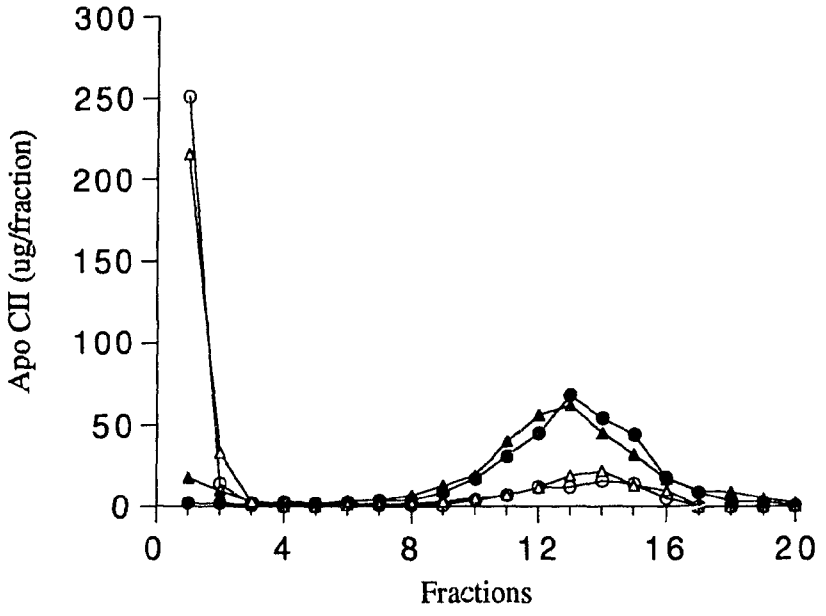
Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein and was carried out for 2 hours at 37°C.

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+HAL indicates the addition of hepatic lipase to the LPL incubation.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.

The conditions of the incubations are represented as:

control: O
LPL: ●
LPL+HAL: X
LPL+LTP: ▲

FIGURE 19: APO CII DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT E



The figure shows the apo CII mass distribution in the fractions isolated by density gradient ultracentrifugation.

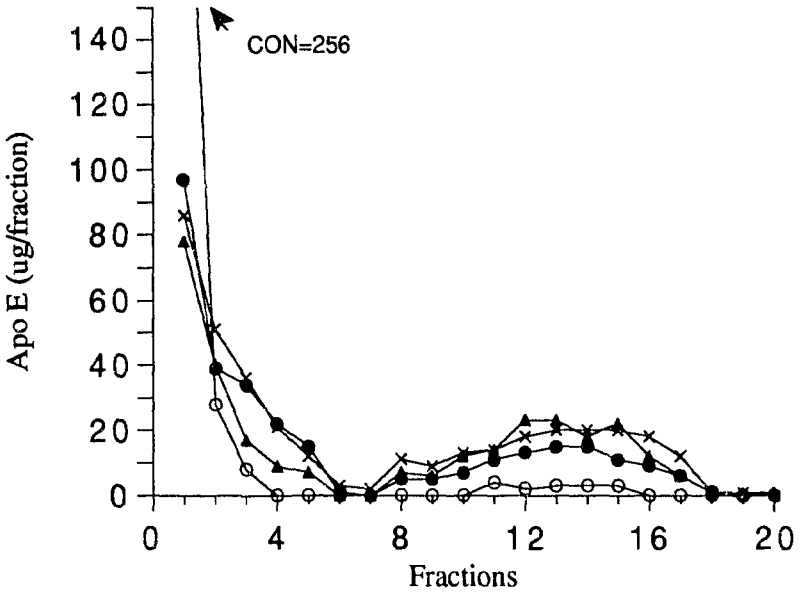
Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein which was carried out for 2 hours at 37°C.

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.
LTP indicates the addition of the lipid transfer protein preparation to the control incubation.

The conditions of the incubations are represented as:

control: O
LPL: ●
LPL+LTP: ▲
LTP: Δ

FIGURE 20: APO E DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT **B**



The figure shows the apo E mass distribution in the fractions isolated by density gradient ultracentrifugation.

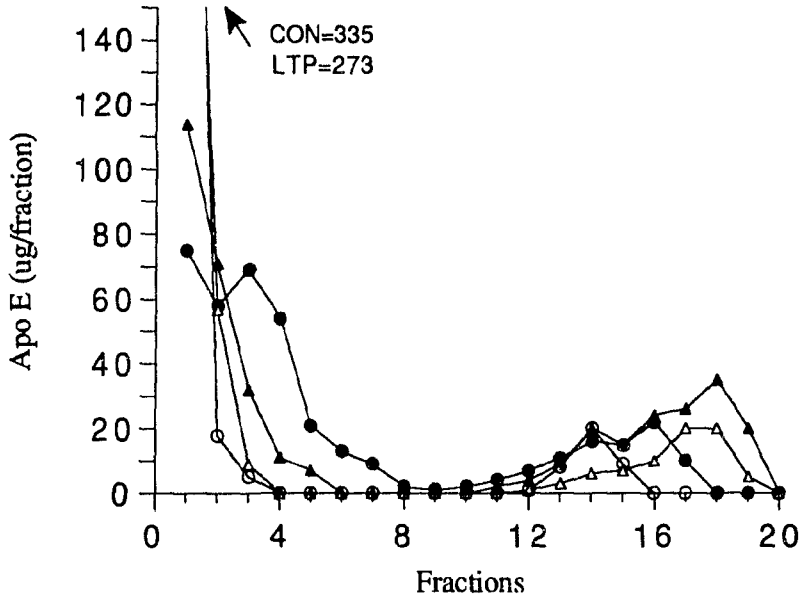
Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein and was carried out for 2 hours at 37°C.

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+HAL indicates the addition of hepatic lipase to the LPL incubation.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.

The conditions of the incubations are represented as:

control: O
LPL: ●
LPL+HAL: ×
LPL+LTP: ▲

FIGURE 21: APO E DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT E



The figure shows the apo E mass distribution in the fractions isolated by density gradient ultracentrifugation.

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein which was carried out for 2 hours at 37°C.

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.
LTP indicates the addition of the lipid transfer protein preparation to the control incubation.

The conditions of the incubations are represented as:

control: O
LPL: ●
LPL+LTP: ▲
LTP: Δ

The apoprotein distributions are presented for apo CII and apo E (Figures 18-21). The two figures for each apoprotein contain the distributions of the apoproteins of experiment B and experiment E. The distributions of apo CIII and apo AI were not included since the distribution of apo CIII was similar to that of apo CII and the apo AI tended to follow the lipid mass and was not involved in mass transfer from VLDL to HDL.

APO CII: (Tables 15 and 16, Figures 18 and 19)

In the LPL incubation, apo CII redistributed extensively from the VLDL/LDL region into the HDL region (Table 15). The LPL+HAL incubation and the LPL+LTP incubation did not demonstrate any difference in the gain of apo CII as compared to the LPL incubation (Table 15). No transfer was observed with LTP alone (Table 15).

The apo CII that was transferred to HDL had a distribution similar to that of the control peak for LPL in experiment B (Figure 18). There was gain on the light side of the control peak in experiment E with a distinct shift in the peak to a lighter fraction.(Figure 19). The distribution of apo CII for the LPL+HAL incubation was the same as that of the LPL incubation (Figure 18).

The distribution of apo CII of the LPL+LTP incubation was on the light side of the control peak and a shift in the peak from fraction 14 into 13 or 12 was observed in three experiments (Figures 18 and 19). The shift to lighter densities was greater than that seen in the LPL incubations. The LTP incubation did not show a difference in distribution from the control in either experiments (Figure 19).

The data indicate that the lipolysis of VLDL by lipoprotein lipase can effect an almost complete transfer of apo CII from VLDL to HDL without the requirement of other factors.

APO CIII: (Tables 15 and 16)

The apo CIII gain by HDL was extensive in the LPL incubation. The LPL+HAL and the LPL+LTP gain was similar to the gain of the LPL incubation. The LTP incubation did not demonstrate a transfer apo CIII to HDL.

The distribution of apo CIII was similar to the distribution of apo CII for all conditions and experiments (results not shown).

As was the case for apo CII, the data indicate that lipolysis of VLDL by lipoprotein lipase alone is sufficient for the transfer of apo CIII from VLDL to HDL.

Apo E: (Tables 15 and 17, Figures 20 and 21)

Apo E was transferred from the VLDL/LDL region to HDL in all experiments but, as compared to the transfer of apo CII and CIII, the transfer was much less extensive. The addition of hepatic lipase consistently resulted in a recovery that was greater than 100% of the apo E mass of the control incubations (Table 17). The increase in apo E was in both the VLDL and HDL region or in the HDL region alone. The increased mass may be due to an effect of hepatic lipase on the expression of the apo E epitopes despite the fact that all samples contained 0.1% Triton. Consequently, the gain by HDL is somewhat of an overestimate. There are reports of an increased expression of apo E epitopes as a result of hepatic lipase activity [99] and the effect may be occurring in the present study. Despite the increased recovery, the gain of apo E in the LPL+HAL incubation was not significantly different from the gain of the LPL incubation.

The LPL+LTP incubation significantly increased the amount of apo E transferred from VLDL to HDL as compared to that of the LPL incubation (Table 15). The increased transfer of apo E to HDL in the LPL+LTP incubation was significantly different relative to the LPL incubation. The LTP incubation also resulted in a small gain of apo E by HDL (Table 15).

The distribution of apo E of the control incubation followed that of the HDL lipid mass. However, the apo E distribution in the LPL, LPL+HAL and LTP incubations did

not mirror the lipid distribution as was noted for apo CII. There was a tendency for the apo E distributions of the LPL, LPL+HAL and LPL+LTP incubations to form two peaks in the HDL region (Figures 20 and 21). One peak occurred in the region of the HDL mass and the other peak was in the dense regions of HDL (fractions 16 and 17). Fractions 16 and 17 are low in lipid and apo AI mass. Apo E was also distributed into the very light region of HDL in many cases (fractions 8, 9 and 10), a region containing nominal amounts of lipid or apo AI.

The LPL+LTP incubation gained apo E in two regions (Figure 20 and 21). The apo E gain was on the light side and over the control peak as well as in the very dense fractions. The increased amount of apo E transferred from VLDL to HDL in the LPL+LTP incubation as compared to the LPL incubation was variable in its distribution (fractions 10, 12, 13 and 15 for experiment B, fractions 13, 14, and 15 for experiment C and fractions 17, 18, and 19 for experiment E, fractions 15, 17, 18 and 19 for experiment D).

APO AI:

The shifts in the lipid mass in the HDL region were more obvious than the shifts in apo AI of the various incubations, particularly in LPL incubations that demonstrated a gain of cholesteryl esters and an increased gain of phospholipid (data not shown).

iii) HDL PARTICLE SIZE: (Table 18, Figures 22 and 23)

Alterations in HDL particle size were assessed by gradient gel electrophoresis and electron microscopy. Figure 22 contains the polyacrylamide gradient gels of fractions 11 and 12 of experiments B and E and fractions 9 and 10 of experiment E. Fraction 12 were specifically selected as an example since it was in the region where the majority of the HDL mass was located and was the fraction where the shift/gain of the HDL mass occurred most frequently in the LPL and LPL+LTP incubations.

The control mass was distributed over a wide range of particle sizes (Figure 22). An increase in mass as well as size was obvious in the LPL+LTP incubation. The LPL+HAL incubation did not demonstrate an increase in HDL particle size in this fraction. An increase in particle size as compared to an increase in the number of particles in this fraction suggests that the process of transfer of mass from the VLDL to the HDL region as a result of lipolysis appeared to be one of transfer of mass to the existing HDL particles rather than the exclusive formation of discrete particles which isolate in the HDL region. This does not preclude the possibility that discrete particles were formed during VLDL lipolysis and were also present in the fraction, nor does it preclude the possibility that discrete particles have exchanged with or donated mass to the existent HDL particles.

The particle sizes of fraction 12 were calculated from the polyacrylamide gradient gels to compare the HDL particle sizes of the various incubation conditions. The values are listed in Table 18 for all of the experiments. The molecular weight standards were in the range of the particle molecular weight of HDL.

Electron microscopy was carried out on a pooled sample of fractions 9-12 of the LPL incubation of experiment E in order to determine if the HDL contained discs or spheres after lipolysis. The electron micrograph showed spherical particles with the majority having diameters of 100-140 Å (Figure 23). Discs were not observed. The stain around the particles is indicative that the particles were spherical shaped since it signifies that the stain was able to partially diffuse under the particle, which would not occur if the morphology were that of a flattened disc.

TABLE 18

PARTICLE SIZE OF FRACTION 12 IN THE HDL REGION BASED ON PAGGE DATA

	Experiment				
	A	B	C	D	E
	Molecular Mass (in k daltons)				
Starting HDL					
Average Mass	n.v		n.v.	288	174-392
Range	n.v	165-504	n.v.	148-445	165-667
Control					
Average Mass					353 344
Range	155-271	202-467	n.v.	173-445	188-433
LPL					
Average Mass				391	362
Range	155-376	192-491	n.v.	182-504	192-467
LPL+HAL					
Average Mass			n.v.	—	—
Range	155-330	192-466	n.v.	—	—
LPL+LTP					
Average Mass	—		n.v.	422	411
Range	—	192-517	n.v.	182-531	192-505
LTP					
Average mass	—	—	—	372	362
Range	—	—	—	173-445	192-467

The conditions of the various experiments are outlined in Table 1.

n.v.= no value available

Starting HDL refers to the HDL used in the incubation.

Average mass refers to the particle size where the mass was most concentrated on the gel.

FIGURE 22

PARTICLE SIZE OF HDL FRACTIONS OF EXPERIMENT **B** AND **E** ON POLYACRYLAMIDE GRADIENT GELS

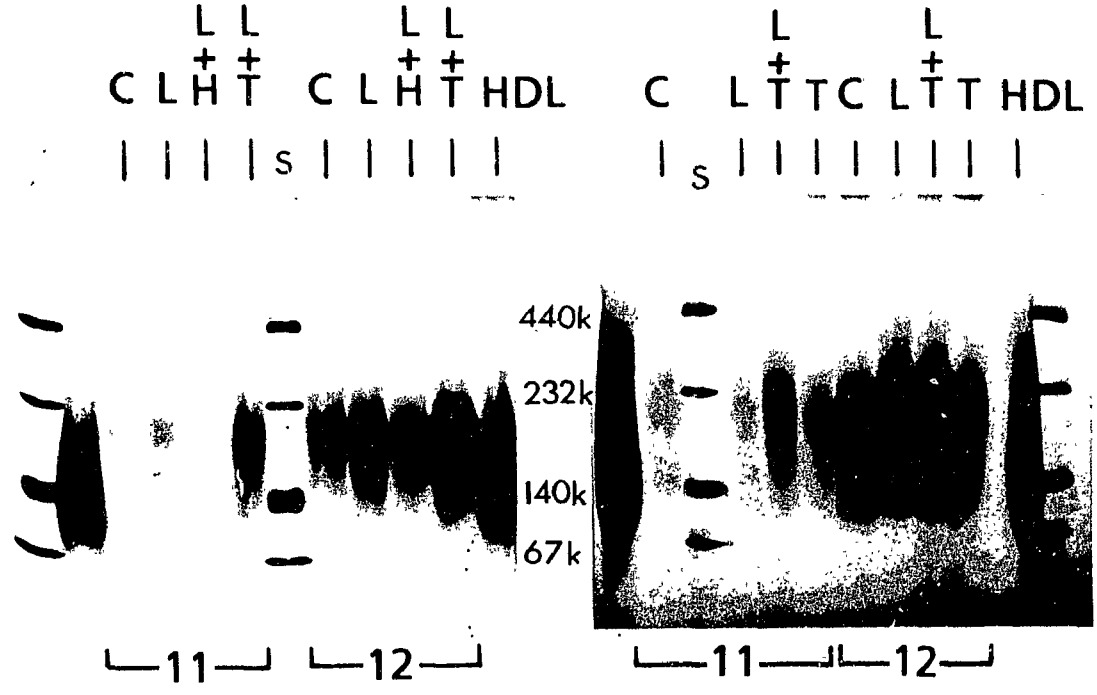
Figure 22 shows 4-30 % polyacrylamide gradient gels. The upper left gel is from experiment B and the upper right gel is from experiment E. Both gels show the HDL particle sizes of fractions 11 and 12. The lower gel shows the HDL particle sizes of fractions 9 and 10 of experiment E. The particle sizes of the original HDL added to the incubations is also shown. Standards are also present.

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein and was carried out for 2 hours at 37°C.

The letters represent the following:
HDL= original HDL of the incubations
C= control = inclusion of heat denatured lipoprotein lipase
L= LPL = inclusion of active lipoprotein lipase
L+H= LPL+HAL = addition of hepatic lipase to the LPL incubation
L+T= LPL+LTP = addition of the lipid transfer protein preparation to the LPL incubation
T= addition of the lipid transfer protein preparation to the control incubation.
S= standards

The standards and their molecular masses are :
Ferritin: 440,000 daltons
Catalase: 232,000 daltons
Lactate Dehydrogenase: 140,000 daltons
Bovine Serum Albumin: 67,000 daltons

FIGURE 22: PARTICLE SIZE OF FRACTIONS 9-12 OF THE EXPERIMENT B AND E INCUBATIONS ON POLYACRYLAMIDE GRADIENT GELS



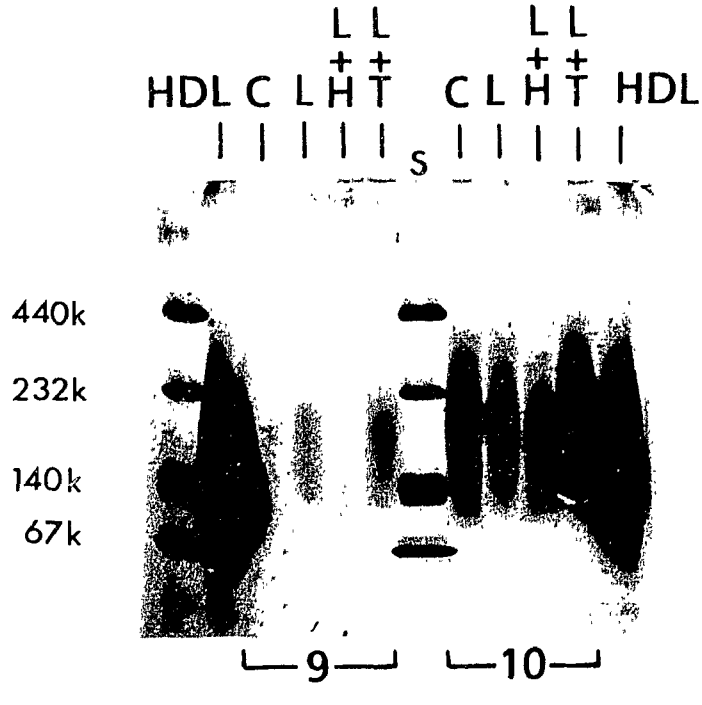


FIGURE 23: ELECTRON MICROGRAPH OF HDL FROM THE LPL INCUBATION OF EXPERIMENT E

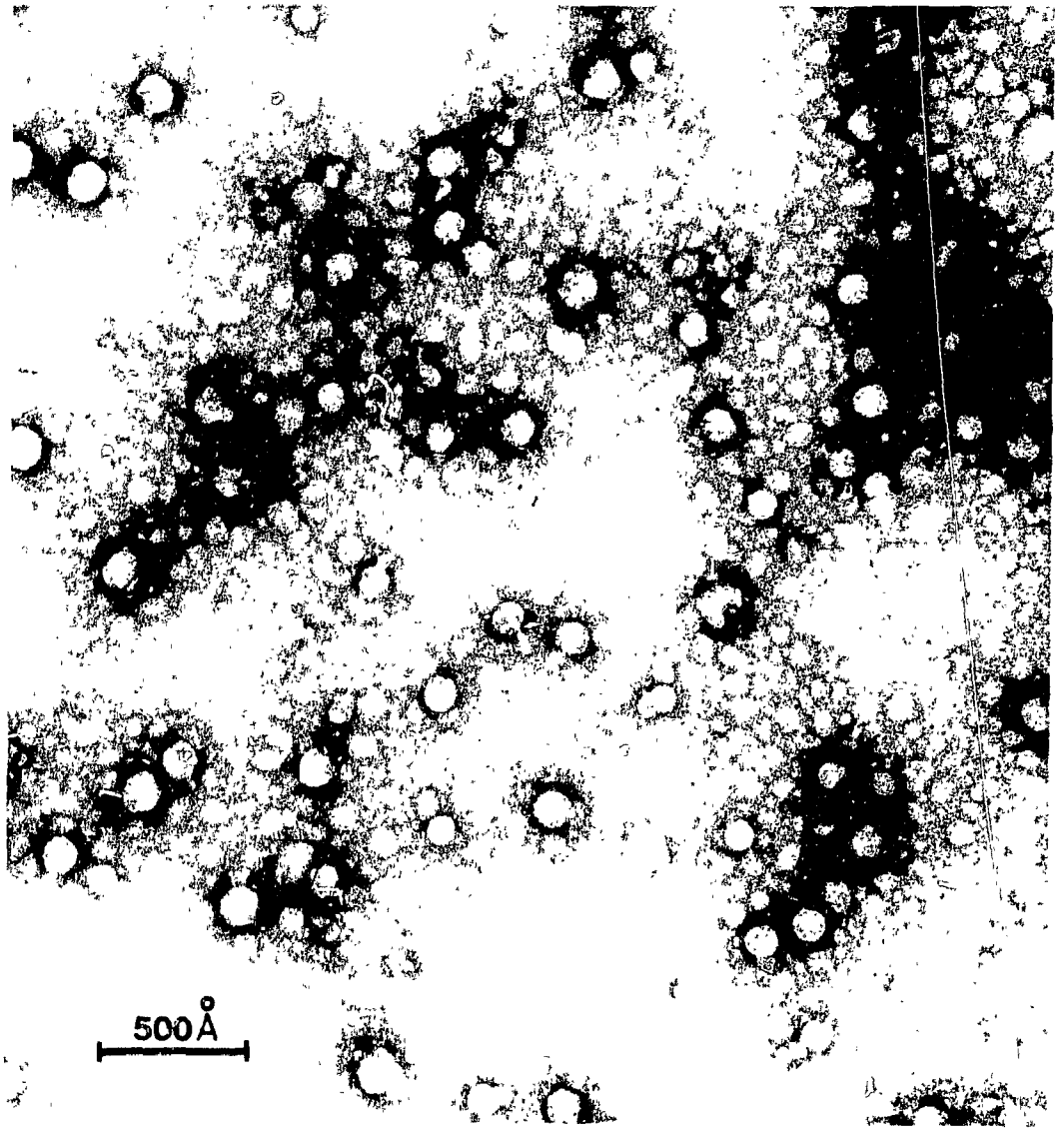


Figure 23 is a negatively stained electron micrograph of the pooled fractions 9-12 of the HDL region of experiment E for the LPL incubation.

The magnification is 307,000 x. The majority of the particles particles were 100-140Å.

c) THE EFFECT ON VLDL/LDL MASS OF LIPOPROTEIN LIPASE AND THE EFFECT OF THE ADDITION OF HEPATIC LIPASE AND LIPID TRANSFER PROTEINS TO THE LIPOPROTEIN LIPASE INCUBATION.

Contrasting reports have been presented concerning the potential of lipoprotein lipase to convert VLDL to LDL. In order to address this issue, the type and distribution of particles in the VLDL/LDL region in the LPL incubation was examined. The effect of the addition of hepatic lipase or the lipid transfer protein to the lipoprotein lipase incubation on the modulation of the particles was investigated. The lipid and apoprotein data have been reported in section b). The redistribution of the VLDL lipid and apoprotein mass in the LDL region of the gradient can be observed in Figures 8 - 21.

i) LIPID DISTRIBUTION: (Figures 8-17, Tables 11 and 13)

The presence of lipoprotein lipase in the incubation resulted in a redistribution of some of VLDL lipid mass into the LDL region (Figures 8-17). However, most of the lipolysed particles were found in fractions 1-3 and the majority of the mass failed to move further down the gradient into the fractions where most of plasma LDL would be located (see Figures 3-6, fractions 4-6). An LDL peak was not formed. Lipid mass could be detected as far as fraction 7 but little mass was present in fraction 5-7. The LPL incubation of experiments D and E demonstrated a greater shift of the lipolysed VLDL mass into the denser fractions (Figures 9,11,13 and 17) than that observed in experiment A and B (Figures 8,10,12 and 16). Experiment D and E also demonstrated more phospholipid transfer from VLDL to HDL and a net gain in cholesteryl ester in the HDL region which was not observed in experiment A and B.

The addition of hepatic lipase to the LPL incubation had little effect on the lipid redistribution as compared to the redistribution observed with the LPL incubation (Figures 8,10, 12 and 16).

The addition of the lipid transfer proteins to the LPL incubation caused an increased retention of the mass in fractions 1 and 2 with less movement of mass into

fractions 3-5 compared to the LPL incubation (Figures 8-17). The effect occurred despite the fact that the addition of the lipid transfer protein to the lipoprotein lipase incubation resulted in an increased transfer of lipid from the VLDL/LDL region to the HDL region. The majority of the mass in the LTP incubations remained in fraction 1 but a small shift from fraction 1 into fraction 2 relative to the control incubation was noticed (Figures 9, 11, 13 and 17).

ii) APOPROTEIN DISTRIBUTION

APO B: (Table 19, Figures 24 and 25)

In the LPL incubation, apo B distributed down the gradient in a manner similar to that observed for the lipid redistributions (Figures 24 and 25). The apo B was distributed further down the gradient in experiments D and E (Figure 25) than in experiments A and B (Figure 24) as was observed for the lipid mass.

The LPL+HAL incubation exhibited a similar redistribution to that of the LPL incubation (Figure 24). Apo B extended marginally more down the gradient than in the LPL incubation.

The LPL+LTP incubation contained more apo B in fractions 1 and 2 and less in fractions 4 and 5 compared to the LPL incubation (Figure 24 and 25). The increased retention of apo B mass in the least dense regions of the gradient (fractions 1 and 2) in the LPL+LTP incubation relative to the distribution in the LPL incubation was similar to that observed for the lipid redistributions. The LTP incubation showed less apo B in fraction 1 and more in fraction 2 as compared to the control (Figure 25).

Apo B recoveries were low relative to the control in some experiments in the incubations that contained active lipoprotein lipase, i.e , the LPL, LPL+HAL or LPL+LTP incubations (Table 19).

TABLE 19

THE APO B MASS IN THE VLDL/LDL REGION

Incubations	Experiment A	B	C	D	E
			µg		
Control	438	300	319	340	325
LPL	419 (-19)	184 (-116)	212 (-107)	266 (-74)	284 (-41)
LPL+HAL	427 (-11)	220 (-80)	255 (-64)	—	—
LPL+LTP	—	218 (-82)	236 (-83)	262 (-78)	358 (+33)
LTP	—	—	—	346 (+6)	326 (+1)

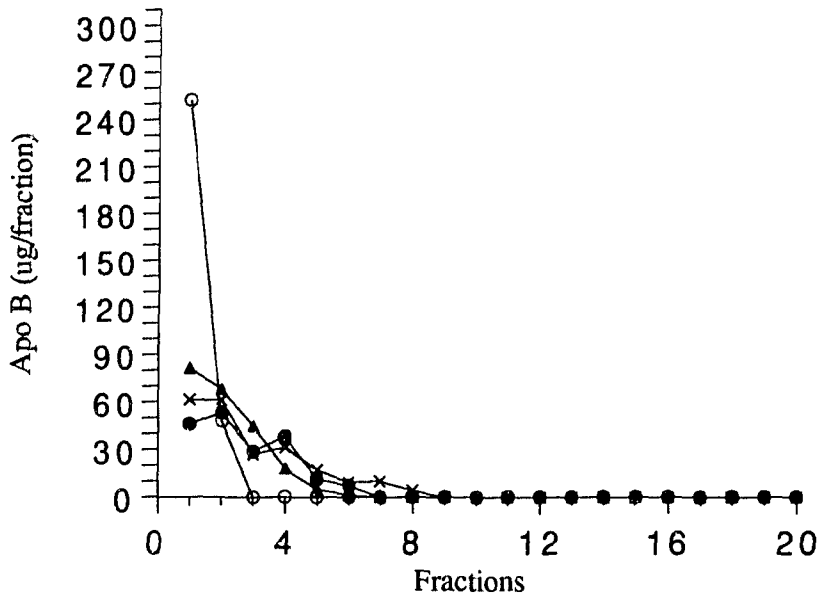
All values are expressed in µg.

The conditions of the various experiments are outlined in Table 1.

VLDL/LDL region includes fractions 1-7.

A net gain or loss in the VLDL/LDL or a difference in recovery of the experimental incubation from the control incubation.

FIGURE 24: APO B DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE INCUBATIONS OF EXPERIMENT **B**



The figure shows the apo B mass distribution in the fractions isolated by density gradient ultracentrifugation.

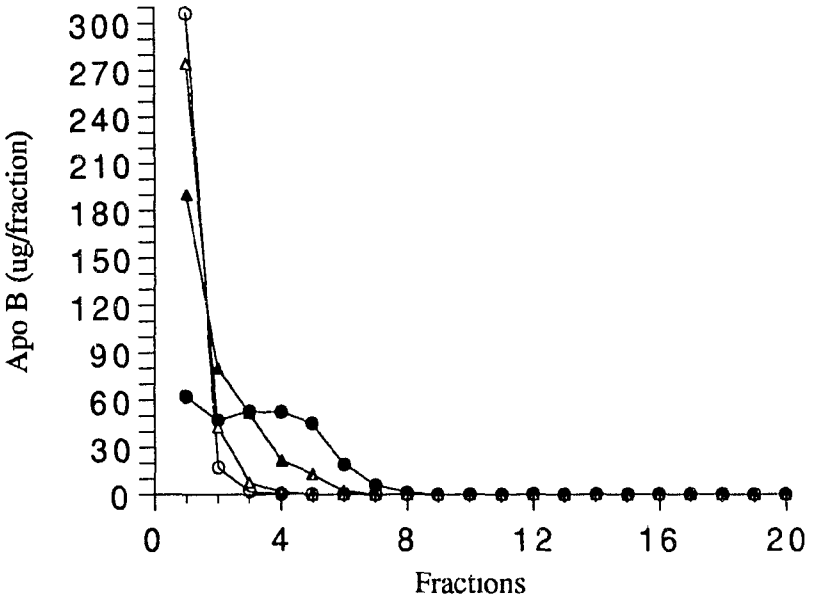
Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein which was carried out for 2 hours at 37°C

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.
LTP indicates the addition of the lipid transfer protein preparation to the control incubation.

