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THE EFFECT OF LIPOPROTEIN STRUCTURE ON INTERLIPOPROTEIN LIPID TRANSFERS BY CHOLESTERYL ESTER TRANSFER PROTEIN

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Thesis submitted to the Department of Biochemistry in partial fulfillment of the requirements for the degree of Master of Science

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Ottawa, Ontario, Canada
May, 1997

Abstract

Alterations in low density lipoprotein (LDL) and high density lipoprotein (HDL) composition that occur in dyslipidemic states may modulate a number of events involved in cholesterol homeostasis. To elucidate the details involved, the relationship between lipoprotein structural properties and cholesteryl ester transfer protein (CETP) mediated neutral lipid exchange has been studied in native lipoproteins, reconstituted apoB (LpB) and homogeneous, recombinant HDL (Lp2A-I) particles containing palmitoyloleoyl phosphatidylcholine (POPC), cholesteryl linoleate (CE) and phosphatidyl inositol (PI). Decreasing the POPC content of Lp2A-I particles, from 150 to 70 molecules/particle confers a small change in surface potential, a decrease in thermodynamic stability and increases CE mass transfer from LDL to Lp2A-I by 30%. Increasing the PI content in Lp2A-I from 0 to 16 molecules/particle increases the surface potential, decreases the thermodynamic stability and significantly increases CE mass transfer to Lp2A-I by 22%. In general, the rates of CE transfer from LDL to Lp2A-I were over 100-fold greater than in the opposite direction. CETP mediated transfer of free cholesterol (FC) from LDL into Lp2A-I was unaffected by changes in Lp2A-I POPC content, while increases in PI content increased FC transfer into Lp2A-I by 40%. Inclusion of 90 molecules of PI into reconstituted LDL (LpB) affected net CE and triacylglycerol (TG) mass transfer to native HDL (nHDL). Addition of PI to LpB essentially abolished the net CE mass transfer from LpB to nHDL. The direction of transfer of TG from LpB to HDL was reversed by the addition of PI. Incubation of PI with plasma caused a decrease in the net mass of CE, TG and FC in apoB-containing lipoproteins. This indicates that the POPC and PI content of lipoprotein particles directly affects CETP mediated neutral lipid exchange. Decreasing

the POPC content in Lp2A-I particles significantly decreases the particle stability. Addition of PI directly increases the particle charge, and also decreases particle stability. This suggests that lipoprotein charge and apoA-I stability modulate neutral lipid transfer by CETP.

Dedications

I dedicate this work to my loving family;
Anne, Baba, and Tunç.

Acknowledgments

I would like to express my appreciation to everyone who has contributed to the successful completion of my education over the last few years of my graduate studies. Particularly, I would like to thank my supervisor, Dr. Dan Sparks who has provided me with the opportunity to explore and develop my research skills. He has taught me more than science, and has helped me develop myself as an honest, communicative human being.

I would also like to acknowledge the assistance of Dr. Ross Milne. Without his expertise, the completion of my studies would have been unfeasible.

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List of Abbreviations and symbols

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apo	apolipoprotein
AVh	average volt-hours
C/EBP	CCAAT / enhancer binding protein
CAD	coronary artery disease
CD	circular dichroism
CE	cholesteryl ester
CETA	cholesteryl ester transfer activity
CETP	cholesteryl ester transfer protein
d	density
$D_{1/2}$	midpoint of denaturation
DG	diglyceride
EDTA	ethylenediaminetetraacetic acid
FC	free cholesterol
FH	familial hypercholesterolemia
FFA	free fatty acid
FPLC	forced pressure liquid chromatography
g	gram
mg	milligram
μg	microgram
ΔG_D	free energy of stability
GdnHCl	guanidine hydrochloride

h	hour
HDL	high density lipoprotein
HL	hepatic lipase
IDL	intermediate density lipoprotein
Ig	immunoglobulin
K	thousand
kbp	kilobase pairs
kDa	kilodalton
L	litre
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
Lp2A-I	recombinant HDL particle
LpB	recombinant LDL particle
LPL	lipoprotein lipase
LTP	lipid transfer protein
M	molar
mAb	monoclonal antibody
mg	milligram
min	minute
ml	millilitre
µl	microlitre
mM	millimolar
µM	micromolar

n	native
Δn	moles of GdnHCl bound during denaturation
NEFA	non-esterified fatty acids
NGGE	non-denaturing gradient gel electrophoresis
nm	nanometer
PAGE	polyacrylamide gel electrophoresis
pH	acidity
pI	isoelectric point
PL	phospholipid
PLTP	phospholipid transfer protein
POPC	1-palmitoyl 2-oleoyl phosphatidylcholine
PI	phosphatidylinositol
RCT	reverse cholesterol transport
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulfate
t	incubation time
TG	triglyceride
TGRL	triglyceride-rich lipoproteins
TP2	anti-transfer protein monoclonal antibody
VLDL	very low-density lipoprotein

1 Introduction

1.1 Overview

In the mammalian circulatory system, lipids are transported in lipoproteins [1]. Lipids are hydrophobic and poorly soluble in aqueous medium. By incorporation into lipoproteins, lipids can be transported through the plasma and lymph, to areas in the body where they can be utilized. Glycerides are transported from the liver and small intestine to muscle and adipose tissue, where they are either stored or metabolized. Other lipids, such as cholesterol, are transported from extra-hepatic tissues to the liver where they can be catabolized. Abnormalities in the lipid composition of lipoproteins can be caused by a number of factors, including diet and genetic predisposition. These abnormalities can lead to dyslipidemic states, which are often associated with premature atherosclerosis and coronary artery disease (CAD) [reviewed in 2].

Lipoprotein structure and function are affected by a number of factors in the plasma. Lipoprotein lipase (LPL) and hepatic lipase (HL) act as lipolytic enzymes and hydrolyze phospholipids (PL), diglycerides (DG) and triglycerides (TG) in lipoproteins. Lecithin:cholesterol acyltransferase (LCAT) esterifies free cholesterol on the surface of lipoproteins. Remodeling of the lipoprotein substrates plays an important role in modulating the interfacial interactions between plasma lipoproteins and various plasma proteins and cell surfaces [1]. Transfer and redistribution of lipids between lipoproteins occurs and is facilitated by specific lipid transfer proteins. Phospholipid transfer protein (PLTP) mediates most of the PL transfer between lipoproteins, although some spontaneous transfer can occur. Cholesteryl ester transfer protein (CETP) also mediates

PL transfer between lipoproteins. CETP is required for the transfer of cholesteryl ester (CE) and TG between lipoprotein particles.

The central role that high-density lipoprotein (HDL) plays in cholesterol metabolism is thought to involve the transport of cholesterol from peripheral tissues to the liver [3]. Several studies have suggested that the efficiency of HDL in mediating this flux may be impaired in hyperlipidemic patients and that this may be related to modifications in HDL composition [reviewed in 4]. These studies have shown that altered HDL composition is associated with altered interactions between HDL and CETP [4, 5, 6, 7]. Specific changes in the conformation and charge of the primary protein of HDL, apoA-I, also occur as a result of alterations in HDL composition. Altered function of the lipoprotein particles appears to be closely correlated with the changes in the molecular properties of HDL.

Both HDL and CETP may play important roles in attenuating the risk of atherosclerosis. This thesis investigates the mechanism and regulation of CETP lipid transfer activity by lipoprotein substrate composition and its role in atherosclerosis and dyslipidemia.

1.2 Lipoprotein Structure

Lipoprotein particles are macromolecular assemblies of specific apoproteins and lipids held together by non-covalent forces [8]. Lipoproteins are generally emulsion or microemulsion particles containing a non-polar lipid core surrounded by a surface of polar lipids and apoproteins. Large very low-density lipoproteins, chylomicrons and some of their remnants belong to the class of emulsion, where particle diameter is in excess of 50

nm. Particles less than 50 nm in diameter, such as low density lipoprotein (LDL), intermediate density lipoprotein (IDL), small very low density lipoprotein (VLDL), and HDL are microemulsions. All have apolar cores of TG and CE, and a surface stabilized by different apoproteins and phospholipids.

Chylomicrons are the largest of the triglyceride-rich emulsion particles and are formed in the intestine during the absorption of fat. They are secreted into the lymph and from there enter the plasma [1]. The core contains mainly TG and a very small amount of CE [8]. Apo B48, apo A-I and A-IV make up the surface proteins. The majority of the surface is made up of phosphatidylcholine [9]. Chylomicrons contain very little free cholesterol (FC). The triglyceride core of chylomicrons is rapidly hydrolyzed in the plasma vascular bed by endothelial bound lipoprotein lipase [10]. This results in the formation of remnant particles, which are rapidly removed from the plasma by specific hepatic receptor-mediated uptake. The cholesterol is either disposed of by the liver, by converting it to bile, or diverted back into the plasma in the form of newly secreted lipoproteins.

VLDL is the term used to describe nascent, TG-enriched particles secreted by the liver. However, particles floating in the VLDL density range can also come from the intestine (e.g. chylomicron remnants). The surface of nascent VLDL is made up largely of phosphatidylcholine, small amounts of cholesterol, apo B_{100} and some of the smaller, exchangeable, apoproteins (apo C, apo E, apo A-I). The core consists mainly of TG, but can have varying amounts of CE, depending on the cholesterol input to the liver [11]. The non-exchangeable apoprotein, apo B_{100}, stays with the VLDL particle from secretion to cellular uptake and catabolism. The other, exchangeable, apoproteins may transfer between different classes of lipoproteins.

In plasma, VLDL is converted by LPL to VLDL remnants and IDL, which contains apoB_{100}, as well as apo C and apo E [11]. IDL contains considerably less TG than VLDL. The IDL particles are either removed by the liver through interaction with the LDL receptor, or are eventually converted to LDL, a microemulsion size particle that is CE-rich and contains almost exclusively apo B_{100}. Conversion of IDL to LDL also is through the action of lipoprotein lipase. LDL is the major carrier of cholesterol and cholesterol ester, in plasma [10]. Delivery of cholesterol to hepatic and extra-hepatic cells is through LDL binding to the LDL receptor on cell surfaces and subsequent receptor-mediated endocytosis.

The HDL lipoproteins are a complex group of particles that have a density range of 1.063 to 1.21 g/ml. The major lipids in order of abundance are PL, CE, cholesterol, DG and TG. The apoproteins are all of the exchangeable type (apo A-I, A-II, A-IV, C-I, C-II, C-III, D and E). The composition of the particles is about 50% protein and 50% lipid [11]. The HDL lipoproteins are further divided into sub-fractions. In humans, these are VHDL, HDL_2 and HDL_3. HDL_2 are larger and contain more core lipids, especially CE and DG. HDL may play an important role in the flux of cholesterol between peripheral cells and the liver, as well as acting as a substrate for numerous plasma enzymes [12]. The extent to which efflux is affected by HDL concentration and composition is still disputed, with different studies using different systems that sometimes contradict one another [13, 14].

1.3 Reconstituted Model Systems

Investigation of the molecular details of the structure/function relationships involved in HDL metabolism requires the use of well-defined, homogeneous substrates to study specific molecular features. Of these reconstituted preparations, the most commonly characterized are simple phospholipid-apolipoprotein mixtures that appear as disc-shaped structures after negative stain electron microscopy. Homogeneous preparations of discoidal LpA-I can be produced by cholate-dispersion methods [reviewed in 15]. These systems have been highly characterized in terms of their metabolic and biophysical characteristics [15-17]. These simple discoidal systems differ substantially from the mature spherical LpA-I particles of the plasma that contain a neutral lipid core, and appear to more closely resemble nascent HDL particles found in the interstitial fluid [18].

Techniques used for the production of spherical HDL particles tend to produce heterogeneous preparations and are not very reproducible [15]. In the early seventies, Scanu *et al* [19] showed that reconstituted spherical LpA-I, A-II particles could be produced from re-purified lipids and apoproteins by co-sonication. Since then, investigations have shown that reconstituted spherical LpA-I, A-II resemble authentic HDL in their ability to interact with LCAT and CETP [6,20], as well as lipoprotein lipase [21] and cultured cells [22]. These spherical reconstituted lipoproteins are much more comparable to the HDL particles found in plasma. Using size characterizations, these reconstituted preparations are shown to be as heterogeneous as their native HDL counterparts. It is this structural heterogeneity that has limited the characterization of the molecular and functional properties of spherical particles. Recently, Sparks *et al* developed a novel sonication technique [23] to produce highly homogeneous spherical

LpA-I and LpA-I, A-II. This method represents a breakthrough for the study of the structure and function of spherical HDL particles, since none of the previously reported techniques used to prepare spherical HDL are capable of producing monodisperse populations of particles. Using this technology, it is now possible to clearly evaluate the effect of HDL structure on CETP function.

1.4 Lipoprotein Remodeling

1.4.1 Lecithin-Cholesterol Acyltransferase

LCAT is synthesized by the liver and secreted into the bloodstream. It catalyzes the hydrolysis of fatty acid from the *sn*-2 position of phosphatidylcholine and transfers the fatty acid to unesterified cholesterol to form CE and lysolecithin [24]. This transesterification reaction occurs predominantly on HDL and provides the majority of plasma CE in man. Cholesterol is removed from the surface of HDL by this transesterification reaction, and is subsequently transferred as CE to acceptor lipoproteins (e.g. LDL and VLDL). This may create a concentration gradient and promote the flux of additional unesterified cholesterol from the surface of cells to HDL and other lipoproteins [25]. Through this mechanism, LCAT is postulated to play a key role in the transport of cholesterol from peripheral tissues to the liver.

1.4.2 Lipoprotein Lipase and Hepatic Lipase

LPL is a lipolytic enzyme that is synthesized by the parenchymal cells of the liver. It is transported and bound as a homodimer to capillary endothelial cells by glycosaminoglycan polymers such as heparan sulphate, where it functions as a TG and PL

hydrolase [26]. The predominant substrate of this enzyme is the TG component of chylomicrons and VLDL. Apolipoprotein C-II significantly increases the activity of LPL on lipoproteins through interaction with an apo C-II binding site [27].

HL is another lipolytic enzyme that is synthesized by hepatocytes and present in the liver sinusoid capillaries. It exerts both glyceride lipase and phospholipase A_1 activities on circulating lipoproteins [28]. HL is involved in the catabolism of apo B-E-containing chylomicron remnants and intermediate density lipoproteins. It is also involved in the remodeling of HDL. Because the substrate specificity of HL is broad, its role in lipoprotein metabolism is unclear. Studies suggest that HL hydrolyzes TG in IDL and chylomicron remnants [29], while hydrolyzing HDL PL [30] and DG [31].

1.4.3 Cholesteryl Ester Transfer Protein

1.4.3.1 Characterization of CETP

CETP is a hydrophobic glycoprotein that is found in many vertebrate species, with varying degrees of expression and activity. Humans and rabbits, for instance, have high activity, while mouse, rat and porcine plasmas do not have effective activity levels [32,33,34]. Human normolipidemic plasma contains about 1-2 mg/l of CETP protein, with women having slightly higher levels [35].

Table I presents the current knowledge about human CETP. CETP contains a high content of hydrophobic amino acids [36] and forms a globular structure, as indicated from NGGE and electron micrographs [37]. It is an acidic glycoprotein [36] that is heat-stable [38]. The gene for CETP is present in humans in a single copy on chromosome 16 in the 16q12-21 region [39]. It is 25 kbp and encompasses 16 exons (35-250 bp) [40].

Table I. Characterization of CETP in human

Amino acids	
-preprotein	493
-mature protein	476
CETP mRNA	1.9 kilobases
Protein classification	Hydrophobic glycoprotein
Translated protein MW (kDa)	53
Apparent MW (kDa)	58-74
Potential N-linked sites	4
Isoelectric point range	4.6 - 5.4
Site of synthesis in human	Liver, spleen small intestine, adrenal glands, adipose tissue

The CETP protein coded by the gene is 476 amino acids, 53 kDa and is synthesized with a postulated hydrophobic prepeptide of 17 amino acids. In plasma, CETP is found to be about 74 kDa with a pI of 4.6-5.4, due to variable sialylation [36]. The discrepancy between the molecular weights of the translated and mature forms of CETP is due to post-translational acquisition of asparagine (N)-linked carbohydrate [41]. CETP has 4 potential sites for N-linked glycosylation [40].

1.4.3.2 Expression of CETP

The CETP gene has low homology with genes coding for enzymes and lipoproteins involved in lipoprotein metabolism. It has 20% homology with PLTP and shares some

sequence homology with the members of a superfamily of gene coding for various proteins, which are able to bind to lipopolysaccharides [42]. Interspecies homology of CETP seems to be high. Antibodies raised against human CETP were able to cross-react with CETP from cynomolgous monkey and rabbit [43, 44]. Expression of the gene in humans was considered for a long time to be exclusively in the liver, where gene expression is high [42]. It has since been revealed that CETP gene expression is ubiquitous with higher expression in spleen and adipose tissue and lower levels within the small intestine, kidneys and heart [45]. CETP biosynthesis is regulated by nutritional factors. A number of studies, in different species, have reported an increase in plasma CETP activity in animals fed high cholesterol, high fat diets or in the postprandial state [reviewed in 46]. Dietary cholesterol appears to be the major factor responsible for these increases. In humans, CETP concentrations in plasma have been shown to increase concomitantly with increased cholesterol consumption [47]. The changes in CETP activity and concentration due to diet appear to be related to alterations in CETP gene expression [48]. Regulation of the expression of the CETP gene may be by a specific transcription factor, the CCAAT/enhancer binding protein (C/EBP). Binding of a sequence contained in the human upstream CETP gene promoter to C/EBP was demonstrated by Agellon *et al.* [49]. The activity of the CETP gene was shown to closely relate to the intracellular level of C/EBP. Sperker *et al* confirmed these findings and also described a concurrent up-regulation of C/EBP and CETP expression by sodium butyrate [50]. Gaudet and Ginsberg have recently described and sequenced 5 Kb of the 5' flanking region of the CETP gene [51]. They have characterized within the proximal promoter region (105bp), three protein binding activities which function synergistically to allow full transcriptional activity and tissue specific

expression of the gene. There are three nuclear hormone receptor response elements present in the CETP promoter, suggesting that the CETP gene may be subject to hormonal regulation.

Transcriptional regulation is not the only way CETP activity is regulated. Inazu *et al* discovered an alternatively spliced variant of CETP that makes up 20% of the total CETP mRNA in the liver and 40-60% in the spleen [52]. Exon 9, which encodes 60 amino acids in the central portion of the CETP molecule, is precisely removed in the variant, leaving exon 8 derived sequences directly linked to exon 10 derived sequences without alteration of the reading frame. The translated protein is inactive and poorly secreted. In addition, the exon 9-deleted variant was shown to form intracellular complexes with full-length CETP molecules, inhibiting secretion of active wild-type CETP [53]. These observations suggest that posttranscriptional events, such as alternative splicing of mRNA could provide a mechanism by which the secretion of active CETP is regulated.

1.4.3.3 Lipid Transfer Activity

CETP facilitates the exchange of neutral lipids and PL between the plasma lipoproteins. It has been shown to be responsible for all the neutral lipid exchange activity in the plasma [54, 55]. CETP is also responsible for about one third the PL transfer occurring in plasma, with the remaining transfer mediated by another transfer protein, PLTP [56].

Virtually all plasma lipoprotein particles may act as donors or acceptors in CETP-mediated lipid transfer reactions. In plasma, CETP can mediate a hetero-exchange of HDL cholesteryl esters with VLDL triglycerides [54]. It was originally thought that transfer of

neutral lipids mediated by CETP occurred as an equimolar exchange, with one molecule of TG transferred for one CE [57]. This was thought to result in a net transfer of CE from HDL to VLDL, and a reciprocal net transfer of triglycerides from VLDL to HDL (Figure 1). A hetero-exchange between VLDL triglycerides and LDL cholesteryl esters is also stimulated by CETP, resulting in a net transfer of TG and CE between these lipoprotein particles [54]. Early studies suggested that exchange of neutral lipids between HDL and LDL occurs without significant net mass transfer, due to similar CE/TG ratios. It has since been established that an equimolar exchange of neutral lipid is not obligatory for a net mass transfer. In lipid transfers between HDL_3 and VLDL, Xiao *et al.* demonstrated that the amount of TG HDL can receive was only one third the amount of CE transferred out in the opposite direction [58]. Other studies have shown that addition of sodium oleate can dissociate the hetero-exchange of CE and TG between HDL and triglyceride-rich lipoproteins [59]. These and other studies have put many of the original hypotheses into question and have prompted a more thorough examination of the factors that regulate the interlipoprotein redistribution of apolar lipids by CETP.

1.4.3.4 Binding of CETP to Lipoprotein Substrates

CETP is known to form complexes with VLDL, LDL and HDL [60]. Although binding to lipoproteins with similar affinities, CETP binds to HDL with the highest avidity. When purifying CETP by ultracentrifugation or immunoaffinity chromatography, CETP recovery is highest in the HDL and density > 1.25 g/ml range. The importance of binding in CETP-lipoprotein interactions is well documented. It is known to be the primary event

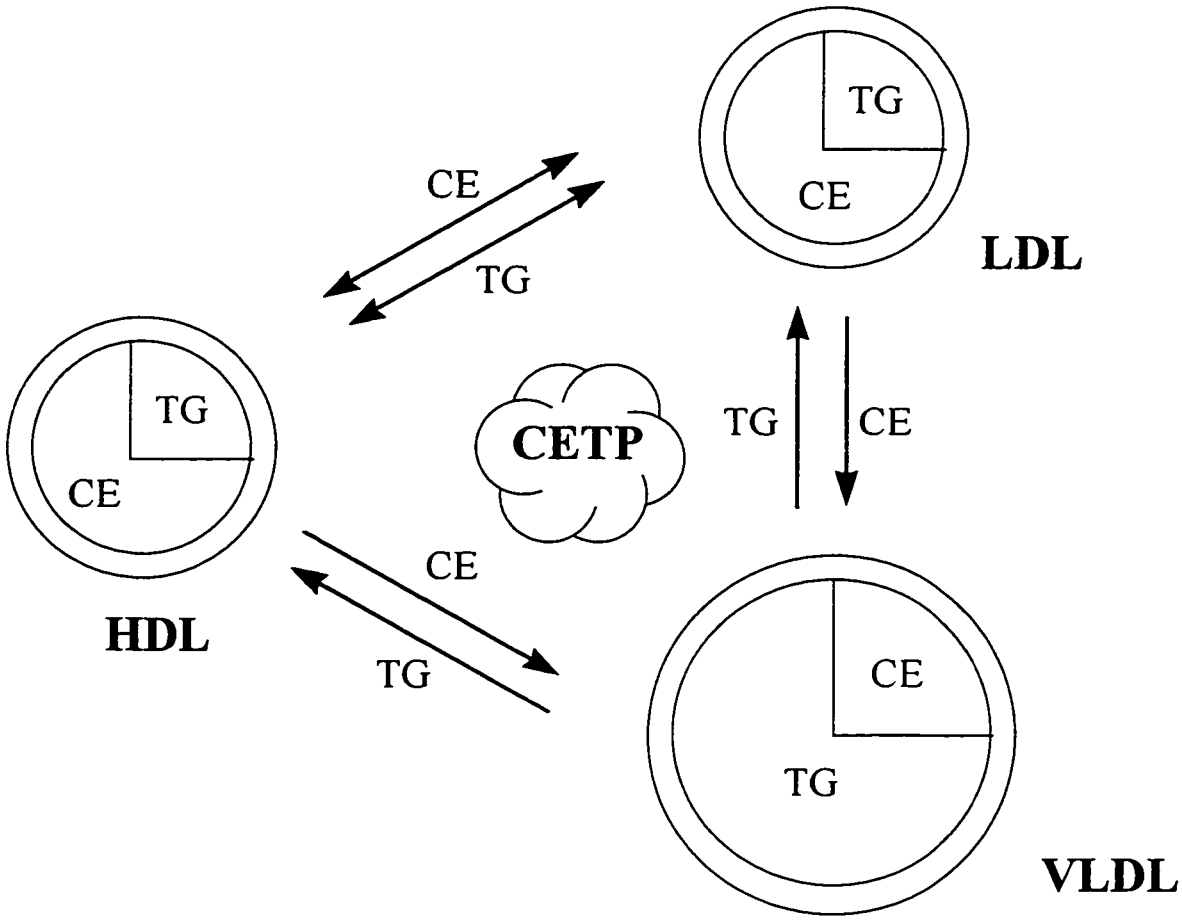


Figure 1. CETP-mediated exchange of CE and TG between lipoprotein fraction in plasma.

of neutral lipid transfer. Pattnaik and Zilversmit first described the binding of CETP to be electrostatic in nature [61]. Wang *et al.* described a putative lipoprotein binding site comprised of a triplet of lysine residues at position 376-378 [62]. This site may be responsible for mediating an electrostatic interaction with negatively charged lipoprotein surface components.

Several groups have demonstrated that the CETA and stability of CETP-lipoprotein interactions are enhanced as a result of increased density of negative charges at the lipoprotein surface. Nishida and coworkers used chemical modification of LDL to increase its net negative charge and demonstrate an increased CE transfer into LDL [63].

The presence of free fatty acids, either directly added [59] or produced by the addition of lipoprotein lipase [64] stimulated CE transfer out of HDL, and TG transfer into HDL. Lagrost demonstrated that the rate of CE transfer from HDL_3 subfractions to LDL and from LDL to HDL_3 was progressively enhanced as the negative charge density of the HDL_3 particles increased [65]. Conversely, an increase in the positive charges was shown to reduce the interaction of CETP with lipoprotein substrates and decrease CETA [63]. These studies demonstrate that subtle variations in lipoprotein charge and CETP binding may produce dramatic alterations in plasma CETP activity.

1.4.3.5 Mechanism of Transfer

Studies into the structure-function relationship of the CETP molecule have involved the production of specific monoclonal anti-bodies [66], epitope analysis by fragmentation and site-directed mutagenesis [67, 68, 69]. These experiments show that the carboxyl-terminal region of the CETP molecule forms a flexible segment [69] involved in the binding of neutral lipid. Deletion of amino acids 465-475 or binding of neutralizing antibodies to amino acids within 463-475 gives rise to a loss of CE and TG transfer activities [67]. Binding of deletional mutants to lipoproteins gives results similar for wild type CETP. By contrast, the binding of both CE and TG to the mutant CETP was found to be markedly impaired. Phospholipid transfer activity remained well preserved, indicating that the C-terminal region of CETP is involved in neutral lipid, but not PL, transfer activities. Both point-mutagenesis of single amino acids between amino acids 468 and 475, as well as circular dichroism (CD) spectroscopy, suggest that the C-terminus of CETP is predominantly α–helix. The amino acids responsible for neutral lipid binding are

clustered on the non-polar face of the helix, while those responsible for binding of the neutralizing antibody TP2 are on the polar face [69]. The TP2 antibody probably binds the hydrophilic face of the helical C-terminal region of CETP and has its neutralizing effect through local steric hinderance. Recent experiments sustain that uptake of neutral lipids occurs by direct binding of CE and TG located closer to the lipoprotein surface, rather then by penetration of CETP into the lipoprotein core [70].

The actual mechanism of CETP-mediated neutral lipid transfer between lipoproteins is still under controversy. Two different mechanisms have been proposed to describe the lipid transfer process (Figure 2). One model describes CETP as a shuttle carrying CE and TG between donor and acceptor lipoprotein fractions [71, 72]. In the other model, CETP acts to mediate the formation of a ternary collision complex of CETP, donor and acceptor lipoproteins [73]. Kinetic analyses of experiments performed with partially purified preparations of CETP have been interpreted to support both carrier-mediated and ternary mechanisms of neutral lipid transfer. Recent studies indicate that both shuttle and ternary complex mechanisms may coexist in vivo [74, 75].

1.5 Reverse Cholesterol Transport

Cholesterol is normally thought to be transported from extra-hepatic tissue to the liver through the plasma and lymph [12]. Cholesterol is then recycled to tissue in the body, or excreted through the intestine in bile. Since non-hepatic tissues are not capable of degrading their own cholesterol, the flux of cholesterol from non-hepatic tissue to the liver is physiologically important to the removal of cholesterol. Susceptibility in individuals to atherosclerosis may be directly affected by events that perturb normal lipid

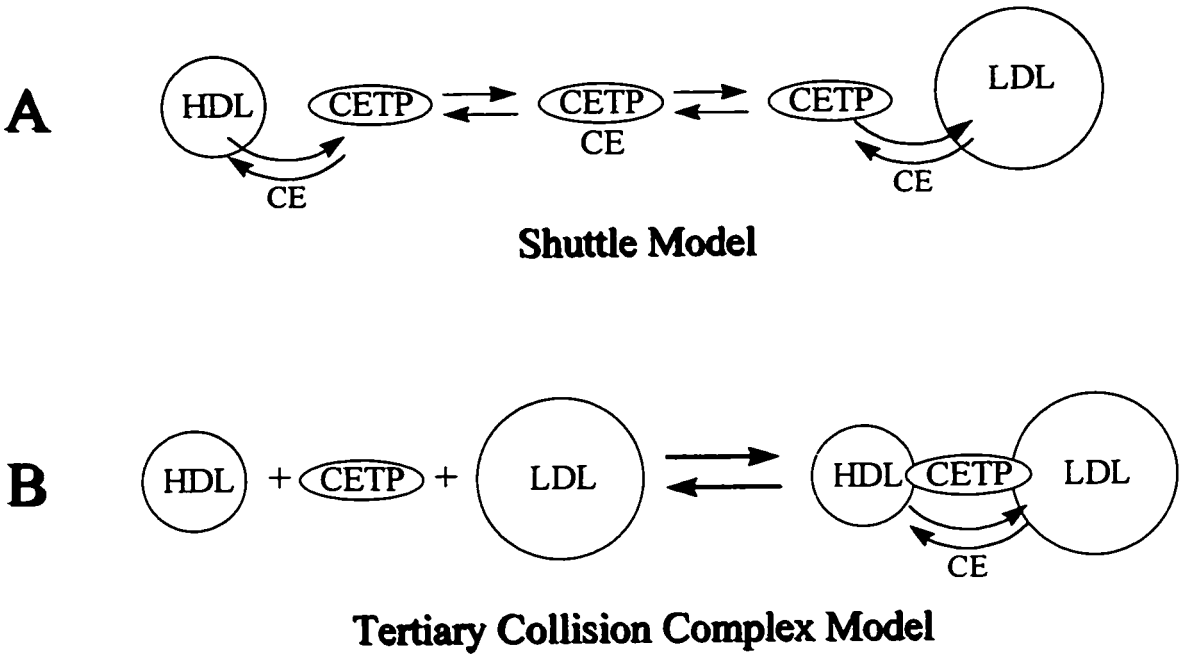


Figure 2. Postulated models of CETP-mediated CE transfer between HDL and LDL
A. CETP would pick up one CE from a donor, dissociate and deliver the CE to the acceptor.
B. CETP would promote the formation of a ternary complex, producing a simultaneous, bi-directional transfer of CE.

transport. There are a number of identifiable steps in reverse cholesterol transport (RCT) (Figure 3); the efflux of FC from cell membranes to HDL [12], the esterification of FC to CE within HDL by LCAT [3], the remodeling of lipoproteins by LPL and HL [26,28], the redistribution of CE and TG among the plasma lipoproteins by CETP [76] and the delivery of cholesteryl esters to the liver [77].

Unesterified cholesterol from peripheral tissue is thought to transfer to HDL through a number of possible avenues, including diffusion [12] and binding of HDL to cellular surfaces [78]. Direct transport of the esterified cholesterol to the liver may occur by direct uptake of the whole HDL particle by receptor-mediated endocytosis [79]. It has been shown recently that some CE can be selectively taken up by the liver without taking up the apolipoproteins of the HDL particle [80]. Alternatively, CETP can transfer

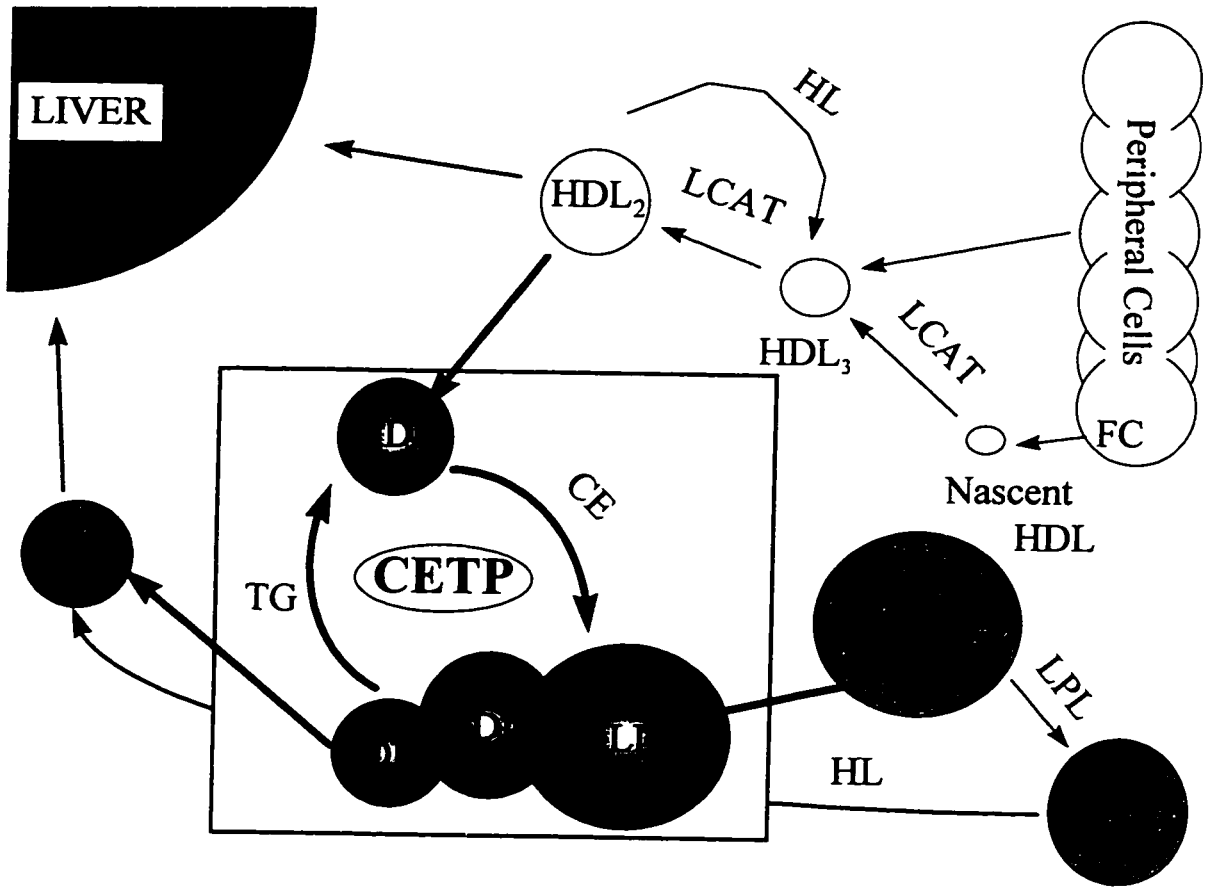


Figure 3. Normal lipid transport.

cholesteryl esters to apoB-containing lipoproteins and CE can be cleared by the liver through hepatic clearance of IDL and LDL involving the LDL receptor [77]. Investigations suggest that during a normolipidemic fasting state, LDL transfers CE to HDL. Studies by Fielding showed that *in vivo* net CE transfer in fasting, normolipidemic baboons was from LDL to HDL [81]. In hyperlipidemic baboons and normolipidemic baboons in a postprandial state, a net transfer of CE occurred from HDL to LDL. During a normolipidemic fasting state, HDL may play more than an intermediary role in transporting cholesterol to the liver, and may be the major source of cholesterol clearance from the plasma. Recent studies have backed up the notion of a reverse cholesterol

transport system and the importance of HDL in that system, showing HDL levels correlating with cholesterol efflux [82]. In contrast, other studies have not found any correlation between HDL or apolipoprotein concentration and the magnitude of cholesterol flux from extra-hepatic organs to the liver [83]. From these studies, it appears the role of HDL in cholesterol flux may not be significant.

1.6 Abnormal Lipid Transport in Hyperlipidemia

Elevated plasma cholesterol can result from a number of different factors, much of which is still poorly understood. Forms of hypercholesterolemia in man and animals have been identified which are separate from other well-defined genetic disorders, such as familial hypercholesterolemia (FH) [84]. FH results from genetic defects in the LDL receptor, inhibiting hepatic LDL uptake. It is characterized by marked elevations in plasma LDL and by abnormalities in the LDL particles. Non-genetic causes include being in a chronic postprandial state and factors which may promote the net transfer of CE from HDL to LDL. Lipemic states such as abnormal TG lipolysis may increase TG concentrations and stimulate CE transfer into the LDL pool. In non-FH hypercholesterolemia, abnormalities in apoB-containing lipoprotein composition may also cause significant changes in the flux of CE from extra-hepatic tissue to the liver for clearance. In these subjects, increased rate of CE mass transfer from HDL to VLDL and LDL appears to be correlated with elevated concentrations of VLDL and LDL [85]. Both CETP concentration and activity are elevated in the plasma of these patients [86]. CETP may therefore contribute to the formation of the CE-enriched apoB-containing subspecies

observed in these subjects and enhance the atherogenicity of LDL and VLDL particles [87].