The conditions of the incubations are represented as:

control: O
LPL: ●
LPL+HAL: X
LPL+LTP: ▲

FIGURE 25: APO B DISTRIBUTION IN THE LIPOPROTEIN FRACTIONS FOR THE EXPERIMENT E INCUBATIONS



The figure shows the apo B mass distribution in the fractions isolated by density gradient ultracentrifugation.

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein which was carried out for 2 hours at 37°C

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.
LTP indicates the addition of the lipid transfer protein preparation to the control incubation.

The conditions of the incubations are represented as:

control: O
LPL: ●
LPL+LTP: ▲
LTP: Δ

APO CII, CIII AND APO E : (Tables 20 and 21)

Since extensive removal of apo CII, CIII and apo E is required for LDL formation, the percentage transfer from the VLDL/LDL region was reported.

Apo CII and CIII were almost completely removed from the VLDL/LDL region in the LPL incubations (Table 20). The average transfer from VLDL to HDL for apo CII was 93% and 98.7% for apo CIII in the LPL incubations. The results were similar in the LPL+HAL incubations with a transfer of 90.7% for apo CII and 99% for apo CIII. The average transfer in the LPL+LTP incubation was 88.5% for apo CII and 90.3% for apo CIII. Incubations with LTP alone did not cause a transfer of apo CII or apo CIII to HDL.

In contrast to the transfer of the apo C's, there was only partial transfer of apo E from VLDL to HDL (Table 21). The incubation with LPL resulted in an average transfer of 28.2%. The LPL+HAL incubation demonstrated a transfer similar to that of the LPL incubation (31.5 %). The average percentage transfer for LPL+LTP was substantially increased compared to the LPL incubation (41.3%). The LTP incubation transferred 17.5%.

Thus the movement of apo E was distinct from that of the apo C's. The lipid transfer protein preparation did stimulate the transfer of apo E from the VLDL/LDL region to the HDL region as compared to that observed in the LPL incubation. However a further transfer of apo E is still required to form LDL.

iii) VLDL/LDL PARTICLE SIZE: (Tables 22 and 23, Figures 26-29)

In order to determine how the various experimental conditions influenced the size of particles in the VLDL/LDL region, the particle size was estimated from non-denaturing polyacrylamide gradient gels and compared to the calculated particle diameters from the lipid data. Figure 26-28 shows the polyacrylamide gradient gels of fractions 1-5 of experiment B and E. Table 22 contains the estimated molecular weights of the particles from the polyacrylamide gradient gels for fractions 1-5 of the experiments

TABLE 20

PERCENT OF VLDL/LDL APO CII AND CIII MASS TRANSFERRED TO HDL

Incubations	Experiment A	B	C	D	E
	Percent				
CII: [1]					
LPL	89.2	92	(73.5)	96.9	93.7
LPL+HAL	89.2	92.4	91.3	——	——
LPL+LTP	——	93.2	90.9	84.9	84.8
LTP	——	——	——	0	6
CIII: [1]					
LPL	97.9	99.5	(68.1)	100	97.9
LPL+HAL	97.9	99.5	100	——	——
LPL+LTP	——	99.5	96.7	80.2 [2]	85.3
LTP	——	——	——	0 [2]	0

The conditions of the various experiments are outlined in Table 1.

The percentage transfer (loss)was calculated by:

$$1 - \frac{\text{sum of fractions 1-7 of experimental incubation}}{\text{sum of fractions 1-7 in control incubation}}$$

[1] Differences in percentage transfer may be partially due to difference in recoveries

[2] The LTP incubation contained an excess of 220 µg CIII. The LPL+LTP incubation had an excess of 397 µg of apo CIII.

TABLE 21

PERCENT OF VLDL/LDL APO E MASS TRANSFERRED TO HDL

Incubations	Experiment A	B	C	D	E
	%	%	%	%	%
Apo E:					
Control	2.3	4.9	8.9	16.3	9.6
LPL	26.3	32.3	(28.7)[1]	31.3	24.5
LPL+HAL	22.1	31.3	20.9	——	——
LPL+LTP	——	50.8	36.9	45.5	40.7
LTP	——	——	——	21.5	14.4

The conditions of the various experiments are outlined in Table 1.

The percentage transferred from VLDL/LDL to HDL was calculated by:

$$1 - \frac{\text{sum of fractions 1-7 of the experimental incubation}}{\text{sum of fractions 1-20 of the control incubation}}$$

[1] The LPL incubation attained 63% VLDL triglyceride hydrolysis rather than the average of 90% in the other incubations with active lipolysis in experiment **C**.

and of a plasma sample isolated using the density gradient. Table 23 contains the calculated particle diameters of the fractions 1-5 derived from the lipid data of Experiments B and E.

The polyacrylamide gradient gels demonstrated that the LPL+LTP incubation retained more mass in fractions 1 and 2 and less mass in fractions 3-5 as compared to the LPL incubation (Figure 26 and 27) which was consistent with the distributions of lipid and apo B on the gradient. The LTP incubation demonstrated less mass in fraction 1 and more in fraction 2 (Figure 27), again in agreement with the lipid and apo B distributions

The particles observed on polyacrylamide gradient gels from fractions 2-5 of the LPL (Figures 26 and 27) and the LPL+HAL incubations (Figure 26) were considerably larger those of plasma LDL and varied widely in size. The particle sizes of fractions 2-5 of the LPL+LTP incubation were smaller and more consistent in size than those of the LPL incubation and approaching the size of plasma LDL in fractions 3-5 (Figure 26 and 27).

A rough estimate of the particle sizes (molecular weight) (Table 22) was made from the standards. Since the highest standard had a lower molecular weight than most of the particles, the values are not accurate and should be used on a comparison basis only. The estimated molecular weight of the plasma VLDL and plasma LDL on the gels were close to reported values. The VLDL was that used in the incubations and the LDL was a plasma preparation of d 1.02-1.05 g/ml which would be the density range of fractions 2-5.

In fraction 1, the control incubation values ranged from 8.2-11.9 million daltons which are representative of a VLDL particle size. The LPL and LPL+HAL incubations demonstrated particles ranging in size from 2.9-11.9 millions daltons. This size is considerably larger than plasma LDL. The LPL+LTP incubations yielded particles with sizes of 2.6-3.6 million daltons which were generally smaller than the particles sizes observed in the LPL or LPL+HAL incubations and approached LDL size (2 million on average). The difference between the LPL+LTP and the LPL particles or the LPL+HAL

TABLE 22

PARTICLE SIZE OF FRACTIONS 1-5 BASED ON PAGGE DATA

The conditions of the various experiments are outlined in Table 1.

The values are crude estimates and should be used for comparison only.

If several values are listed and a star * is absent, this indicates that the mass was equally distributed between the different sized particles. The presence of a star * indicates that the majority of the mass was of this size.

n.d.= not determinable due to too little mass

n.v.= no value available

Starting VLDL refers to the VLDL used in the incubation.

LDL was obtained from plasma at densities d 1.02-1.05 gm/ml.

The plasma value was obtained from a density gradient sample from subject **B**.

TABLE 22
PARTICLE SIZE OF FRACTIONS 1-5 BASED ON PAGGE DATA

Incubations	Experiment A	B	C	D	E
	Molecular Mass Estimate (in millions of daltons)				
Starting VLDL	n.v.	8.2	8.0*, 37.2	11.9	8.5
Range	n.v.	2.9-35.3	4.8-13.4	4.9-25.8	4.1-24.6
LDL	2.1(1.7 and 2.4)	2.1	2.0	1.9	n.v.
Range	1.5-4.2	2.1-3.3	1.8-2.8	1.5-2.9	n.v.
Fraction 1	Plasma= 13.3, range 6.6-55.8				
Control	10.9	8.2	8.7*, 37.2	11.9	8.5
LPL	4.2, 4.7, 5.9	2.9*, 7.2	(5.9)	11.9	4.1
LPL+HAL	4.2, 4.7, 5.9	2.9*, 6.8	3.8	—	—
LPL+LTP	—	2.6	3.3	4.2	3.6
LTP	—	—	—	11.9	7.8
Fraction 2	Plasma=1.8*, 2.4*, 15.2				
Control	10.9	7.2	11.2	14.0	10.5
LPL	3.2, 5.0	2.2, 2.9, 5.4	(6.5)	7.1	4.6
LPL+HAL	3.2, 5.2	2.2, 2.9, 4.9	4.5	—	—
LPL+LTP	—	2.1	2.6	3.4	2.8
LTP	—	—	—	10.3	7.8
Fraction 3	Plasma=1.7, range 1.5-2.7				
Control	n.d.	n.d.	n.d.	n.d.	n.d.
LPL	4.2	2.1, 3.9*	(6.8)	5.7	4.6
LPL+HAL	4.2	2.1, 3.9*	4.6	—	—
LPL+LTP	—	1.8	2.5	3.0	2.5
LTP	—	—	—	n.d.	n.d.
Fraction 4	Plasma=1.7, 2.1*				
Control	n.d.	n.d.	n.d.	n.d.	n.d.
LPL	3.5	3.1	(n.d.)	5.2	4.0
LPL+HAL	2.1, 3.5*	2.1, 2.5*	4.2	—	—
LPL+LTP	—	2.0	2.4	2.8	2.5
LTP	—	—	—	n.d.	n.d.
Fraction 5	Plasma=1.9*, faint 3.9				
Control	n.d.	n.d.	n.d.	n.d.	n.v.
LPL	3.2	4.4	(n.d.)	3.8	n.v.
LPL+HAL	1.5, 3.0	2.8, 4.4*	3.1	—	—
LPL+LTP	—	2.6	2.5	3.1	n.v.
LTP	—	—	—	n.d.	n.v.

TABLE 23

CALCULATED PARTICLE DIAMETERS OF FRACTIONS 1-5 BASED ON LIPID DATA

	Experiment						
	A	B	C	D	E	Mean ± SD	Plasma Average
				(in Angstroms)			
Fraction 1							
Control	289	238	282	337	291	287 ±35	355
LPL	168	155	(179)	163	175	165 ±8	
LPL+HAL	162	148	153	—	—	154 ±7	
LPL+LTP	—	146	151	202	184	160 ±21	
LTP	—	—	—	319	297	308	
Fraction 2							
Control	289	256	244	264	267	264 ±17	272
LPL	161	136	(195)	114	144	139±20	
LPL+HAL	161	140	138	—	—	146 ±13	
LPL+LTP	—	161	161	105	166	163 ±2	
LTP	—	—	—	292	289	291	
Fraction 3							
Control	139	143	277	211	194	193 ±57	220
LPL	135	115	(196)	130	118	125 ±10	
LPL+HAL	134	113	130	—	—	126 ±11	
LPL+LTP	—	141	169	174	167	159 ±16	
LTP	—	—	—	224	182	203	
Fraction 4							
LPL	117	114	(174)	115	109	114 ±3	208
LPL+HAL	109	112	132	—	—	118 ±13	
LPL+LTP	—	152	182	98	135	156 ±24	
LTP	—	—	—	84	94	89	
Fraction 5							
LPL	126	155	(107)	117	108	127 ±20	206
LPL+HAL	124	161	127	—	—	137 ±21	
LPL+LTP	—	165	111	89	91	122 ±38	
LTP	—	—	—	94	78	86	

The conditions of the various experiments are outlined in Table 1.
Particle Diameter is calculated as outlined in Appendix B.
The plasma average is the average value for the fraction of 4 samples of normolipidemic plasma.

FIGURE 26

PARTICLE SIZE OF FRACTIONS 1-4 OF THE EXPERIMENT **B** INCUBATIONS ON POLYACRYLAMIDE GRADIENT GELS

Figure 26 shows 2-16 % polyacrylamide gradient gels of fractions 1-4 of the experiment **B** incubations and of the starting VLDL.

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein and was carried out for 2 hours at 37°C.

The letters represent the following:
V= starting VLDL of the incubations
C= control = inclusion of heat denatured lipoprotein lipase
L= LPL = inclusion of active lipoprotein lipase
L+H= LPL+HAL = addition of hepatic lipase to the LPL incubation
L+T= LPL+LTP = addition of the lipid transfer protein preparation to the LPL incubation
S= high molecular weight standards

The standards and their molecular masses are :
Thyroglobulin Dimer: 1,340,000 daltons
Thyroglobulin: 669,000 daltons
Ferritin: 440,000 daltons
Catalase: 232,000 daltons

FIGURE 26: PARTICLE SIZE OF FRACTIONS 1-4 OF THE EXPERIMENT B INCUBATIONS ON POLYACRYLAMIDE GRADIENT GELS

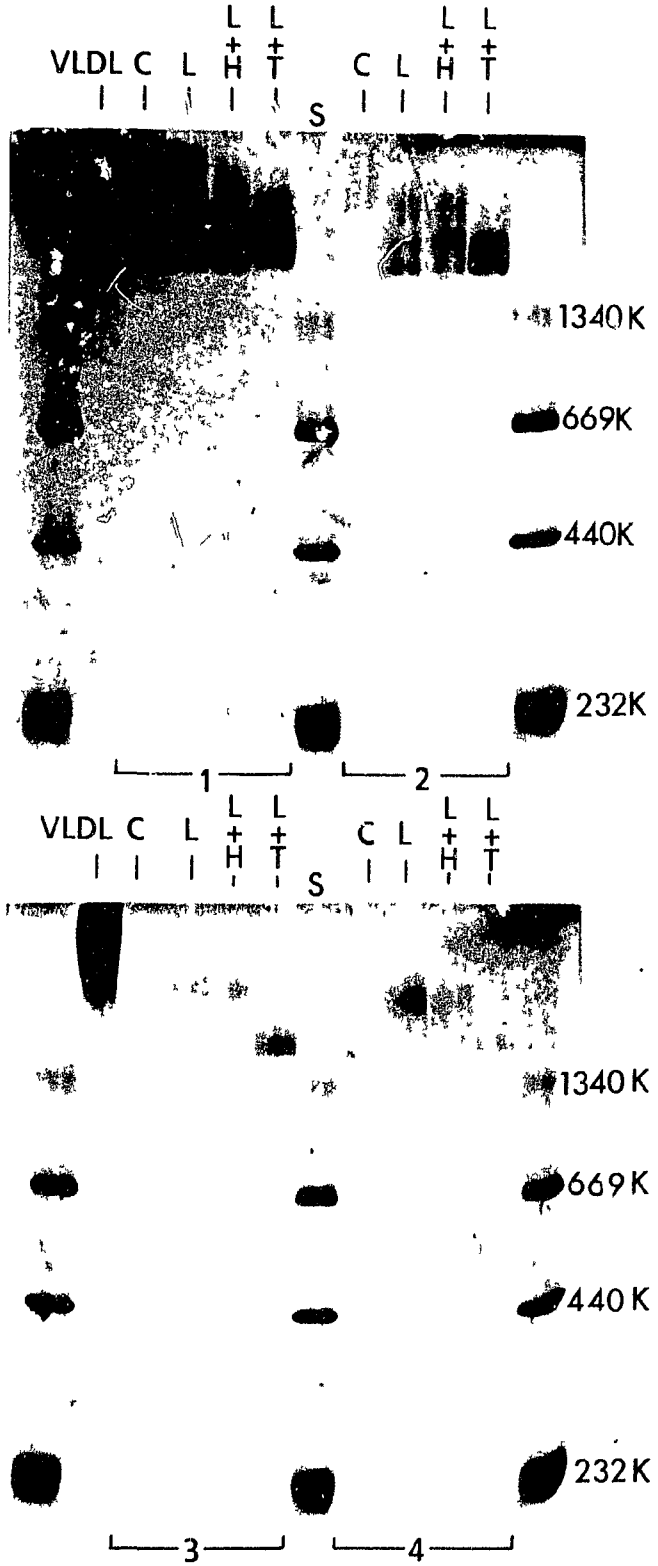


FIGURE 27

PARTICLE SIZE OF FRACTION 1-4 OF THE EXPERIMENT E INCUBATION ON POLYACRYLAMIDE GRADIENT GELS

Figure 27 shows 2-16 % polyacrylamide gradient gels of fractions 1-4 of the experiment E incubations, of the starting VLDL and of a plasma LDL sample.

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein and was carried out for 2 hours at 37°C.

The letters represent the following:
V = starting VLDL of the incubations
C = control = inclusion of heat denatured lipoprotein lipase
L = LPL = inclusion of active lipoprotein lipase
L+T= LPL+LTP = addition of the lipid transfer protein preparation to the LPL incubation
T = LTP = addition of the lipid transfer protein preparation to the control incubation
LDL = a plasma preparation of LDL, d 1.02-1.05 gm/ml
S = high molecular weight standards

The standards and their molecular masses are :
Thyroglobulin Dimer: 1,340,000 daltons
Thyroglobulin: 669,000 daltons
Ferritin: 440,000 daltons
Catalase: 232,000 daltons

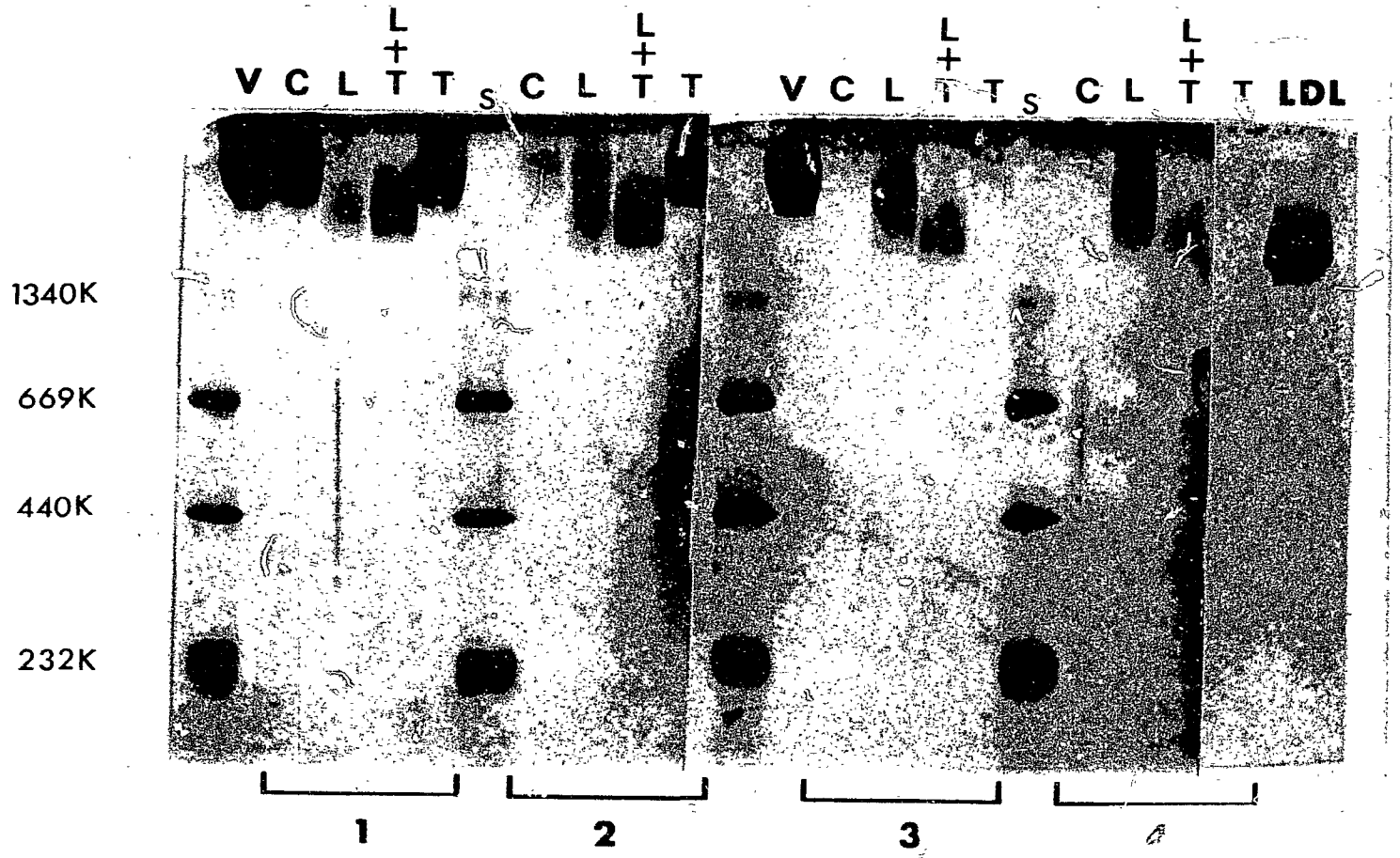


FIGURE 27: PARTICLE SIZE OF FRACTION 1-4 OF THE EXPERIMENT E INCUBATION ON POLYACRYLAMIDE GRADIENT GELS

FIGURE 28

PARTICLE SIZE OF FRACTIONS 4 AND 5 OF THE EXPERIMENT A INCUBATIONS ON POLYACRYLAMIDE GRADIENT GELS

Figure 28 shows 2-16 % polyacrylamide gradient gels of fractions 4 and 5 of the experiment **A** incubations.

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein and was carried out for 2 hours at 37°C.

The letters represent the following:
C= control = inclusion of heat denatured lipoprotein lipase
L= LPL = inclusion of active lipoprotein lipase
L+H= LPL+HAL = addition of hepatic lipase to the LPL incubation
S= high molecular weight standards

The standards and their molecular masses were :
Thyroglobulin Dimer: 1,340,000 daltons
Thyroglobulin: 669,000 daltons
Ferritin: 440,000 daltons
Catalase: 232,000 daltons

FIGURE 28: PARTICLE SIZE OF FRACTIONS 4 AND 5 OF THE EXPERIMENTAL INCUBATIONS ON POLYACRYLAMIDE GRADIENT GELS

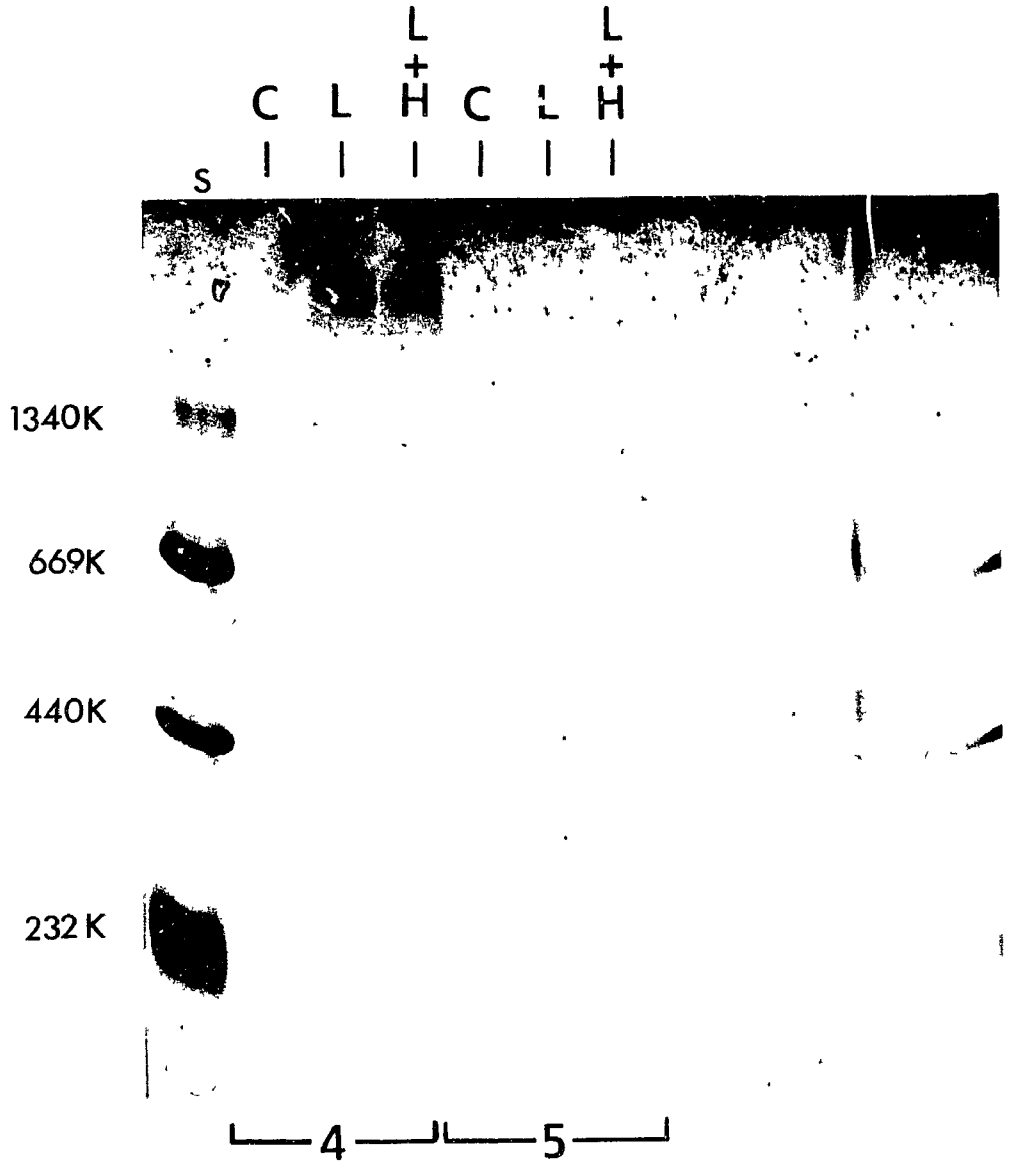


FIGURE 29

EFFECT OF CELLOBIOSE ON PARTICLE SIZE OF FRACTIONS 2 AND 3 OF THE EXPERIMENT C INCUBATIONS AS DEMONSTRATED ON POLYACRYLAMIDE GRADIENT GELS

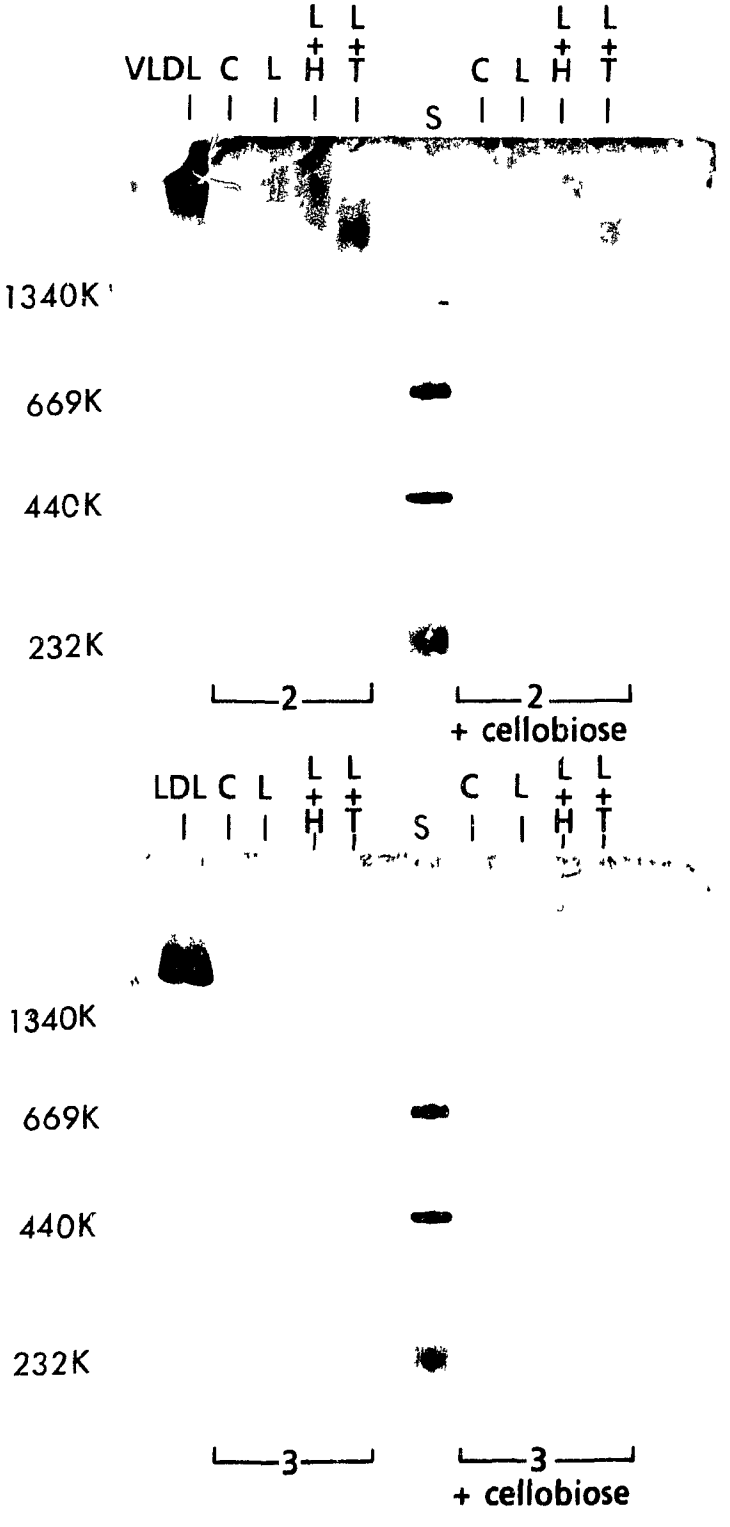
Figure 29 shows 2-16 % polyacrylamide gradient gels of fractions 2 and 3 of the experiment C incubations, of the starting VLDL and of a plasma sample of LDL. The left side of each gel demonstrates the particle size of the fractions when applied in the absence of cellobiose and the right side of the gel shows the fractions when cellobiose was present.

Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein and was carried out for 2 hours at 37°C.