1.7 Atherosclerosis

Over the years, a lot of attention has been paid to CETP's role in the flux of lipids, to elucidate its role in atherogenesis. CETP transfers newly esterified CE from HDL to other plasma lipoproteins, and may therefore be involved in the accumulation / elevation of CE in the plasma. The major pathway for removal of CE from the plasma is transfer to apolipoprotein B (apoB)-containing lipoproteins, and by hepatic clearance of IDL and LDL [77]. In this sense CETP seems to play an anti-atherogenic role, facilitating the removal of cholesterol from the plasma. Other investigations with human CETP deficients [88] and hypertriglyceridemic mice expressing a CETP transgene [89] have linked the activity of CETP with protection against atherosclerosis.

HDL cholesterol has long been identified as a protective factor against CAD, independent of the level of LDL [90]. Although generally recognized, it is not fully explained. Investigations into Japanese families deficient in CETP due to a splicing defect, suggest that CETP may be an atherogenic element [91]. In its absence, patients exhibited no increase in CAD and the individuals' lipoprotein profiles fit that of a reduced risk for CAD. The importance of these studies is questionable as Japanese diets are known to be anti-atherogenic and may be the reason why these individuals are less susceptible to CAD. Japanese-Americans deficient in CETP living off of a western diet were found to have a higher incidence of CAD over Japanese-Americans without CETP gene mutations [88]. In this study, genetic CETP deficiency was found to be an independent risk factor for CAD.

On the other hand, the action of CETP may produce CE enriched apoB-containing lipoproteins, a lipoprotein profile that is correlated with higher incidence of CAD [92]. Studies with mice expressing a simian CETP transgene [93] and with Japanese families deficient in CETP [91] determined CETP as an independent risk factor for atherosclerosis. The studies of Zhong *et al.* [88] and Hirano *et al.* [94] both illustrate the relationship between lipoproteins (particularly HDL) and CETP. They show that lowering CETP levels and increasing HDL cholesterol concentration does not necessarily provide protection against atherosclerosis. This is an important observation as some researchers see the lowering of CETP and increasing of HDL levels in the plasma as a key strategy against CAD [83]. It appears that the determination of the factors that regulate CETP and inhibit transfer of CE into LDL will be important if we are to effectively design a therapy to control the accumulation of CE in the plasma.

1.8 Rationale for this Study

During the past few years, a number of studies have suggested that CETP concentration [95] and lipoprotein composition [96,97] may affect the cholesteryl ester transfer reaction. Although there is no general agreement concerning the kinetic model of the CETP-mediated lipid transfer reaction, it is clear that one of the key steps of the transfer process is the interaction of CETP with the lipoprotein surface [98]. Although we know electrostatic interactions may be important, there is no clear understanding of how the cholesteryl ester transfer mechanism is controlled. Although there have been many hypotheses, all have been proven not to be valid. The mechanism and regulation of CETP activity needs to be further characterized as a whole picture. Many of the studies have

only looked at one direction of the cholesteryl ester transfer reaction. CETP mediates a bi-directional transfer of neutral lipid, and to fully understand the mechanisms involved, one must look at the transfer from donor lipoprotein to acceptor lipoprotein, from acceptor to donor and the resultant net mass transfer. Well defined, reconstituted lipoprotein particles need to be utilized to resolve specific structural questions and map changes in lipoprotein composition to changes in function.

The primary aim of this study is to elucidate the effect of lipoprotein structure and charge on CETP mediated CE and TG transfer. An ultracentrifugal method was developed to separate donor and acceptor particles after incubation with CETP. Using a radiolabeled tracer, both directions of neutral lipid transfer can be directly studied. Reconstituted Lp2A-I particle series were developed to directly study the effect of variations in lipoprotein composition on the ability of these particles to act as a substrate for purified CETP.

1.9 Specific Aims

1. Characterize the factors that regulate efflux / influx of neutral lipids and give rise to mass transfer of CE.
2. Characterize the effects of variations in surface lipids of reconstituted Lp2A-I on its ability to act as a substrate for purified CETP.

2 Experimental Procedures

2.1 Materials

Cholesteryl oleate and [^{3}H]cholesteryl oleate (specific activity 48.0 Ci/mmol) were obtained from Amersham (Oakville). Bovine serum albumin, cholesteryl linoleate, triolein and cholesterol were purchased from Sigma (St. Louis, MO). 1-palmitoyl 2-oleoyl phosphatidylcholine and phosphatidyl inositol were obtained from Avanti Polar Lipids (Birmingham, AL.). Triglyceride and total and free cholesterol determination reagent kits were purchased from Boehringer Mannheim (Indianapolis, IN). CNBr-activated Sepharose and Protein G Sepharose were obtained from Pharmacia Biotech (Uppsala, Sweden). All other reagents were analytical grade.

2.2 Lipoproteins

2.2.1 Lipoprotein Isolation

VLDL, LDL and HDL were isolated from plasma by sequential ultracentrifugation at densities 1.006, 1.006-1.063 and 1.063-1.210 g/ml, respectively [99]. Density was adjusted by adding 1mM EDTA, 0.02% NaN_3 and dry KBr. Ultracentrifugation at 114,000 x g was performed at 8°C for 20h (VLDL and LDL) and 48h (HDL). The lipoproteins were dialyzed two to three times for 4-12 h against 100-fold greater volumes of NaCl/Tris buffer (150 mM NaCl, 10 mM Tris/HCL, 0.3 mM EDTA, 4.6 mM NaN_3, pH 7.4) before use.

2.2.2 Incorporation of Radiolabeled Lipids into Lipoproteins

[^{3}H]cholesterol oleate (270 μCi) and POPC (15 mg) were dried under N_2 and suspended in 3 ml Tris/saline buffer (pH 8.0), and sonicated for 8 x 30s using a Branson 450 sonicator with one-eighth inch tapered microtip probe at an output control setting of 3 (manufacturer rated output of 40 watts), with 30s rests in between. The lipid vesicles were added to 120 ml VLDL-depleted plasma, incubated overnight at 37°C and then [^{3}H]cholesteryl ester labeled LDL and HDL were obtained by sequential ultracentrifugation as previously described.

2.2.3 Incorporation of PI into Lipoproteins and Plasma

Various amounts of PI were dried under N_2 into 12 x 75 mm test-tubes and 1ml of Tris/saline, pH 8.0, was added. This solution was vortexed for two minutes, and an aliquot of LDL, HDL or plasma was added. This solution was gently swirled by hand and incubated at 37°C for 1 to 16h. Controls containing only plasma and Tris/saline were incubated similarly. Lipoproteins were isolated and characterized.

2.2.4 Isolation of HDL and Purification of Apoprotein A-I

HDL was isolated from fresh plasma of normolipidemic donors by sequential ultracentrifugation [99]. HDL was delipidated [100], and purified apoA-I was isolated by gel filtration [101] on Sephacryl S-200. Prior to use, purified apoA-I was resolubilized in 6 M guanidine HCL and dialyzed extensively against Tris/saline buffer, pH 8.0.

2.2.5 Preparation of Recombinant HDL Complexes

Spherical recombinant LpA-I were prepared by co-sonication of POPC, cholesteryl linoleate, cholesteryl oleate, PI and apoA-I using a technique similar to that originally described by Hirz and Scanu [102]. POPC and other purified lipids in chloroform (see Table 2 for starting concentrations) were dried under N_2 in a 12 x 75 mm test-tube, and 800 µl of Tris/saline, pH 8.0, was added. All sonications were performed in the 12 x 75 mm test-tube suspended in a 15°C water bath and under nitrogen. The lipids-buffer solution was initially sonicated for 1 min using a Branson 450 sonicator with one-eighth inch tapered microtip probe at an output control setting of 3 (manufacturer rated output of 40 watts). This suspension was then incubated for 30 min at 37°C and sonicated again for 5 min using a 95% duty cycle. ApoA-I (2 mg of a 1.4 mg protein/ml Tris/saline solution) was added to the lipid suspension and the protein-lipid mixture was sonicated for 4 x 1 min (with maximal output of 40 watts and 90% duty cycle) punctuated by 1 min cooling periods. LpA-I complexes were then filtered through a 0.22 µm syringe tip filter and re-isolated by size exclusion chromatography on a Superose 6 column [103].

2.2.6 Preparation of Reconstituted LDL

2.2.6.1 Delipidation of LDL

The apoB from LDL was isolated using a delipidation procedure essentially by the method developed by Lundberg and Suominen [104]. LDL was dialyzed against 50 mM NaCl-50 mM Na_2CO_3 (pH 10) overnight. This native LDL was incubated with sodium deoxycholate (NaDOC) at a weight ratio of 1:12 (apoB:cholate) for 30 min. in the dark.

After 30 min. incubation at room temperature, the solubilized protein was separated from lipid and detergent by gel filtration on a sepharose CL-4B column (1.6cm×60cm). The column was equilibrated and eluted with 50 mM sodium chloride-50 mM sodium carbonate-10 mM NaDOC (pH=10) at 4°C. Three milliliter fractions were collected and analyzed for protein, cholesterol and phospholipid. The protein peaks were concentrated using centriprep concentrators. To remove detergent, the concentrated sample was passed through a Sephadex G-75 column (1.6×30 cm) and eluted with 0.01 M Tris-HCL buffer, pH=9. Three milliliter fractions were collected, the protein eluted in the void volume and the bile salt close to the total volume of the column. The protein peak was further concentrated using a centriprep concentrator. To determine the amount of detergent associated with apoB after gel filtration various amounts of sodium deoxycholate were incubated with apoB and the mixtures were electrophoresed on 0.5% agarose gels. A relationship was observed between apoB surface charge and concentration of sodium deoxycholate, from which we estimated the isolated apoB to have less than 0.04 molecules of sodium deoxycholate per molecule of protein.

2.2.6.2 ApoB Aggregation

Immulon II Removawells (Dynatech Laboratories, Chantilly, VA) were coated by an overnight incubation with 100 µl of anti-human apoB monoclonal antibody 1D1 (5 µg/ml, 5 mM glycine, pH 9.2) and subsequently saturated by incubations for 1 h with 100 µl of 1% bovine serum albumin-PBS, pH 7.4. Serial dilution of test and control apoB containing

samples (4 μg/ml) were prepared in microtiter plates and added to the plates and incubated for 3 h. The plates were washed four times with wash buffer a solution of 0.15 M NaCl containing 0.025% Tween 20. Iodine-labeled anti-human apoB monoclonal antibodies (100 μl) 1D1, 2D8 and 3F5 (1/100 dilution) were added to the 1D1-apoB captured mixtures and incubated for 4 h and washed with Tween-saline solution as above and counted for bound radioactivity [105].

2.2.6.3 Production of Homogeneous LpB

The isolated apoB was incubated with microemulsions of defined lipid composition. The microemulsions were prepared by co-sonication of various lipids in chloroform (4 mg POPC : 2 mg cholesteryl linoleate: 1 mg triolein). All sonications were performed in flat bottom 25 X 55 mm glass vials in the presence of tris/saline pH 8.0 under nitrogen, suspended in a 15°C water bath. The lipid-buffer solution was sonicated for 30-45 min using a Branson 450 sonicator and with 3/8" microtip probe at a output control setting of 2. After sonication, the micoremulsion were complexed to apoB (2.4 mg protein) by a further 20 min. sonication of the mixture under nitrogen and in a 40°C bath sonifer. The reconstituted particles were then isolated by size exclusion chromatography on a Superose 6 column.

2.2.7 Characterization of Lipoproteins

2.2.7.1 Composition

Total cholesterol, free cholesterol and triglycerides were determined enzymatically using Boehringer Mannheim kits and manufacturers suggested procedures. Cholesterol assays were based upon the oxidation of cholesterol, in the presence of cholesterol oxidase, to produce hydrogen peroxide. A further reaction of hydrogen peroxide, 4-aminophenazone and phenol, in the presence of peroxidase, produces a coloured compound 4-(p-benzoquinone-monoimino) phenazone [106]. Total cholesterol was measured in the same manner by first cleaving fatty acids from cholesterol esters with cholesterol esterase [107]. Esterified cholesterol was then determined by subtracting free from total cholesterol. Triglyceride assays were based upon the lipase hydrolysis of triglycerides to glycerol, subsequent action by glycerol kinase and glycerol phosphate oxidase to produce hydrogen peroxide, and a final colourimetric reaction with peroxidase 4-aminophenazone and 4-chlorophenol to produce a pink coloured compound, 4-(p-benzoquinone-monoimino) phenazone. Phospholipids were determined by phospholipase D hydrolysis to produce choline and production of hydrogen peroxide by the action of choline oxidase [108, 109]. The further colourimetric reaction of hydrogen peroxide is as previously described. Proteins were determined by the Lowry method as modified by Markwell *et al.* [110].

2.2.7.2 Determination of LpA-I Physical and Structural Characteristics

The size and homogeneity of apoA-I complexes were estimated by non-denaturing gradient gel electrophoresis [111] on precast 8-25% acrylamide gels (Pharmacia Phastgel)

after protein staining and densitometric scanning. Lipoprotein particle electrophoretic mobility, valence and surface charge characteristics were determined by electrophoresis on precast 0.5% agarose gels (Beckman, Paragon Lipo kit) as previously described [112].

2.2.8 Circular Dichroism

The average secondary structures of LpA-I apoA-I were monitored by CD spectroscopy on a Jasco J41A spectropolarimeter calibrated with a 0.1% (w/v) D-10-Camphorsulfonic acid solution [113]. CD spectra were measured at 24^{0}C in a 0.1 cm path length quartz cell with a sample protein concentration of 67 mg/ml buffer. The percent α-helix in apoA-I was calculated from the molar ellipticity at 222 nm. Isothermal denaturations were performed to determine the thermodynamic stability of α-helices in apoA-I. Aliquots of each complex (33mg of protein/ml of buffer) were incubated with from 0-6 M GdnHCL in 0.05 M phosphate buffer, pH 7.2, for 72 h at 4 ^{0}C. The free energy of unfolding of apoA-I on the surface of LpA-I complexes was calculated as described previously [113].

2.3 Cholesteryl Ester Transfer Protein Purification

2.3.1 Purification of CETP by Immunoaffinity Chromatography

Purified TP2 [115] anti-CETP monoclonal antibody (mAb) was purified from mouse ascites fluid using protein G sepharose and coupled to CNBr-activated sepharose according to the manufacturers instructions.

Human plasma was apoB precipitated by adding 1% w(v) dextran sulphate and 10 mM $CaCl_2$ in 500 mmol $MgCl_2$ to a 10 times volume of plasma, incubated at room temperature for 20 minutes. The supernatant was loaded on to a phenyl-sepharose CL-4B column and eluted with distilled water. Fractions containing CETP activity were pooled and loaded onto the TP2 immunoaffinity column using a flow rate of 0.2 ml/min. The column was then washed with PBS (pH 8.0) until the OD_{280} reached less than 0.1. The column was eluted with either acetic acid (0.1 M, pH 2.5) or 3 M thiocyanate until the OD_{280} reached less than 0.1. The eluted fractions were pooled and dialyzed against Tris/saline (pH 7.4), and the column was washed and stored at 4^0C with PBS/NaN_3.

2.3.2 Purification of CETP by Affinity Chromatography

CETP was purified using a modification of the method described by Kato *et al* [114]. Human plasma was acquired from the Canadian Red Cross and CETP was purified through several steps. Apo B was precipitated by adding 1% w(v) dextran sulphate in 500 mmol $MgCl_2$ to a 10 times volume of plasma, incubated at room temperature for 20 minutes and centrifuged at 3500 rpm in a Sorvall RC-5B Superspeed Centrifuge. The supernatant was then loaded on to a phenyl-sepharose CL-4B column and eluted with distilled water. Fractions containing activity were dialyzed against 1 mM Tris/saline buffer containing 5 mM EDTA and 25 mM NaCl, pH 7.4, pooled, and loaded onto a DEAE cellulose column. The CETP was eluted with a linear NaCl-Tris gradient containing 5 mM EDTA (25mM NaCl and 1mM Tris to 200 mM NaCl and 10 mM Tris). The CETP fraction was then dialyzed in 39 mM phosphate buffer containing 0.025% EDTA and applied to a succinylated LDL-Sepharose column. The column was washed with 39 mM

sodium phosphate buffer containing 0.025% EDTA, and eluted with 4mM sodium phosphate buffer containing 0.025% EDTA. Fractions containing CETP activity were collected and concentrated. Activity was characterized and the enzyme was stored at 4°C until use. CETP activity was characterized using a stock substrate (POPC:CE:apoA-I ratio of 100:20:2, mol/mol/mol) and quantitated into units of enzyme activity, where 100 unit = 1 nmol cholesteryl ester transferred/h.

2.4 Transfer Assays

CETP activity was determined using two different methods. One assay was used during CETP purification to determine the cholesteryl ester transfer activity (CETA) in affinity and immunoaffinity column eluted fractions, using HDL as the acceptor and [^{3}H]cholesteryl ester-labeled LDL as the donor particles. Determinations by this assay reflected the activity of CETP and used a shorter incubation period to minimize back transfer. The other assay was used to monitor interlipoprotein lipid transfers by utilizing CETP purified from plasma, native LDL and Lp2A-I complexes as donor and acceptor particles, and ultracentrifugal separation of the particles. This allowed for the determination of cholesteryl ester, cholesterol and triglyceride mass transfer, as well as the rates of cholesteryl ester transfer between acceptor and donor particles.

2.4.1 CETA Determination Through Precipitation of Apolipoprotein B-Containing Lipoproteins

CETA was determined using a modification of the method originally described by Kato and Nishida [114]. The assay mixture consisted of [^{3}H]cholesteryl ester labeled LDL (150 μg as protein, p=1.006-1.063), HDL (100 μg as protein, p=1.21), 100 μl column

purified fraction, and made up to 300 µl with PO_4 buffer (39 mM phosphate buffer, 60 mM NaCl, 0.025% EDTA, pH 7.4). Following 1h incubation at 37°C, 50 µl was removed and counted. The remainder in the tubes was immediately placed in an ice bath and LDL present in the assay mixture was then precipitated by adding 30 µl of 60 mM $MgCl_2$ and 30 µl of 0.1% dextran sulfate in phosphate buffer containing 60 mM NaCl and EDTA. After standing for 20 min, the assay mixtures were centrifuged at 15,890 x g for 15 min, and 100 µl of the supernatant was removed for counting, leaving the precipitate undisturbed. The CETA was computed by subtracting blank values, which contained PO_4 buffer, in place of column purified fraction.

2.4.2 Ultracentrifugal Assay of CETP Activity

Spherical Lp2A-I complexes (25 µg apoA-I) containing [^{3}H]cholesteryl oleate, plasma purified LDL (d=1.019-1.063 g/ml, 10-50 µg apoB) and purified CETP (10 µL in standardized activity) were incubated for 2-6 hours at 37°C with PO_4 buffer at this volume. Incubation mixtures were made up to 600 µl (d=1.05 g/ml) and centrifuged at 100k rpm for 2-8 hours in a TLA 100.1 rotor, using a Beckman tabletop ultracentrifuge. 300 µL from the top and bottom of the polycarbonate tubes were removed to eppendorfs and 100 µl counted for ^{3}H. The rate of cholesteryl ester transfer by CETP was determined by the amount of ^{3}H counted in the upper fraction. Cholesterol and triglyceride mass transfer was determined enzymatically using commercially available enzyme kits. All values were computed against zero time samples, which took into account transfer that occurred during ultracentrifugation.

2.5 Assay of LCAT Activity

Reconstituted Lp2A-I were characterized as substrates for purified LCAT using a standard assay system, similar to that described in [116]. Each enzyme assay contained the lipoprotein substrate, 0.3 units of purified LCAT, bovine serum albumin (2.5 mM), and Tris/NaCl buffer (final volume, 500 μL). Incubations were carried out for 10 min at 37°C and were terminated with the addition of 2 ml of ethanol. Reaction products were extracted in hexane, and then the amount of ^{3}H associated with cholesteryl ester was determined by thin layer chromatography using a solvent system composed of hexane/diethyl ether/acetic acid, 90:10:1 (v/v/v).

3 RESULTS

3.1 Characterization of Lipid Transfer Assay

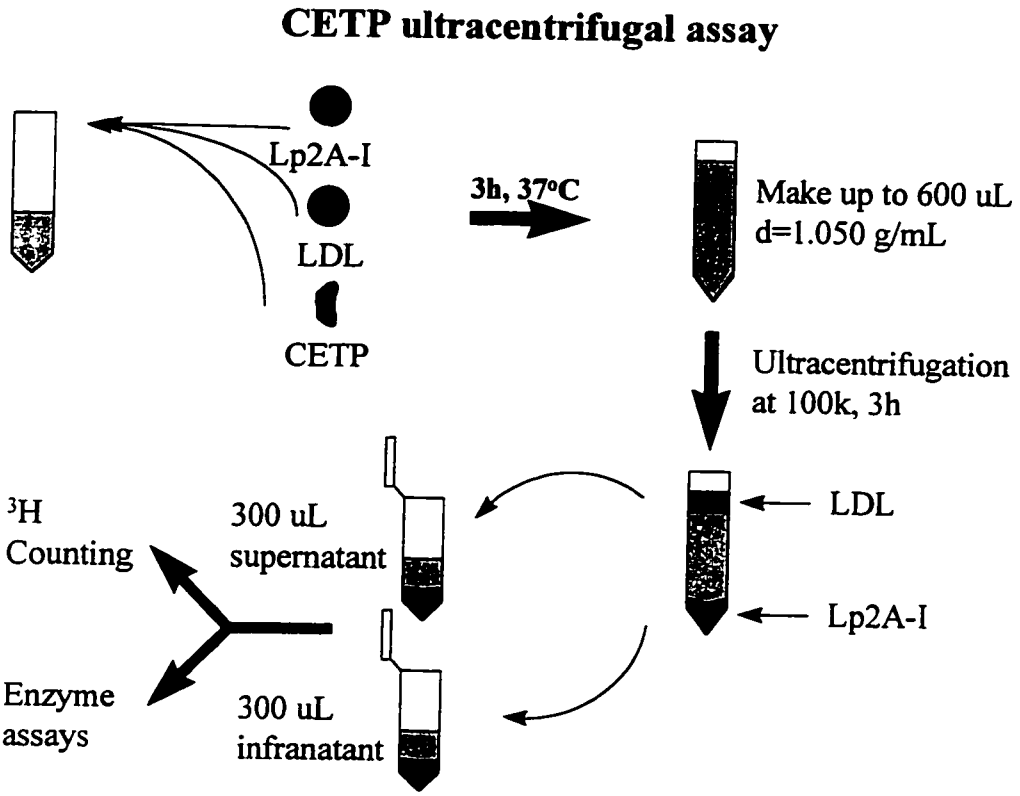


Figure 4. Ultracentrifugal assay of cholesteryl ester transfer activity.

An ultracentrifugal assay was developed to measure the rates of lipid exchange between LDL and HDL and the resultant mass transfer catalyzed by CETP (Figure 4). HDL and LDL were isolated by sequential ultracentrifugation and dialyzed against Tris buffer (pH 7.4, d=1.006 g/ml). To characterize the effects of solution density on the ultracentrifugal separation of HDL and LDL, 100 μg of protein of each was added to ultracentrifugal tubes and made up to 600 μl with KBr buffers of differing densities. These preparations were ultracentrifuged for 6 hours and the separation profiles of HDL and LDL measured (Figure 5). A density of d=1.05 g/ml was found to separate the HDL and LDL satisfactorily. Over 95% of the LDL located at the top 300 μl the preparation

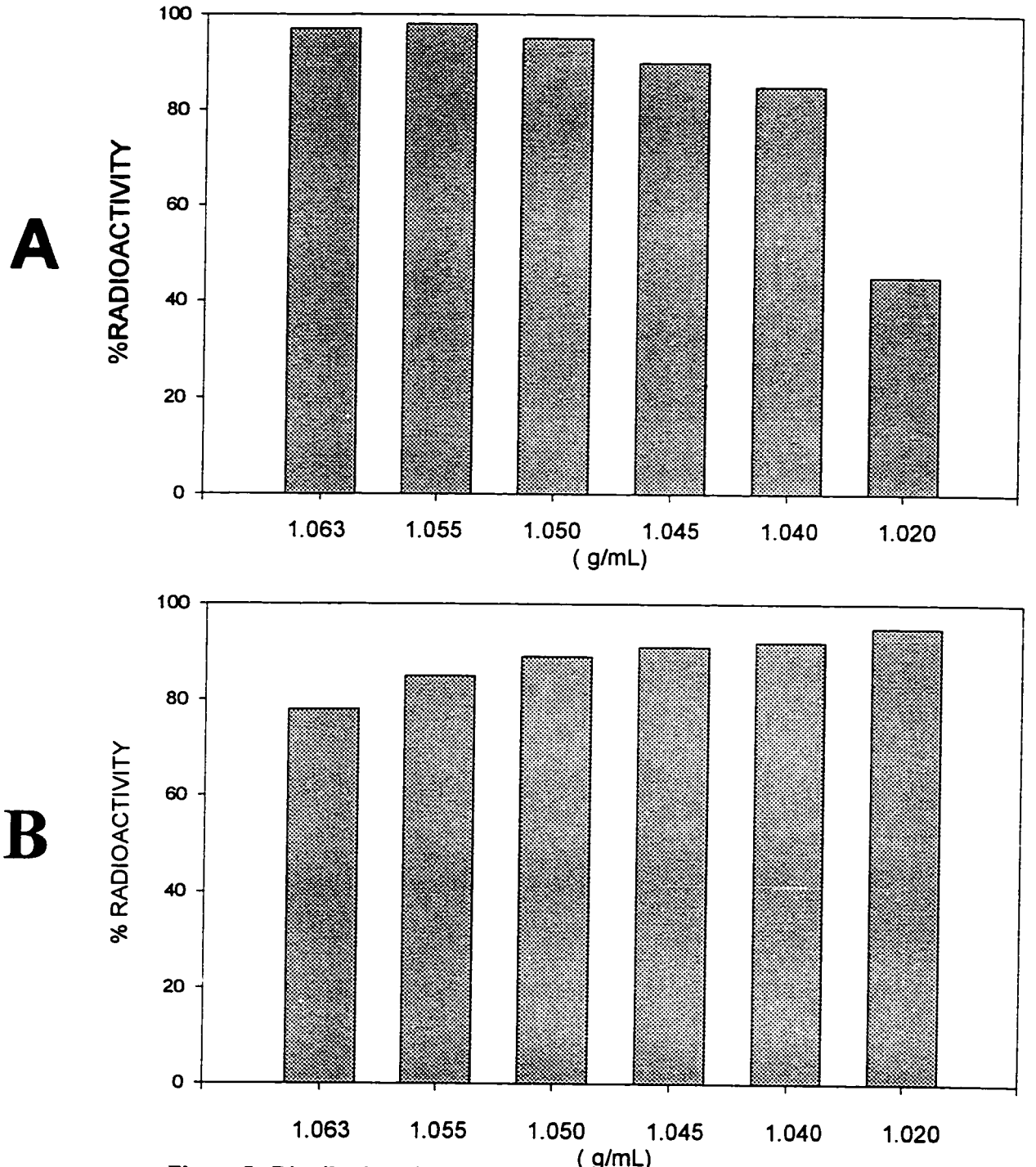


Figure 5. Distribution of LDLand HDL during ultracentrifugation at different densities.

(H^3) CE-labeled LDL (A) and HDL (B) were ultracentrifuged for 3h in a Beckman Tabletop Ultracentrifuge. Spins were done with solution densities set from 1.063 to 1.020 g/mL using various amounts of KBr. The top 300 µl (for LDL; A) and bottom 300 µl (for HDL; B) fractions were collected and counted.

and almost 90% of the HDL located at the bottom 300 µl. Radioactivity measurement analyses of radiolabeled lipoproteins were corroborated by non-denaturing gradient gel electrophoresis (NGGE) and scanning densitometry (data not shown).

The amount of time necessary to achieve optimal separation was also examined (Figure 6). At 3 hours, the separation of lipoproteins was almost identical to 6 hours, with a separation difference of only 1.5% for each lipoprotein. The 3h ultracentrifuge time was therefore used for all subsequent experiments, as the small difference in separation was justified by the amount of time saved.

3.2 Purification of CETP

3.2.1 Purification of CETP by Immunoaffinity Chromatography

An immunoaffinity column was constructed by coupling the anti-CETP mAb TP2 to CNBr-activated sepharose. Dextran sulphate precipitated plasma was loaded onto the column and eluted with 0.1 M acetic acid. CETA was not detectable in the eluent. A western blot of the acetic acid elution showed no evidence of CETP protein being eluted. In an attempt to more efficiently release CETP from the TP2 immunoaffinity column, thiocyanate was used for elution. Western blot analysis of the thiocyanate eluted fraction showed significant amounts of CETP (Figure 7). However, no CETA was detectable. Although the thiocyanate elution yielded CETP, modification of the protein may have resulted in a loss of activity.

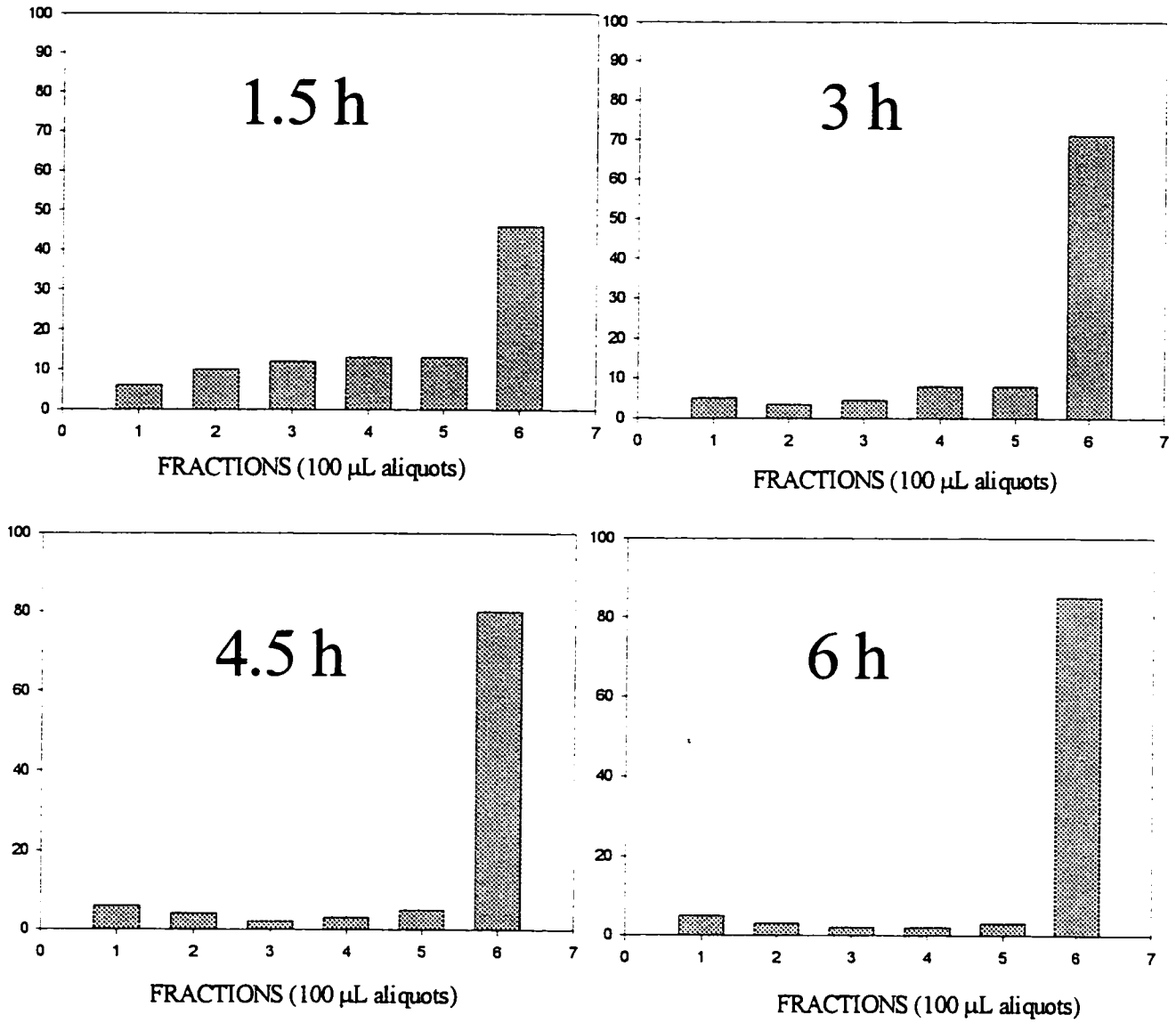


Figure 6. HDL distribution during ultracentrifugation at different times

[^{3}H] labeled HDL was adjusted to a volume of 600 µL and a density of d=1.050 g/mL using the KBr density buffer. The solution was then ultracentrifuged in a Beckman Tabletop ultracentrifuge for 1.5, 3, 3.5 and 6 hours. Sequential 100 µL aliquots were taken and counted for radioactivity.

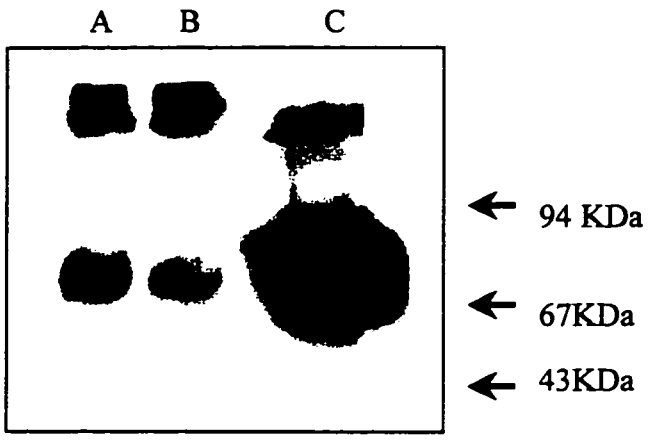


Figure 7. Purification of CETP by immunoaffinity chromatography. Dextran sulphate precipitated plasma was loaded onto a TP2 immunoaffinity column and eluted with 3M thiocyanate. The eluent was analyzed by 8% SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-CETP TP2 antibody. Autoradiography was carried out after incubation with I^{125}-labeled goat anti-mouse IgG. Lanes A and B contain the 1st and 2nd eluted protein-containing fractions, respectively. Lane C contains control recombinant CETP.

3.2.2 Purification of CETP by Succinylated LDL Chromatography

Purification of CETP followed the method of Kato *et al* [114] with only slight modification. Plasma was incubated for 20 minutes with dextran sulphate and ultracentrifuged. The precipitate was loaded onto a phenyl-sepharose column and eluted with dH_2O. Phenyl-sepharose chromatography elution profiles (Figure 8) showed that CETP activities eluted in fractions 15 to 48. Subsequently, DE-52 chromatography (Figure 9) of these fractions separated CETP activity from LCAT activity. The CETP activity was eluted with a salt gradient from 5mM to 200 mM NaCl. CETA eluted at approximately 30mM NaCl and continued until 100mM. This elution profile was partially overlapped with the elution of LCAT activity, which began eluting at 70mM NaCl. CETP

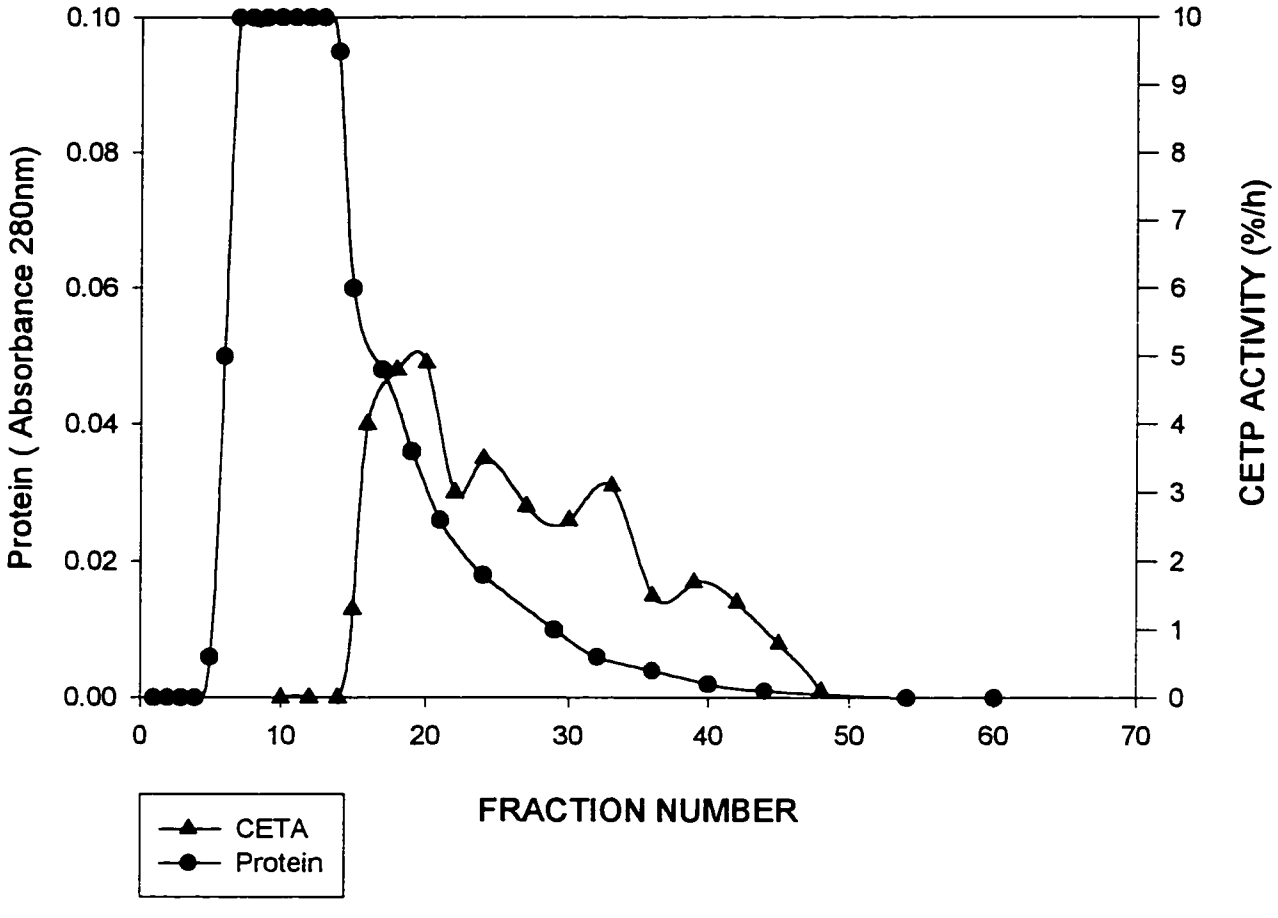


Figure 8. Phenyl-sepharose column chromatography of CETP activities.