The letters represent the following:
V= starting VLDL of the incubations
C= control = inclusion of heat denatured lipoprotein lipase
L= LPL = inclusion of active lipoprotein lipase, 63% triglyceride lipolysis
L+H= LPL+HAL = addition of hepatic lipase to the LPL incubation
L+T= LPL+LTP = addition of the lipid transfer protein preparation to the LPL incubation
LDL = a sample of plasma LDL, d1.02-1.05 g/ml
S= high molecular weight standards

The standards and their molecular masses were :
Thyroglobulin Dimer: 1,340,000 daltons
Thyroglobulin: 669,000 daltons
Ferritin: 440,000 daltons
Catalase: 232,000 daltons

FIGURE 29: EFFECT OF CELLOBIOSE ON PARTICLE SIZE OF FRACTIONS 2 AND 3 OF THE EXPERIMENT C INCUBATIONS AS DEMONSTRATED ON POLYACRYLAMIDE GRADIENT GELS



particles was more apparent in the denser fractions (fractions 2-5). A small difference in the particles of LPL+HAL incubation compared to the particles of the LPL incubation was noted in fractions 4 and 5 (Figure 28). Smaller particles were present in fractions 4 and 5 of the LPL+HAL incubation as compared to the particles of the LPL incubation, resulting in the formation of a lower band on polyacrylamide gradient gels (Table 22). There was slightly more mass in fractions 4 and 5 of the LPL+HAL incubation as compared to the LPL incubation (Figure 28).

In order to compare lipoproteins from lipolysis with those present in plasma, fractions 1-5 of a plasma sample from subject B were subjected to polyacrylamide gradient gel electrophoresis and particle size was estimated (Table 22). Through a comparison the particle size of the LDL fraction rather than the particle size of an LDL density cut (d 1.019-1.063 g/ml), it was found that the lipolysed particles still differed from the particle size of the corresponding plasma fraction. The distribution of the lipoproteins of subject B demonstrated that VLDL was in fraction 1 and the majority of the LDL mass was in fractions 4 and 5 (Figure 4). Comparing the particles sizes observed *in vitro* to those of the plasma, in fraction 1 the control particle sizes were in the range of the plasma value reflecting the presence of VLDL particles. The majority of the LPL and LPL+HAL particles were smaller than the those of the plasma but about twice the molecular weight of plasma LDL. The particles of the LPL+LTP were all substantially smaller than the particle size of the plasma. For fractions 2-5, the LPL and LPL+HAL particles were larger than the corresponding size of the plasma fraction while the LPL+LTP values were closer to and, in some cases, almost equal to the size in plasma. The lower band that appeared with LPL+HAL in fractions 4 and 5 was in the size range of the LPL+LTP particles and approached LDL size.

It was possible that the large sized particles of fractions 1-5 of the LPL and LPL+HAL incubations as well as in the LPL+LTP incubation which were observed on the polyacrylamide gradient gels were due to the presence of vesicles that formed during

lipolysis. To determine whether vesicles were present, cellobiose was added to fractions 1-5 of all the incubations in order to disrupt any vesicles that may have been present. The addition of cellobiose did not effect the particle size in any incubation indicating that vesicles were not the cause of the large particles in fractions 1-5. Figure 29 contains the results of the addition of cellobiose to fractions 2 and 3 of experiment C. It is also possible that vesicles would not enter the gel, in any case, if they were similar in charge to that of LpX. Lp X demonstrates a net positive charge and runs in the opposite direction to that of the plasma lipoproteins on agarose gels [25] (i.e., to the cathode rather than the anode).

The particle size (diameters) of the VLDL/LDL fractions were calculated from the lipid data based on the relationship between the surface area and core volume of a sphere which is characteristic of spherical plasma lipoproteins. The calculated particle diameters differed greatly from the size determined by polyacrylamide gradient gel electrophoresis. Fractions 1-5 of the LPL incubation had a calculated particle diameter that was extremely small relative to the average particle diameters of the plasma fractions (Table 23). The calculated diameters were small relative to those of plasma remnants, IDL or LDL (average diameter 200-220Å). Smaller particle diameters were most apparent in fractions 2-5. The calculated particle diameters for the LPL+HAL incubation were similar to those of the LPL incubation. The particle diameters of the LPL+LTP incubation were the same in fraction 1 as those of the LPL incubation but were consistently higher in fractions 2, 3 and 4. The values were lower than the corresponding plasma value. These results are the converse of those observed with polyacrylamide gradient gel electrophoresis where the LPL+LTP incubation demonstrated particle sizes that were markedly less than those observed in the LPL incubation or the LPL+HAL incubation. The difference between the observed particle size and the calculated particle size is consistent with the concept that the particles in the incubations had not equilibrated and that the small calculated particle diameters were due to an excess of surface relative to core in the LPL and LPL+HAL

incubations. The surface was not as excessive in the LPL+LTP incubation, thus the particles were calculated to be larger than those of the LPL or LPL+HAL incubation. An excess surface relative to core was corroborated by the calculation of the surface lipid to core lipid ratio (S/C) (see following section iv) which was very high in the LPL or LPL+HAL incubations relative to plasma LDL. The S/C ratio was lower in the LPL+LTP incubation as compared to the LPL or LPL+HAL incubations, due to the increase in the loss of unesterified cholesterol and phospholipid observed in the LPL+LTP incubation. Thus the use of a calculated particle size is not accurate when the particles have not equilibrated.

iv) A COMPARISON OF THE VLDL/LDL FRACTIONS OF THE LPL INCUBATION WITH THOSE OF THE LPL+LTP INCUBATION.

In order to further delineate the differences between the particles formed in the VLDL and LDL region of the gradient in the presence of lipoprotein lipase as compared to those formed in the presence of lipoprotein lipase and the lipid transfer protein preparation, several lipid and apoprotein ratios were calculated to determine how one component changed with respect to the other. The control values are listed for fractions 1 and 2 reflecting the ratio of the starting VLDL. The data were also used to compare how particles differed from plasma LDL as an average value for the LDL fractions was calculated from the density gradient data of subjects A, C and D. The majority of the plasma LDL is in fractions 4-6 in the density gradient, therefore the values of fractions 4 and 5 are considered characteristic of LDL particles.

UNESTERIFIED CHOLESTEROL TO PHOSPHOLIPID RATIO(UC/PL): (Table 24)

The UC/PL ratios of the LPL incubation and the LPL+LTP were generally lower than the plasma LDL values indicating a requirement for a greater loss of phospholipid from these fractions if the fractions are to become LDL particles.

CHOLESTERYL ESTER TO TRIGLYCERIDE RATIO(CE/TG): (Table 25)

The CE/TG ratios in the LPL incubations were low in fractions 1 and 2 relative to the plasma LDL values of 12-16 (plasma fractions 4 and 5) but were more similar to plasma LDL in fractions 3-5. The difference between the LPL value and the plasma value progressively decreased from fraction 1 to fraction 5. The triglyceride enrichment in fractions 1 and 2 suggests that a greater amount of triglyceride hydrolysis is necessary in order to achieve a plasma LDL ratio. The CE/TG ratio of the LPL+LTP incubation was higher than that of the LPL incubation in fraction 1, the same in fraction 2 and tended to be lower in fractions 3-5. The difference in the ratio compared to the LPL incubation may reflect enrichment in cholesteryl ester in fraction 1 and enrichment in triglyceride in fractions 3-5. The converse may be true of a greater hydrolysis or loss of triglycerides from fraction 1 and a loss of cholesteryl esters from fraction 3-5. Relative to plasma LDL values, the CE/TG ratio was consistently lower indicating the requirement of a greater loss of triglyceride from all fractions for formation of plasma LDL.

SURFACE LIPID TO CORE LIPID RATIO (S/C): (Table 26)

The LPL incubation consistently demonstrated surface to core ratios that were excessively high in all fractions compared to the ratio of plasma LDL (0.73-0.74). The LPL+LTP incubation exhibited surface to core ratios that were noticeably lower than those of the LPL incubation (in 80% of comparable cases). The ratios remained higher than that of plasma LDL. The high S/C ratio indicated that the LPL particles had an excess of surface relative to core and a reduction of this ratio is required in order to approach the values of plasma LDL. The ratio was reduced in the LPL+LTP incubation but a further reduction would be required in order to achieve a ratio that is similar to that of the plasma LDL.

LIPID TO APO B RATIO AND APO E TO APO B RATIO: (Figures 30 and 31)

Since apo B is considered to be the one constituent which remains with the VLDL particle as it is lipolysed and modified to form LDL, the ratios of unesterified cholesterol

TABLE 24

UC/PL RATIOS OF FRACTIONS 1-5 OF THE LPL AND LPL+LTP INCUBATIONS

	Experiment					Plasma
	A	B	C	D	E	Average
Fraction 1						
Control	.589	.571	.505	.495	.463	.653
LPL	.952	.887	.704(.499)	.629	.604	
LPL+LTP	—	.789	.672	.760	.677	
Fraction 2						
Control	.583	.514	.561	.397	.478	.805
LPL	.987	.784	.660(.568)	.620	.658	
LPL+LTP	—	.822	.655	.429	.552	
Fraction 3						.822
LPL	.806	.713	.686(.605)	.804	.655	
LPL+LTP	—	.736	.608	.603	.653	
Fraction 4						.863
LPL	.806	.769	.622(.616)	.722	.707	
LPL+LTP	—	.705	.701	.487	.625	
Fraction 5						.849
LPL	.967	.667	1.809(1.309)	.742	.796	
LPL+LTP	—	.549	1.439	.395	.410	

The conditions of the control, LPL and LPL+LTP experiments are outlined in Table 1.

The plasma average is the average value for the fraction of 3 samples of normolipidemic plasma.

UC/PL= unesterified cholesterol to phospholipid molar ratio

In the case of experiment **C**, the value for the LPL+HAL incubation was listed and the value for the LPL incubation at 63% VLDL triglyceride hydrolysis is in brackets.

TABLE 25

CE/TG RATIOS OF FRACTIONS 1-5 OF THE LPL AND LPL+LTP INCUBATIONS

Incubations	Experiment A	B	C	D	E	Plasma Average
Fraction 1						
Control	.568	.506	.394	.389	.441	.403
LPL	5.546	2.513	2.388(1.054)	2.501	2.818	
LPL+LTP	—	4.289	3.630	3.197	4.193	
Fraction 2						
Control	.640	.419	.457	.474	.440	.972
LPL	7.708	2.740	3.184(.830)	3.282	4.247	
LPL+LTP	—	2.786	3.114	2.752	3.910	
Fraction 3						5.197
LPL	14.530	9.605	3.889(.924)	6.766	9.465	
LPL+LTP	—	6.044	2.502	2.497	4.450	
Fraction 4						11.953
LPL	54.923	11.308	3.720(1.030)	15.893	10.422	
LPL+LTP	—	3.923	2.297	4.839	4.305	
Fraction 5						16.163
LPL	43.154	3.487	7.659(4.359)	15.692	9.385	
LPL+LTP	—	3.696	14.385	5.044	4.969	

The conditions of the control, LPL and LPL+LTP incubations are outlined in Table 1.

The plasma average is the average value for the fraction of 3 samples of normolipidemic plasma.

CE/TG= cholesteryl ester to triglyceride molar ratio

In the case of experiment **C**, the value for the LPL+HAL incubation was listed and the value for the LPL incubation at 63% VLDL triglyceride hydrolysis is in brackets.

TABLE 26

SURFACE TO CORE RATIOS OF FRACTIONS 1-5 OF THE LPL AND LPL+LTP INCUBATIONS

	Experiment					Plasma
	A	B	C	D	E	Average
Fraction 1						
Control	.580	.743	.603	.490	.569	.518
LPL	.997	1.167	1.150(.966)	1.041	.930	
LPL+LTP	—	1.193	1.126	.789	.858	
Fraction 2						
Control	.572	.674	.714	.625	.634	.623
LPL	1.039	1.358	1.286(.900)	1.702	1.195	
LPL+LTP	—	1.085	1.045	1.871	.976	
Fraction 3						.697
LPL	1.268	1.613	1.390(.890)	1.379	1.528	
LPL+LTP	—	1.213	.982	.949	.971	
Fraction 4						.727
LPL	1.540	1.642	1.349(1.032)	1.574	1.744	
LPL+LTP	—	1.124	.917	2.067	1.301	
Fraction 5						.740
LPL	1.417	1.106	1.576(2.048)	1.545	1.797	
LPL+LTP	—	.975	1.855	2.386	2.279)	

The conditions of the control, LPL and LPL+LTP incubations are outlined in Table 1.

The plasma average is the average value for the fraction of 3 samples of normolipidemic plasma.

S/C= surface to core molar ratio =

$$\frac{\text{(moles of unesterified cholesterol + moles of phospholipid)}}{\text{(moles of cholesteryl ester + moles of triglyceride)}}$$

In the case of experiment C, the value for the LPL+HAL incubation was listed and the value for the LPL incubation at 63% VLDL triglyceride hydrolysis is in brackets.

FIGURE 30

MOLAR RATIOS OF
UC/APO B, PL/APO B, CE/APO B, TG/APO B AND APO E/APO B
OF FRACTIONS 1-5 OF THE LPL AND LPL+LTP INCUBATIONS OF
EXPERIMENT **B**

The figure contains the graphs depicting the ratios of:

unesterified cholesterol to apo B = UC/apo B
phospholipid to apo B = PL/apo B
cholesteryl ester to apo B = CE/apo B
triglyceride to apo B = TG/apo B

These ratios are graphed for fractions 1-5 of the LPL and LPL+LTP incubations of experiment **B**.

LPL refers to the inclusion of active lipoprotein lipase in the incubation.
LPL+LTP refers to the addition of the lipid transfer proteins to the LPL incubation.

The dark hatched bars represent the value for the LPL incubation and the light hatched bars represent the value for the LPL+LTP incubation.

FIGURE 30: MOLAR RATIOS OF UC/APO B, PL/APO B, CE/APO B, TG/APO B AND APO E/APO B OF FRACTIONS 1-5 OF THE LPL AND LPL+LTP INCUBATIONS OF EXPERIMENT B

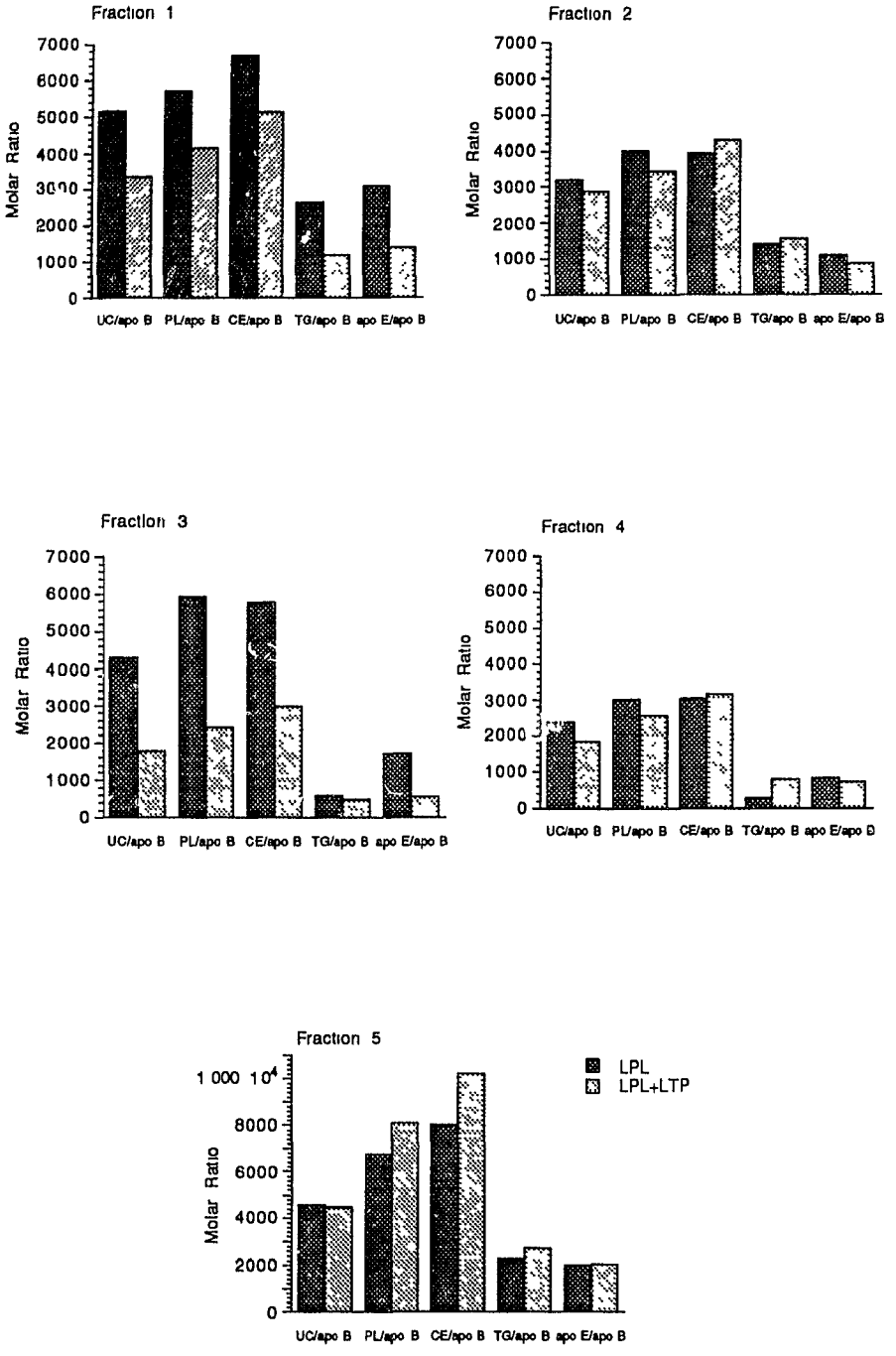


FIGURE 31

MOLAR RATIOS OF
UC/APO B, PL/APO B, CE/APO B, TG/APO B AND APO E/APO B
OF FRACTIONS 1-5 OF THE LPL AND LPL+LTP INCUBATIONS OF
EXPERIMENT E

The figure contains the graphs depicting the ratios of:

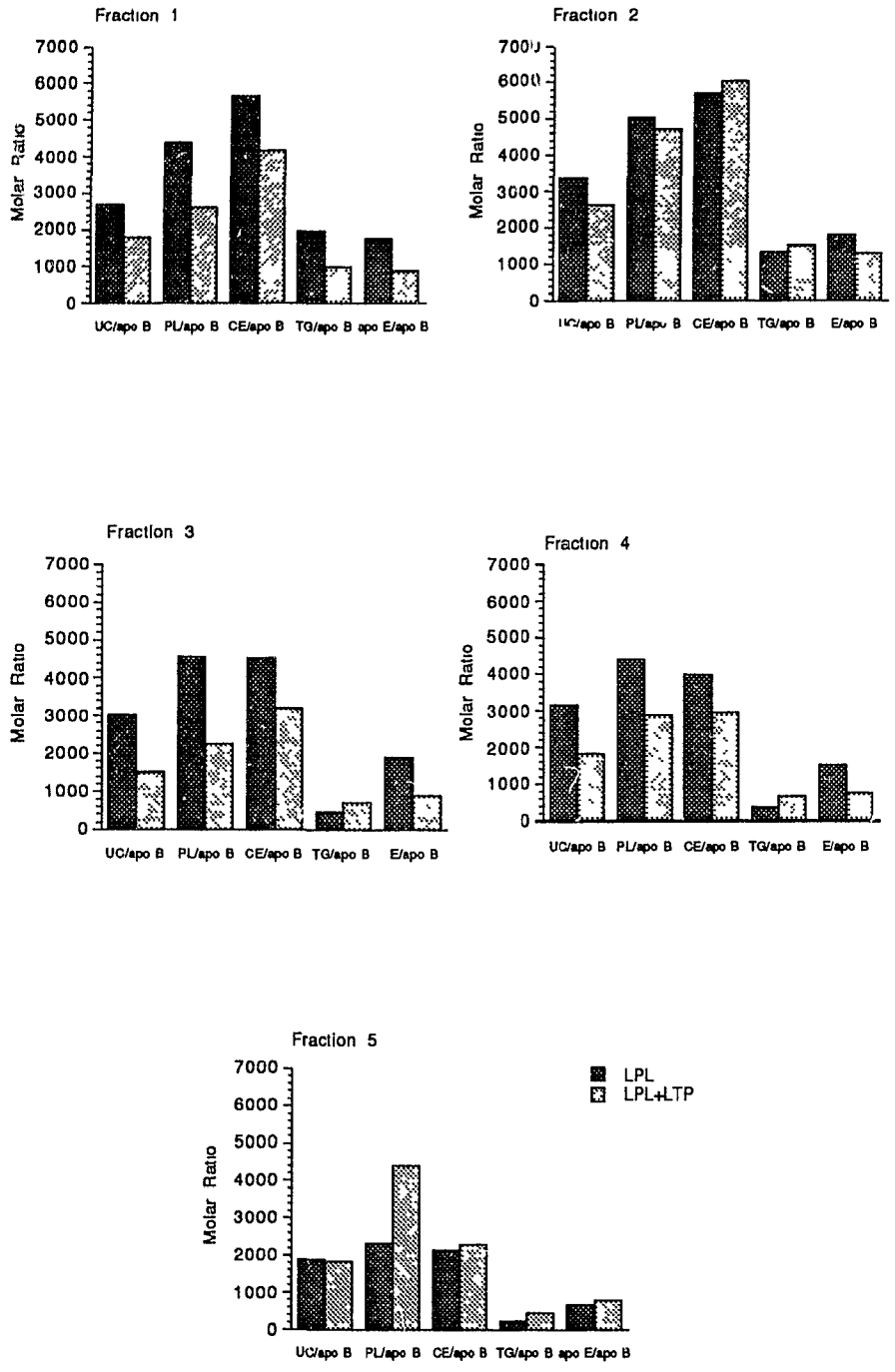
unesterified cholesterol to apo B = UC/apo B
phospholipid to apo B = PL/apo B
cholesteryl ester to apo B = CE/apo B
triglyceride to apo B = TG/apo B

These ratios are graphed for fractions 1-5 of the LPL and LPL+LTP incubations of experiment E.

LPL refers to the inclusion of active lipoprotein lipase in the incubation.
LPL+LTP refers to the addition of the lipid transfer proteins to the LPL incubation.

The dark hatched bars represent the value for the LPL incubation and the light hatched bars represent the value for the LPL+LTP incubation.

FIGURE 31: MOLAR RATIOS OF UC/APO B, PL/APO B, CE/APO B, TG/APO B AND APO E/APO B OF FRACTIONS 1-5 OF THE LPL AND LPL+LTP INCUBATIONS OF EXPERIMENT E



how the lipid transfer protein preparation, in the presence of lipoprotein lipase, affected to apo B (UC/apo B), phospholipid to apo B (PL/apo B), cholesteryl ester to apo B (CE/apo B), triglyceride to apo B (TG/apo B) and apo E to apo B (apo E/apo B) were calculated for fractions 1-5 for the LPL and LPL+LTP incubations. The ratios of the LPL+LTP incubation were compared to those of the LPL incubation in order to establish the particles that isolated in the VLDL/IDL/LDL range. Figures 30 and 31 graphically demonstrate how these ratios varied for the LPL and LPL+LTP incubations in experiment B and E, respectively.

In the LPL+LTP incubation as compared to the LPL incubation, the unesterified cholesterol, phospholipid and apo E content of the fraction 1-4 were reduced with respect to the apo B content. The decrease in the ratio reflects a loss of surface components in the LPL+LTP incubation relative to the LPL incubation.

The triglyceride content was higher in most cases in the LPL+LTP incubation as compared to the LPL incubation indicating more core relative to apo B in the LPL+LTP versus the LPL incubation. However, in fraction 1 the ratio was lower in the LPL+LTP incubation.

The cholesteryl ester content relative to apo B was variable. Using the results of experiments B and E, the CE/apo B ratio was reduced in fractions 1 and 3, higher in fractions 2 and 5 and either the same or lower in fraction 4.

Overall the ratios indicate that the addition of the lipid transfer protein activity to that of the lipoprotein lipase activity resulted in a loss of all surface and core components from the particles in fraction 1 relative to apo B and a loss of surface with a retention or increase in triglyceride core in fractions 2-4. This conclusion assumes that the immunological expression of apo B was the same for the LPL and the LPL+LTP incubations.

C) *IN VIVO* EXPERIMENTS:

In vivo heparin-induced lipolysis was studied to compare the results to those of the *in vitro* incubations. The *in vivo* conditions were similar to the *in vitro* conditions in that the lipase was released from the endothelium and was therefore acting in a "solution" rather than being bound to the capillary endothelium. The lipolysis of triglycerides was rapid and the time span for sampling was similar. The subjects' plasma lipids were in the low-normal range for triglycerides and total cholesterol as were the lipids in the *in vitro* situation. *In vivo* heparin-induced lipolysis differed from the *in vitro* situation in that the lipoprotein mass distribution was affected not only by the lipolysis by lipoprotein lipase and hepatic lipase but ongoing uptake as well as synthesis of lipoproteins during the experimental time period may have been occurring. The *in vivo* situation also contains the combination of LPL+HAL+LTP which was not a condition in the *in vitro* situation and LCAT was presumably present in the *in vivo* studies. These differences must be kept in mind when interpreting the data and comparing the results to the *in vitro* situation. Therefore a net gain or loss in the entire gradient *in vivo* may not only reflect a difference in the recovery/hydrolysis of the lipids and apoproteins but may reflect the presence of newly synthesized lipoproteins entering the plasma or uptake of lipoproteins from the plasma compartment. In the case of cholesteryl ester, an increase may be due to the activity of LCAT in synthesizing cholesteryl ester from unesterified cholesterol and phospholipid, particularly in the HDL region.

The results of *in vivo* heparin-induced lipolysis for two normolipidemic subjects are outlined in Figures 32-41. The pre-heparin blood sample was taken at 0 time and the post-heparin sample was taken after 1-1.5 hours of heparin infusion. Each figure contains the pre- and post-heparin distribution of subject A and subject B. The changes in lipids in the VLDL/LDL region and in the HDL region and the net changes in the post-heparin sample relative to the pre-heparin sample are listed in Table 27. The corresponding values for the apoproteins are listed in Table 29. The unesterified cholesterol to

phospholipid ratio (UC/PL), the cholesteryl ester to triglyceride ratio (CE/TG) and the surface to core ratio (S/C) were calculated for fractions 1-5 and are listed in Tables 30-32. All data are listed in appendix A.

1) LIPID DISTRIBUTION:

UNESTERIFIED CHOLESTEROL: (Table 27, Figures 32 and 33)

There was a net loss of unesterified cholesterol from the VLDL/LDL region and a net gain in the HDL region. The transfer was similar to the *in vitro* results for the LPL, LPL+HAL and LPL+LTP incubations. The amount transferred to HDL was similar to that transferred with the LPL and LPL+HAL incubation but less than that observed with the LPL+LTP incubation (70-100 µg) (Table 10). The loss in the VLDL/LDL region was in fraction 1, i.e., from VLDL (Figure 32 and 33). A slight increase was seen in unesterified cholesterol in the LDL peak (Figure 32 and 33).

The gain of unesterified cholesterol in the HDL region was in the region of the peak mass in subject A (fractions 12-15) and on the light side of the peak mass in subject B (fractions 11-13) (Figures 32 and 33). No shift in the HDL peak was observed. This gain on the light side without a shift in the HDL peak in subject B was similar to the trend observed in the *in vitro* situation in the LPL incubation (Figures 8 and 9).

PHOSPHOLIPID: (Table 27, Figures 34 and 35)

There was a marked loss of phospholipid from the VLDL/LDL region and a substantial gain in the HDL region (Table 27). These results were similar to the LPL, LPL+HAL and the LPL+LTP incubations with respect to the amount lost from the VLDL/LDL region and the transfer being from the VLDL/LDL region to the HDL region (Table 10). The loss from VLDL was due to transfer to HDL as well as VLDL phospholipid hydrolysis as observed in the *in vitro* situation. The estimated amounts of hydrolysis were calculated from the net loss in the gradient of the post-heparin sample relative to the pre-heparin sample divided by the phospholipid mass in fractions 1 and 2

TABLE 27

NET GAIN OR LOSS OF LIPIDS IN THE VLDL/LDL AND HDL REGIONS IN SUBJECTS A AND B

	Subjects					
Lipid:	A	B	A	B	A	B
	VLDL/LDL µg		HDL µg		Net Change µg	
Unesterified Cholesterol	-99	-127	+76	+42	-23	-85
Phospholipid	-920	-990	+512	+670	-408	-320
Cholesteryl Ester	-577	-875	+504	+159	-73	-716
Triglyceride	-2894	-2333	-389	-380	-3283	-2713

Subjects A and B uderwent a heparin infusion and blood samples were taken at time 0 (pre-heparin sample) and one to one and a half hours later (post-heparin sample).

The value for the gain or loss was arrived at by summing the changes between the pre- and post-heparin samples for fractions 1-7 for VLDL/LDL, fractions 8-20 for HDL and fractions 1-20 for the net change.

FIGURE 32

THE UNESTERIFIED CHOLESTEROL DISTRIBUTION AMONG THE LIPOPROTEINS AND THE CHANGE ACROSS THE GRADIENT OF SUBJECT A

The subject underwent a heparin infusion for a period of 1-1.5 hours. A blood sample was taken at time 0 and at the end of the infusion period and the samples were referred to as the pre-heparin and post-heparin sample, respectively.

The plasma from the blood samples was subjected to density gradient ultracentrifugation and each fraction from the gradient was analysed for lipid mass by gas liquid chromatography.

Figure a depicts the pre- and post-heparin distribution of unesterified cholesterol among the lipoproteins of subject **A** .
For figure a the samples are represented as:

pre-heparin = O
post-heparin = ●

Figure b depicts the net change (post-heparin value - pre-heparin value) in each fraction of the gradient.

FIGURE 32: THE UNESTERIFIED CHOLESTEROL DISTRIBUTION AMONG THE LIPOPROTEINS AND THE CHANGE ACROSS THE GRADIENT OF SUBJECT A

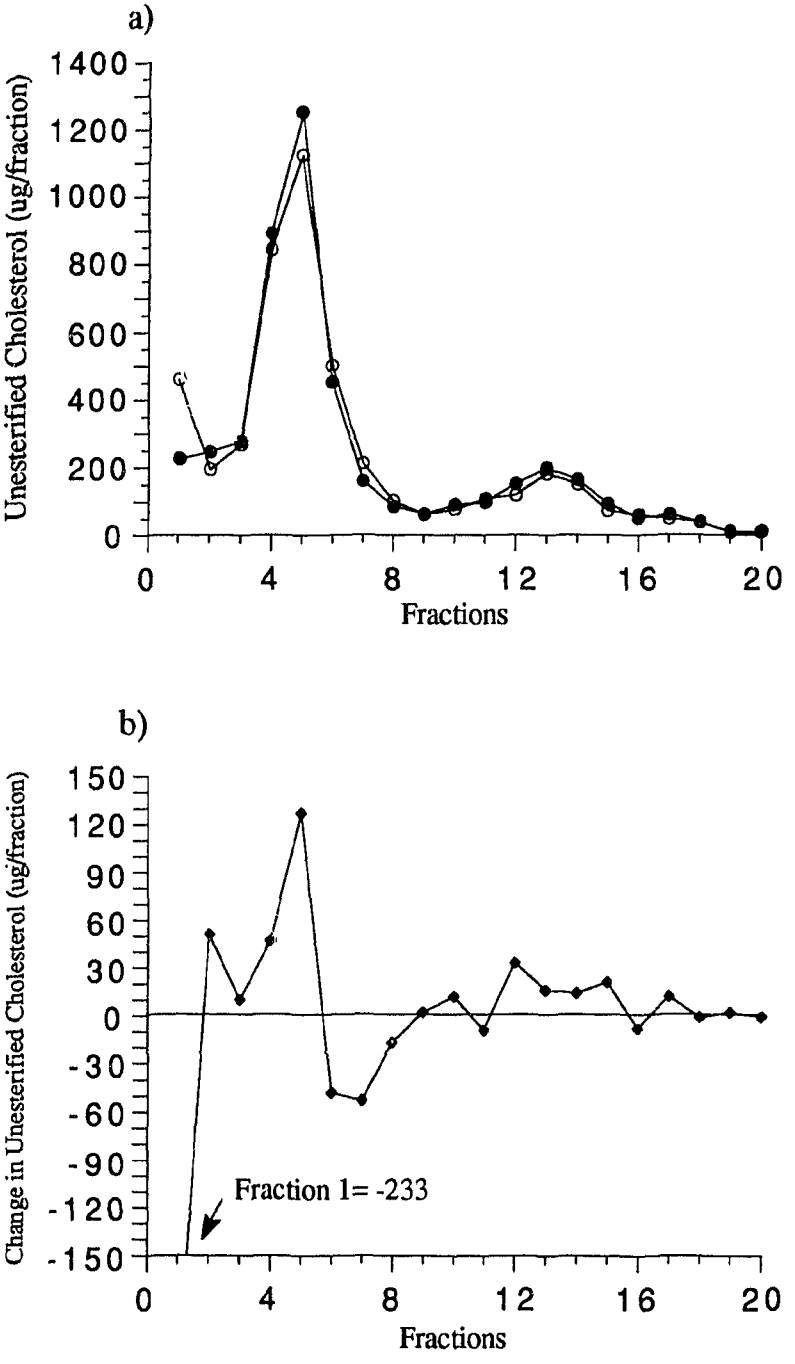


FIGURE 33

THE UNESTERIFIED CHOLESTEROL DISTRIBUTION AMONG THE LIPOPROTEINS AND THE CHANGE ACROSS THE GRADIENT OF SUBJECT **B**

The subject underwent a heparin infusion for a period of 1-1.5 hours. A blood sample was taken at time 0 and at the end of the infusion period and the samples were referred to as the pre-heparin and post-heparin sample, respectively.

The plasma from the blood samples was subjected to density gradient ultracentrifugation and each fraction from the gradient was analysed for lipid mass by gas liquid chromatography.

Figure a depicts the pre- and post-heparin distribution of unesterified cholesterol among the lipoproteins of subjects B .

For figure a the samples are represented as:

pre-heparin = O

post-heparin = ●

Figure b depicts the net change (post-heparin value - pre-heparin value) in each fraction of the gradient .

FIGURE 33: THE UNESTERIFIED CHOLESTEROL DISTRIBUTION AMONG THE LIPOPROTEINS AND THE CHANGE ACROSS THE GRADIENT OF SUBJECT B

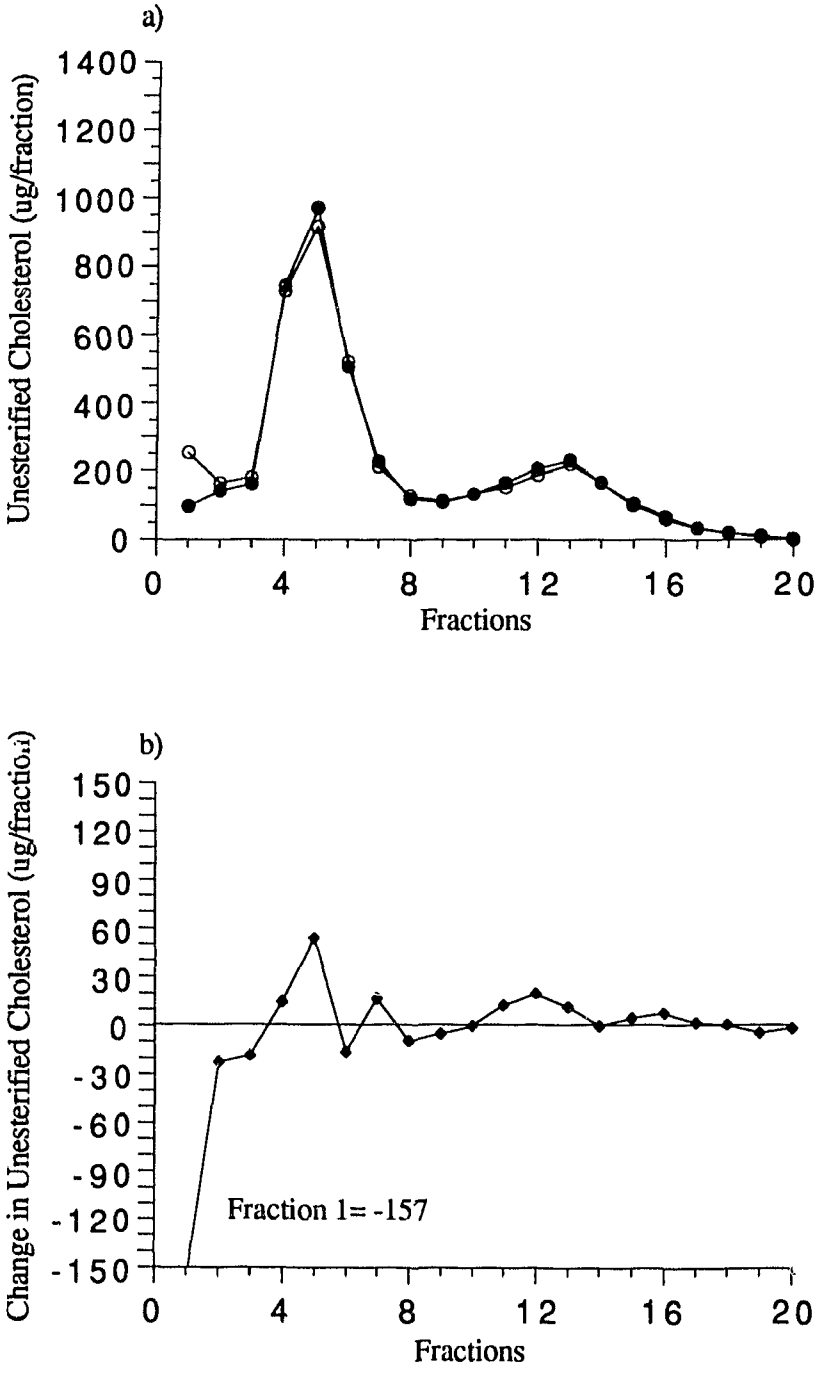


FIGURE 34

THE PHOSPHOLIPID DISTRIBUTION AMONG THE LIPOPROTEINS AND CHANGE ACROSS THE GRADIENT OF SUBJECT A

The subject underwent a heparin infusion for a period of 1-1.5 hours. A blood sample was taken at time 0 and at the end of the infusion period and the samples were referred to as the pre-heparin and post-heparin sample, respectively.

The plasma from the blood samples was subjected to density gradient ultracentrifugation and each fraction from the gradient was analysed for lipid mass by gas liquid chromatography.

Figure a depicts the pre- and post-heparin distribution of phospholipid among the lipoproteins of subject **A** .

For figure a the samples are represented as:

pre-heparin = O

post-heparin = ●

Figure b depicts the net change (post-heparin value - pre-heparin value) in each fraction of the gradient.

FIGURE 34: THE PHOSPHOLIPID DISTRIBUTION AMONG THE LIPOPROTEINS AND CHANGE ACROSS THE GRADIENT OF SUBJECT **A**

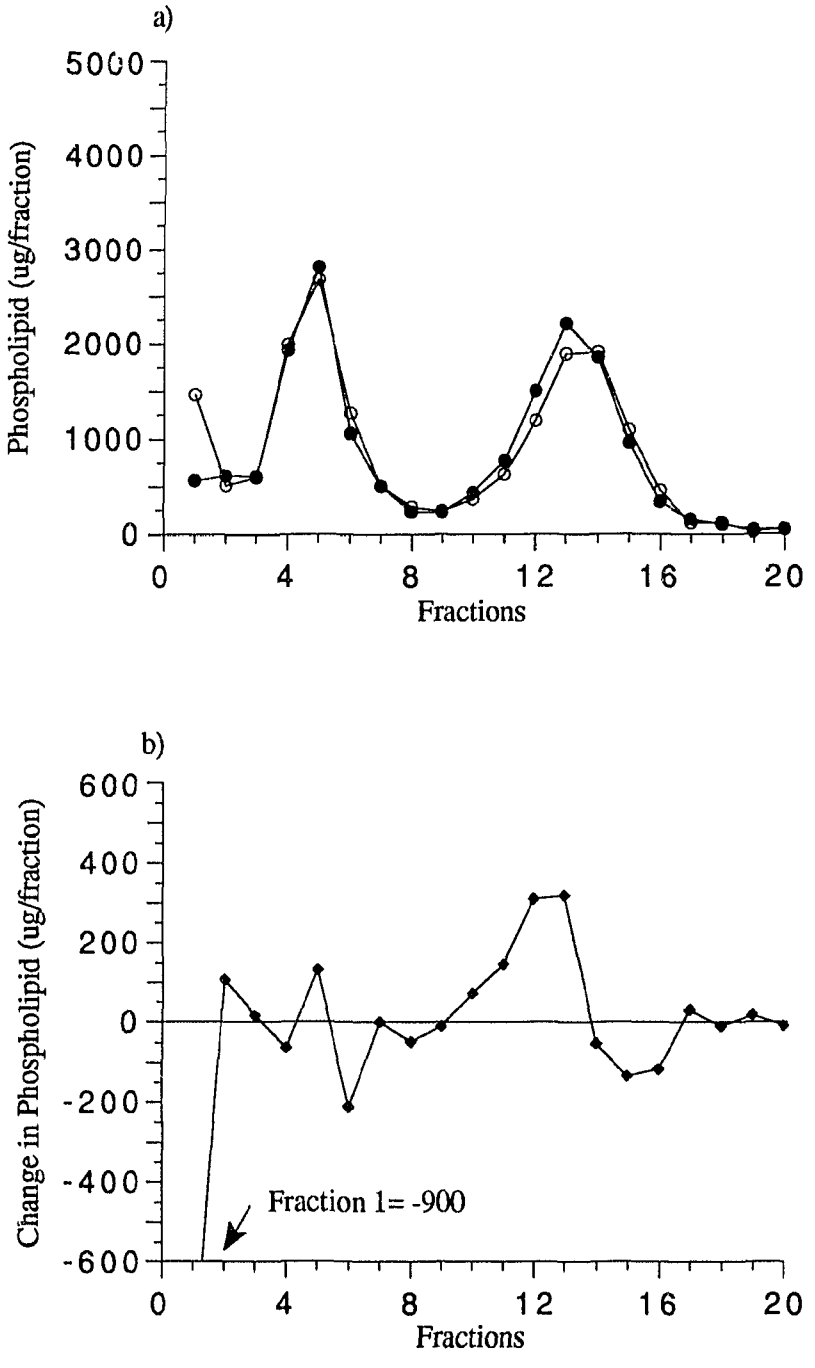


FIGURE 35

THE PHOSPHOLIPID DISTRIBUTION AMONG THE LIPOPROTEINS AND CHANGE ACROSS THE GRADIENT OF SUBJECT **B**

The subject underwent a heparin infusion for a period of 1-1.5 hours. A blood sample was taken at time 0 and at the end of the infusion period and the samples were referred to as the pre-heparin and post-heparin sample, respectively.

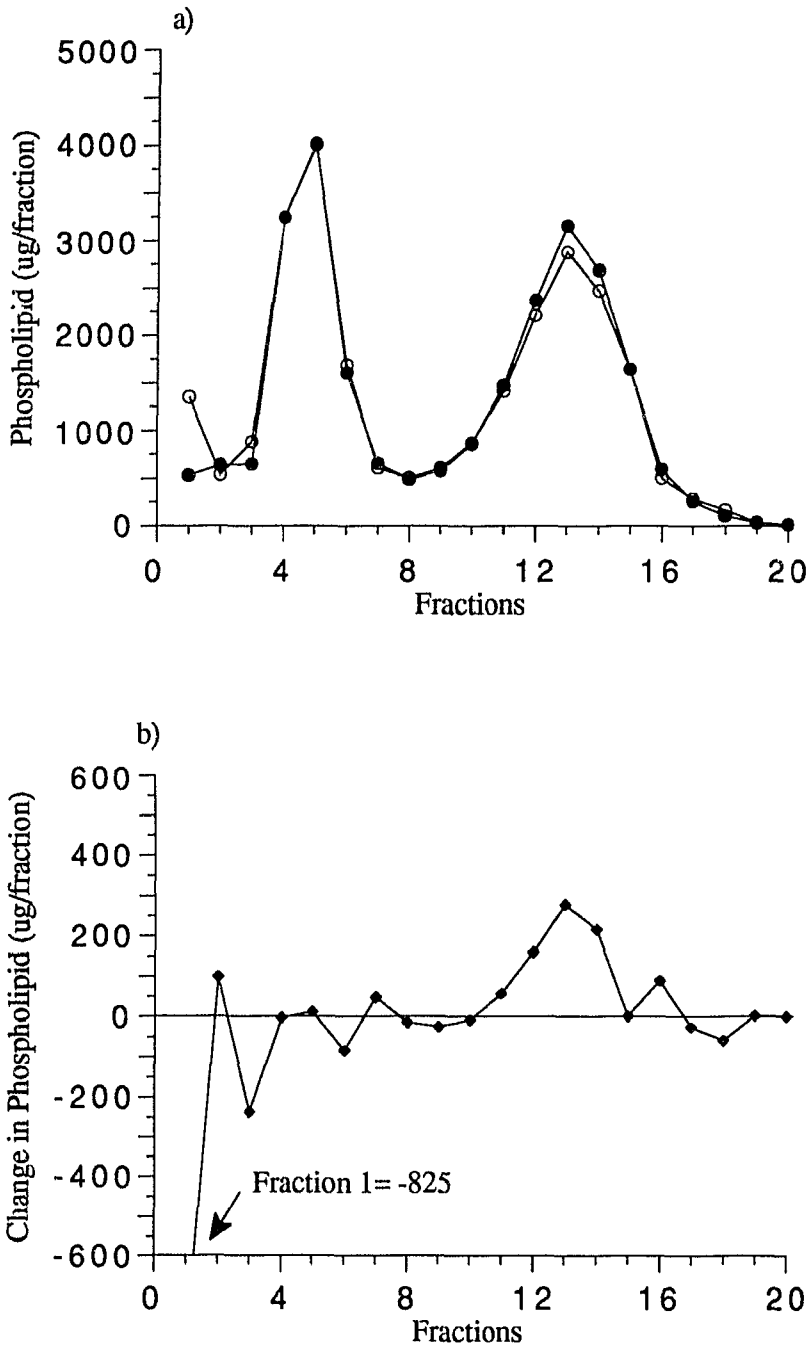
The plasma from the blood samples was subjected to density gradient ultracentrifugation and each fraction from the gradient was analysed for lipid mass by gas liquid chromatography.

Figure a depicts the pre- and post-heparin distribution of phospholipid among the lipoproteins of subject **B**.
For figure a the samples are represented as:

pre-heparin = O
post-heparin = ●

Figure b depicts the net change (post-heparin value - pre-heparin value) in each fraction of the gradient.

FIGURE 35: THE PHOSPHOLIPID DISTRIBUTION AMONG THE LIPOPROTEINS AND CHANGE ACROSS THE GRADIENT OF SUBJECT **B**



of the pre-heparin sample. The estimated amount of hydrolysis for subjects A and B was 21.6% and 16.9%, respectively which was less than that observed in the LPL incubation but similar to that of the LPL+LTP incubation (Table 8). The similarity in the percentage of hydrolysis of phospholipid of the *in vivo* situation with that of the LPL+LTP incubations suggests that the phospholipid was transferred to HDL before it could be hydrolysed on VLDL by lipoprotein lipase *in vivo* which was observed *in vitro* when the lipid transfer proteins were present in the incubation with lipoprotein lipase. The loss of phospholipid that occurred in the VLDL/LDL region was from fraction 1, i.e., from VLDL and a loss in the LDL region was not obvious (Figures 34 and 35). It should be noted that the percentage of hydrolysis calculated from the net loss of phospholipid may also reflect hydrolysis by hepatic lipase, LCAT activity or lipoprotein uptake.

The amounts of phospholipid transferred to the HDL region (+512 µg and +670 µg) in subjects A and B (Table 27) were higher than that observed in the LPL incubations (Table 10). When compared to the LPL+LTP incubation, the amounts transferred to HDL in the two subjects were greater than the amount transferred in Experiments B and C (+419 µg and +476 µg) and equal or less than the amount transferred in experiments D and C (+682 µg and +885 µg). The phospholipid transfer from VLDL to HDL in the *in vivo* situation may therefore reflect the combined activity of the lipid transfer proteins and the action of lipoprotein lipase. The gain in phospholipid mass in subject A was on the light side of the HDL peak and a shift in the peak from fractions 13-14 to fraction 13 occurred (Figure 34). These results were similar to the results of the LPL incubation in terms of a gain on the light side of the peak (Figure 11) and were similar to the results of LPL+LTP incubation in terms of a gain on the light side with a shift in the peak. In subject B the gain was in the HDL peak fractions 13, 14 and 15 and did not tend to distribute to a particular region of the HDL peak (Figure 35).

CHOLESTERYL ESTER: (Table 27, Figures 36 and 37)

Cholesteryl ester was lost from the VLDL/ LDL region (Table 27). Subject A lost cholesteryl ester from fraction 1 (VLDL) and in the dense fractions of LDL (6 and 7) (Figure 36) . A gain was observed on the light side (fraction 4) and in the peak of LDL. Subject B lost cholesteryl ester mass from fractions 1 and 2 but some loss from the LDL peak region was observed as well. There was a relatively large net loss of cholesteryl ester from the plasma in subject B which was not observed in subject A. (This loss only represents 3% of the subject's VLDL cholesteryl ester and may be due to uptake.)

The HDL region gained cholesteryl ester in both subjects (Table 27). The gain in subject A was in the range of that gained in the LPL incubation in experiment E (Table 12). The gain in subject B was in the range of the LPL incubation in experiment D and of the LPL+LTP incubation of experiment E (Table 12). The gain was on the light side of the peak with a shift in the peak to fraction 13 in subject A who demonstrated a greater gain in cholesteryl ester than subject B (Figure 36). There was a small loss in the dense region (fraction 16). These results are similar to the results of the LPL and LPL+LTP incubations of experiment E where a net gain in cholesteryl ester occurred (Figure 11). Subject B gained on the light side and in the peak but there was no shift in the peak location (Figure 36). This gain on the light side without a shift in peak was similar to distribution of the LPL incubation when a net gain in cholesteryl ester was absent (experiments A, B) (Figure 12). It is possible that a net gain of cholesteryl ester in the HDL region may be due to LCAT activity in the *in vivo* situation which was not present in the *in vitro* incubations. The net gain in the HDL region corresponds to an LCAT activity of 26.5 and 12.5 µg cholesteryl ester/ml/hr which is in the range of the plasma activity of 19-56 µg cholesteryl ester/ml/hr when estimated with an ideal synthetic substrate.

FIGURE 36

THE CHOLESTERYL ESTER DISTRIBUTION AMONG THE LIPOPROTEINS AND CHANGE ACROSS THE GRADIENT OF SUBJECT A

The subject underwent a heparin infusion for a period of 1-1 1.5 hours. A blood sample was taken at time 0 and at the end of the infusion period and the samples were referred to as the pre-heparin and post-heparin sample, respectivcly.

The plasma from the blood samples was subjected to density gradient ultracentrifugation and each fraction from the gradient was analysed for lipid mass
by gas liquid chromatography.

Figure a depicts the pre- and post-heparin distribution of cholesteryl ester among the lipoproteins of subject A .
For figure a the samples are represented as:

pre-heparin = O
post-heparin = ●

Figure b depicts the net change (post-heparin value - pre-heparin value) in each fraction of the gradient.

FIGURE 36: THE CHOLESTERYL ESTER DISTRIBUTION AMONG THE LIPOPROTEINS AND CHANGE ACROSS THE GRADIENT OF SUBJECT A

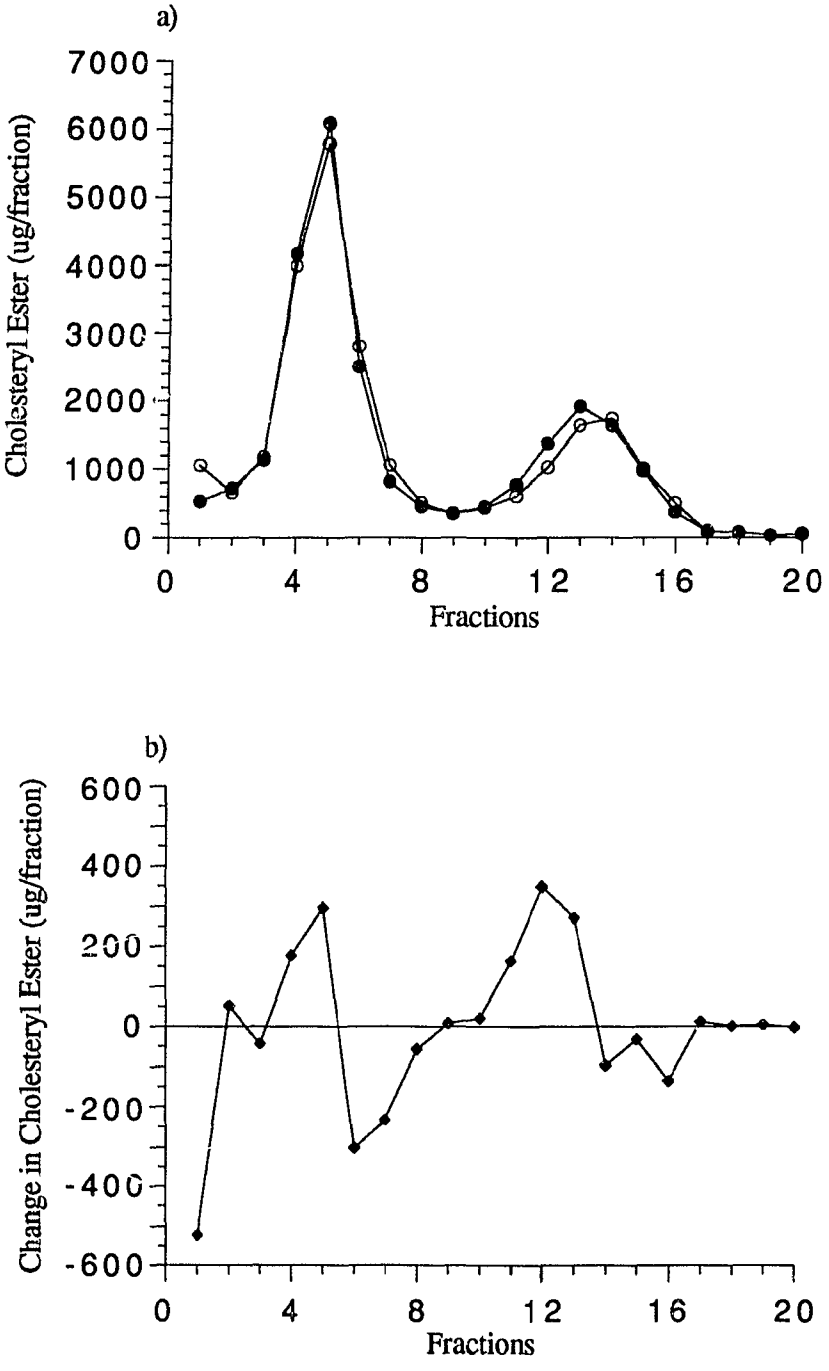


FIGURE 37

THE CHOLESTERYL ESTER DISTRIBUTION AMONG THE LIPOPROTEINS AND CHANGE ACROSS THE GRADIENT OF SUBJECT **B**

The subject underwent a heparin infusion for a period of 1-1.5 hours. A blood sample was taken at time 0 and at the end of the infusion period and the samples were referred to as the pre-heparin and post-heparin sample, respectively.

The plasma from the blood samples was subjected to density gradient ultracentrifugation and each fraction from the gradient was analysed for lipid mass by gas liquid chromatography.

Figure a depicts the pre- and post-heparin distribution of cholesteryl ester among the lipoproteins of subject **B** .

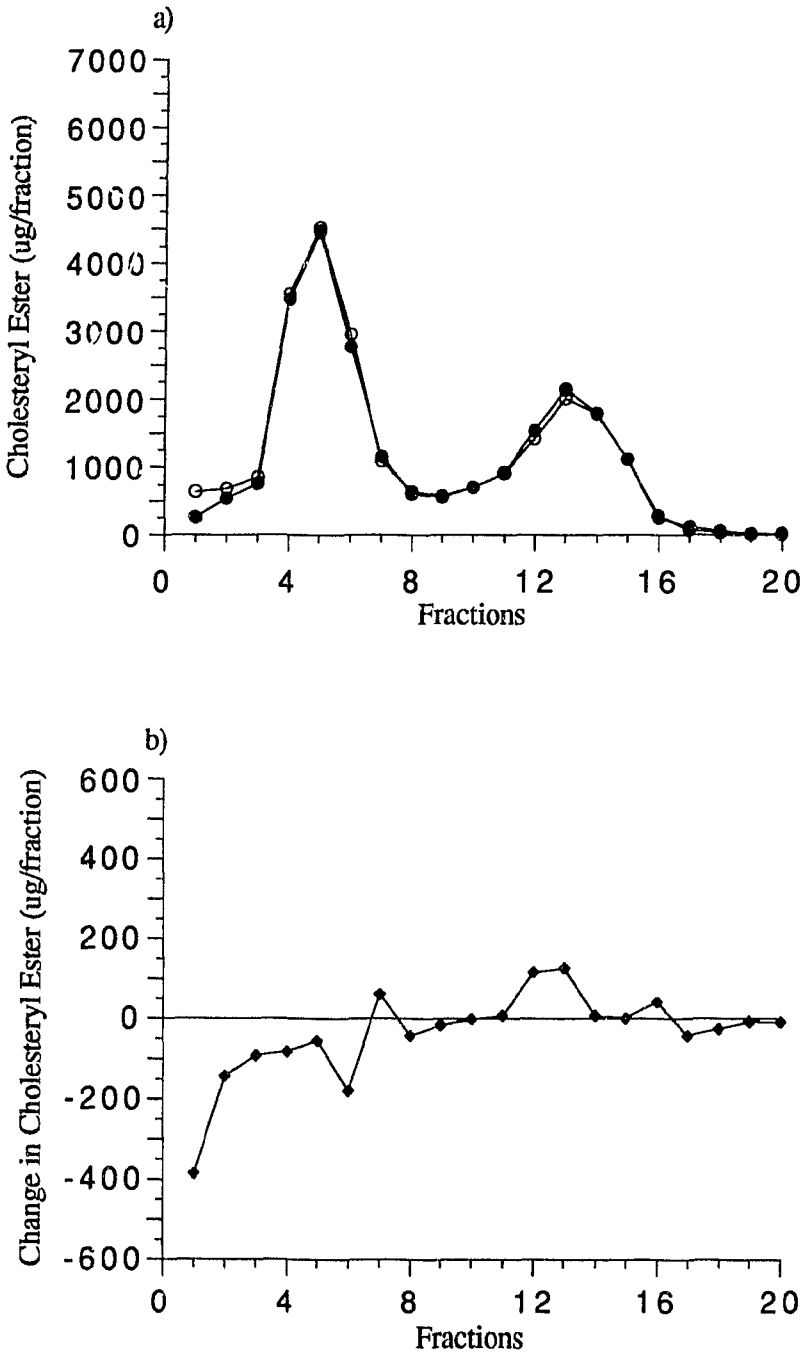
For figure a the samples are represented as:

pre-heparin = O

post-heparin = ●

Figure b depicts the net change (post-heparin value - pre-heparin value) in each fraction of the gradient.

FIGURE 37: THE CHOLESTERYL ESTER DISTRIBUTION AMONG THE LIPOPROTEINS AND CHANGE ACROSS THE GRADIENT OF SUBJECT B



TRIGLYCERIDE: (Tables 27 and 28, Figures 38 and 39)

Triglycerides were lost from the VLDL/LDL region (Table 27). The major loss was in fraction 1. The percentage triglyceride hydrolysis was 75.6% and 80.8% for subjects A and B respectively (83.9% and 86.5% if the loss from fraction 1 rather than fraction from fractions 1 and 2 was used). The percentage hydrolysis was 7-16% lower than that of the *in vitro* incubations that contained active lipoprotein lipase (88-93%). The HDL fractions demonstrated a loss of triglycerides throughout (Table 28, Figures 38 and 39). Triglyceride hydrolysis in the HDL region, based on the *in vitro* results, reflects the activity of hepatic lipase rather than lipoprotein lipase. The percentage of HDL hydrolysis was 74.8% and 48% for subjects A and B, respectively. This corresponded to the hydrolysis reported in the LPL+HAL incubations of 71.8%, 73.1% and 56.7%. Table 28 lists the percentage hydrolysis of the triglycerides in fractions 8-20 for subjects A and B. The hydrolysis was greatest in fractions 14-16 of subject A and 13-15 of subject B. Both these regions include the HDL triglyceride peak fraction and fractions on the dense side of the peak. The same trend was apparent in the *in vitro* LPL+HAL incubation of experiment A but differed in experiment B and C. 2)

APOPROTEIN DISTRIBUTION:

APO CII : (Table 29, Figure 40)

Apo CII, present primarily in fraction 1, was extensively transferred to the HDL region (Table 29). The percentage transfer from the VLDL/LDL region was 84.7% and 97.7%. The values were similar to the percentage transfer in the LPL, LPL+HAL and LPL+LTP incubations (Table 20), indicating that lipoprotein lipase activity in plasma was responsible for the transfer of apo CII from VLDL to HDL.