Apo-B precipitated plasma was loaded onto a phenyl-sepharose column and washed extensively with phenyl-sepharose buffer. Bound proteins were subsequently eluted with dH_20. 90 x 10 mL fractions were collected at a flow rate of 0.9 mL/min. CETP activities were determined as described in section 2.4.3.

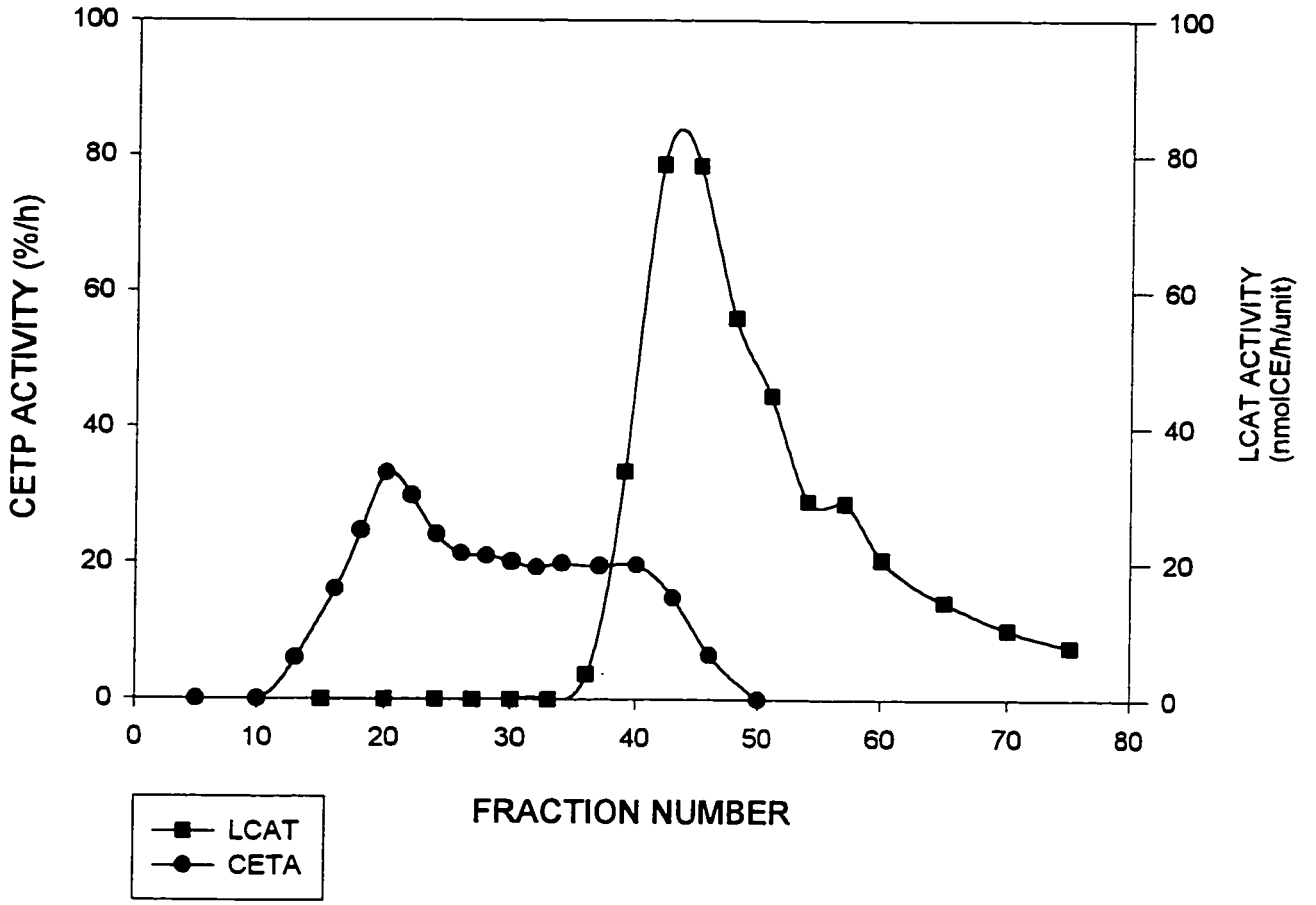


Figure 9. DE-52 column chromatography of CETP and LCAT activities.

The Phenyl-sepharose eluted fractions containing CETP activity were loaded onto a DE-52 and washed extensively with 1mM Tris/saline buffer (25mM NaCl, pH 7.4). Bound proteins were subsequently eluted with a gradient from 1 mM Tris/saline to 10mM Tris/saline (200mM NaCl, pH 7.4). 90 x 10 mL fractions were collected at a flow rate of 0.9 mL/min. CETP and LCAT activities were determined as described in sections 2.4.1 and 2.4.3 respectively.

was then collected and loaded onto a succinylated-LDL column that was prepared by first coupling LDL to CNBr-activated sepharose and then modifying the LDL with succinic anhydride. After loading the CETP, the column was washed with 39 mM PO_4, eluted with a 4mM PO_4 solution and 5 ml fractions were collected. The CETA eluted at 55 ml (Figure 10). CETP was routinely purified approximately 100-fold by the phenyl-sepharose column and 200-fold by the DE-52 column. These values are both very similar to the literature values for this method of purification. After chromatography on the succinylated-LDL column, CETP was purified approximately 18 000 fold, similar to that reported by Kato *et al* [114].

3.3 Reconstituted HDL Studies

3.3.1 Spherical Lp2A-I

Spherical rHDL particles were prepared by co-sonication of apoA-I and pure lipids as described in section 2.2.5. The compositions of particles varying in their POPC and PI contents (Table 2) were measured after re-isolation of the Lp2A-I by gel filtration. POPC particles were found to vary in their final POPC composition, from 46 to 82 moles of POPC per 2 moles of apoA-I. The PI series particles varied in PI content from 0 to 28 moles of PI per 2 moles of apoA-I. It is important to note that the PI series particles all have a similar initial composition of 100 moles of POPC to 40 moles of CE to 2 moles of apoA-I, but contain increasing amounts of PI. Particles were run on NGGE (Figure 11) to measure their hydrated diameter. A small increase in size was observed for the POPC particles, as POPC content increased. A slight increase in the size of particles with increasing amount of PI was also observed, but this was likely due to an artifact of the

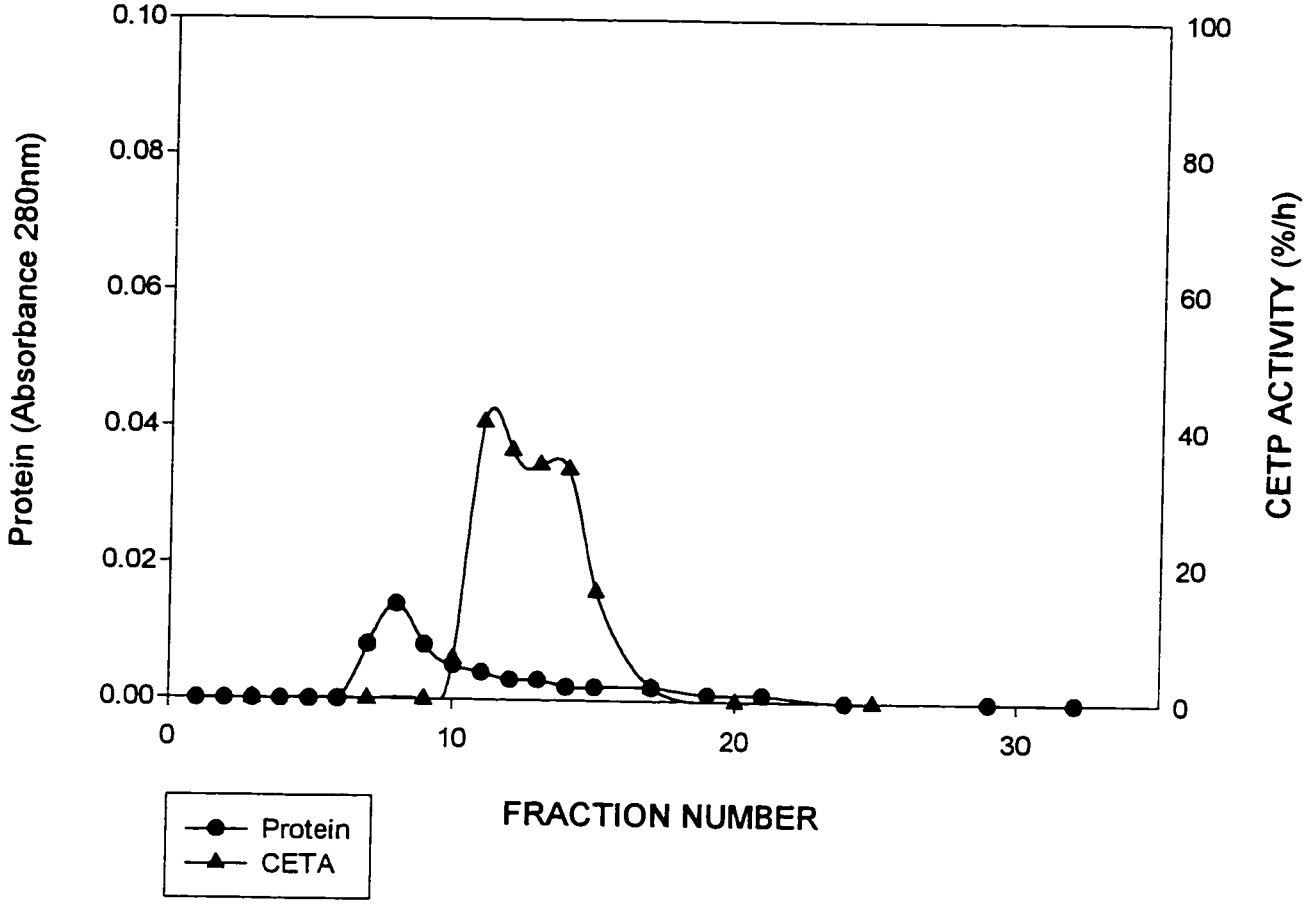


Figure 10. Succinylated-LDL column chromatography of CETP.

The DE52 eluent was loaded onto a succinylated-LDL column and washed extensively with 39 mM PO_4 buffer. Bound proteins were subsequently eluted with 4 mM PO_4. 40 x 5 mL fractions were collected at a flow rate of 0.9 mL/min. CETP activities were determined as described in section 2.4.1.

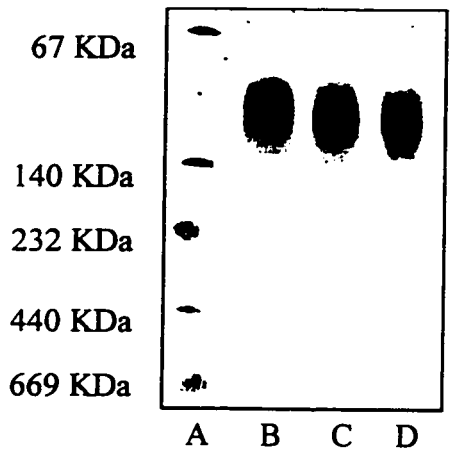


Figure 11. Effect of POPC content on Lp2A-I hydrated diameter Lp2A-I particles varying in POPC content were run on NGGE for 350 AVh and visualized by staining with coomassie blue. Lane A contains high molecular weight markers, B. 46:14:2, C. 54:14:2, D. 82:16:2 (POPC:CE:apoA-I).

increased negative charge of these particles. This increased electrophoretic mobility was not observed when the PI series particles were run to 700 AVh, instead of the standard 350 AVh.

3.3.2 Effect of Particle Composition on the Secondary Structure of ApoA-I in Lp2A-I

Differences in apoA-I organization on the POPC and PI varying particles are evident in terms of the amount of amphipathic α-helical structure in apoA-I on the various particles. Particles varying in PI content exhibit a significantly reduced α-helical content in apoA-I as compared to particles varying in POPC (Table 2). Addition of 8 molecules of

Table 2. Composition, size and α-helix content of POPC and PI series Lp2A-1

Variation in Phosphatidylcholine Content

Initial Composition POPC:CE:A-I (mol/mol)	Final Composition[a] POPC:CE:ApoA-I (mol/mol)	NGGE Size[b] (nm)	α-Helix[c] %
70:40:2	46:14:2	7.8	96.9
100:40:2	54:14:2	7.8	89.8
150:40:2	82:16:2	7.9	92.0

Variation in Phosphatidylinositol Content

Initial Composition POPC:CE:PI:A-I (mol/mol)	Final Composition[a] POPC:CE:PI:ApoA-I (mol/mol)	NGGE Size[b] (nm)	α-Helix[c] %
100:40:0:2	54:14:0:2	7.8	89.8
100: 40:8:2	61:14:8:2	7.7	63.8
100: 40:16:2	48:11:16:2	7.7	77.4
100: 40:32:2	40:10:28:2	7.6	79.8

[a] Composition determined as described in section 2.2.6.1.
[b] Average of 3 runs ± 0.5 nm (S.D.).
[c] Determined from molar ellipticities at 222nm ± 4% (S.D.).

PI to an Lp2A-I particle causes a significant reduction in α-helical content, while further addition of PI appears to promote an increase in the α-helicity of apoA-I. Varying the POPC content of Lp2A-I particles has no significant effect on the amount of amphipathic α-helical structure in apoA-I.

3.3.3 Effect of Particle Composition on the Net Charge of Lp2A-I

The net surface charge of Lp2A-I was calculated after electrophoresis on 0.5% precast agarose gels using the method described by Sparks [112] (Table 3). A very small

Table 3. Surface potential of POPC and PI series Lp2A-1

Variation in Phosphatidylcholine Content	
Composition POPC:CE:A-I (mol/mol)	Surface Potential[a] (-mV)
46:14:2	10.9
54:14:2	10.7
82:16:2	10.5

Variation in Phosphatidylinositol Content	
Composition POPC:CE:PI:A-I (mol/mol)	Surface Potential[a] (-mV)
54:14:0:2	10.5
61:14:8:2	11.8
48:11:16:2	13.4
40:10:28:2	14.7

[a] Charge potential at the particle surface ± 0.2 (S.D.)

increase in the net negative charge was observed for POPC particles as POPC content decreased. This is apparently due to the change in conformation of the apoA-I molecule on the surface of the particle. For the PI particle series, a clear and consistent increase in the net negative charge was observed as the PI content was increased in the Lp2A-I particles (Figure 12).

Figure 12.. Addition of PI increases Lp2A-I net surface negative charge. Lp2A-I particles varying in PI content were run on precast 0.5% agarose gels for 30 minutes at 100V and visualized by staining with Beckman Paragon Lipostain lipid stain. Lane A contains apoA-I, B. 54:14:0:2, C. 61:14:8:2, D. 48:11:16:2, E. 40:10:28:2 (POPC:CE:PI:apoA-I)

3.3.4 Isothermal Denaturation of ApoA-I in Lp2A-I

Variations in the diameter and core composition of Lp2A-I particles have significant effects on the denaturation of lipid-bound apoA-I by GdnHCl. Figure 13 shows the GdnHCl denaturation curves for the Lp2A-I particles varying in POPC content. The differences in the estimated midpoints of denaturation, $D_{1/2}$ can be seen from Table 4. ApoA-I on the 82:16:2 particle appears to be more resistant to GdnHCl denaturation than apoA-I on the 54:14:2 or 46:14:2 because the $D_{1/2}$ values are higher. Increasing the POPC content of Lp2A-I appears to have an affect on the unfolding of apoA-I by GdnHCl (Figure 13), and gives rise to a substantial change in $D_{1/2}$ values (Table 4). Analysis of GdnHCl denaturation curves (Figure 13, *inset*) using the GdnHCl binding model as described previously [113] shows that changes in denaturation curves correspond to significant changes in the estimated free energies of unfolding, ΔG_D, of apoA-I (Table 4). Increasing the POPC content of Lp2A-I significantly increases the ΔG_D of apoA-I on the particle.