TABLE 28

HYDROLYSIS OF HDL TRIGLYCERIDES IN SUBJECTS **A** AND **B**

	Subject					
	A			**B**		
Fraction #	Pre-heparin Mass	Post-heparin	% Loss	Pre-heparin Mass	Post-heparin	% Loss
	μg			μg		
8	36	14	61	73	63	14
9	20	6	70	79	65	18
10	19	6	68	65	37	43
11	29	8	72	99	38	62
12	46	10	78	91	67	26
13	111	31	72	139	49	65
14	119	22	81.5	118	36	69.5
15	61	11	82	109	42	61.5
16	36	6	83	14	9	36.2
17	17	5	71	1	2	(50)
18	11	5	54.5	1	1	0
19	10	3	70	1	0	0
20	5	4	20	1	2	(50)

Subjects **A** and **B** underwent a heparin infusion and blood samples were taken at time 0 (pre-heparin sample) and 1 to 1.5 hours later (post-heparin sample).

The values for the losses were arrived at by subtracting the pre-heparin value from the and post-heparin value.

FIGURE 38

THE TRIGLYCERIDE DISTRIBUTION AMONG THE LIPOPROTEINS AND THE CHANGE ACROSS THE GRADIENT OF SUBJECT A

The subject underwent a heparin infusion for a period of 1-1.5 hours. A blood sample was taken at time 0 and at the end of the infusion period and the samples were referred to as the pre-heparin and post-heparin sample, respectively.

The plasma from the blood samples was subjected to density gradient ultracentrifugation and each fraction from the gradient was analysed for lipid mass by gas liquid chromatography.

Figure a depicts the pre- and post-heparin distribution of triglyceride among the lipoproteins of subject **A** .
For figure a the samples are represented as:

pre-heparin = O
post-heparin = ●

Figure b depicts the net change (post-heparin value - pre-heparin value) in each fraction of the gradient.

FIGURE 38: THE TRIGLYCERIDE DISTRIBUTION AMONG THE LIPOPROTEINS AND THE CHANGE ACROSS THE GRADIENT OF SUBJECT A

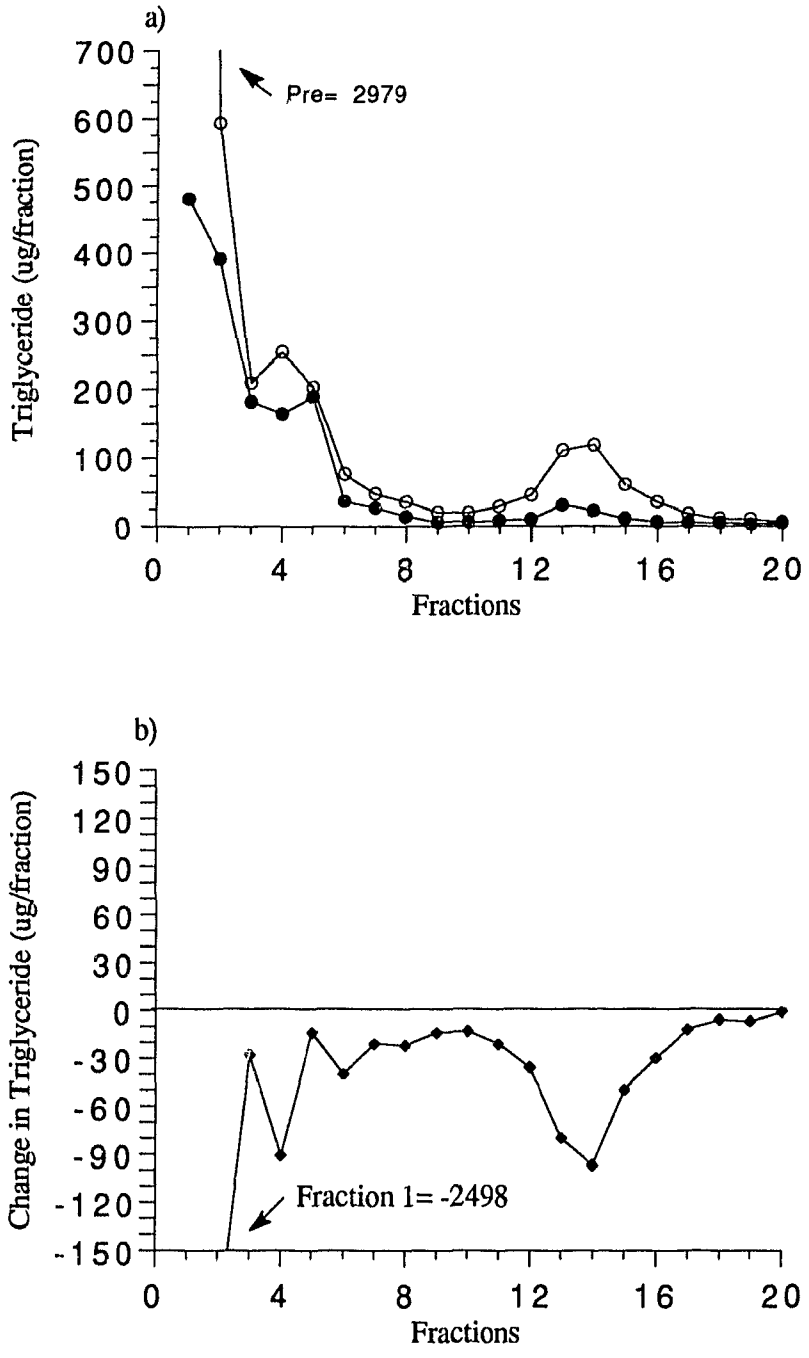


FIGURE 39

THE TRIGLYCERIDE DISTRIBUTION AMONG THE LIPOPROTEINS AND THE CHANGE ACROSS THE GRADIENT OF SUBJECT **B**

The subject underwent a heparin infusion for a period of 1-1.5 hours. A blood sample was taken at time 0 and at the end of the infusion period and the samples were referred to as the pre-heparin and post-heparin sample, respectively.

The plasma from the blood samples was subjected to density gradient ultracentrifugation and each fraction from the gradient was analysed for lipid mass by gas liquid chromatography.

Figure a depicts the pre- and post-heparin distribution of triglyceride among the lipoproteins of subject **B** .
For figure a the samples are represented as:

pre-heparin = ○
post-heparin = ●

Figure b depicts the net change (post-heparin value - pre-heparin value) in each fraction of the gradient.

FIGURE 39: THE TRIGLYCERIDE DISTRIBUTION AMONG THE LIPOPROTEINS AND THE CHANGE ACROSS THE GRADIENT OF SUBJECT **B**

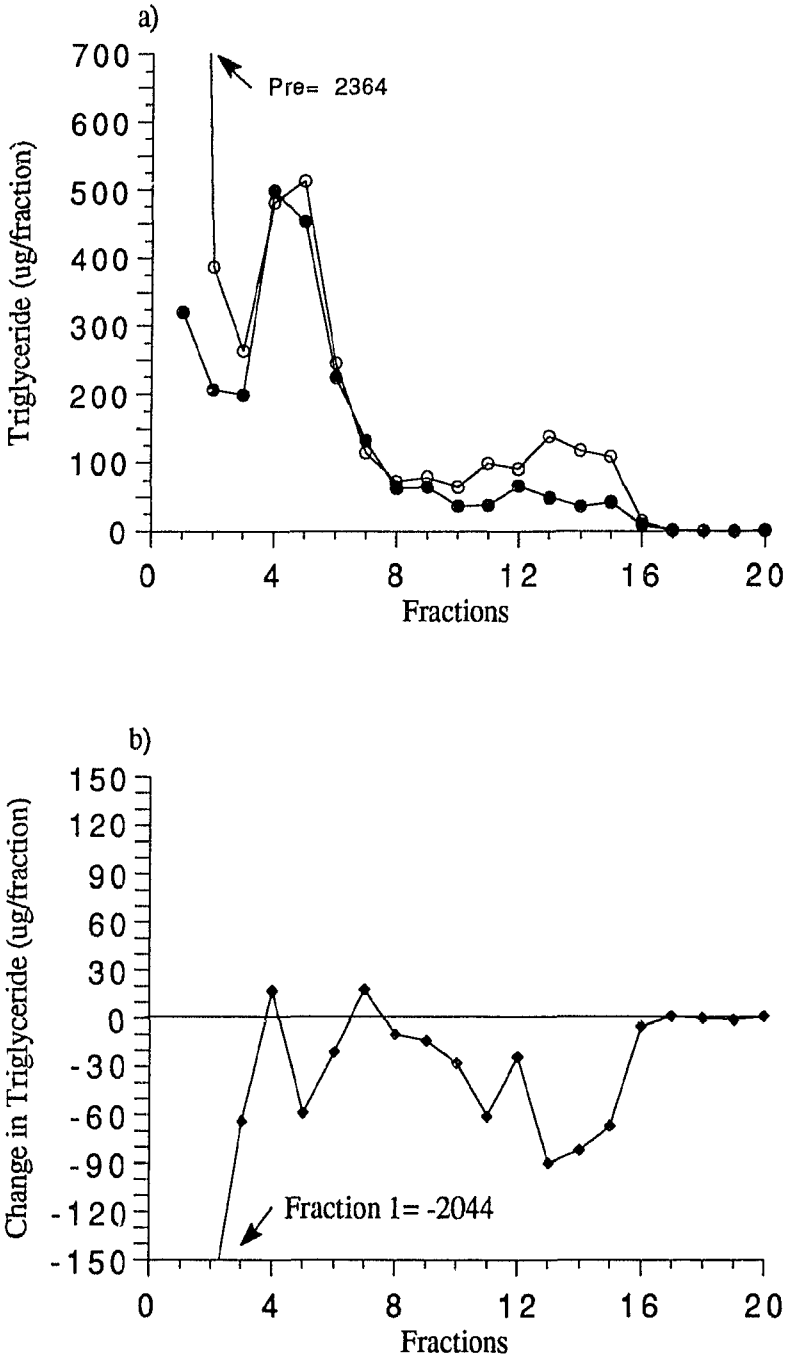


TABLE 29

NET GAIN OR LOSS OF APOPROTEINS IN THE VLDL/LDL AND HDL REGIONS FOR SUBJECTS A AND B

	Subjects					
Apoprotein	A	B	A	B	A	B
	VLDL/LDL µg		HDL µg		Net Change µg	
Apo E	-61	-74	+129	+28	+68	-46
Apo CII	-111	-84	+100	+162	-11	+78
Apo CIII	-190	-210	+26	+360	-164	+150

Subjects **A** and **B** uderwent a heparin infusion and blood samples were taken at time 0 (pre-heparin sample) and one to one and a half hours later (post-heparin sample).

The values for the gain and loss was arrived at by summing the changes between the pre- and post-heparin samples for fractions 1-7 for VLDL/LDL, fractions 8-20 for HDL and fractions 1-20 for the net change.

FIGURE 40

THE APO CII DISTRIBUTION AMONG THE LIPOPROTEINS OF SUBJECTS **A** AND **B**

Each subject underwent a heparin infusion for a period of 1-1.5 hours. A blood sample was taken at time 0 and at the end of the infusion period and these samples were referred to as the pre-heparin and post-heparin sample, respectively.

The plasma from these blood samples was subjected to density gradient ultracentrifugation and each fraction from the gradient was analysed for apo CII by electroimmunoassay.

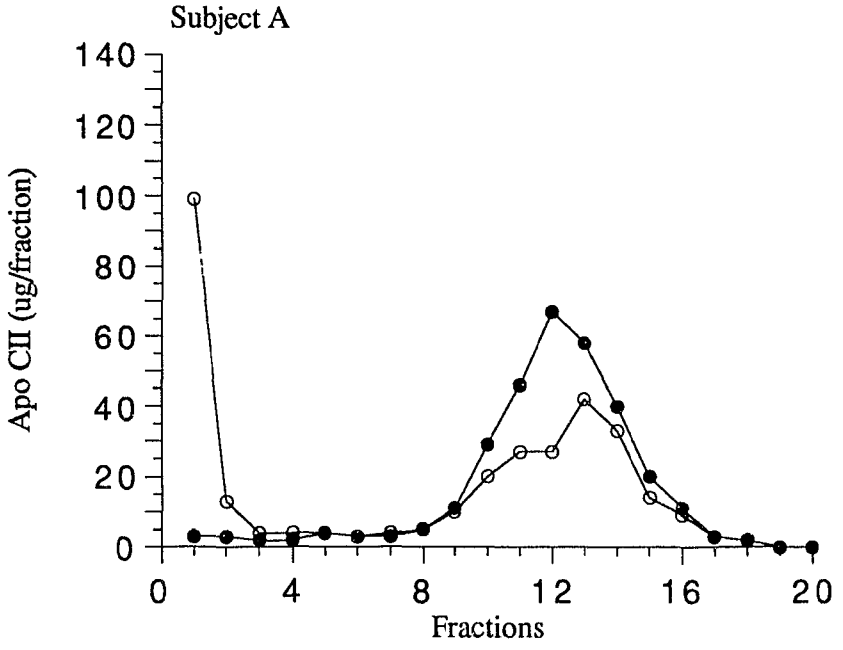
The figures depict the pre- and post-heparin distribution of apo CII among the lipoproteins of subjects **A** and **B**.

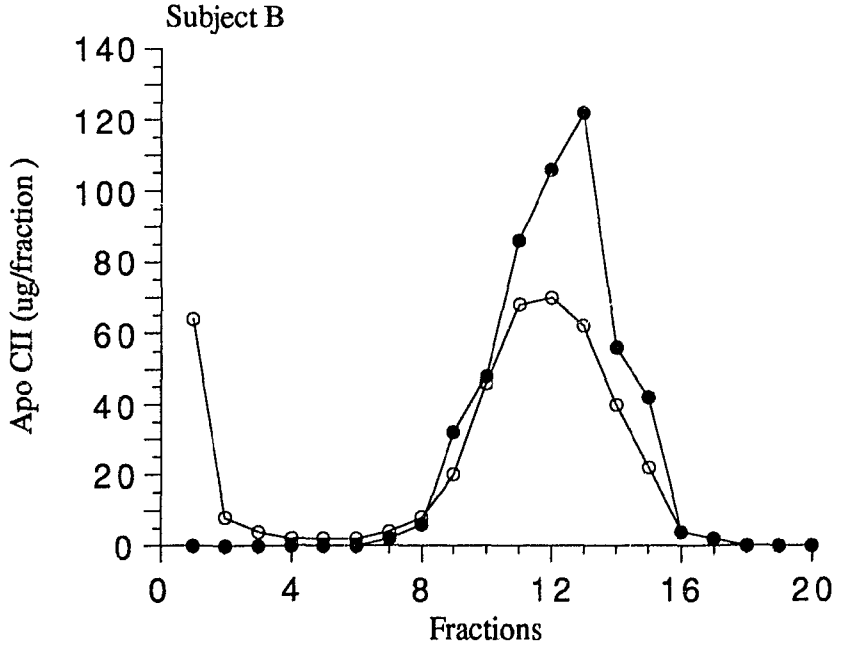
For each figure, the samples are represented as:

pre-heparin = O

post-heparin = ●

FIGURE 40: THE APO CII DISTRIBUTION AMONG THE LIPOPROTEINS OF SUBJECTS **A** AND **B**





APO CIII: (Table 29)

Apo CIII, which was present mainly in fraction 1 was also extensively transferred from VLDL to the HDL region (Table 29). The percentage transfer from the VLDL/LDL region was 81.2% and 70.5% in subjects A and B, respectively . This was somewhat less than the transfer observed in the LPL, LPL+HAL and LPL+LTP incubations which was in the 96-100% range in most cases (Table 20).

APO E: (Table 29, Figure 41)

Apo E was transferred mainly from fraction 1 to the HDL region. Both subjects lost a similar amount from the VLDL/LDL region (Table 29). The loss differed when expressed as a percentage loss of the pre-heparin apo E mass (100% and 37% in subjects A and B, respectively). The apo E transferred was substantially higher than the percentage transfer observed in the LPL incubations (average = 28%) indicating that the action of lipoprotein lipase alone was not adequate to achieve the substantial loss of apo E which occurred in *vivo* during lipolysis (Table 21). The percentage of apo E transferred in subject B was in the range observed for the LPL+LTP incubation (average = 41%) indicating a possible role for lipid transfer protein activity in augmenting apo E transfer from VLDL to HDL *in vivo* as was observed *in vitro* (Table 21).

Apo E in the HDL region in the pre-heparin samples distributed in two peaks (Figure 41). The peaks were in the light (fractions 8-10 or 9-13) and very dense region of HDL (fractions 15-18 and 14-17) for subjects A and B. Subject A had a third peak where most of the HDL mass was located(fractions 11-14). In the post-heparin sample, the apo E that was transferred to HDL was gained in two peaks, in the light and in the dense regions. The *in vitro* gain of apo E by HDL in the LPL, LPL+HAL and LPL+LTP incubations occurred in two peaks in some experiments, in the region of HDL mass and in the dense regions of HDL. A gain in the very dense region was particularly obvious in the LPL+LTP incubation of experiment D and E (Figure 21).

FIGURE 41

THE APO E DISTRIBUTION AMONG THE LIPOPROTEINS
OF SUBJECTS **A** AND **B**

Each subject underwent a heparin infusion for a period of 1-1.5 hours. A blood sample was taken at time 0 and at the end of the infusion period and these samples were referred to as the pre-heparin and post-heparin sample, respectively.

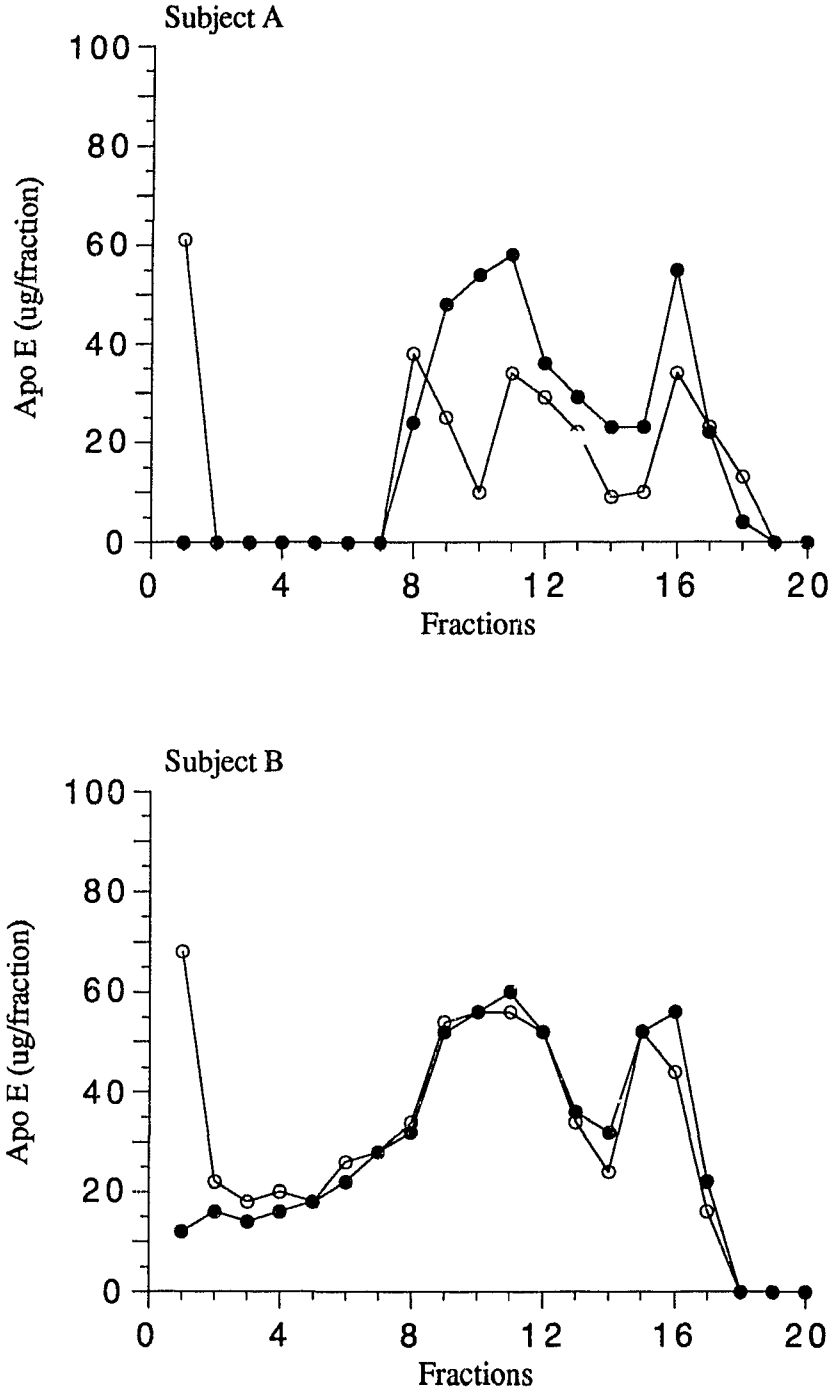
The plasma from these blood samples was subjected to density gradient ultracentrifugation and each fraction from the gradient was analysed for apo E by electroimmunoassay.

The figures depict the pre- and post-heparin distribution of apo E among the lipoproteins of subjects **A** and **B**.

For each figure, the samples are represented as:

pre-heparin = O
post-heparin = ●

FIGURE 41: THE APO E DISTRIBUTION AMONG THE LIPOPROTEINS OF SUBJECTS **A** AND **B**



3) LIPID RATIOS:

UNESTERIFIED CHOLESTEROL/PHOSPHOLIPID RATIO (UC/PL): (Table 30)

The UC/PL ratio demonstrated small increase in the post-heparin fractions relative to the pre-heparin fractions in subject A, most notably in fraction 1 (Table 30). Relative to the plasma LDL ratio, all post-heparin fractions demonstrated ratios (0.76-0.9) which were close or in the LDL range. The VLDL/LDL fractions of subject B had a high phospholipid content resulting in a low UC/PL ratio throughout and the ratio persisted in the post-heparin samples.

CHOLESTERYL ESTER/TRIGLYCERIDE RATIO (CE/TG): (Table 31)

The CE/TG ratio in the post-heparin sample increased in all fractions relative to the pre-heparin value (Table 31). Relative to plasma LDL, the ratios of fraction 1 and 2 were low, more like IDL ratios as was observed in the LPL and LPL+LTP incubations while fractions 3-5 were in the LDL range, as observed in the LPL but not the LPL+LTP incubation (Table 25).

SURFACE TO CORE RATIO : (Table 32)

The surface/core ratio increased markedly in the post-heparin samples in fractions 1 and 2 in both subjects (Table 32). The S/C ratios were higher than those of plasma LDL. The S/C ratio did not appear to change in the LDL fractions (fraction 3-5) as compared to the pre-heparin sample. In the *in vitro* experiments, the LPL incubations demonstrated high S/C ratios in all fractions relative to the LDL ratio (Table 26). Compared to the post-heparin ratios, the ratios of the LPL incubation in fraction 1 were similar. The ratio in fraction 2 of the LPL incubation was generally higher than those of the post-heparin samples. The ratios of the LPL incubation remained much higher than those of the post-heparin samples in fractions 3-5. Therefore, particles were formed in *vivo* with a high surface to core ratio in the VLDL to IDL/light LDL density range as observed *in vitro* . The presence of particles with a high S/C ratio in the LDL fractions (3-

5) could not be established due to the large mass of LDL in the fractions which may mask a relatively small population of such particles.

The post-heparin ratios of both fractions 1 and 2 were similar to the S/C ratios of the LPL+LTP incubations (Table 26). The observation that the ratio in the post-heparin sample in fraction 2 was similar to that of the LPL+LTP incubation but was lower than that of the LPL incubation may indicate that the high ratio in fractions 1 and 2 in the post-heparin sample relative to plasma LDL was due to the action of lipoprotein lipase as a result of hydrolysis of the triglyceride core while the lipid transfer proteins play a role in reducing the high S/C ratio in plasma. Further activity of the lipid transfer protein may be necessary to reduce the S/C ratios in fractions 1 and 2 of the post-heparin samples as well as in all of the VLDL/LDL fractions where mass was present (1-5) in the LPL+LTP incubation.

TABLE 30

UC/PL RATIOS OF FRACTIONS 1-5 OF SUBJECTS **A** AND **B**

	Subject		Plasma
	A	**B**	Average
Fraction 1			.653
Pre-heparin	.627	.375	
Post-heparin	.800	.365	
Fraction 2			.805
Pre-heparin	.761	.614	
Post-heparin	.795	.450	
Fraction 3			.822
Pre-heparin	.900	.408	
Post-heparin	.910	.501	
Fraction 4			.863
Pre-heparin	.845	.450	
Post-heparin	.921	.460	
Fraction 5			.849
Pre-heparin	.839	.459	
Post-heparin	.890	.485	

Subjects **A** and **B** underwent a heparin infusion and a blood sample was taken immediately before the infusion (pre-heparin sample) and 1 to 1.5 hours after the infusion (post-heparin sample).

The plasma average is the average value for the fraction of 4 samples of normolipidemic plasma.

UC/PL= unesterified cholesterol to phospholipid molar ratio

TABLE 31

CE/TG RATIOS OF FRACTIONS 1-5 OF SUBJECTS A AND B

	Subject		Plasma
	A	**B**	Average
Fraction 1			.403
Pre-heparin	.463	.358	
Post-heparin	1.446	1.067	
Fraction 2			.972
Pre-heparin	1.458	2.325	
Post-heparin	2.385	3.443	
Fraction 3			5.197
Pre-heparin	7.329	4.246	
Post-heparin	8.148	5.007	
Fraction 4			11.953
Pre-heparin	20.462	9.649	
Post-heparin	33.227	9.104	
Fraction 5			16.163
Pre-heparin	37.240	11.452	
Post-heparin	42.040	12.889	

Subjects **A** and **B** underwent a heparin infusion and a blood sample was taken immediately before the infusion (pre-heparin sample) and 1 to 1.5 hours after the infusion (post-heparin sample).

The plasma average is the average value for the fraction of 4 samples of normolipidemic plasma.

CE/TG= cholesteryl ester to triglyceride molar ratio

TABLE 32

SURFACE TO CORE RATIOS OF FRACTIONS 1-5 OF SUBJECTS **A** AND **B**

	Subject		Plasma
	A	**B**	Average
Fraction 1			.518
Pre-heparin	.600	.635	
Post-heparin	.949	1.193	
Fraction 2			.623
Pre-heparin	.680	.740	
Post-heparin	.922	1.101	
Fraction 3			.697
Pre-heparin	.710	.989	
Post-heparin	.770	.891	
Fraction 4			.727
Pre-heparin	.738	1.007	
Post-heparin	.725	1.031	
Fraction 5			.740
Pre-heparin	.697	.998	
Post-heparin	.717	1.040	

Subjects **A** and **B** underwent a heparin infusion and a blood sample was taken immediately before the infusion (pre-heparin sample) and 1 to 1.5 hours after the infusion (post-heparin sample).

The plasma average is the average value for the fraction of 4 samples of normolipidemic plasma.

S/C= surface to core molar ratio =

$$\frac{\text{(moles of unesterified cholesterol + moles of phospholipid)}}{\text{(moles of cholesteryl ester + moles of triglyceride)}}$$

CHAPTER V

DISCUSSION

A. IN VITRO STUDIES:

The *in vitro* studies were carried out to investigate the role of lipoprotein lipase on the augmentation of HDL mass and the formation of LDL during VLDL lipolysis under conditions that were more physiological than those used in previous studies. VLDL and HDL were included in a ratio and concentration similar to that of normolipidemic plasma. Adequate albumin was included in the incubations to prevent an artifactual effect due to the accumulation of free fatty acids during triglyceride and phospholipid hydrolysis. The study was designed to dissect out the roles of hepatic lipase and the lipid transfer proteins (cholesteryl ester transfer protein and phospholipid transfer protein) in the modulation of the products formed during lipolysis of VLDL by lipoprotein lipase in the presence of HDL.

The plasma concentration of HDL has demonstrated a strong inverse relationship with the incidence of coronary heart disease. There are two general theories to account for the inverse relationship. The first theory suggests a direct effect by HDL in preventing the development of the atherosclerotic plaque by removing cholesterol or preventing its uptake. This theory infers that HDL plays a role in the transport of cholesterol from the periphery to the liver for excretion and some researchers suggest that this is accomplished by cholesteryl ester transfer protein through transfer of HDL cholesteryl esters to VLDL. The VLDL is converted to LDL which is removed by the liver. The second theory, which suggests a "non-causative" relationship of HDL with the prevention of atherosclerosis, proposes that high HDL concentrations reflect the activity of an ongoing process that is in itself anti-atherogenic. One mechanism that has been suggested is the lipolysis of VLDL by lipoprotein lipase since an augmentation of HDL mass has been reported to occur during this process. In this proposal, a high HDL reflects rapid and efficient lipolysis of

VLDL with a high transfer of lipids and apoproteins from VLDL to HDL. Such a process of rapid lipolysis should reduce the half life of VLDL or triglyceride rich VLDL remnants (partially lipolysed particles) in the plasma and consequently reduce the accumulation of cholesteryl ester in these lipoproteins due to CETP activity. Lipolysis can also stimulate the transfer of phospholipid from VLDL to HDL. Remnants or IDL particles have been associated with the development of atherosclerosis. According to this theory more cholesteryl ester would remain in HDL rather than be carried by VLDL, remnants or IDL and LDL which are all lipoproteins that have been related to the development of atherosclerosis under certain conditions.

Since apo AI concentrations have demonstrated a strong inverse relationship with the incidence of premature atherosclerosis and reduced levels of lipolysis have been found to increase apo AI catabolism [247], it has been suggested that the lipolytic rate or capacity may play an important role in achieving an elevated HDL concentration and possibly an anti-atherogenic lipoprotein profile.

How the process of lipolysis causes an increase in HDL mass, affects the VLDL/LDL cascade, reduces HDL catabolism and plays an anti-atherogenic role in lipoprotein metabolism is unclear. The results of the present study elucidate some of the effects of lipoprotein lipase on lipoprotein interconversion and clarifies the effect of the inclusion of hepatic lipase and the lipid transfer proteins on HDL and LDL formation during lipolysis. The results suggest a new role for the interaction of lipolysis and the lipid transfer proteins in plasma resulting in an effect on plasma lipoprotein metabolism that is desirable with respect to the prevention of atherosclerosis.

1. THE EFFECT OF LIPOPROTEIN LIPASE ON HDL MASS AND DISTRIBUTION.

The action of lipoprotein lipase on VLDL resulted in an increase of HDL mass due to a transfer of unesterified cholesterol, phospholipid, apo CII, CIII, E to HDL. The transfer of unesterified cholesterol was consistent while the phospholipid transfer varied being 1-2 times the molar mass of unesterified cholesterol (2-4 times the mass in μg).

Previous *in vitro* studies have demonstrated the transfer of the apo C's to HDL during VLDL lipolysis, the transfer being directly related to the extent of triglyceride hydrolysis [75,77,78], as found in the present study since the percentage hydrolysis of VLDL triglycerides and percentage transfer of apo CII and CIII from VLDL to HDL were similar (88-94% for triglycerides and 88-100% for the apo C's).

The transfer of apo E from to HDL averaged 28.5% of the original VLDL apo E in the LPL incubation. The transfer of apo E has not been frequently reported for *in vitro* studies. In one study that attained 50% VLDL triglyceride hydrolysis, 30% of the VLDL apo E was found in the HDL density range (in the absence of HDL) [78] which is similar to the amount reported in the present study. In another study of VLDL lipolysis in which lipoprotein lipase was added to a plasma sample, 40-50% transfer of apo E mass from VLDL to HDL was observed [164]. This percentage of apo E transfer was higher than that observed in the lipoprotein lipase incubation of the present study but was similar to the percentage transfer in the incubation containing both lipoprotein lipase and the lipid transfer proteins. Plasma contains the lipid transfer proteins which might have been responsible for the additional apo E transfer from VLDL to HDL.

The transfer of unesterified cholesterol averaged 18% of the original VLDL mass for the LPL incubation. The percentage of unesterified cholesterol transferred from VLDL to the HDL density is higher than that observed in studies that attained the same degree of hydrolysis but were carried out in the absence of HDL [76]. The percentage transfer is in agreement with the 16% transfer reported in a study which included HDL in the incubation at a similar VLDL to HDL ratio used in the present study [79]. The

increased transfer in the presence of HDL suggests an influential role for HDL as an acceptor of lipid from VLDL during lipolysis.

The transfer of phospholipid to HDL averaged 9% of the original VLDL mass in experiments A and B and 14% in experiments D and E. It was difficult to compare the percent of phospholipid transfer to that other studies. Some studies determined phospholipid transfer to VLDL to HDL by measuring the lipid phosphorus in the HDL density range [76,79,83]. Lipid phosphorus would include the lysophosphatidylcholine formed as a result of VLDL phospholipid hydrolysis which can isolate in the very dense regions of HDL. Other studies used a density cut of 1.04-1.21 which includes the LDL range [77].

The amount of phospholipid transferred from VLDL to HDL in the present study was different in experiments A and B as compared to experiments D and E. The composition of the original VLDL and HDL and the percentage of VLDL triglyceride and phospholipid hydrolysis were compared for experiments A and B versus D and E to determine if there was a difference. While most parameters were similar, it was noted that the HDL in experiments D and E had a lower UC/PL ratio (0.11 and 0.13) than the HDL of experiments A, B and C (0.17, 0.21 and 0.17) (Table 6). This observation suggests that the lower UC/PL may play a role in causing an increased transfer of VLDL phospholipid to HDL. A low UC/PL ratio in HDL is indicative of an greater surface "fluidity" as compared to HDL with a higher UC/PL ratio. A more fluid surface may facilitate the incorporation of lipid from the lipolysing VLDL particle into the existing HDL particle or facilitate the donation of material from the existing HDL particle to a newly formed HDL particle derived from VLDL. Since it was the HDL that differed in composition rather than the VLDL, this is an indication that HDL plays a role in accepting lipid mass from VLDL or controlling the transfer of mass from VLDL to the HDL density range. The increased transfer of phospholipid was not accompanied by an increased transfer of unesterified cholesterol and apo E .

The LPL incubations that demonstrated the greater transfer of phospholipid (experiments D and E) also demonstrated a gain in cholesteryl esters. A gain of cholesteryl ester was not observed in experiments A and B but rather a small loss of cholesteryl ester was observed in the HDL region as compared to the control. As described above, experiments D and E differed from A and B in the composition of the HDL, having a lower UC/PL ratio in the experiments that demonstrated a gain of cholesteryl ester by HDL as well as an increased phospholipid transfer to HDL. A gain of cholesteryl esters in experiments D and E was not due to LCAT activity as the HDL had been assayed for the activity which was found to be negligible. The percentage of cholesteryl ester transferred to HDL in the present study was 8 and 20.5% of the starting VLDL. Transfer of cholesteryl ester from the VLDL region has been reported in many studies [23,77,82,83,161,213] but in several studies the appearance of cholesteryl ester in the HDL density was not discussed. The percentage of VLDL cholesteryl ester transferred to that HDL regions were 2% [83], 12%[78], 14% [160], 10% [23] and 10% (d 1.04-1.063 g/ml) [77], which are in the range of the present study. The observed transfer of cholesteryl ester from the VLDL to the HDL density range in these studies, in the absence of HDL[23,78,83,89], is indicative of the formation of a spherical particle of an HDL density. The presence of spherical particle is indicated as the cholesteryl ester must be present as a neutral lipid core shielded from the hydrophilic aqueous environment by a bilayer, presumably containing unesterified cholesterol and phospholipid. There is evidence of the formation of spherical HDL particle derived from lipolysing VLDL (in the absence of HDL in the incubation) [23]. When the newly formed HDL particle was incubated with a preparation of plasma HDL similar to that used in the present study, in that it lacked apo E, the newly formed particle gained apo AI and cholesteryl ester while the plasma HDL gained phospholipid, triglyceride, apo CII and apo CIII [119]. It would be interesting to determine if a spherical particle was formed from VLDL when cholesteryl ester was transferred to HDL in the LPL incubation in the

present study. However, with large amounts of HDL present in the incubation it is difficult to determine if the gain in mass observed in the HDL region is due to the incorporation of lipids and apoproteins into the existing HDL or due to the formation of a "new" HDL particle. In the presence of HDL, as in the present study, it is not clear whether the presence of cholesteryl ester indicates the formation of discrete newly formed particle that exchanges its components with the original HDL or if the cholesteryl ester becomes incorporated along with the phospholipids and unesterified cholesterol gained in the HDL particle already present. The observation of a decrease in mass in the dense region and an increase in the light region, which was observed in the LPL incubation in the experiments where cholesteryl ester transfer was absent, suggests that the original HDL was gaining the transferred mass and the gain of lipid resulted in a lower density of the HDL particles. In the experiments that demonstrated a gain in cholesteryl esters in the HDL density range, a decrease in the dense region was not usually observed. The lack of a decrease in the dense region with a substantial gain in the light region suggests the formation of a new particle or the incorporation of the transferred mass in the HDL particles that were originally present in the light region of the gradient. A differentiation between these two effects cannot be made with the present study design.

When the VLDL cholesteryl esters were labeled with [^{3}H]cholesteryl esters, a small amount of transfer of cholesteryl ester label relative to the control was present in experiments A and B even though a small net decrease in cholesteryl esters was measured. The appearance of label in the HDL region relative to the control increased in the experiments that demonstrated a net gain of cholesteryl ester. This represents a trend for cholesteryl ester transfer from VLDL to the HDL region. However, since the average mass gain of the four was not significant relative to the control due to the high variability of the results in the present study, the effect of LPL on cholesteryl ester gain by HDL is inconclusive at present requiring more experiments to demonstrate whether a gain by

HDL could be repeatedly observed and attain significance. The use of an HDL preparation with a low UC/PL ratio as compared to one with a high ratio would be an interesting study to determine if the ratio did have an effect on the cholesteryl ester transfer from VLDL to HDL.

HDL triglyceride mass did not change significantly relative to the control suggesting that lipoprotein lipase has little hydrolysing activity on HDL triglycerides when VLDL is present. Hydrolysis of HDL triglycerides by lipoprotein lipase has been reported but the hydrolysis occurred in the absence of VLDL in the incubation [243].

The gain in mass in the HDL region of the LPL incubation was in the less dense (light) region of the HDL distribution of the control incubation. A small shift in the mass to the light side of the control peak with a small decrease in the dense region was observed in the experiments that did not gain cholesteryl ester. The increase in HDL mass in the light region was not accompanied by a shift in the location of the peak . The gain occurred in fractions 8-14 which is the HDL_2 density range. A gain in the HDL_2 region may indicate a moderate conversion of dense HDL to light HDL in the LPL incubation since a small decrease in the dense fractions (14-18) accompanied the increase in the light region. A small shift of HDL mass to a lighter density is in agreement with the observations of Taskinen [79] and Zechner [161] of a slight shift in the HDL_3 to a lower density distribution without a complete conversion to HDL_2 but is in contradiction with the data of Patsch [160] who reported complete conversion. Patsch et al included a d>1.21 g/ml plasma preparation in their study which may be responsible for the greater shift in HDL mass since in the present study, with the addition of the lipid transfer proteins to the lipoprotein lipase incubation, a greater shift in HDL mass to a lighter density was observed as compared to the distribution with lipoprotein lipase alone. The shift in HDL mass was accompanied by a change in the location of the HDL peak. LCAT, which is present in plasma, may also be involved in the complete conversion of HDL_3 to HDL_2 as suggested by the data of Zechner [161]. In the LPL incubations that

gained cholesteryl ester and more phospholipid, the gain in the light region was not usually accompanied by a loss in the dense region.

The apo E redistribution from VLDL to HDL in the LPL incubation differed greatly from the redistribution of the apo CII, CIII or lipid. Rather than tending to redistribute to the light region of the HDL mass, as was observed for the apo C's, the apo E was found in: 1) the very light region ; 2) in the region where the majority of HDL mass is located; and 3) in the very dense region of HDL. The distribution of the apo E that transferred to the HDL region was similar to that reported in the study of Rubinstein which observed 40-50% transfer in the presence of plasma [164]. Notably, apo E was also observed in the HDL region where very small and presumably dense particles would isolate. In the present study, the very dense fractions of HDL have been noted to contain lysophosphatidylcholine in the incubations that included active lipoprotein lipase. It is possible that the apo E, which isolated in these same fractions, may be associated with the lysophosphatidylcholine but this was not determined in the present study.

The HDL fractions of the LPL incubation, as observed on polyacrylamide gels, contained particles that were similar or larger than the control HDL particles. The electron micrograph demonstrated spherical particles in the HDL size range of 100-140 Å and no discs were observed. The presence of spherical particles and the absence of discs is in agreement with the observations of Tam and Breckenridge [23] and Caturpso [159] and is contrary to the reports of several other studies [75-77]. The different observations may be due to the need for adequate albumin in the studies that observed discs since it is possible that the discs were actually dehydrated vesicles. It has been observed that the addition of albumin can greatly reduce the production of vesicles during VLDL lipolysis by lipoprotein lipase *in vitro* [23]. The present study contained albumin in a concentration that was equal to that of plasma and contained VLDL triglyceride similar to that of normal plasma. The amount of albumin included was calculated to be more than

adequate to bind the free fatty acids released during the hydrolysis of the VLDL triglycerides and phospholipids which may account for the absence of "discs."

2. THE EFFECT OF THE ADDITION OF HEPATIC LIPASE TO THE LPL INCUBATION ON HDL MASS AND DISTRIBUTION.

The inclusion of hepatic lipase resulted in extensive hydrolysis of the HDL triglyceride and some HDL phospholipid. The gain of unesterified cholesterol, apo CII, CIII and apo E in HDL observed in the LPL incubation was not further affected by the addition of hepatic lipase. A small gain of the lipid could be observed in the light region of the HDL distribution with a small decrease in the dense but as compared to the LPL incubation the shifts were less obvious. There was a tendency for the HDL mass of the LPL+HAL incubation to maintain a position on the gradient similar to that of the control incubation. The mass transferred from VLDL was frequently found in the region over the control peak rather than an addition to the light side as observed with the LPL incubation. It has been suggested that hepatic lipase plays a role in the conversion of (light) HDL_2 to dense HDL_3 since the activity is inversely correlated with HDL_2 concentrations [244]. In the present study a conversion of light HDL particles to dense HDL particles was not obvious when lipoprotein lipase was also present in the incubation with hepatic lipase.

A narrowing of the peak was observed in some of the distributions of the LPL+HAL incubations. The narrowing of the HDL peak and gain in mass over the control peak in preference to a gain in mass on the light side may be due to the opposing effects of lipoprotein lipase and hepatic lipase on the HDL distribution. The gain of unesterified cholesterol and phospholipid due to the LPL activity would decrease the density of particles and may therefore cause a slight shift of HDL mass from the dense side of the control peak to a lighter density. However, the extensive hydrolysis of HDL triglyceride would increase the density of the particles and counteract a gain in mass on the light side of the control peak as observed with lipoprotein lipase.

Analysis of HDL on polyacrylamide gels indicated that the LPL+HAL incubation had less mass in the lighter fractions than that observed in the LPL incubation. The tendency for larger particles to be present as observed with the LPL incubation was not as obvious and the particles were similar in size to those of the control incubation in many cases. This may indicate that hydrolysis of the core triglyceride by hepatic lipase may have counteracted the effect of lipoprotein lipase on increasing the particle size by the addition of unesterified cholesterol and phospholipid while the gain in mass caused by lipoprotein lipase may have prevented the particles from becoming smaller than the control particles. Therefore the overall effect of the addition of hepatic lipase to the LPL incubation was a tendency to prevent an decrease in HDL density as well as an increase in HDL particles size as compared to the effect of lipoprotein lipase. In the presence of lipoprotein lipase, the HDL particles were not converted to denser particles by hepatic lipase.

3. THE EFFECT OF THE ADDITION OF THE LIPID TRANSFER PROTEINS TO THE LPL INCUBATION ON HDL MASS AND DISTRIBUTION.

The addition of the lipid transfer protein preparation to the LPL incubation resulted in an increased transfer of VLDL phospholipid, unesterified cholesterol and apo E to the HDL region relative to that observed in the LPL incubation.

The gain in unesterified cholesterol and phospholipid was consistently twice the amount observed in the LPL incubations. Although there was variability in the amount of phospholipid gained by HDL from VLDL in the LPL incubations, there was a consistent doubling of this amount in the LPL+LTP incubations. The transfer of unesterified cholesterol relative to phospholipid was high relative to their concentrations on the original VLDL in the LPL+LTP incubations of experiments B and C. With the increased transfer of phospholipid in experiments D and E, the unesterified cholesterol and phospholipid were transferred in a similar proportion to that of the original VLDL.

The gain in apo E averaged 1.5 times the amount transferred in the LPL incubation. This observation indicates that the lipid transfer proteins plays a role in augmenting the amount of transfer of apo E from VLDL or from lipolysing particles to HDL which could not be achieved by lipoprotein lipase alone.

The addition of the lipid transfer protein preparation to the LPL incubation resulted in a greater shift to the lighter regions of the HDL distribution as compared to the LPL incubation. The shift to a lighter fraction occurred particularly in fractions 10-13 corresponding to approximate densities of 1.08-1.11 g/ml, which is the HDL_2 density range. A corresponding decrease in lipid mass in the dense fractions 15-18 which have an estimated density of 1.13-1.18 g/ml was observed in most experiments.

In contrast to the LPL, the incubation of LPL+LTP resulted in a shift of the location of the peak of the HDL distribution. The increase in the light density range with a corresponding decrease in the dense region is indicative of a net addition of lipid to the starting HDL mass causing the HDL to become lighter. The substantial gain/shift in mass to the light fractions may be due to a stimulation of phospholipid transfer activity of PLTP or of CETP since more phospholipid was transferred in the LPL+LTP incubations than the LPL incubation. The observation of a stimulation of phospholipid transfer activity which was essentially absent without lipolysis is in agreement of the investigation of Tall of the activity of PLTP or of a d>1.21 plasma preparation [89]. This study reported an increase in the mass of VLDL phospholipid transferred to HDL when lipoprotein lipase was active but no significant increase in the transfer of unesterified cholesterol was observed. The cause of the increased transfer of unesterified cholesterol and apo E observed in the present study is not clear but may be due to a surface effect that accompanies the greater loss of phospholipid when lipolysis of VLDL is carried out in the presence of the lipid transfer proteins.

The LPL+LTP incubation demonstrated reduced or absent transfer of cholesteryl esters from HDL to VLDL in exchange for triglyceride. In the study of Patsch [160]

lipolysis of VLDL in the presence of HDL_3 and a d>1.21 plasma preparation, a significant enrichment of the HDL particle with unesterified cholesterol and phospholipid was observed. An exchange of HDL cholesteryl ester with VLDL triglyceride was not apparent. The converse occurred in that VLDL lost cholesteryl esters while HDL gained cholesteryl esters. The observation of a lack of transfer of cholesteryl esters from HDL to VLDL in the study of Pastch et al is in agreement with the present observation of a reduced or lack of transfer of cholesteryl ester from HDL to VLDL and a stimulation of phospholipid and unesterified cholesterol ester transfer from VLDL to HDL when lipoprotein lipase was included in the lipid transfer protein incubation. In contrast, Zechner [161] did not report a shift in the HDL density or a gain of phospholipid and unesterified cholesterol during VLDL lipolysis by lipoprotein lipase in the presence of cholesteryl ester transfer protein (CETP) when compared to the effect of lipoprotein lipase alone. Possibly the phospholipid transfer protein exclusively or in concert with CETP rather than CETP alone is required for the increased transfer of phospholipid and unesterified cholesterol and for the significant shift in HDL mass to a lighter density. The relative rates of the phospholipid transfer activities of CETP and PLTP may also differ and effect the outcome. A reduced transfer of triglyceride from VLDL to HDL in exchange for cholesteryl ester was noted by Zechner [161]. However, a net transfer of cholesteryl esters from HDL to VLDL was reported in this study which was much greater than that observed in the present study. The reason for the greater cholesteryl ester transfer from HDL to VLDL during lipolysis may be due to the fact that the amount of lipoprotein lipase added was sufficient to hydrolyse 60-70% of the VLDL triglycerides in a 24 hour period. This rate of lipolysis is very slow relative to that of the present study where the lipoprotein lipase activity was sufficient to achieve 88-93% hydrolysis in a 2 hour period. The half life of VLDL is normally 2-4 hours so this study actually demonstrates the result of the prolonged presence of VLDL due to ineffective lipolysis. A slow rate of lipolysis would allow for the exchange of VLDL triglyceride for HDL

cholesteryl esters as observed in the study of Zechner [161]. In contrast, effective lipolysis prevents the enrichment of VLDL with cholesteryl esters by rapidly hydrolyzing the VLDL to particles that have a markedly reduced triglyceride content relative to cholesteryl ester and are therefore poor triglyceride donors. The transfer of cholesteryl ester from HDL to these lipolysed particles may not take place or may be greatly reduced as was observed in the present study.

Polyacrylamide gradient gels demonstrated an increased mass in the lighter fractions of the LPL+LTP incubation relative to the other incubation conditions and the presence of larger particles. A recent report by Barter [245] on the effect of a lipid transfer protein preparation (plasma d> 1.21 g/ml) on HDL particle size during VLDL lipolysis by lipoprotein lipase indicated that the particle size of HDL decreased at low levels of lipolysis but the decrease was not observed at high levels of lipolysis. Furthermore, when adequate amounts of albumin were added to completely bind the released free fatty acids, a decrease in particle size was not observed. The presence of excess free fatty acids during lipolysis of VLDL has been reported to increase the transfer of cholesteryl ester from HDL to VLDL[88] and the transfer of triglycerides from VLDL to HDL. Hydrolysis of HDL triglycerides, which has been reported *in vitro* with lipoprotein lipase when VLDL was absent, would decrease the particle size of the HDL by decreasing the core, similar to the process described for combined effect of CETP and hepatic lipase [243]. The lack of a decrease in HDL particle size in the presence of adequate albumin observed in the study of Barter is in agreement with the results of the present study where the albumin concentration was adequate to bind the released free fatty acids and the particles failed to decrease in size but rather increased. Thus it appears that the lipid transfer proteins play a role in the conversion of dense HDL particles to lighter HDL particles when lipolysis of VLDL by lipoprotein lipase is ongoing in the presence of adequate albumin. In the study of the effect of lipolysis on the phospholipid transfer protein activity, it was observed that the particle diameters increased as well [89].

Due to the observed augmented transfer of apo E to HDL in the LPL+LTP and in the LTP incubations as compared to the LPL incubation, the lipid transfer protein preparation may play a role in the formation/augmentation of HDL with apo E. HDL with apo E can bind to hepatic receptors and be removed from the plasma. This route has been suggested for the removal of cholesterol/cholesteryl esters from plasma in rats. The role of HDL with apo E in humans is not clear. The present observation of increased transfer of apo E to HDL contrasts with reports of patients with CETP deficiency who demonstrate high concentrations of HDL including substantial amounts of apo E-enriched HDL particles [192]. The presence of high amounts of HDL containing apo E with CETP deficiency suggests that the increased transfer of apo E to HDL observed in the present study due to the addition of the lipid transfer proteins may be due to the action of PLTP rather than CETP. In the study that observed an increase in PLTP activity in the presence of lipoprotein lipase, the effect on the transfer of apo E was not reported [89]. PLTP activity has not been studied in relation to apo E transfer between lipoproteins.

A small gain of apo E by HDL was also observed in the LTP incubation as compared to the control. The amount of apo E gained in the LTP incubation did not account for the total increase in transfer of apo E observed in the LPL+LTP incubation relative to the transfer observed in the LPL incubation. This suggests that lipoprotein lipase activity was required for part of the lipid transfer protein-stimulated transfer of apo E in the LPL+LTP incubation.

When referring to the activity of the lipid transfer protein preparation it should be noted that this was a crude preparation containing CETP and PLTP activity. LCAT activity in the lipid transfer protein preparation was essentially inhibited so it is unlikely that LCAT had any effect on the results but other unknown constituents may have been present in the transfer protein preparation whose effect cannot be determined.

It is interesting to note that in experiment D, the apo AI and lipid present in the lipid transfer protein preparation did not appear to affect the transfer of mass from the

VLDL region to the HDL region since the gain of unesterified cholesterol and apo E did not differ from that observed in the LPL+LTP incubations of experiments B, C and E. The phospholipid transfer was higher in experiment D than in experiments B and C but was less than that of experiment E. The lipid transfer protein preparation of experiment E did not demonstrate the presence of any excess lipid and contained one-half of the amount of apo AI relative to the amount in the lipid transfer protein preparation of experiment D. Therefore, there was no obvious relationship between the amount of apo AI (or lipid) added and the augmented transfer of mass observed in the LPL+LTP incubation as compared to the LPL incubation. Furthermore, the transfer of phospholipid in the LPL incubations of experiments D and E also demonstrated an increased amount of phospholipid transfer from VLDL to HDL and the LPL incubation did not contain any additional apo AI or lipid.

The most noticeable effect of the LTP incubation, i.e. of the lipid transfer protein preparation in the absence of lipoprotein lipase activity, was a substantial transfer of cholesteryl esters from HDL to VLDL in exchange for transfer of triglyceride from VLDL to HDL. This activity is characteristic of the reported activity of CETP by some investigators but transfer of cholesteryl esters from LDL to HDL was the major route of cholesteryl ester transfer reported in other studies. The present study was carried out in the absence of LDL so the characteristics of cholesteryl ester transfer may differ when only HDL and VLDL are present. The lipid transfer protein preparation did not demonstrate net phospholipid transfer in the absence of lipoprotein lipase. The LTP incubation demonstrated a small gain of unesterified cholesterol by HDL as compared to the control incubation. The activity of the lipid transfer protein preparation alone (LTP incubation) was remarkably different from the activity in the presence of lipoprotein lipase (LPL+LTP incubation). The LPL+LTP incubation demonstrated a substantial reduction in the cholesteryl ester transfer activity and a marked stimulation of phospholipid transfer activity as compared to the LTP incubation. Thus, with active

lipolysis, the transfer of cholesteryl ester from HDL to VLDL was substantially reduced while the transfer of phospholipid to HDL was stimulated. Lipolysis has been reported to decrease the catabolism of HDL. It is possible that it is the reduction of the cholesteryl ester transfer activity during lipolysis that decreases the catabolic rate of the HDL since subjects with a CETP deficiency demonstrated high levels of HDL and a reduced catabolic rate. The observation of a reduction in the cholesteryl ester transfer activity from HDL to VLDL is in contrast to the other studies [87,88] that reported a stimulation of cholesteryl ester transfer from HDL to VLDL during lipolysis of VLDL by bovine skim milk lipoprotein lipase. The major difference between these studies and the present study was that the increased cholesteryl ester transfer to VLDL was observed only when the amount of albumin in the incubation was inadequate for binding the released free fatty acids. The increased activity was demonstrated to be due to the negative charge on the free fatty acid which increased the binding of the transfer protein to VLDL [88]. In the present study, the amount of albumin was more than adequate to bind the free fatty acids and the VLDL and HDL were present in proportions and concentrations that were more physiological than those used in the previous studies [87,88]. These differences may account for the present observations of a reduction in the transfer of cholesteryl esters from HDL to VLDL as compared to the increase observed in the earlier work.

A free fatty acid concentration which exceeds the binding capacity of albumin is unlikely to occur in the fasting situation during hydrolysis of normal concentrations of VLDL. However, this situation may occur during alimentary lipemia particularly if the diet contained a high fat content and yielded a high concentrations of triglyceride rich chylomicrons in the plasma. It may also occur in individuals who have elevated plasma triglycerides. Under these conditions the free fatty acid may increase the binding of the transfer proteins to VLDL and HDL and stimulate a gain of cholesteryl ester by VLDL.

In summary, lipoprotein lipase caused a transfer of apo CII, CIII, apo E, unesterified cholesterol, phospholipid and in some cases cholesteryl ester from VLDL to

HDL. The HDL distribution demonstrated a small shift of the mass to a lighter density or simply an increase in mass in the light density range if cholesteryl esters were gained. Hepatic lipase caused extensive hydrolysis of HDL triglycerides and some phospholipid hydrolysis. Hepatic lipase activity did not result in the formation of denser HDL particles in the presence of lipoprotein lipase nor was the small shift in HDL mass to a lighter density as apparent as compared to the LPL incubation. The addition of the lipid transfer proteins to the lipoprotein lipase incubation resulted in an increased amount of transfer of phospholipid, unesterified cholesterol and apo E from VLDL to HDL as compared to that observed with lipoprotein lipase alone. The transfer of cholesteryl esters from HDL to VLDL, which was observed in the incubation of the lipid transfer proteins alone, was reduced or absent in the incubation containing the lipid transfer proteins and lipoprotein lipase. Thus when VLDL was being lipolysed by lipoprotein lipase, the phospholipid transfer activity was stimulated and the cholesteryl ester transfer from HDL to VLDL was reduced.

4. THE EFFECT OF LIPOPROTEIN LIPASE ON VLDL/LDL MASS AND DISTRIBUTION

Lipoprotein lipase activity resulted in extensive hydrolysis of VLDL triglycerides and some phospholipid hydrolysis. The hydrolysed VLDL particles demonstrated a redistribution of some of the mass into the light region of the plasma LDL distribution. However the majority of the mass was retained in the fraction where VLDL and IDL(fractions 1 and 2) and very light LDL(fraction 3) would isolate on the gradient. The majority of the mass failed to move into the fractions of the gradient where most of the LDL is isolated (fractions 4-6) and a true LDL peak was not formed. This observation is contrary to some reports that indicate a conversion of most of VLDL into LDL solely as a result of VLDL lipolysis by lipoprotein lipase [158]. Possibly the difference between the studies is the fact that human skim milk lipoprotein lipase was used which results in more extensive phospholipase activity (70%) than the lipoprotein lipase obtained from rat

hearts which was used in the present study. The VLDL phospholipid hydrolysis observed with lipoprotein lipase in the present study averaged 30.6%. Furthermore the LDL was isolated by sequential ultracentrifugation which isolates an LDL preparation that covers a density range of 1.019-1.063 g/ml. The retention of mass in the light region of LDL without a shift or formation of a typical LDL peak which was noted in the present study would not be observed if LDL was isolated by sequential ultracentrifugation. A greater shift in the apo B and lipid mass was observed in experiments D and E which also demonstrated a greater transfer of phospholipid and cholesteryl ester out of the VLDL/LDL region into the HDL region. The greater shift of mass into the LDL density range in experiments D and E suggests that the lipolysed particles of experiments A and B require loss of cholesteryl esters and phospholipid to achieve an LDL density. A further loss of lipid in experiments D and E would be required for all the lipolysed mass to shift to an LDL density. The loss of cholesteryl ester is suggested to contribute more to the shift in mass to a higher density rather than phospholipid since the density of cholesteryl esters is 0.97 gm/ml which is much lighter than the density of LDL (d 1.019-1.063 gm/ml) while phospholipids have a density of 1.04 g/ml which is in the LDL density range.