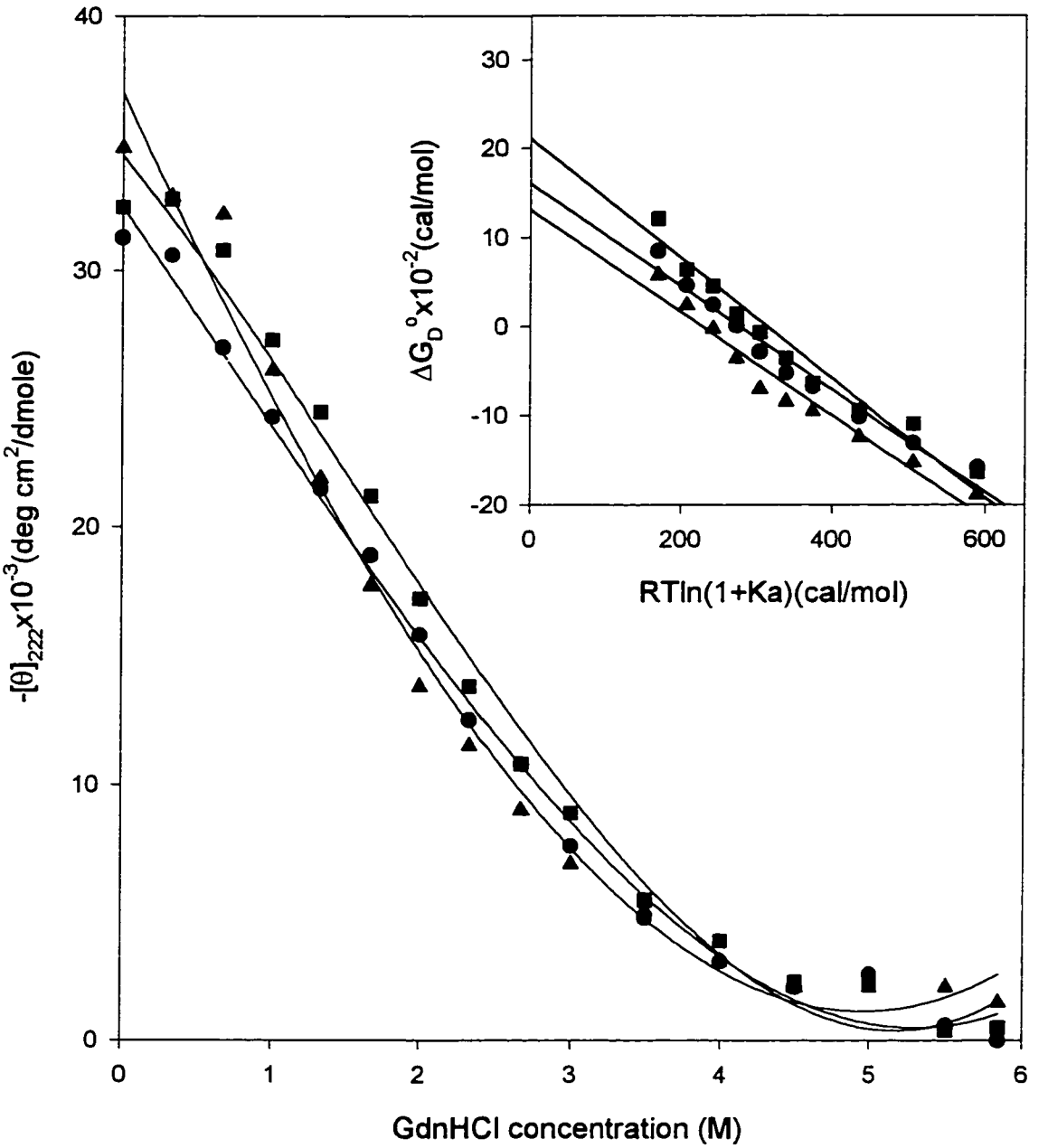


Figure 13. Effect of GdnHCl concentration on the molar ellipticityof apoA-I on Lp2A-I varying in POPC content. Aliquots of Lp2A-I (POPC:CE:A-I molar ratios: ▲, 70:40:2; ●, 100:40:2; ■, 150:40:2) were incubated with 0-6M GdnHCL,and CD spectra were measured as described in section 2.2.5. *Inset*, linear regression plots are shown of the observed free energies of denaturation (ΔG_D) against RTln(1+Ka).

Table 4. Denaturation characteristics of LP2A-I particles

Variation in Phosphatidylcholine Content			
Particle	$D_{1/2}$[a]	ΔG_D[b]	Δn[c]
POPC:CE:A-I	M GdnHCl	*kcal/mol apoA-I*	*mol GdnHCl/ mol apoA-I*
46:14:2	1.63	1.14	5.1
54:14:2	1.98	1.60	5.7
82:16:2	2.14	1.84	6.2

Variation in Phosphatidylinositol Content			
Particle	$D_{1/2}$[a]	ΔG_D[b]	Δn[c]
POPC:CE:PI:A-I	M GdnHCl	*kcal/mol apoA-I*	*mol GdnHCl/ mol apoA-I*
54:14:0:2	1.98	1.60	5.7
61:14:8:2	2.05	1.91	6.5
48:11:16:2	1.95	1.58	5.3
40:10:28:2	1.97	1.38	4.7

[a] Midpoint of GdnHCL denaturation ± 0.03 M (S.D.).
[b] Standard charge in free energy of denaturation ± 0.05 kcal (S.D.).
[c] GdnHCl bound during denaturation ± 0.5 (S.D.).

Figure 14 shows GdnHCl denaturation curves for Lp2A-I varying in PI content. Variation in PI content seems to have no significant affect on the midpoint of denaturation of Lp2A-I varying in PI. Analysis of the GdnHCl denaturation curves shows that, in contrast to Lp2A-I varying in POPC, changes in the ΔG_D do not parallel $D_{1/2}$ values (Table 4 and Figure 14, *inset*). Further increases in PI content of the particle is associated with a decrease in ΔG_D values (Table 4). It is evident that increasing the PI content in Lp2A-I corresponds to a significant decrease in the ΔG_D value. Increasing the PI content in Lp2A-I therefore appears to decrease the conformational stability of apoA-I.

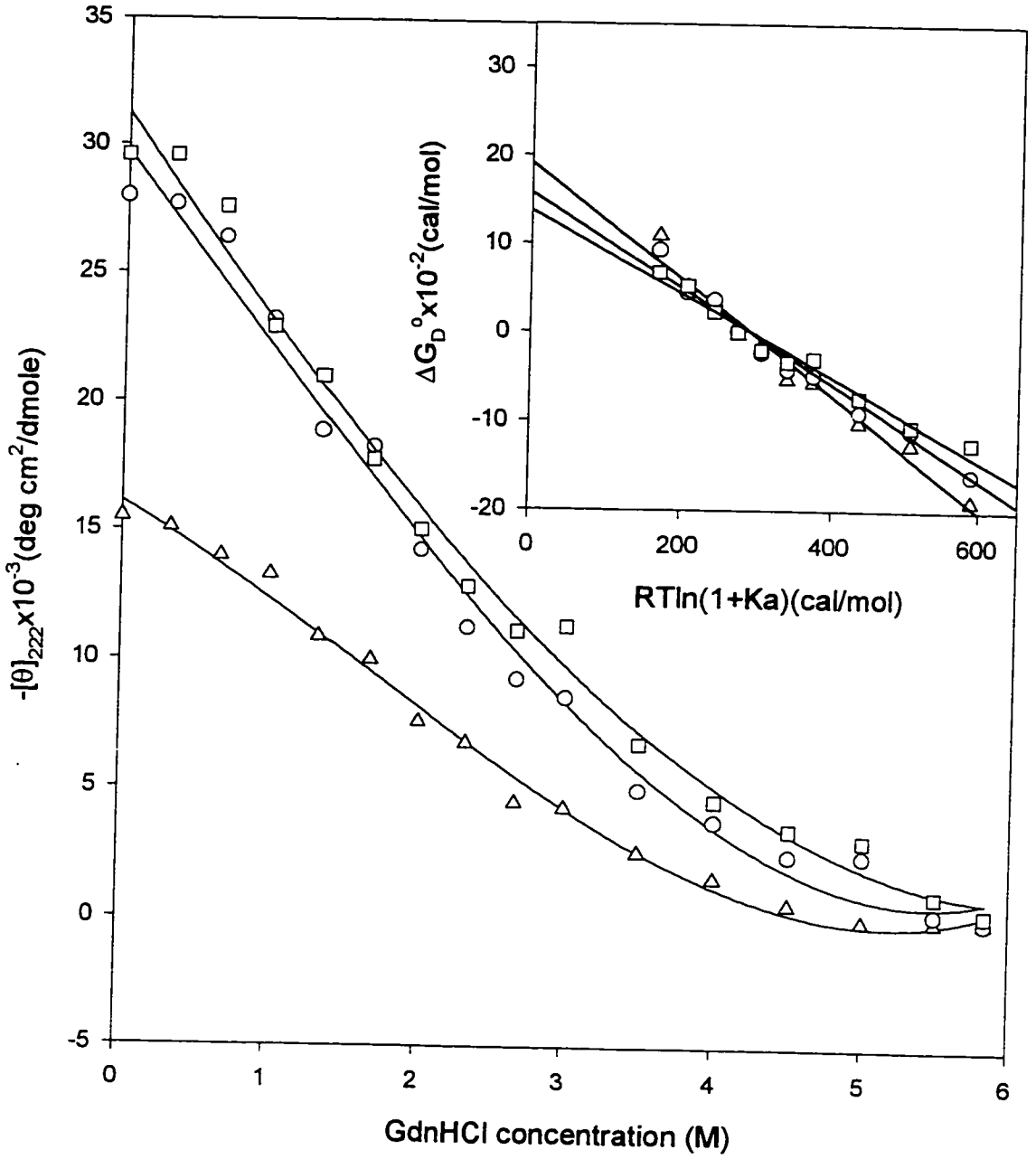


Figure 14. Effect of GdnHCl concentration on the molar ellipticityof apoA-I on Lp2A-I varying in PI content. Aliquots of Lp2A-I (POPC:CE:PI:A-I molar ratios: Δ, 100:40:8:2; O, 100:40:16:2; □, 100:40:32:2) were incubated with 0-6M GdnHCL,and CD spectra were measured as described in section 2.2.5. *Inset*, linear regression plots are shown of the observed free energies of denaturation (ΔG_D) against RTln(1+Ka).

3.4 CETP Assays

3.4.1 Lp2A-I Particles

Lp2A-I particles varying in POPC content were incubated with LDL and CETP as described in section 2.4.2. 25 μg of protein of Lp2A-I was incubated with 10 μg, 20 μg and 50 μg of LDL protein, giving LDL to HDL ratios of 0.4, 0.7 and 2.0; a ratio of 0.7 representing a normolipidemic ratio and the higher and lower ratios relating to hyper- and hypo-betalipoproteinemia. As the ratio of LDL to HDL increased, the rate of transfer of ^{3}H-labeled CE from the Lp2A-I particles to the LDL particles increased (Figure 15). The experiment also shows that at each of the LDL to HDL ratios, the transfer of ^{3}H-labeled CE from the Lp2A-I particles to the LDL particles decreased as POPC content increased. CE transfer was highest for the 46:14:2 particle, and lowest for the 82:16:2 particle.

The mass transfer of CE from LDL to the Lp2A-I particles was also determined (Figure 16) and showed that as Lp2A-I POPC content was increased, the amount of CE transferred out of LDL was reduced. The highest amounts of CE were transferred into the 46:14:2 particle from LDL. Next was the 54:14:2 particle, and the 82:16:2 particle promoted the lowest amount of CE transfer from LDL.

In the particles varying in POPC, the particle with the least amount of POPC, had the highest rates of CE transfer into and out of that particle. It is of consequence to note the difference in scale between Figures 15 and 16. The rate of CE transfer from LDL into the Lp2A-I particles as approximately 100 fold-greater than the CE transfer in the opposite direction.

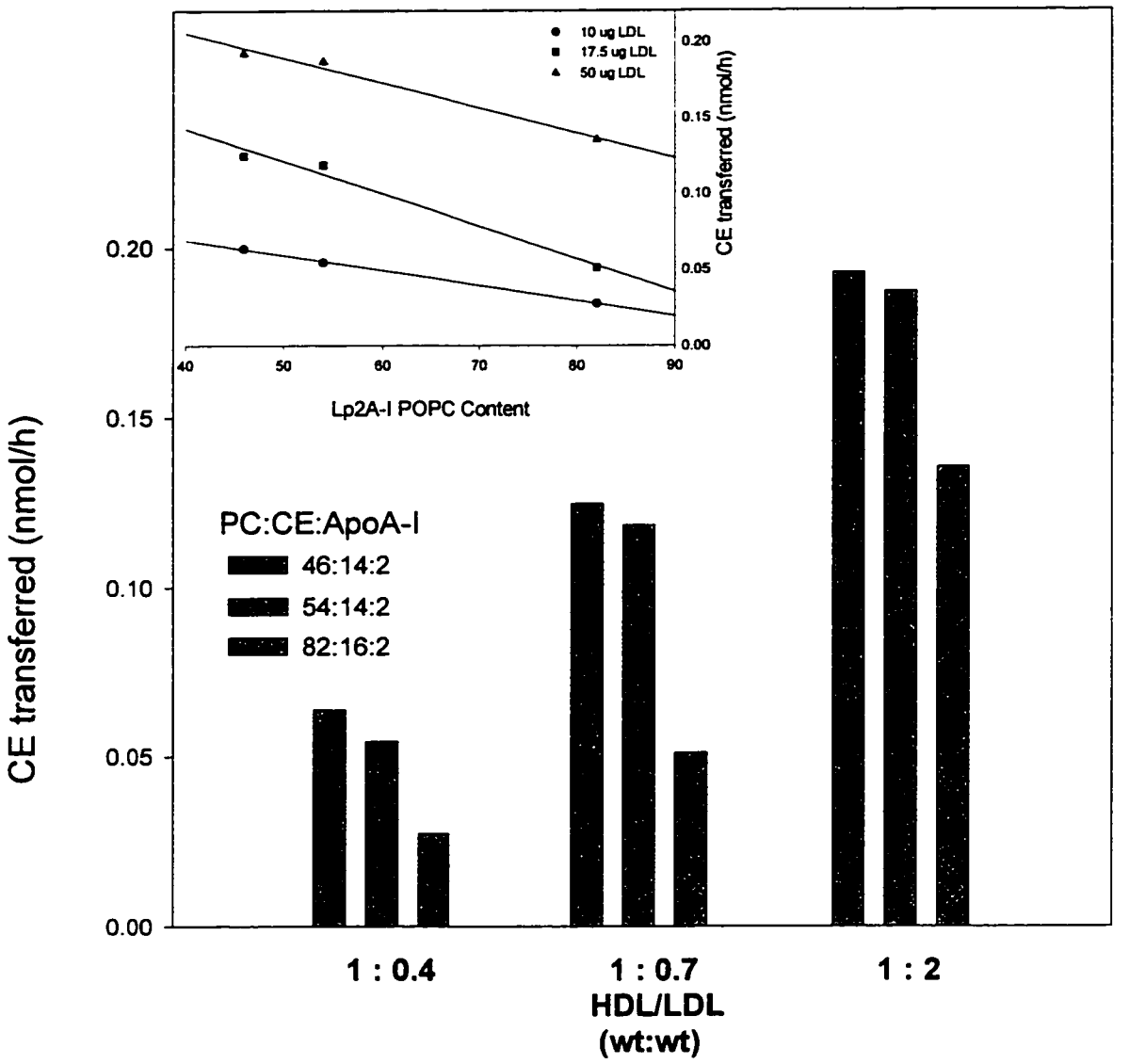


Figure 15. Effect of variations in Lp2A-I POPC content on the rate of CE transfer to LDL. 25 μg of protein of Lp2A-I particles varying in POPC content (see Table 2 for compositions) were incubated with 10 μg, 17.5 μg and 50 μg of native LDL protein for 3h at 37°C and then separated by ultracentrifugation. Samples were then counted to determine the amount of [^{3}H] CE transferred to LDL. Control samples that were not incubated were subtracted to determine CETP dependant CE transfer. Values are the average of duplicate determinations. *Inset*, shows that an apparent relationship exists between Lp2A-I POPC content and their ability to promote the transfer of CE to LDL.

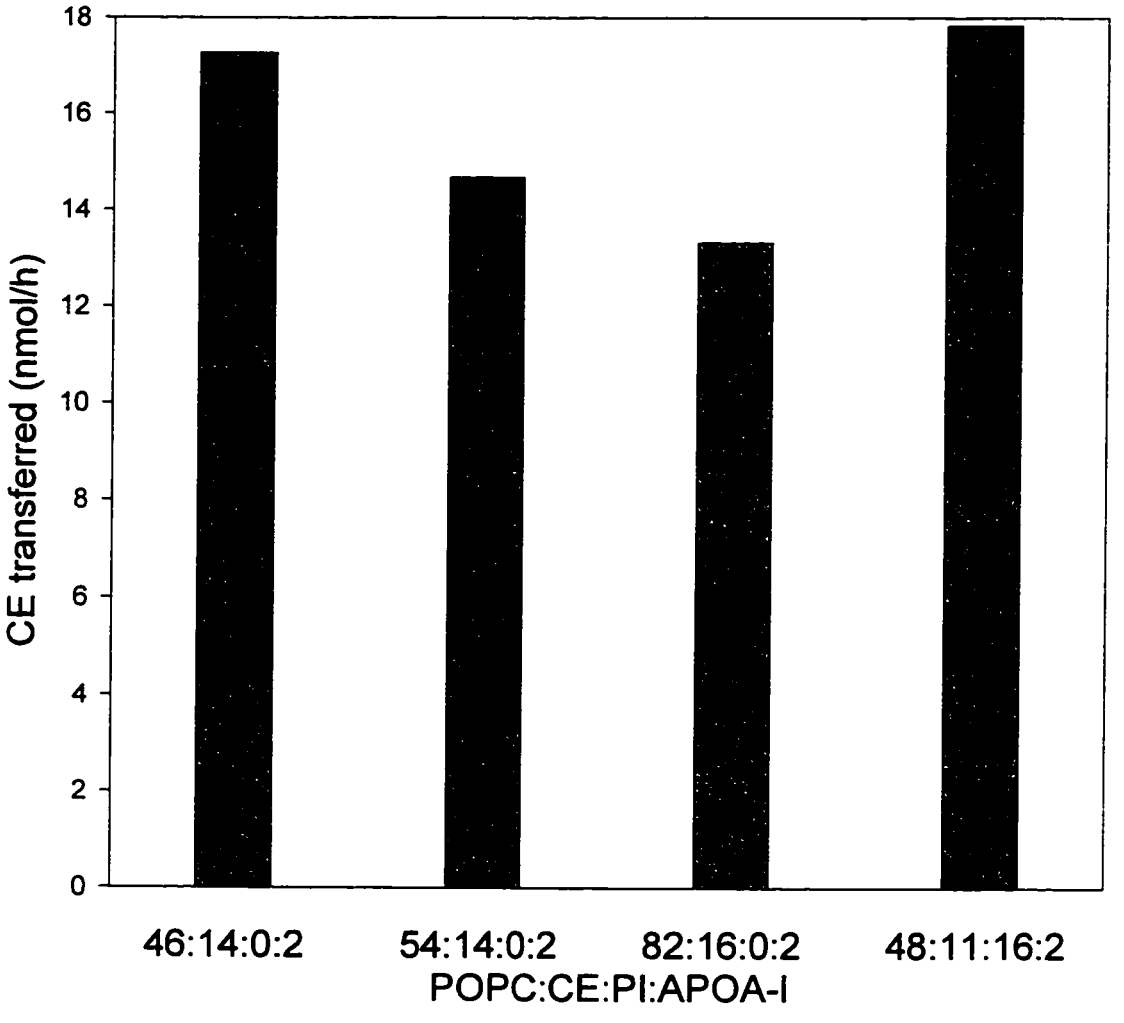


Figure 16. Effect of variation in Lp2A-I POPC and PI content on CE mass transfer from LDL to Lp2A-I.