By studying the composition and size of the lipolysed VLDL particles in the LPL incubation, it was determined that the particles differed markedly from plasma LDL and were more like IDL particles in many ways. The particles contained very little apo CII and CIII which is characteristic of LDL particles. The apo E content was very high relative to plasma LDL which contains little apo E. Relative to plasma LDL or the LDL which isolates in fractions 4 and 5 on the gradient, the particles contained an excess of phospholipid relative to unesterified cholesterol (low UC/PL ratio) in several cases since the ratio was more like the VLDL and IDL ratio (0.6-0.8) rather than the ratio for plasma LDL (0.8-1). Fractions 1 and 2 were enriched in triglyceride relative to cholesteryl esters (low CE/TG ratio) and were similar to IDL in their ratio (2.5-4). A greater loss of

triglycerides from fractions 1 and 2 is therefore also necessary if the particles are to become similar in composition to plasma LDL. The fractions in the LDL region (fraction 3-5) were similar to plasma LDL in most cases and therefore a loss of triglyceride is not required in these fractions. None of the fractions in any experiment were found to be enriched in cholesteryl esters relative to triglycerides which has been reported for the LDL formed as a result of lipolysis of VLDL by bovine or human milk lipoprotein lipase [75,77]. There was a substantial excess of surface relative to core in all fractions of all experiments and the ratio was frequently very high (1.1-1.8) relative to that of plasma LDL (0.7-0.8). The surface to core ratio in the experiments of Deckelbaum [82] was 1.07. This ratio is low compared to the surface to core ratio observed in the present study since many of the ratios were in the 1.3-1.7 range. Possibly the greater phospholipase activity of bovine skim milk lipase which was used in the study of Deckelbaum accounted for the lower surface to core ratio observed. Since the formation of vesicles as a result of VLDL lipolysis by lipoprotein lipase has been reported in several studies, it was possible that the larger particles and high surface to core ratio was due to the presence of vesicles. Cellobiose which disrupts vesicles did not alter the distribution of particle sizes on polyacrylamide gels in fractions 1-5. The surface to core ratio, which normally reflects the particle size in plasma lipoproteins, increases as the particle size decreases due to a decrease in the size of the neutral core lipid. Plasma HDL which is the smallest of the lipoproteins demonstrates a ratio of 1-1.5 as compared to the ratio for plasma LDL which is in the 0.7-0.8 range. Theoretically, from the surface to core ratio of the lipolysed VLDL particles, their size should be in the range of HDL (molecular weight = 200,000-400,000) and smaller than plasma LDL which has an average molecular weight in the 2 million range. However, when polyacrylamide gels were used to estimate the molecular weight the particles were substantially larger than plasma LDL in fractions 1-5. The estimated molecular weight of the lipolysed VLDL particles was in the IDL range of 4-5 million in most fractions. Thus, considerable reduction in the mass would be required to

achieve the size of plasma LDL. However when the particle diameters of fractions 1-5 were calculated from the lipid data, they were all smaller than that of LDL and were in the HDL size range. The calculation of the particle diameter predicts the size based on the proportion of the surface area in relation to the core volume and is therefore based on the assumption that the surface components have equilibrated and cover the core. The probable explanation for the small calculated particle diameter of the lipolysed particles is that there is excess surface relative to core which results in an underestimate of the calculated particle size. It appears that the lipolysed particles have not equilibrated and do not follow the same physicochemical parameters used to define plasma lipoproteins. Therefore the use of calculated particle diameters from lipid data for particles that have undergone metabolic alterations such as these lipolysed particles may not be an accurate method of determining the particle size. The particle diameter that Deckelbaum et al [82] observed of the post-lipolysis LDL with electron microscopy was in the range of 270 Å and the molecular weight was calculated be approximately 5 million. This molecular weight is similar to that estimated for the particles in the LDL fractions of the LPL incubation.

Overall, the particles that were formed in the presence of lipoprotein lipase tend to be more like IDL than LDL in size, CE/TG ratio in fraction 1 and 2, and like VLDL and IDL in UC/PL ratio. The high amount of apo E remaining also suggests a large IDL particle. However the high surface to core ratio is unlike VLDL, IDL or LDL.

5. THE EFFECT OF THE ADDITION OF HEPATIC LIPASE TO THE LPL INCUBATION ON VLDL/ LDL MASS AND DISTRIBUTION.

Hepatic lipase was initially investigated in this study as it was thought that hepatic lipase may affect the hydrolysis of phospholipids and apo E or cholesteryl ester removal from the lipolysing VLDL particles. However, this effect was not observed.

The addition of hepatic lipase to the lipoprotein lipase incubation resulted in minimal changes in the VLDL/LDL lipid or apoprotein distributions. It did not increase

the amount of VLDL triglycerides or phospholipids hydrolysed nor affect the transfer of apo CII, CIII and apo E to HDL. There is evidence in the literature to suggest that hepatic lipase plays a role in the removal of apo E from IDL [166] but this was not observed in the present study. The lack of an effect of hepatic lipase is in agreement with some studies [174,175] but contrary to the studies of hepatic lipase deficiency that suggest a significant role in the conversion of IDL to LDL [45,84,174]. The lack of an effect in the present study and in other *in vitro* studies may be due to the fact that, *in vivo*, hepatic lipase is present in the liver while lipoprotein lipase is an extra-hepatic enzyme. As a consequence, *in vivo*, the VLDL particles may be acted on by lipoprotein lipase in the periphery, modified in the circulation and, on transport through the hepatic circulation, subsequently acted upon by hepatic lipase. Since lipoprotein lipase and hepatic lipase were present in the same incubation in the present study, it is possible that hepatic lipase may not compete successfully with lipoprotein lipase for binding to VLDL or to the lipolysed VLDL particles and therefore not demonstrate an effect. There is some evidence to suggest that hepatic lipase may not bind as well to VLDL as compared to lipoprotein lipase as it has been reported the binding of hepatic lipase was reduced more that of lipoprotein lipase when the surface pressure of an artificial emulsion was increased [154]. The authors suggested that the higher surface pressure in VLDL relative to HDL would therefore result in the preferential binding of lipoprotein lipase to VLDL and of hepatic lipase to HDL [154]. If this is the case, one can speculate that hepatic lipase may reduce the excess triglyceride observed in fractions 1 and 2 of the incubation containing lipoprotein lipase alone if the lipolysed particles were re-isolated and hepatic lipase was able to act in the absence of lipoprotein lipase.

A slight decrease in particle size was noted in fractions 4 and 5 in incubations that included hepatic lipase as compared to the lipoprotein lipase alone. The smaller particles were in the size range of the particles of the LPL+LTP incubation and approached that of plasma LDL. As discussed earlier, it is clear that hepatic lipase was

highly active on HDL triglycerides. It is possible that a greater amount of hepatic lipase activity may be required to demonstrate a substantial effect on the conversion of lipolysed VLDL/IDL particles into LDL.

6. THE EFFECT OF THE ADDITION OF THE LIPID TRANSFER PROTEINS TO THE LPL INCUBATION ON VLDL/ LDL MASS AND DISTRIBUTION.

The addition of the lipid transfer proteins to the lipoprotein lipase incubation resulted in the formation of particles that demonstrated a reduced content of apo E, less surface relative to core and a substantially smaller size as compared to the particles formed with lipoprotein lipase. These modifications resulted in the particles of the LPL+LTP incubation being more characteristic of plasma LDL than those resulting from the lipolysis of VLDL in the absence of the lipid transfer protein preparation. Large LDL particles have been reported in cases of subjects demonstrating CETP deficiency [86]. The presence of the large LDL particles may reflect the activity of lipoprotein lipase in the absence of the modifying effect of CETP which has both phospholipid transfer activity as well as cholesterol ester transfer activity.

The particles of the LPL+LTP incubation lost more unesterified cholesterol, phospholipid and apo E relative to apo B, as compared to the particles of the LPL incubation. The increased loss of unesterified cholesterol and phospholipid was reflected by the lower surface to core ratio in the LPL+LTP incubation as compared to the LPL incubation.

Cholesteryl ester was decreased relative to apo B in several of the fractions (1, 3 and 4) in the LPL+LTP incubation relative to the LPL incubation but was slightly increased or the same in others. The particles were triglyceride depleted relative to apo B in fraction 1 and enriched in fractions 2-5 compared to those of the LPL incubation. The particles varied in their triglyceride content relative to cholesteryl ester as demonstrated by the differences in the CE/TG ratio in the LPL+LTP incubation as compared to the LPL incubation. The first fraction of the gradient was poorer in triglyceride relative to

cholesteryl ester, as compared to the first fraction of the LPL incubation. The second fraction did not differ while fractions 3-5 demonstrated an enrichment in triglyceride relative to the LPL incubations. The CE/TG ratios of fractions 3-5 were similar to plasma LDL in the LPL incubation while those of the LPL+LTP incubation remained in the IDL range. These results correspond to the change observed in the CE/apoB and TG/apo B ratios of the fraction 3-5 of the LPL+LTP incubation.

It is possible that a transfer of cholesteryl ester from fractions 3-5 to fraction 1 in exchange for triglyceride had occurred in the LPL+LTP incubation to allow for the triglyceride enrichment relative to cholesteryl ester in these fractions. Such a transfer has been suggested by Deckelbaum [212] as a mechanism for formation of LDL. It was suggested that this exchange would provide a means of decreasing the core of lipolysed VLDL particles by the loss of cholesteryl esters and hydrolysis of the triglycerides which were gained in exchange. It is unlikely that this process was occurring in the present study because fraction 1 demonstrated a preferential loss of cholesteryl ester and triglyceride relative to apo B in the LPL+LTP incubation as compared to the LPL incubation. Thus a loss of both core constituents was occurring in the particles of this fraction. A transfer of cholesteryl ester from HDL in exchange for triglyceride in the lipolysed particles particularly in fractions 3-5 is another possible mechanism. In the LPL incubation, fractions 3-5 demonstrated CE/TG ratios that were frequently in the LDL range (9-15) which are higher than the ratio in HDL (8-10). Possibly the consequent gain of cholesteryl ester by HDL in exchange for transfer of triglyceride to these particles was not noted in some experiments because there is little mass in fractions 3-5 so the gain would be small. A role for hepatic lipase in the hydrolysis of these particles which are relatively enriched in triglycerides and which are formed in the presence of the lipid transfer proteins and lipoprotein lipase can suggested. Further studies which include hepatic lipase in the incubation of the lipid transfer proteins and lipoprotein lipase or

studies that incubate hepatic lipase with the re-isolated lipolysed particles of the lipid transfer protein and lipoprotein lipase incubation would be required.

The finding of particles in the LPL+LTP incubations that were smaller than those of the LPL incubation yet were isolated at a lighter density and demonstrated a lower surface to core ratio than those of the LPL incubation is contrary to the principles that are considered to govern lipoprotein size. Normally, lighter (less dense) particles are larger and have a lower surface to core ratio. The paradox of a smaller particle isolating in a lighter density with a reduced surface to core ratio may reflect the loss of excess surface components of a higher density (phospholipid = d 1.04 g/ml, unesterified cholesterol = d 1.067 g/ml, protein = d 1.35 g/ml) and the augmentation of core components of a lighter density (triglyceride = d 0.91 g/ml), in particles that have not equilibrated. Thus, the particles may not follow the typical physicochemical characteristics of spherical plasma lipoproteins where a smaller sized particle corresponds to an increase in the surface to core ratio and an increase in density.

For conversion into particles that are similar to LDL, the LPL+LTP particles in the VLDL/LDL region would require further loss of surface relative to core, an additional loss of apo E, loss of phospholipid relative to unesterified cholesterol and a decrease in triglyceride content relative to cholesteryl ester. In order to allow for an increase in density and decrease in particle size further removal of lipid is required. Possibly, when adequate lipid is removed, the contribution of apo B to the density of the particle increases resulting in a particle that is the density of plasma LDL. However, the conditions used in the present study were not sufficient to accomplish this conversion.

The LTP incubation demonstrated a slight shift in mass from fraction 1 into fraction 2. The shift is presumably due to the gain in cholesteryl ester and loss of triglycerides that occurred due to the action of the cholesteryl ester transfer protein in the absence of lipolysis. Cholesteryl esters have an approximate density of 0.98 g/ml while triglycerides have a lower density of 0.91 g/ml

As discussed in the section on the effect of the addition of the lipid transfer protein on HDL formation, previous studies of the effect of lipolysis on CETP activity have reported a stimulation of the transfer of cholesteryl esters from HDL to VLDL when the concentration of albumin was low [87,88]. We did not observe this stimulation but rather a reduced rate of transfer possibly because the albumin concentration was that of human plasma and was adequate in binding the free fatty acids produced by lipolysis. We suggest that the transfer was reduced due to the rapid and effective hydrolysis of VLDL triglycerides which caused the formation of lipolysed particles with a high cholesteryl ester/triglyceride ratio relative to VLDL and also a high ratio relative to HDL. These conditions would be sub-optimal for the transfer of triglyceride to HDL in exchange for cholesteryl ester. We did confirm the reported stimulation of phospholipid transfer activity with lipolysis [89] which could have been due to PLTP and/or CETP since both proteins have been reported to demonstrate phospholipid transfer activity. This activity may have been responsible for the increased loss of surface lipid relative to core lipid in the particles isolating in the VLDL/LDL region of the LPL+LTP incubation as compared to the LPL incubation due to the increased transfer of unesterified cholesterol and phospholipid to HDL. The increased loss of apo E may be related to this increased loss of surface mass.

The results of this study indicate that the lipid transfer proteins appear to play a role in the conversion of lipolysed VLDL particles, i.e. remnants, into LDL. This conclusion is based on the observed effects of adding the lipid transfer proteins to the lipoprotein lipase incubation under conditions where the proportions and concentrations of VLDL and HDL, activity of LTP, concentration of albumin and pH are all in the normal physiological range.

CETP has been suggested to be atherogenic due to its role in the cholesterol enrichment of VLDL. We suggest that the lipid transfer proteins do not necessarily play an atherogenic role when lipolysis is rapid and effective since VLDL would not be

available for prolonged exposure to CETP, preventing any extensive exchange of VLDL triglyceride for HDL cholesteryl ester. Under these circumstances, in the presence of active lipolysis and the lipid transfer proteins, the cholesteryl ester-triglyceride exchange would be reduced while PLTP and/or CETP phospholipid transfer activity would aid in the conversion of post-lipolysis remnant particles which may be atherogenic into LDL particles.

Therefore, based on the present studies, it is reasonable to suggest that rapid lipolysis of normal amounts of triglyceride-rich lipoproteins is desirable since it would: 1) avoid the enrichment of VLDL with cholesteryl esters; 2) prevent the resultant formation of cholesteryl ester rich remnants; 3) maintain cholesteryl ester in HDL; 4) augment HDL mass. Rapid lipolysis of normal concentrations of triglyceride-rich lipoproteins would have to be accompanied by a dietary intake that is small (with a low to moderate fat content) but frequent, which thus avoids an acute fat load, in order to prevent the accumulation of excess free fatty acids in the plasma during lipolysis. The positive relationship of the development of atherosclerosis with elevated plasma triglyceride concentrations and the inverse relationship of HDL mass with triglycerides may be related to this phenomenon.

B. *IN VIVO* STUDIES:

1. THE EFFECT OF POST-HEPARIN LIPOLYSIS ON THE HDL AND VLDL/LDL MASS AND DISTRIBUTION.

In vivo lipolysis of VLDL, induced by the release of lipoprotein lipase and hepatic lipase from endothelial cells as a result of a heparin infusion, demonstrated many similarities to the results of the *in vitro* incubations. The comparison of the results of the *in vitro* incubations provided indications as to the cause of the various redistributions of the lipid and apoprotein mass observed *in vivo.*

The results were similar to the *in vitro* studies in terms of a transfer of apo CII and CIII from VLDL to HDL, a transfer of unesterified cholesterol and large transfer of

phospholipid, a hydrolysis of triglyceride and a shift in the density of the HDL to a lighter density with a change in the location of the peak in one subject. There was an equal or greater transfer of apo E to HDL and a substantial gain of cholesteryl esters by HDL with a corresponding decrease in the VLDL region. The conditions which caused these effects can be suggested from the results of the various conditions used in the *in vitro* studies. The apo CII and CIII transfer, the unesterified cholesterol transfer, some of the phospholipid transfer and the small shift/increase in mass in the light region of HDL mimics the action of lipoprotein lipase observed *in vitro*. The extensive hydrolysis of HDL triglycerides can be attributed to hepatic lipase activity. The greater gain in phospholipid, the greater transfer of apo E and the shift of the HDL peak to a lighter density, relative to that observed *in vitro* with lipoprotein lipase, may be due to the action of the lipid transfer proteins in the presence of lipoprotein lipase. The VLDL/LDL region did not gain cholesteryl ester *in vivo* but demonstrated a substantial decrease. This corresponds with the results of the *in vitro* incubation of the action of the lipid transfer proteins in the presence of highly active lipoprotein lipase in that transfer of cholesteryl ester form HDL to VLDL was reduced relative to the activity observed in the absence of lipoprotein lipase or was completely absent. A gain in cholesteryl ester in the HDL region was similar to that gained in two of the lipoprotein lipase experiments and may be related to this activity. However, unlike the in *vitro* incubation the activity of LCAT cannot be precluded and may account for the increase.

In the VLDL/LDL region, a substantial decrease in the VLDL mass was observed but mass did remain in fractions 1 and 2 as was observed in all of the *in vitro* incubations. An increase in mass and characterization of the mass in the LDL region (fractions 3-7) in the post-heparin sample was difficult to observe and analyze due to the magnitude of the LDL mass present in this region. It is interesting to note that the surface to core ratio of fractions 1 and 2 was elevated as compared to plasma LDL and was in the range of the values of the LPL and LPL+LTP incubation in fraction 1 and in the range of the

LPL+LTP incubation in fraction 2. This indicates that 1 to 1 1.5 hours of lipolysis/modification through release of the lipases is inadequate for the particles to equilibrate and that the lipid transfer protein may play a role in this process. Possibly, as previously suggested a longer incubation time would have allowed for a greater loss of surface and improved particle equilibration.

In vivo studies have reported a transfer of apo C mass from VLDL to HDL [246] in one normal subject. They report that the percentage lost was similar for apo CII and CIII and corresponded with the percentage of VLDL triglyceride hydrolysis which is similar to the observations of the present study. The results of Eisenberg [80] for hypertriglyceridemics indicate a shift of the VLDL mass from VLDL I and II to VLDL III and IDL after 45 minutes of a heparin infusion. Mass on the light side of the LDL peak was also augmented. HDL mass was augmented with a shift in the peak into a lighter density, with significantly more mass in the HDL_2 range. Much of the transfer of lipid and apoprotein mass was carried out by following the movement of radioactivity. There was extensive transfer to HDL of the apo CII and CIII. Transfer of cholesterol and phospholipid to HDL was reported.

The study of the *in vivo* heparin induced lipolysis points out the usefulness of the *in vitro* incubations in that conditions can be dissected out and recombined in order to determine the individual and combined effects of the enzymes or lipid transfer proteins on lipoprotein metabolism. The comparison of the *in vitro* results to the *in vivo* results is particularly applicable when the conditions of the incubation are as close to the physiological situation as possible. The *in vitro* experiments cannot provide all the conditions present *in vivo*, but as demonstrated in this study, can suggest how certain processes are functioning *in vivo*.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The role of lipoprotein lipase in the formation of LDL and HDL as a result of lipolysis of VLDL was the focus of the study. The effect of hepatic lipase and the lipid transfer proteins on the modulation of the process or on the products of VLDL lipolysis was also investigated. The following conclusions were made.

IN VITRO STUDIES:

LIPOPROTEIN LIPASE

1. Lipoprotein lipase caused an increase in HDL mass and a slight shift of the mass to a lighter density.

2. The HDL gained unesterified cholesterol, phospholipids and in some experiments cholesteryl ester. The gain in cholesteryl ester by HDL from VLDL may be due to the low unesterified cholesterol/phospholipid ratio of the original HDL.

3. HDL triglycerides were unaffected by lipoprotein lipase.

4. The transfer of apo CII and CIII from VLDL to HDL was extensive (88-95%) indicating that lipoprotein lipase alone is responsible for the removal of these apoproteins from VLDL during lipolysis. The transfer of apo E (31%) was much lower than that observed for the apo CII and CIII indicating that factors other than lipoprotein lipase in plasma are responsible for the complete removal of apo E for formation of LDL.

5. The lipolysed VLDL particles did not form true plasma LDL. They were lighter and substantially larger than plasma LDL and had an extremely high surface to core ratio compared to LDL.

LIPOPROTEIN LIPASE PLUS HEPATIC LIPASE:

1. Hepatic lipase in the presence of lipoprotein lipase caused extensive hydrolysis of HDL triglycerides as well as some phospholipid hydrolysis.

2. The HDL distribution on the gradient did not change.

3. There was minimal further effect on the conversion of VLDL to LDL over that noted for lipoprotein lipase alone.

LIPOPROTEIN LIPASE PLUS LIPID TRANSFER PROTEINS:

1. The lipid transfer proteins in the presence of lipoprotein lipase caused an augmentation of phospholipid, unesterified cholesterol and apo E transfer to HDL. The amount transferred was approximately double that transferred to HDL in the presence of lipoprotein lipase alone.

2. The HDL distribution showed a marked shift to the lighter density range which was substantially greater than that observed with lipoprotein lipase alone.

3. The transfer of cholesteryl ester from HDL to VLDL, that was observed when VLDL and HDL were incubated with the lipid transfer protein alone, was greatly reduced.

4. The lipolysed VLDL particles were smaller and had a reduction in the surface relative to core lipids as compared to the particles formed in the presence of lipoprotein lipase alone. The particle sizes approached that of plasma LDL but complete conversion of the particles to LDL was not achieved.

IN VIVO STUDIES:

1. Heparin-induced lipolysis resulted in a transfer of apo CII, apo CIII, apo E, phospholipid and unesterified cholesterol from the VLDL/LDL region to HDL. A gain in HDL cholesteryl ester was also observed. HDL triglycerides were extensively hydrolysed.

2. By comparing the extent of transfer of mass from VLDL to HDL and the amount of HDL triglyceride hydrolysis of the *in vivo* data to that of the in *vitro data*, it can be inferred that:

a) Lipoprotein lipase activity alone can account for the transfer of apo CII and apo CIII from VLDL to HDL.

b) Hepatic lipase activity alone can account for the HDL triglyceride hydrolysis.

c) Lipid transfer protein activity, in the presence of lipoprotein lipase activity, can account for some of the augmented transfer of phospholipid and apo E from VLDL to HDL.

In conclusion, the results of the present study suggest several mechanisms whereby lipoprotein lipase can contribute to the formation of an anti-atherogenic profile.

1. Lipoprotein lipase caused an increase in HDL mass. The increase in HDL mass occurred in the light region of the HDL distribution which is indicative of a role for lipoprotein lipase in the conversion of HDL_3 to HDL_2 or augmentation of HDL_2 mass. HDL_2 is the subfraction which is inversely related to the incidence of coronary heart disease.

2. Lipoprotein lipase prevented the hepatic lipase-induced conversion of the original HDL to denser particles which has been reported by others. Consequently, the HDL catabolic rate may be reduced when lipoprotein lipase is active since the conversion of HDL_2 to HDL_3, by the combined action of hepatic lipase and cholesteryl ester transfer protein, has been postulated to increase the rate at which the HDL is catabolized.

3. Lipoprotein lipase stimulated phospholipid transfer from VLDL to HDL when present with the lipid transfer proteins. The lipid transfer proteins did not cause net transfer of phospholipid in the absence of lipoprotein lipase. Transfer of unesterified cholesterol to HDL was also stimulated by the combination of the lipid transfer proteins

and lipoprotein lipase. The increased transfer of VLDL mass to HDL was accompanied by a substantial increase in the light region of HDL on the gradient reflecting an increased conversion of dense HDL (HDL_3) to HDL_2 as compared to that observed with lipoprotein lipase alone. This result suggests that there is a concerted effect of lipoprotein lipase and the lipid transfer proteins.

4. The markedly reduced transfer of cholesteryl ester from HDL to VLDL which was observed with the lipid transfer proteins when lipoprotein lipase was present would reduce the enrichment of HDL with triglycerides and VLDL with cholesteryl esters. Since enrichment of HDL_2 with triglycerides is required for the conversion of HDL_2 to HDL_3 by hepatic lipase, this process may be reduced in the presence of lipoprotein lipase and HDL catabolism may be slowed. Most importantly, the formation of cholesteryl ester-enriched VLDL was prevented. Therefore, in plasma, rapid lipolysis may prevent enrichment of VLDL with cholesteryl esters resulting in a decreased amount of cholesteryl esters being present in VLDL remnants, IDL and LDL. Thus a greater proportion of the plasma cholesteryl esters would transported by HDL and the amount of cholesteryl esters transported by the atherogenic lipoproteins would be reduced.

CHAPTER VII

APPENDICES

APPENDIX A

Appendix A contains the raw data for the lipid and apoprotein values used in the study. The data are listed for each fraction of the density gradient, numbered 1-20. The data for the *in vitro* experiments and for the *in vivo* experiments are included.

The *in vitro* data contain the values for all of the control (CON), lipoprotein lipase (LPL), lipoprotein lipase and hepatic lipase (LPL+HAL) and lipoprotein lipase and lipid transfer protein (LPL+LTP) incubations.

The *in vivo* data contains the pre-heparin (pre) and post-heparin (post) values of subjects A (A) and B (B).

The lipid data lists the values for unesterified cholesterol (UC), cholesteryl ester (CE), phospholipid (PL) and triglyceride (TG). The apoproteins are listed as A, B, CII, CIII and E. The values are expressed in micrograms.

The [^{3}H]cholesteryl ester radioactivity is listed in counts per minute (cpm).

DATA FROM EXPERIMENT A

	UC CON	UC LPL	UC LPL+HAL	CE CON	CE LPL	CE LPL+HAL	PL CON	PL LPL	PL LPL+HAL	TG CON	TG LPL	TG LPL+HAL	AI CON	AI LPL	AI LPL+HAL
1	626	384	404	1773	1124	1127	2130	808	824	4082	265	237	18	6	6
2	37	174	181	115	501	518	127	353	401	235	85	121	1	1	1
3	9	72	71	14	200	193	26	179	175	16	18	19	1	3	3
4	6	35	43	9	84	79	9	87	95	7	2	8	10	7	8
5	0	14	18	0	33	36	0	29	31	0	1	2	16	17	16
6	2	11	12	10	14	35	8	21	23	4	1	5	20	24	25
7	4	8	12	18	31	21	16	16	21	6	1	1	28	31	31
8	6	9	10	29	33	31	32	35	38	18	0	1	50	53	53
9	7	11	10	26	43	30	37	62	43	9	16	5	80	81	74
10	7	13	17	29	39	39	50	57	49	3	3	3	154	179	146
11	17	32	29	105	125	114	141	185	210	31	52	23	288	345	330
12	29	51	47	201	225	219	319	399	396	46	45	15	705	725	750
13	48	71	71	432	393	393	571	593	581	114	88	31	1240	1240	1240
14	59	75	81	514	464	533	667	665	734	113	89	25	1520	1410	1520
15	40	48	47	404	353	345	445	456	438	83	63	20	1340	1470	1500
16	19	24	20	211	186	116	221	213	182	35	26	0	732	680	468
17	18	13	6	54	24	31	175	168	164	16	0	0	158	124	139
18	8	12	7	20	52	16	94	126	122	2	0	6	100	67	94
19	0	6	2	13	42	17	25	10	9	2	6	3	70	85	67
20	0	0	4	8	8	12	12	12	6	0	1	1	0	0	0

	B CON	B LPL	B LPL+HAL	CII CON	CII LPL	CII LPL+HAL	CIII CON	CIII LPL	CIII LPL+HAL	E CON	E LPL	E LPL+HAL	CON DIAM	LPL DIAM	LPL+HAL DIAM
1	408	208	207	142	6	6	604	12	12	390	167	182	289	168	162
2	30	112	105	5	1	1	1	0	0	21	68	73	289	161	161
3	0	51	57	1	1	1	0	0	0	9	37	34	139	135	134
4	0	29	37	0	1	1	0	0	0	0	19	19	159	117	109
5	0	6	8	0	1	1	0	0	0	0	9	10	0	126	124
6	0	13	130	0	1	1	0	0	0	0	10	9	198	91	164
7	0	0	0	0	5	5	0	1	1	0	7	8	175	183	112
8	0	0	0	0	5	5	0	1	1	0	10	10	182	122	113
9	0	0	0	0	6	6	0	10	10	0	12	12	130	132	114
10	0	0	0	0	9	6	0	21	12	0	9	9	103	107	110
11	0	0	0	4	17	19	1	55	55	0	6	6	138	133	105
12	0	0	0	9	35	37	15	100	121	0	14	15	121	108	99
13	0	0	0	8	38	38	17	138	168	3	20	21	140	122	113
14	0	0	0	8	30	30	16	154	195	4	10	11	138	125	117
15	0	0	0	5	21	23	21	118	129	3	9	13	154	133	124
16	0	0	0	3	8	5	1	34	22	0	7	15	156	141	104
17	0	0	0	0	2	2	0	0	1	0	3	5	82	56	61
18	0	0	0	0	0	0	0	0	1	0	0	4	55	83	60
19	0	0	0	0	0	0	0	0	0	0	0	0	109	363	242
20	0	0	0	0	0	0	0	0	0	0	0	0	115	126	179

DATA FROM EXPERIMENT B

	UC CON	UC LPL	UC LPL+HAL	UC LPL+LTP	CE CON	CE LPL	CE LPL+HAL	CE LPL+LTP	PL CON	PL LPL	PL LPL+HAL	PL LPL+LTP	TG CON	TG LPL	TG LPL+HAL
1	561	179	218	204	1186	392	487	528	1967	404	561	518	3067	204	261
2	113	128	160	147	245	264	326	371	440	327	392	358	764	126	163
3	11	94	92	61	28	213	198	171	75	264	242	166	47	29	22
4	5	68	53	25	8	147	111	72	20	177	125	71	4	17	7
5	6	38	32	17	35	112	110	65	36	114	107	62	46	42	40
6	7	24	26	14	22	54	41	34	40	70	65	32	4	3	1
7	6	16	15	13	17	38	36	44	39	59	39	59	14	16	0
8	7	14	15	11	22	46	42	38	35	59	77	56	5	10	1
9	10	15	13	15	49	56	48	65	75	87	75	98	35	11	0
10	11	17	17	27	77	86	64	114	90	107	82	157	16	23	2
11	23	33	30	57	161	167	154	254	200	202	175	351	45	38	10
12	39	59	51	94	268	300	273	447	321	387	303	568	50	50	17
13	58	73	77	107	426	476	449	606	544	620	554	803	58	53	13
14	67	88	99	102	578	543	636	547	689	742	780	867	78	64	18
15	58	69	73	62	674	598	708	434	582	621	693	440	96	75	25
16	37	27	23	23	331	282	253	167	385	325	184	155	27	15	15
17	13	10	2	1	62	50	15	23	125	138	16	12	10	36	9
18	1	0	1	1	8	10	11	11	28	9	5	11	5	5	5
19	3	1	0	1	4	4	0	4	32	4	0	13	3	5	0
20	2	0	1	1	9	0	9	7	9	0	4	3	0	0	0