25 μg of protein of Lp2A-I particles varying in POPC and PI content (see Table 2 for compositions) were incubated with 17.5 μg of native LDL protein for 3h at 37°C and separated by ultracentrifugation. CE mass was measured by enymatic kits as described in section 2.2.4.1. Control samples that were not incubated were subtracted to determine CETP dependant CE transfer. Values are the average of duplicate determinations.

The rate of transfer of ^{3}H-labeled CE from the Lp2A-I particles to the LDL particles was studied in particles varying in PI (Figure 17). Increasing the LDL to HDL ratio had a similar effect with POPC particles; the rate of transfer of ^{3}H-labeled CE from the Lp2A-I particles to the LDL particles increased. As well, the particles containing PI had higher rates of transfer than the control 54:14:0:2 particle. The 48:11:16:2 particle had a higher rate of transfer than the 61:14:8:2 particle at each of the different LDL to HDL ratios. The data for the 40:10:28:2 particle was spurious and is not shown.

PI incorporation also affected CE mass transfer from LDL (Figure 16). The 48:11:16:2 particle exhibited the highest ability to receive CE from LDL. The amount of CE received was approximately 20% higher than the control particle.

Addition of PI had a similar effect with POPC particles. Both rates of CE transfer between the Lp2A-I particle and LDL were increased.

Free cholesterol (Figure 18) and phospholipid mass transfer was measured. Free cholesterol mass transfer from LDL to the POPC series of Lp2A-I particles averaged approximately 7 nmol/h. There was no significant difference between the different particles. On the other hand, the particle containing 8 molecules of PI, had a 40% higher mass transfer of free cholesterol from LDL to the reconstituted particle, than the control 54:14:0:2 particle. Similar to that reported by Kato *et al* [114], no phospholipid transfer activity was observed in these experiments.

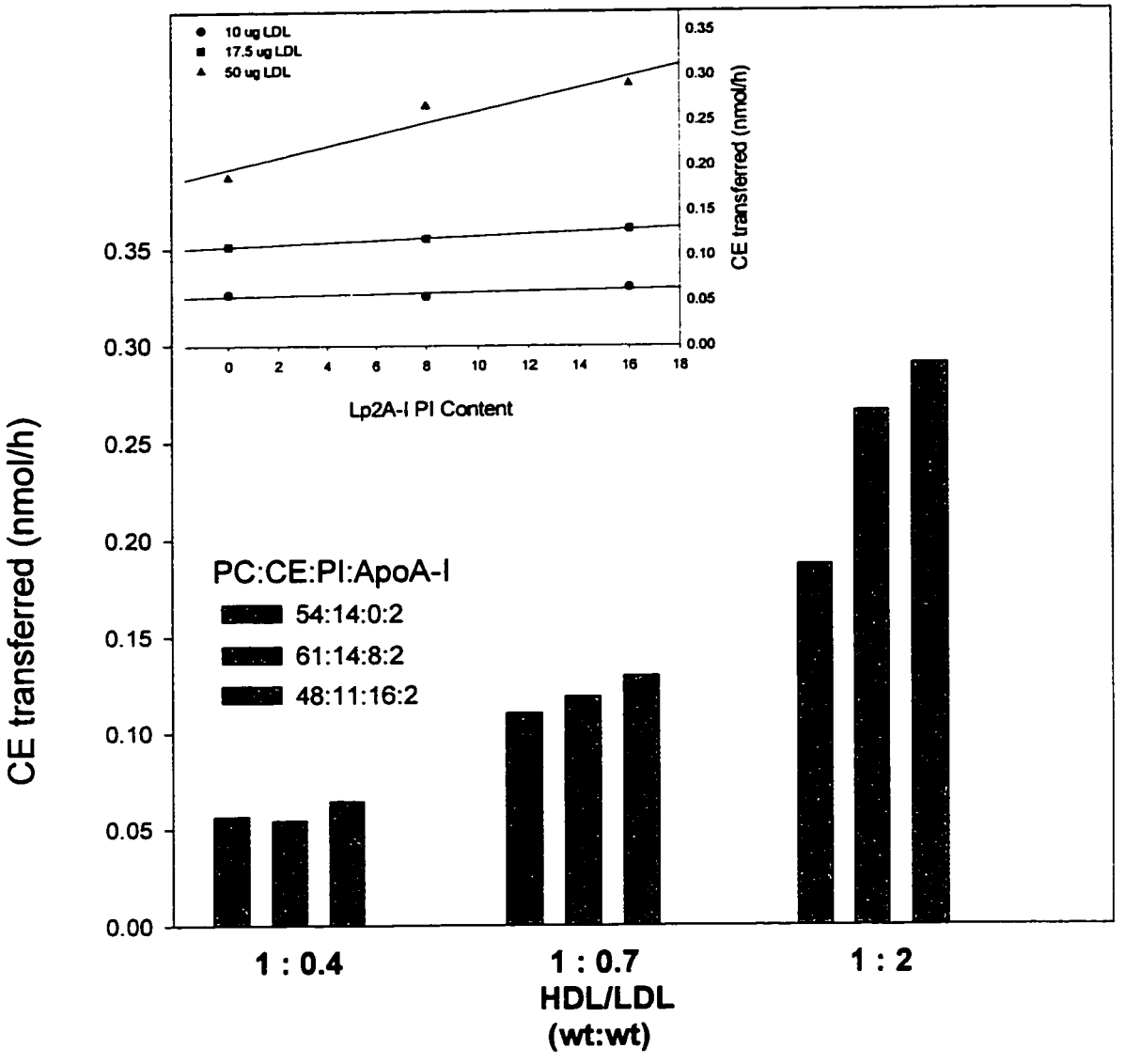


Figure 17. Effect of variations in Lp2A-I PI content on the rate of CE transfer to LDL
25 μg of protein of Lp2A-I particles varying in PI content (see Table 2 for compositions) were incubated with 10 μg, 17.5 μg and 50 μg of native LDL protein for 3h at 37°C and then separated by ultracentrifugation. Samples were then counted to determine the amount of [^{3}H] CE transferred to LDL. Control samples that were not incubated were subtracted to determine CETP dependant CE transfer. Values are the average of duplicate determinations. *Inset*, shows that an apparent relationship exists between Lp2A-I PI content and their ability to promote the transfer of CE to LDL.

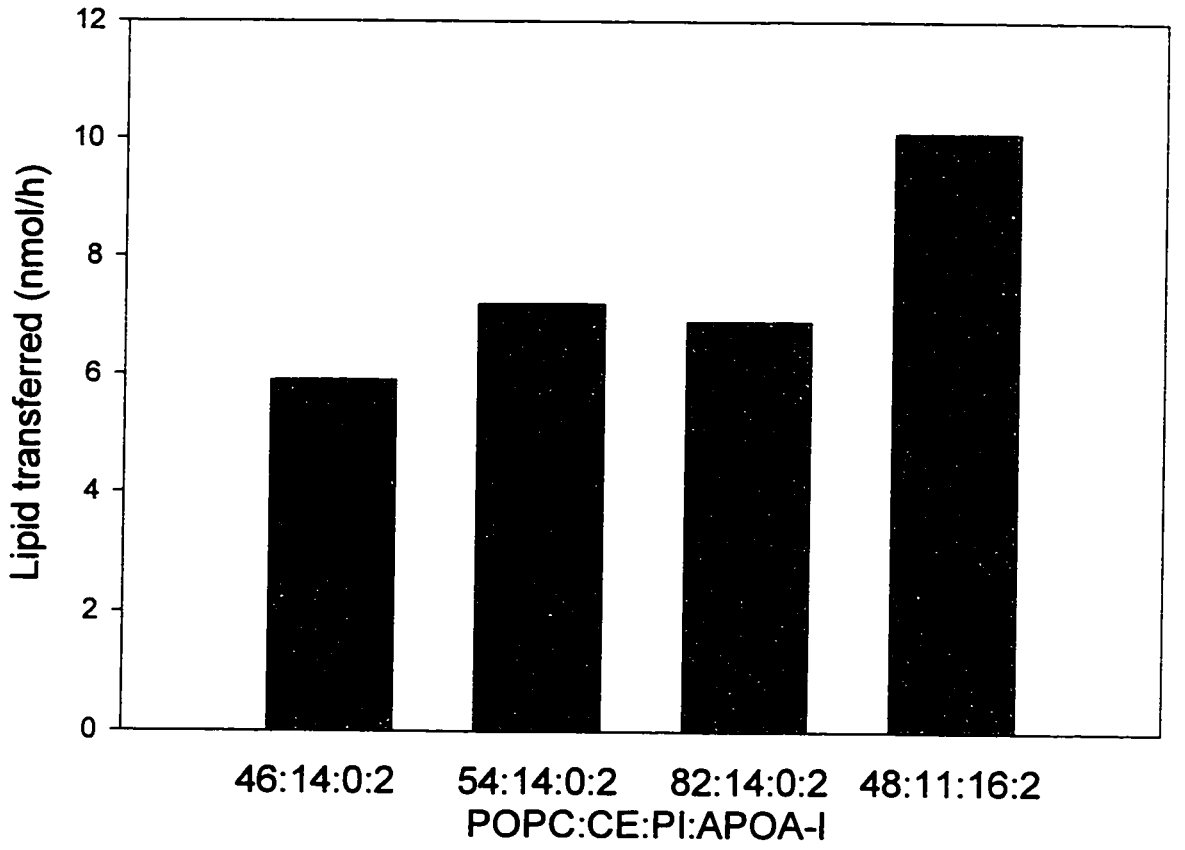


Figure 18. Effect of variation in Lp2A-I POPC and PI content on free cholesterol mass transfer from LDL to Lp2A-I.

25 μg of protein of Lp2A-I particles varying in POPC and PI content (see Table 2 for compositions) were incubated with 17.5 μg of protein of native LDL for 3h at 37^0C and separated by ultracentrifugation. FC mass was measured by enymatic kits. Control samples that were not incubated were subtracted to determine CETP dependant CE transfer. Values are the average of duplicate determinations.

3.4.2 Studies with Reconstituted LpB

To characterize the effect of LDL charge on CETA, collaborative studies were undertaken with Vinita Chauhan to explore the effect of the inclusion of PI into a reconstituted LDL (LpB) particle. Apo B was delipidated and reconstituted with sonicated microemulsions containing POPC, CE and PI as described in the methodology. Native HDL_3 was incubated with purified CETP and native LDL, or LpB ± PI and CE mass transfer was measured (Figure 19). The difference between the incubations with native LDL and LpB (no PI) indicate the direction of mass transfer. With native LDL, a total mass transfer of 4.5 nmol/ml/h from LDL to HDL_3 was observed. With LpB, the direction of mass transfer was 22.4 nmol/ml/h into the reconstituted lipoprotein. The inclusion of 90 molecules of PI almost completely inhibited the mass transfer of CE from HDL_3 into the reconstituted lipoprotein.

Figure 20 shows the TG mass transfer between the different lipoproteins. TG mass transfer was observed to be 34.9 nmol/ml/h from native LDL into HDL_3 and 11.5 nmol/ml/h from LpB (-PI). With the inclusion of PI into the LpB particle, the direction of mass transfer was reversed, with 4.9 nmol/ml/h of TG transferred from HDL_3 into LpB (+PI). These observations indicate that the direction of CETP neutral lipid transfer is affected by the anionic lipid content of LDL.

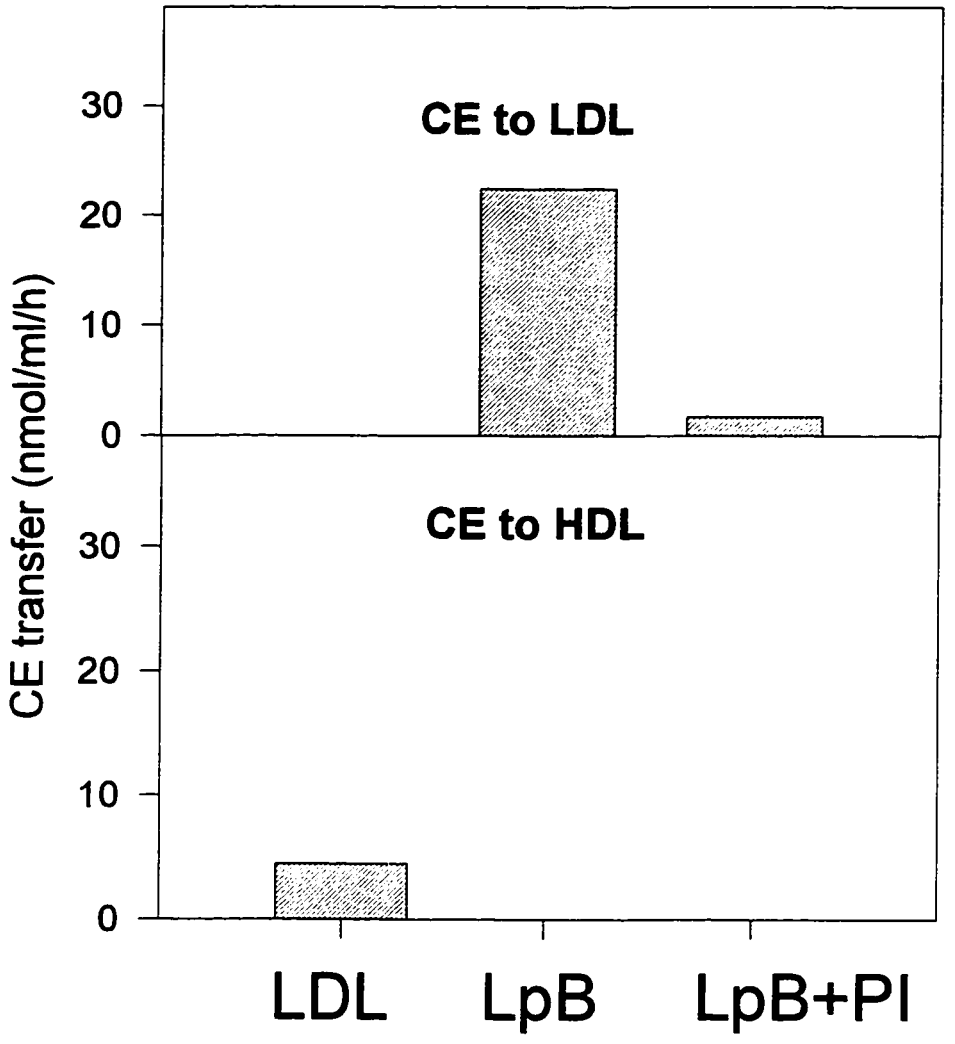


Figure 19: Effect of LpB PI content on CE transfer between LpB and HDL. Native HDL_3 was combined with LDL or reconstituted LpB particles (± 90 moles PI) and incubated for 3 hours at 37°C, with CETP. Lipid mass transfers were calculated after ultracentrifugal separation of LDL and HDL. Mass transfer was measured by enymatic kits. Control samples that were not incubated were subtracted to determine CETP dependant CE transfer. Values are the average of duplicate determinations.

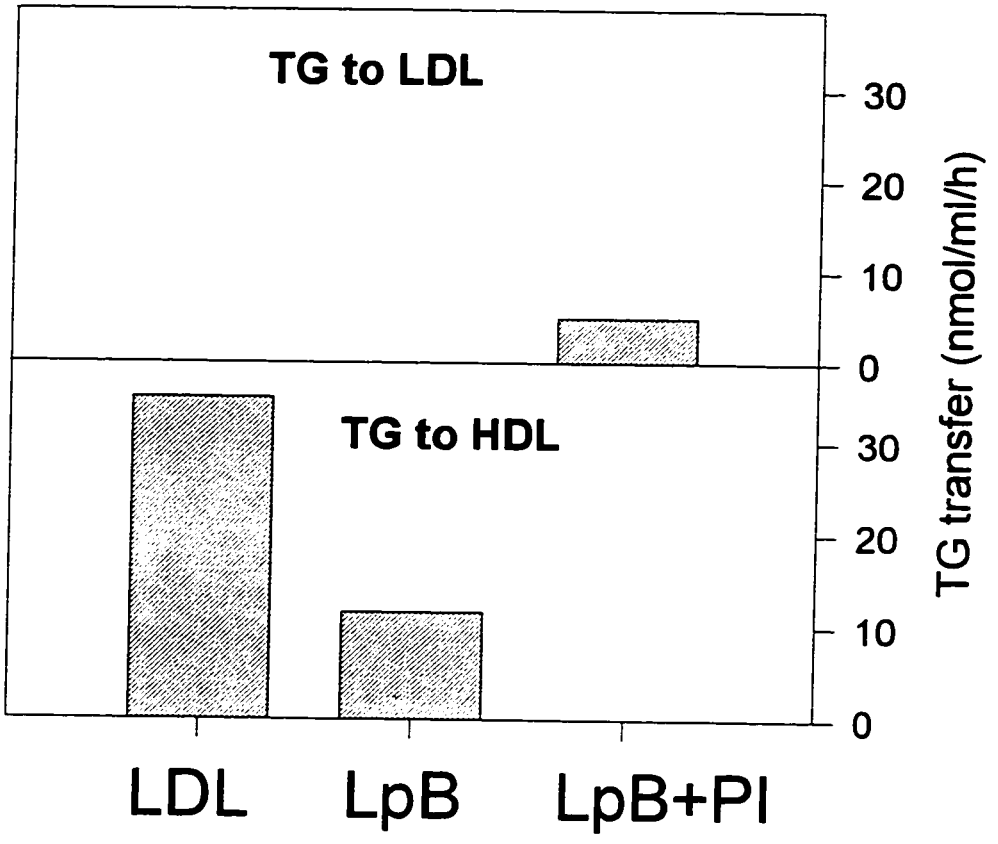


Figure 20: Effect of LpB PI content on TG transfer between LpB and HDL. Native HDL_3 was combined with LDL or reconstituted LpB particles (± 90 moles PI) and incubated for 3 hours at 37°C, with CETP. Lipid mass transfers were calculated after ultracentrifugal separation of LDL and HDL. Mass transfer was measured by enymatic kits. Control samples that were not incubated were subtracted to determine CETP dependant CE transfer. Values are the average of duplicate determinations.