	TG LPL+LTP	AI CON	AI LPL	AI LPL+HAL	AI LPL+LTP	B CON	B LPL	B LPL+HAL	B LPL+LTP	CII CON	CII LPL	CII LPL+HAL	CII LPL+LTP	CIII CON	CIII LPL
1	161	24	5	5	4	252	46	61	61	211	4	4	3	315	0
2	178	5	5	4	4	48	53	61	68	35	4	4	4	52	0
3	37	4	4	4	5	0	29	27	45	3	4	4	3	0	0
4	24	6	11	7	8	0	38	31	18	0	0	0	0	0	0
5	23	12	15	11	13	0	11	17	5	0	0	0	0	0	0
6	3	22	23	18	27	0	7	9	1	0	3	2	2	0	1
7	8	40	37	25	45	0	0	10	0	0	5	5	5	0	1
8	3	56	64	44	66	0	0	4	0	0	5	5	8	0	2
9	18	93	73	68	105	0	0	0	0	0	7	6	8	0	6
10	18	167	154	107	215	0	0	0	0	3	11	9	16	1	21
11	48	438	285	277	436	0	0	0	0	6	24	18	38	18	41
12	79	748	687	578	932	0	0	0	0	10	39	39	61	19	65
13	88	1240	1240	1153	1469	0	0	0	0	13	50	59	74	37	103
14	60	1659	1645	1754	1754	0	0	0	0	17	56	63	62	42	102
15	56	1727	1686	1909	1634	0	0	0	0	14	41	48	36	29	74
16	12	908	799	775	688	0	0	0	0	9	18	16	12	19	35
17	15	416	196	175	175	0	0	0	0	1	4	3	4	0	0
18	5	87	81	112	104	0	0	0	0	0	1	1	1	0	0
19	5	91	53	102	56	0	0	0	0	0	0	0	0	0	0
20	0	33	17	38	32	0	0	0	0	0	0	0	0	0	0

DATA FROM EXPERIMENT B (CONT'D)

	CIII LPL+HAL	CIII LPL+LTP	E CON	E LPL	E LPL+HAL	E LPL+LTP	CPM CON	CPM LPL	CPM LPL+HAL	CPM LPL+LTP	CON DIAM	LPL DIAM	LPL+HAL DIAM	LPL+LTP DIAM
1	0	0	256	97	86	78	326200	109476	126000	128769	238	154	148	146
2	0	0	28	39	51	40	53811	61517	70024	68289	256	136	139	161
3	0	0	8	34	36	17	8742	42247	39646	29225	143	115	112	141
4	0	0	0	22	21	9	2070	30600	21146	10849	95	114	112	152
5	0	0	0	15	12	7	3078	15722	9790	5235	265	153	161	165
6	1	1	0	1	3	0	1998	8593	5206	3282	102	107	91	128
7	1	1	0	0	2	0	2997	4578	2998	3818	120	122	113	121
8	2	2	0	5	11	7	2276	4234	3106	2558	113	126	92	108
9	0	6	0	5	9	6	2005	2347	1576	2490	155	114	101	123
10	17	22	0	7	13	12	800	1777	1198	3360	144	139	114	121
11	35	56	4	11	14	14	1490	1693	1341	6326	145	138	129	123
12	65	105	2	13	18	23	1839	2008	1602	10530	140	128	131	129
13	104	113	3	15	20	23	1619	1697	1606	13957	131	126	122	126
14	114	99	3	15	20	18	1405	1498	1441	13890	138	122	123	111
15	90	74	3	11	20	22	1316	1282	1138	8993	175	150	147	149
16	38	31	0	9	18	12	1243	1274	1061	3610	135	135	184	152
17	0	0	0	6	12	6	931	734	416	1450	99	108	194	378
18	0	0	0	1	1	1	507	460	444	1038	93	234	342	193
19	0	0	0	0	1	1	644	439	674	624	64	249	0	116
20	0	0	0	0	0	1	710	245	341	446	130	0	236	229

DATA FROM EXPERIMENT C

	UC CON	UC LPL	UC LPL+HAL	UC LPL+LTP	CE CON	CE LPL	CE LPL+HAL	CE LPL+LTP	PL CON	PL LPL	PL LPL+HAL	PL LPL+LTP	TG CON	TG LPL	TG LPL+HAL
1	557	377	227	268	1308	1011	566	780	2211	1513	646	799	4341	1254	310
2	56	101	150	124	115	236	375	381	200	356	455	379	329	372	154
3	11	32	97	48	20	77	229	155	41	106	283	158	84	109	77
4	5	12	50	21	14	26	128	65	14	39	161	60	44	33	45
5	11	17	28	23	20	20	41	33	14	26	31	32	15	6	7
6	9	9	17	12	22	23	43	17	21	27	47	24	13	4	4
7	9	8	15	13	18	13	26	20	23	15	42	27	1	4	1
8	9	21	12	13	45	67	22	53	67	89	32	52	11	22	1
9	15	22	17	20	74	88	59	67	82	103	66	82	21	27	13
10	25	25	27	33	123	101	105	112	144	130	115	161	43	35	20
11	33	40	41	58	161	165	170	204	163	209	194	289	30	43	29
12	58	64	60	85	263	252	269	330	407	450	380	590	115	116	25
13	62	98	92	110	408	398	433	453	624	651	676	822	139	142	47
14	73	92	113	113	606	625	635	590	792	900	950	970	161	175	108
15	53	95	113	108	501	460	561	428	658	786	885	862	113	111	45
16	30	27	28	33	168	93	87	90	366	308	297	335	24	11	2
17	18	16	13	16	33	39	24	13	300	267	213	132	33	30	3
18	15	23	21	7	13	25	13	2	238	247	175	55	9	30	10
19	3	4	3	5	1	5	3	3	82	57	21	55	0	2	0
20	3	5	3	4	6	3	3	3	35	32	13	29	0	0	0

	TG LPL+LTP	AI CON	AI LPL	AI LPL+HAL	AI LPL+LTP	B CON	B LPL	B LPL+HAL	B LPL+LTP	CII CON	CII LPL	CII LPL+HAL	CII LPL+LTP	CIII CON	CIII LPL
1	281	50	8	0	0	299	166	94	109	263	49	5	7	326	95
2	160	6	3	0	0	20	40	53	65	4	9	3	3	12	13
3	81	9	5	4	3	0	6	45	31	3	3	3	3	0	0
4	37	16	6	5	7	0	0	43	22	0	1	2	1	0	0
5	3	24	8	9	10	0	0	7	5	0	3	3	3	0	0
6	4	25	18	16	19	0	0	8	2	2	3	4	3	0	0
7	2	57	34	18	32	0	0	5	2	3	5	4	5	0	0
8	3	91	97	38	65	0	0	0	0	3	6	6	6	0	4
9	25	109	114	48	80	0	0	0	0	3	8	8	8	0	10
10	32	247	120	104	158	0	0	0	0	5	11	14	17	7	11
11	59	297	255	220	337	0	0	0	0	9	22	25	34	14	20
12	113	683	680	406	777	0	0	0	0	11	30	38	48	25	46
13	126	1075	1075	1060	1463	0	0	0	0	13	39	48	44	46	65
14	185	1613	1497	1929	1785	0	0	0	0	21	43	52	45	46	91
15	113	1584	1728	2073	2390	0	0	0	0	16	31	47	34	40	73
16	12	714	507	461	1175	0	0	0	0	6	20	18	17	13	30
17	17	218	143	151	219	0	0	0	0	0	5	6	4	0	10
18	2	109	120	59	168	0	0	0	0	0	0	2	0	0	0
19	0	113	45	62	118	0	0	0	0	0	0	0	0	0	0
20	3	64	44	43	87	0	0	0	0	0	0	0	0	0	0

DATA FROM EXPERIMENT C (CONT'D)

	CIII LPL+HAL	CIII LPL+LTP	E CON	E LPL	E LPL+HAL	E LPL+LTP	CPM CON	CPM LPL	CPM LPL+HAL	CPM LPL+LTP	CON DIAM	LPL DIAM	LPL+HAL DIAM	LPL+LTP DIAM
1	0	8	307	201	109	129	143900	165681	69026	51728	282	179	153	151
2	0	0	21	31	69	48	14765	26970	35378	25922	244	195	138	161
3	0	0	8	15	50	23	5124	5414	18523	9491	277	196	130	170
4	0	0	0	7	30	11	1936	1524	9272	4070	398	174	132	182
5	0	0	0	3	14	7	2539	1869	4653	2078	195	107	127	111
6	0	0	0	3	10	7	3728	1094	2987	1809	171	123	121	104
7	0	3	0	3	10	8	1333	1562	1754	1449	105	122	92	100
8	3	8	0	5	10	10	1313	2622	1538	2485	124	131	97	135
9	11	12	0	0	0	0	1104	2280	1500	2489	151	144	137	142
10	18	31	0	0	0	0	754	1602	2590	4376	151	139	138	124
11	27	56	0	0		0	1097	1095	2496	6237	149	134	135	126
12	60	96	0	0	0	0	762	833	1244	9838	133	122	115	115
13	103	123	1	1	12	32	903	949	1003	13369	131	122	110	111
14	132	117	23	22	21	38	710	932	1095	14418	141	132	119	122
15	134	101	9	15	25	22	682	781	1081	11263	138	114	108	104
16	38	30	0	14	17	13	822	629	1110	3228	95	76	71	72
17	10	0	0	12	14	8	577	483	586	915	65	69	54	65
18	0	0	0	0	0	0	271	372	329	992	51	65	55	48
19	0	0	0	0	0	0	430	354	460	682	42	54	55	47
20	0	0	0	0	0	0	434	341	326	446	58	50	61	62

DATA FROM EXPERIMENT D

	UC CON	UC LPL	UC LPL+LTP	UC LTP	CE CON	CE LPL	CE LPL+LTP	CE LTP	PL CON	PL LPL	PL LPL+LTP	PL LTP	TG CON	TG LPL	TG LPL+LTP
1	508	125	272	442	1474	373	1022	1603	2056	398	717	1929	4956	195	418
2	46	127	46	69	140	251	101	278	232	410	215	352	386	100	48
3	9	100	25	11	18	238	84	26	35	249	83	42	46	46	44
4	8	66	18	10	13	158	37	13	33	183	74	57	10	13	10
5	6	30	13	7	13	72	27	12	28	81	66	29	5	6	7
6	7	23	13	9	20	58	23	27	45	77	48	59	8	6	3
7	9	18	13	11	20	59	42	25	65	104	95	57	12	30	43
8	8	20	15	12	34	86	43	20	38	127	60	50	9	53	60
9	13	23	16	11	64	100	55	43	96	120	92	74	28	43	39
10	18	35	27	25	92	158	92	53	118	189	139	82	23	57	43
11	44	51	65	39	180	228	152	103	206	249	211	136	66	52	42
12	45	67	76	47	298	328	277	208	332	386	408	263	79	64	72
13	58	86	100	69	521	489	458	346	544	559	658	423	157	86	97
14	58	71	120	89	560	560	592	512	580	651	844	615	129	101	124
15	33	36	156	95	364	304	473	507	383	395	824	636	60	62	70
16	17	13	58	100	75	24	66	168	207	177	321	481	29	8	11
17	3	10	16	20	10	23	30	27	62	181	179	211	10	4	11
18	3	2	11	12	19	32	34	40	109	55	145	186	0	8	9
19	4	6	7	5	24	30	19	15	69	57	60	66	3	0	1
20	3	1	4	2	15	15	21	16	45	20	28	33	0	0	15
21															

	TG LTP	AI CON	AI LPL	AI LPL+LTP	AI LTP	B CON	B LPL	B LPL+LTP	B LTP	CII CON	CII LPL	CII LPL+LTP	CII LTP	CIII CON	CIII LPL
1	3999	79	0	0	51	316	66	151	298	303	0	31	280	393	0
2	622	13	8	0	13	24	73	74	47	15	4	10	32	15	0
3	57	12	9	5	10	0	65	26	1	0	0	0	1	0	0
4	12	27	25	22	25	0	37	10	0	0	0	0	0	0	0
5	5	42	40	36	51	0	23	1	0	0	0	0	0	0	0
6	18	50	53	47	60	0	2	0	0	0	3	3	0	0	0
7	22	79	79	67	95	0	0	0	0	0	3	4	0	0	0
8	41	115	116	110	134	0	0	0	0	0	7	6	0	0	5
9	38	166	172	153	214	0	0	0	0	0	12	9	0	8	16
10	23	254	339	290	321	0	0	0	0	0	18	16	0	13	38
11	56	469	516	538	521	0	0	0	0	5	36	32	7	11	72
12	79	593	633	721	802	0	0	0	0	11	72	67	13	59	114
13	124	1439	1449	1791	1731	0	0	0	0	17	79	74	15	74	152
14	204	1912	1862	2774	2887	0	0	0	0	15	49	56	12	70	149
15	154	1442	1058	5659	5580	0	0	0	0	9	14	19	15	48	103
16	63	552	295	4927	5149	0	0	0	0	7	11	14	9	15	35
17	4	261	143	1993	1657	0	0	0	0	0	3	5	0	0	15
18	13	107	63	1323	954	0	0	0	0	0	0	4	0	0	0
19	8	97	60	508	362	0	0	0	0	0	0	3	0	0	0
20	5	30	24	182	192	0	0	0	0	0	0	0	0	0	0
21															

DATA FROM EXPERIMENT D (CONT'D)

	CIII LPL+LTP	CIII LTP	E CON	E LPL	E LPL+LTP	E LTP	CPM CON	CPM LPL	CPM LPL+LTP	CPM LTP	CON DIAM	LPL DIAM	LPL+LTP DIAM	LTP DIAM
1	72	388	305	69	140	279	69562	17703	30289	39356	337	163	202	319
2	8	58	25	96	51	30	4802	16457	6290	5433	264	114	105	292
3	0	0	5	53	19	5	857	12679	1696	774	212	130	174	224
4	0	0	0	25	8	0	360	7494	706	252	105	115	98	84
5	0	0	0	12	0	0	241	3464	462	250	100	117	89	94
6	0	0	0	11	0	0	206	1769	413	228	102	111	88	117
7	1	0	0	9	0	0	189	941	450	298	90	124	132	120
8	11	0	0	7	0	0	157	692	486	334	145	149	200	156
9	23	10	0	11	0	0	171	594	728	511	137	154	141	151
10	41	14	0	9	0	0	261	606	1327	931	136	149	133	120
11	89	33	0	1	1	0	297	630	2622	1567	151	145	119	143
12	152	51	0	6	4	0	368	747	4272	2677	153	137	122	145
13	199	84	1	13	15	1	453	836	6418	4728	168	140	123	149
14	204	95	34	32	31	5	534	886	8086	6729	162	143	124	156
15	185	101	30	27	30	24	338	540	7062	7015	155	136	102	143
16	58	61	0	23	25	25	277	371	1555	3187	94	60	64	86
17	27	16	0	10	23	21	294	352	794	714	77	57	65	56
18	25	15	0	0	33	28	233	154	583	550	60	121	72	71
19	8	0	0	0	23	0	166	138	295	268	82	93	74	75
20	0	0	0	0	0	0	62	81	185	154	76	120	170	109
21								0	0	0				

DATA FROM EXPERIMENT E

	UC CON	UC LPL	UC LPL+LTP	UC LTP	CE CON	CE LPL	CE LPL+LTP	CE LTP	PL CON	PL LPL	PL LPL+LTP	PL LTP	TG CON	TG LPL	TG LPL+LTP
1	655	126	258	530	1870	446	1010	2082	2833	418	763	2492	5542	207	315
2	48	119	159	109	120	341	613	387	201	362	577	484	357	105	205
3	16	121	59	16	36	304	211	37	76	370	181	78	84	42	62
4	5	124	29	10	6	263	79	11	16	351	93	45	12	33	24
5	8	64	18	12	9	122	38	14	30	161	88	66	22	17	10
6	11	35	14	9	20	75	25	11	44	125	44	25	21	17	7
7	11	27	11	10	35	35	19	12	66	59	39	59	16	6	7
8	11	20	16	17	36	36	38	61	76	46	67	111	8	9	23
9	14	24	26	12	48	82	79	36	76	141	161	84	41	23	33
10	19	35	36	22	94	133	111	66	126	203	209	172	36	26	34
11	40	50	66	34	212	194	230	133	313	294	467	236	55	37	75
12	52	75	104	56	344	357	370	268	510	580	765	478	87	75	119
13	68	91	107	76	629	869	839	501	707	958	1149	666	129	171	236
14	70	99	108	87	806	863	743	676	872	1018	1097	871	166	160	191
15	46	66	65	66	570	604	518	527	651	623	574	562	127	174	244
16	19	18	21	28	128	152	77	170	321	362	225	446	34	59	12
17	17	16	31	25	25	26	39	30	261	241	260	241	36	16	34
18	3	2	3	10	12	10	29	16	132	15	13	130	9	11	10
19	2	3	3	3	6	14	12	6	69	16	15	39	0	0	0
20	0	1	2	3	3	3	5	2	13	16	10	23	0	0	0
21															

	TG LTP	AI CON	AI LPL	AI LPL+LTP	AI LTP	B CON	B LPL	B LPL+LTP	B LTP	CII CON	CII LPL	CII LPL+LTP	CII LTP	CIII CON	CIII LPL
1	4522	58	0	0	62	306	62	190	274	251	2	18	216	512	10
2	865	10	0	0	18	17	47	80	43	14	2	10	33	17	0
3	76	11	7	7	9	2	53	52	8	2	1	3	3	0	0
4	14	14	13	11	14	0	52	21	1	0	3	1	0	0	0
5	11	19	15	16	24	0	45	13	0	0	2	1	0	0	0
6	18	68	25	30	48	0	19	2	0	2	3	4	1	0	0
7	6	58	36	44	70	0	6	0	0	1	4	4	1	0	1
8	45	80	63	89	112	0	1	0	0	1	4	7	2	0	2
9	24	150	125	162	154	0	0	0	0	1	9	13	3	4	7
10	30	275	251	284	280	0	0	0	0	4	17	19	5	9	24
11	65	460	309	520	506	0	0	0	0	7	31	40	7	36	57
12	138	882	770	1150	1194	0	0	0	0	12	45	56	12	70	98
13	225	1533	1364	1845	1942	0	0	0	0	12	68	62	19	71	156
14	287	2293	1913	2111	3102	0	0	0	0	16	54	45	22	77	213
15	358	1368	1418	3177	3878	0	0	0	0	14	44	32	13	45	151
16	83	491	317	2359	1991	0	0	0	0	5	18	17	10	16	43
17	21	180	144	1091	757	0	0	0	0	0	9	10	0	0	18
18	10	149	72	900	521	0	0	0	0	0	3	9	0	0	11
19	0	81	53	301	222	0	0	0	0	0	3	5	0	0	0
20	0	22	9	95	75	0	0	0	0	0	0	2	0	0	0
21															

DATA FROM EXPERIMENT E (CONT'D)

	CIII LPL+LTP	CIII LTP	E CON	E LPL	E LPL+LTP	E LTP	CPM CON	CPM LPL	CPM LPL+LTP	CPM LTP	CON DIAM	LPL DIAM	LPL+LTP DIAM	LTP DIAM
1	60	462	335	75	114	273	330862	73228	96247	188337	292	175	184	297
2	15	78	18	58	71	57	22210	65465	57196	33972	267	144	165	289
3	0	0	5	69	32	9	5161	72858	21478	3703	194	118	167	182
4	0	0	0	54	11	0	799	70505	6790	1006	140	109	134	94
5	0	0	0	21	7	0	662	33755	3830	1147	136	108	91	78
6	0	0	0	13	0	0	2144	15102	2678	1029	127	104	102	139
7	3	0	0	9	0	0	536	6761	2632	1122	116	93	99	71
8	9	0	0	2	0	0	410	3921	2988	1474	09	116	124	136
9	17	2	0	1	0	0	454	3201	5032	2334	155	112	109	113
10	44	6	0	2	0	0	509	3174	8413	4538	142	115	107	97
11	94	23	0	4	2	0	765	2562	16403	9966	126	116	105	124
12	143	34	1	7	4	1	1149	2706	27574	18980	127	115	104	128
13	146	77	8	11	9	3	1522	3135	34280	26366	151	152	138	152
14	160	75	20	16	20	6	1675	3298	36447	34579	157	144	128	155
15	117	60	9	15	15	7	1130	2152	25931	28064	154	168	177	203
16	38	24	0	22	24	10	690	1326	7311	8735	95	104	81	102
17	18	9	0	10	26	20	600	1221	2759	2481	67	60	70	63
18	16	0	0	0	35	20	555	771	2103	2130	59	185	314	62
19	0	0	0	0	20	5	279	606	990	935	50	121	114	57
20	0	0	0	0	0	0	111	167	601	351	67	61	86	49
21								0	0	0				

DATA FROM SUBJECTS "A" AND "B"

	UC pre "A"	CE pre "A"	PL pre "A"	TG pre "A"	UC post "A"	CE post "A"	PL post "A"	TG post "A"	E pre "A"	CII pre "A"	CIII pre "A"	E post "A"	CII post "A"	CIII post "A"	UC pre "B"
1	459	1055	1466	2979	226	532	566	481	61	99	127	0	3	3	253
2	195	661	513	593	246	713	620	391	0	13	38	0	3	8	165
3	268	1177	596	210	278	1134	612	182	0	4	25	0	2	4	180
4	842	3990	1995	255	889	4167	1932	164	0	4	11	0	2	5	729
5	1124	5781	2682	203	1251	6076	2814	189	0	4	12	0	4	7	918
6	500	2811	1271	77	452	2509	1059	37	0	3	9	0	3	7	522
7	212	1050	507	48	159	817	507	27	0	4	12	0	3	10	211
8	102	508	286	36	85	451	237	14	36	5	20	24	5	17	127
9	60	348	243	20	62	356	232	6	25	10	38	48	11	35	112
10	77	432	367	19	89	452	439	6	10	20	76	54	29	94	132
11	107	608	630	29	98	772	776	8	34	27	81	58	46	135	152
12	118	1022	1189	46	151	1370	1499	10	29	27	127	36	67	156	186
13	183	1642	1892	111	199	1913	2211	31	22	42	183	29	58	164	220
14	150	1740	1910	119	164	1643	1857	22	9	33	155	23	40	122	166
15	72	1002	1092	61	93	970	958	11	10	14	63	23	20	61	100
16	60	503	460	36	52	367	344	6	34	9	42	55	11	28	58
17	48	77	122	17	60	89	152	5	23	3	8	22	3	7	31
18	39	70	116	11	38	71	105	5	13	2	0	4	2	0	19
19	9	24	29	10	11	28	47	3	0	0	0	0	0	0	10
20	9	52	56	5	8	50	47	4	0	0	0	0	0	0	2

	CE pre "B"	PL pre "B"	TG pre "B"	UC post "B"	CE post "B"	PL post "B"	TG post "B"	E pre "B"	CII pre "B"	CIII pre "B"	E post "B"	CII post "B"	CIII post "B"
1	647	1352	2364	96	261	527	320	68	64	240	12	0	0
2	688	538	387	143	545	637	207	22	8	24	16	0	0
3	854	884	263	162	762	647	199	18	4	12	14	0	0
4	3549	3244	481	744	3467	3241	498	20	2	4	16	0	24
5	4519	4005	512	972	4465	4017	453	18	2	4	18	0	24
6	2950	1690	245	506	2771	1606	224	26	2	4	22	0	18
7	1093	602	115	228	1154	650	133	28	4	10	28	2	22
8	654	500	73	118	612	484	63	34	8	24	32	6	42
9	581	601	79	107	566	576	65	54	20	92	52	32	104
10	708	865	65	132	707	856	37	56	46	210	56	48	210
11	912	1414	99	165	919	1471	38	56	68	340	60	86	360
12	1424	2209	91	206	1541	2369	67	52	70	420	52	106	460
13	2016	2874	139	232	2143	3153	49	34	62	400	36	122	480
14	1788	2468	118	166	1795	2685	36	24	40	240	32	56	320
15	1115	1638	109	105	1116	1639	42	52	22	124	52	42	212
16	252	503	14	66	293	592	9	44	4	46	56	4	68
17	113	284	1	33	71	258	2	16	2	18	22	2	18
18	58	164	1	20	33	105	1	0	0	0	0	0	0
19	16	29	1	6	9	31	0	0	0	0	0	0	0
20	13	16	1	1	4	16	2	0	0	0	0	0	0

APPENDIX B

CALCULATION OF PARTICLE DIAMETER FROM LIPID DATA.

The particle diameters were calculated from the lipid data, obtained by gas chromatography, based on a calculation of the radius of the neutral lipid core [226, 227]. The radius of the core is considered to be the measurement that varies as the particle changes in size since the radius of the surface lipid monolayer of phospholipid and unesterified cholesterol which also contains protein is assumed to remain constant (2.05 nm). It was determined that the protein on the surface did not contribute to the particle diameter [227]. Therefore the radius of the core can be obtained in the following manner:

$$\frac{4\pi r^2_{core}}{4/3\,\pi r^3_{core}} = \frac{\text{surface area of core}}{\text{core volume}} = \frac{k[\,0.685\ (\text{moles \% PL}) + 0.391\ (\text{moles \% UC})]}{k[1.556\ (\text{moles \% TG}) + 1.068\ (\text{moles \% CE})]}$$

where 0.685, 0.391, 1.556 and 1.068 are the molecular volumes (expressed in nm^2) of phospholipids (PL), unesterified cholesterol (UC), triglycerides (TG) and cholesteryl esters (CE), respectively. Using the above equation and solving for r_{core}, the equation becomes:

$$r_{core} = \left[\,3 \times \frac{\text{core volume}}{\text{surface area of core}}\,\right] = 3x\ \frac{[\,0.685\ (\text{moles \% PL}) + 0.391\ (\text{moles \% UC})]}{[1.556\ (\text{moles \% TG}) + 1.068\ (\text{moles \% CE})]}$$

The radius of the particle can therefore be arrived at by adding the radius of the surface monolayer (2.05 nm) to the calculated radius of the core. By multiplying the value for the radius of the particle by 2 x 10, the calculated diameter of the particle, expressed in angstroms, is attained.

APPENDIX C

DISTRIBUTION OF PHOSPHOLIPID AND CHOLESTERYL ESTER OF EXPERIMENT D

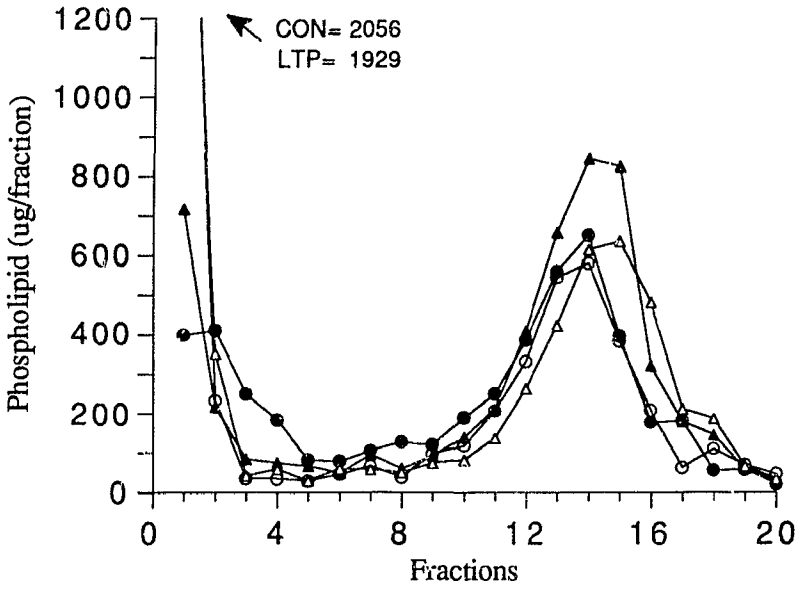
Appendix C contains the phospholipid and cholesteryl ester distributions of experiment D.

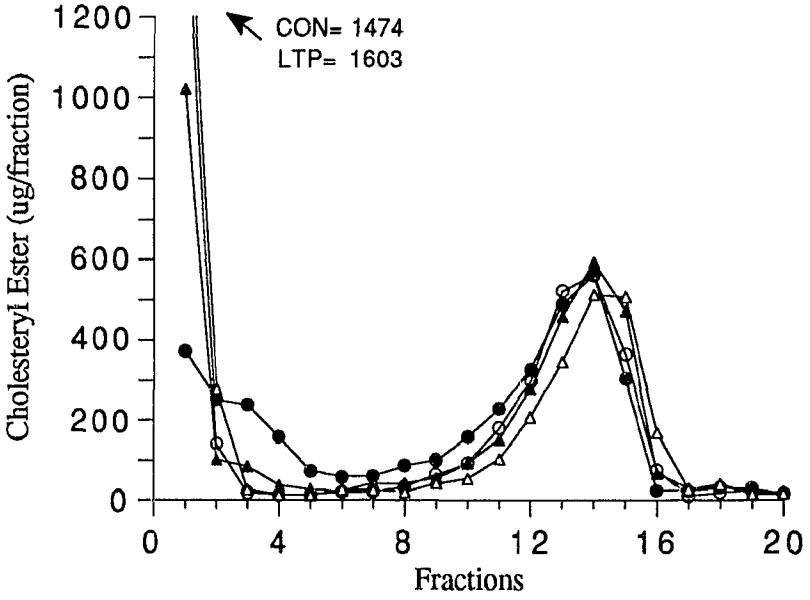
Each incubation contained VLDL+HDL in a ratio of 2 mg VLDL protein to 10 mg HDL protein which was carried out for 2 hours at 37°C

Control refers to the inclusion of heat denatured lipoprotein lipase.
LPL refers to the inclusion of active lipoprotein lipase.
LPL+LTP indicates the addition of the lipid transfer protein preparation to the LPL incubation.
LTP indicates the addition of the lipid transfer protein preparation to the control incubation.

The conditions of the incubations are represented as:

control: ○
LPL: ●
LPL+LTP: ▲
LTP: △


CON= 2056
LTP= 1929
Phospholipid (ug/fraction)
1200
1000
800
600
400
200
0
0
4
8
12
16
20
Fractions


CON= 1474
LTP= 1603
Cholesteryl Ester (ug/fraction)
1200
1000
800
600
400
200
0
0
4
8
12
16
20
Fractions

CHAPTER VIII

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