3.4.3 Studies with PI and whole plasma

To determine whether we could incorporate PI into plasma lipoproteins by simple incubation, we dried various amounts of PI into a tube and incubated with LDL and HDL overnight at 4°C. After electrophoresis on agarose, both LDL (Figure 21) and HDL (not shown) electrophoretic mobilities were shown to increase in proportion to the amount of PI added. To label both lipoproteins simultaneously, a fasting plasma sample was incubated with PI similarly. We then determined the effect of lipid mass transfer between the d<1.063 g/ml (VLDL, LDL, IDL) and d>1.063 g/ml (HDL) fractions. After incubation for 16h at 37^{0}C, the plasma was ultracentrifugally separated and lipid mass measured. Figure 22 shows the lipid mass measurements for the d<1.063 g/ml fraction. No significant difference was found between plasma that was incubated at 4^{0}C or 37^{0}C for any of the lipids measured. In contrast, the mass of CE was significantly lower in the d<1.063 g/ml fraction of plasma after incubation with PI. Similar observations were made for TG lipid mass and FC lipid mass transfers. A decrease in CE, TG and FC lipid mass was observed in the d<1.063 g/ml (i.e. LDL / VLDL) fraction of plasma incubated with PI relative to plasma incubated in the absence of PI (Figure 22). As expected, the opposite was observed for the d>1.063 g/ml (i.e. HDL) fraction. Incubations in the presence of PI increased the ratio of CE/protein by 31%, FC/protein by 14% and TG/protein by13%in the HDL fraction.

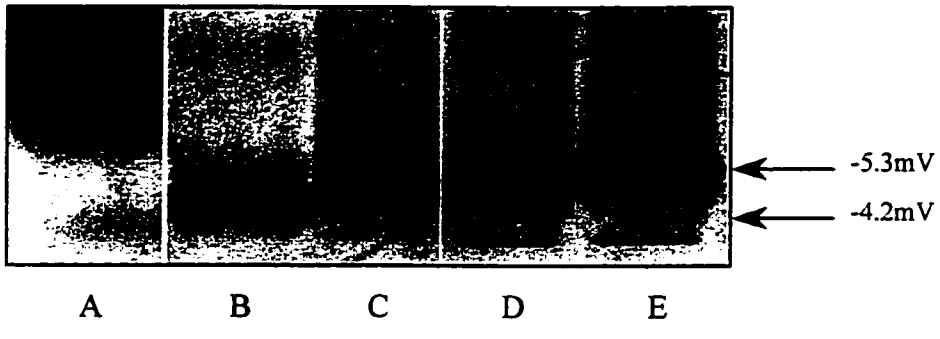


Figure 21: Addition of PI increases LDL net negative surface charge. LDL was incubated with PI for 16h at 37^{0}C. Pi mass added was equivalent to 5%, 10% and 15% of the total mass of phospholipid in the LDL. After incubation, LDL samples were run on precast 0.5% agarose gels for 30 minutes at 100V and visualized by staining with Beckman Paragon Lipostain lipid stain. Lane A contains apoA-I, B. native LDL, C. LDL +5% PI, D. LDL + 10% PI, E. LDL + 15% PI.

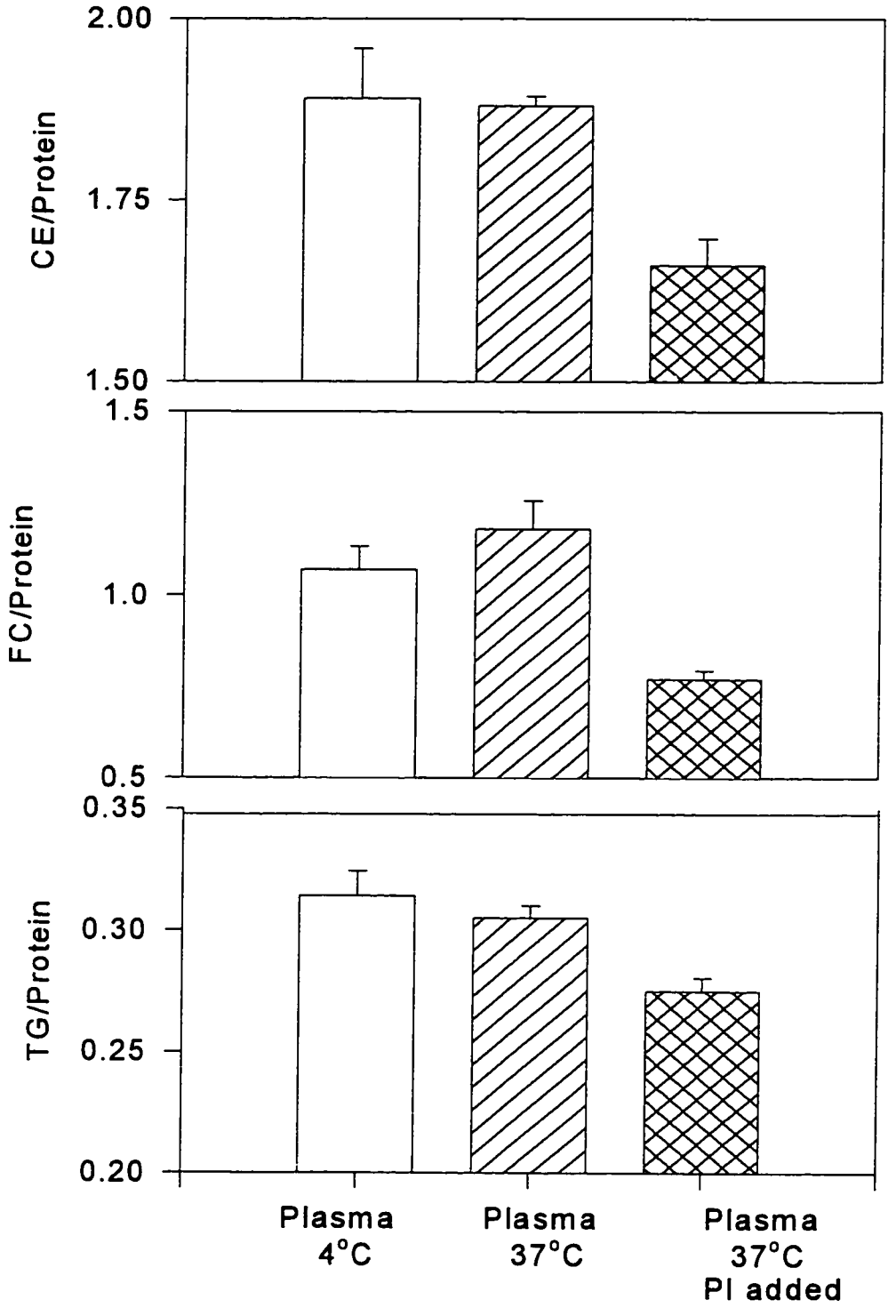


Figure 22. Effect of PI on lipid mass measurements in the d<1.063 g/ml plasma fraction.
Plasma was incubated for 16 hours at 4°C, at 37°C and 37°C with the addition of PI.
Lipid mass measurements were made in the d<1.063 g/ml fraction after ultracentrifugal separation.

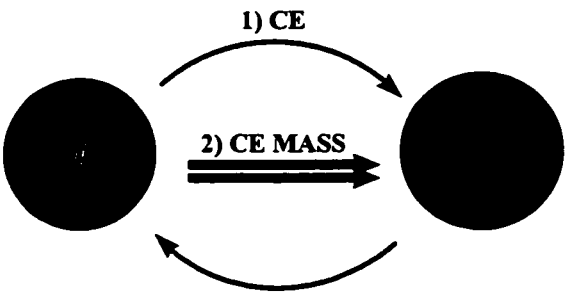
4.0 Discussion

4.1 Experimental rationale

The principal goal of this research was to determine the effects of Lp2A-I surface charge and structural properties on the regulation of CETP-mediated neutral lipid transport. Observations from previous studies need to be interpreted with caution. Heterogeneous preparations of native lipoproteins, as well as chemical modification of lipoprotein substrates can confuse estimations of what may be regulating the activity of CETP under *in vivo* conditions. Since the composition and structure of lipoproteins influence the enzymes that act at a lipoprotein interface, an unambiguous characterization of the regulation of CETP will require the use of homogeneous lipoprotein substrates with well-defined compositions and structures. In this study, we have tested the hypothesis that Lp2A-I composition-dependent changes in apoA-I stability and charge modulate the interfacial interactions and actions of CETP.

The overall objective was to determine how variations in lipoprotein composition would affect its ability to act as an acceptor or donor of transferred CE or TG. Although the effect of lipoprotein composition on CETP activity has been measured in other studies, using a variety of methods, few studies have looked at all components of CETP activity simultaneously. Studies by some investigators, i.e. Lagrost [65] and Nishida [63], have monitored the movement of [^{3}H]CE to estimate the rate of CE transfer from donor lipoprotein to acceptor. Alternatively, studies by Bagdade [58] and Rajaram [59] have measured the total mass transfer that occurs in a CETP mediated reaction. The transfer of CE mass between lipoproteins is a result of differences in the bi-directional exchange of this lipid (Figure 23). Because most previous studies have only measured the rate of

transfer in one direction or the mass transfer, they cannot resolve mechanistic questions on how CETP is regulated. Isotopic transfer from a donor lipoprotein to acceptor particles reflects the rate of transfer in one direction only. The total mass transfer that occurs can be determined simply by measuring the total mass of lipids in the lipoprotein particles before and after a transfer reaction. The reciprocal back-transfer that occurs can then be calculated from the difference between the total mass transferred and the rate of transfer from donor to acceptor. To characterize these events we developed an assay system that facilitates the measurement of all three parameters.



1) Rate of CE transfer from donor to acceptor lipoprotein

2) Overall Net Mass Transfer

3) Rate of CE backtransfer from acceptor to donor lipoprotein

Figure 23. Transfer of CE between donor and acceptor lipoproteins.

This assay works equally well with native or reconstituted lipoproteins. Utilizing [^{3}H]CE in the neutral lipid core of the Lp2A-I allows for the simultaneous measurement of both rates of CETP transfer and the overall mass transfer. This system separates the substrates without modification of the lipoproteins involved.

4.2 *Effect of Lp2A-I composition on the structure and stability of apoA-I*

In the present study, a detailed characterization of two different Lp2A-I particle series has been undertaken. Table 2 shows that particle size does not significantly change when POPC content of Lp2A-I is decreased. The α-helical content of Lp2A-I is also not affected by the changes in POPC content observed in this study (Table 2). This suggests that apoA-I on these particles does not adopt a different conformation as the POPC content is varied. This is consistent with our observation that the net negative surface charge conferred to the lipoprotein particle by the conformation of apoA-I is only slightly modified when POPC content is increased (Table 3). However, major differences between the stability of apoA-I on the various particles are evident when POPC is varied. Increasing POPC content leads to an increased $D_{1/2}$ and an increased conformational stability (Table 4). Although POPC content does not affect the α-helical content of Lp2A-I, it appears to affect its thermodynamic stability. The number of POPC molecules that interact with the apoprotein may affect the ability of GdnHCl to bind to and denature apoA-I. As POPC content decreases, the particle size and α-helical content of the Lp2A-I remain constant. This means a lower POPC density would surround the apolipoprotein, making it more accessible to GdnHCl. This should result in a reduced Δn, which is what is observed in Table 4.

The different PI particles observed in this study also do not appear significantly different in their size. The major change in their net surface charge is directly due to the addition of negatively charges moieties (PI) to the surface of the lipoprotein particle. In contrast to Lp2A-I varying in POPC, a change in the α-helical content of PI containing particles is evident. The change in α-helical content in the PI particles appears to be due to

the direct addition of the anionic lipid. The negatively charged PI molecules may alter the conformation of apoA-I on these particles by modifying the attractive and repulsive forces on different parts of the apoprotein. Addition of a small amount of PI induces a large drop in the α-helical content of Lp2A-I. Since apoA-I is relatively flexible on the surface of the lipoprotein, a small amount of negative charge may affect the global organization of the apoprotein. Further addition of PI promotes an increase in the particle's α-helical content. As the amount of negative charge on the surface increases, the apoproteins are forced to adopt a novel conformation. This is consistent with previous suggestions that apoA-I electrostatic properties affect its organization on Lp2A-I particles [113].

In contrast to the POPC series, increased PI content is associated with a decreased conformational stability of apoA-I, while the denaturation midpoint is unaffected by the addition of PI. Variations in PI have an affect on apoA-I α-helix content and thermodynamic stability, however, the α-helical content and thermodynamic stability of Lp2A-I particles are inversely related. An initial drop in α-helical content coincides with an increased thermodynamic stability. But, as the thermodynamic stability decreases, the α-helical content increases. The binding of apoA-I to lipid is thought to be mediated by its amphipathic α-helices [23, 113]. Although one might expect ΔG_D values to increase with increased α-helical content, the effect of increased net negative charge on an apoprotein conformation has not been well characterized. The global negative charge increase may force the apoprotein into a specific conformation that, although increased in amphipathic α-helical content, is less stable due to changes in secondary structure that are not thermodynamically favourable. It can be concluded that the conformational stability of apoA-I is affected by POPC and PI content, with each having quite opposite effects.

4.3 Effect of HDL charge on CETA

In this investigation, reconstituted Lp2A-I particles were used to determine the effects of varying Lp2A-I POPC and PI content on CETP activity. A relationship was shown to exist between POPC content and CETP activity. As POPC content decreased, the CE transfer rate from these particles into acceptor LDL increased. CE transfer between Lp2A-I particles containing PI and LDL was also shown to increase as PI content increased. The mass transfer of CE into the Lp2A-I from LDL was also shown to increase as POPC content decreased. The relatively large, 100-fold difference in CE transfer may be attributed to the difference in neutral lipid composition of the particles involved. The net mass transfer of CE from the cholesteryl ester rich LDL to the relatively lipid-poor Lp2A-I appears to be independent of a reciprocal exchange for TG. CETP neutral lipid exchange was originally thought to be equimolar, with one CE or TG being transferred in exchange for a CE or TG [57]. Recent studies have shown that CETP-mediated neutral lipid transfer can occur without a concomitant back transfer. Xiao and Bagdade [58] found that a heteroexchange during CE mass transfer is not obligatory. They showed that HDL_3 could promote a net mass transfer of CE to LDL and HDL_2 without accepting any TG. Barter *et al* proposed that in lipoproteins with similar neutral lipid composition, such as HDL and LDL in human plasma, no net transfer of CE occurs [117]. In contrast, mass transfer of CE can occur between lipoproteins of similar core content and this transfer is affected by the surface lipid composition of the lipoproteins involved.

CE transfer appears to be related to an increase in the net negative charge of these particles. Figure 24 compares the lipid transfer activities for both particle series to their negative surface charges. Although only two particles are shown for the PI series of

particles, there appears to be a relationship between the charge of the particle and CE transfer activity. For the POPC particles, the small change in charge of these particles is related to a large change in the particles ability to transfer CE. The effect of changes in the net negative charge of a lipoprotein particle on CETP activity is well documented. Pattnaik and Zilversmit were the first to describe the binding of CETP to form lipoprotein complexes as electrostatic in nature [61]. Wang *et al* described a putative lipoprotein binding site, a triplet of lysine residues at position 376-378 [62]. This region has been proposed to mediate an electrostatic interaction with negatively charged lipoprotein surface components. Other studies have shown that chemical modification of lipoproteins to increase their negative surface charge density will affect CETP. Nishida *et al* used succinylation and acetylation to increase the net negative charge of LDL and HDL [63]. They showed that an increase in negative charge could be both stimulatory and inhibitory to unidirectional lipid transfer rates. Other studies using less harsh methods of altering lipoprotein surface charge have also described directional specificity in enhancing CETP mediated neutral lipid transfer. Lipoprotein surface charge can be made more negatively charged by the inclusion of negatively charged moieties such as free fatty acids (FFA). Sammett and Tall used lipoprotein lipase to increase the content FFA in lipoproteins and observed a stimulation of CE transfer from HDL to VLDL [64]. Barter and Rajaram included oleate in mixtures of HDL, CETP and triglyceride-rich lipoproteins (TGRL) [74]. They noticed increased transfer of CE out of HDL and decreased transfer of TG into HDL

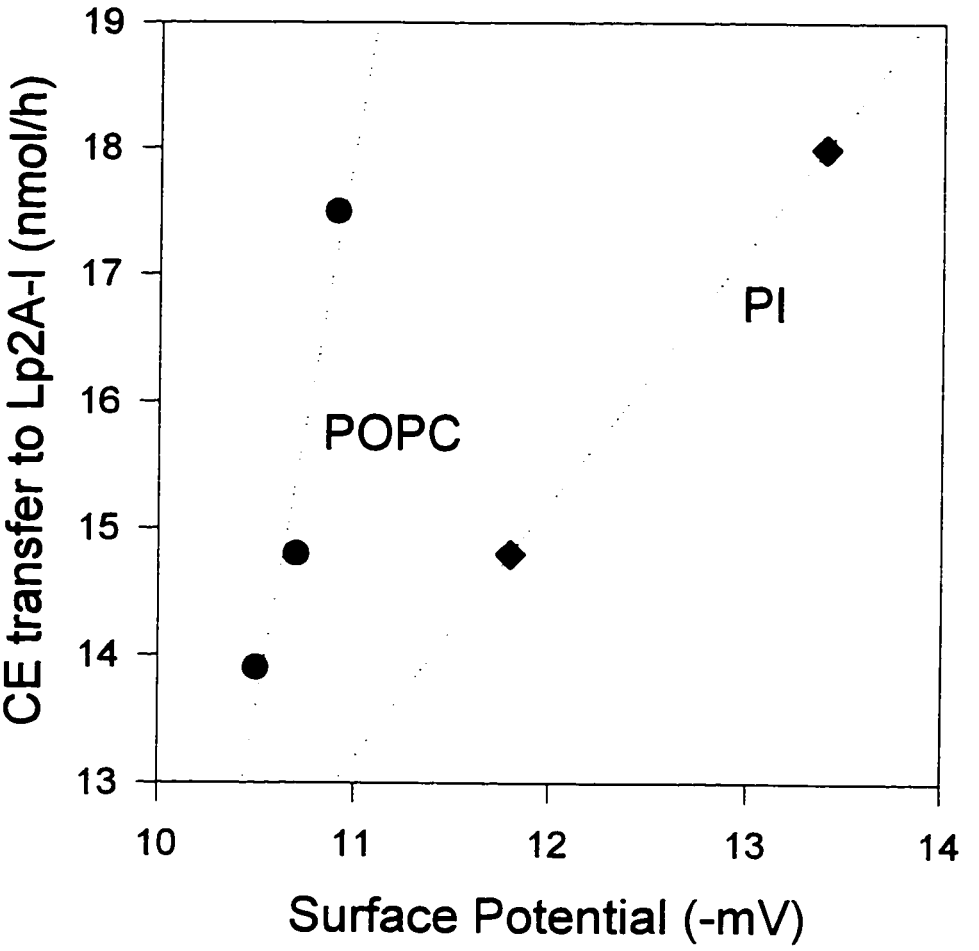


Figure 24. Effect of LpA-I Charge on CE Transfer

The rates of CE transfer from LDL into acceptor Lp2A-I varying in POPC content (46:14:2, 54:14:2, 82:16:2) and PI content (61:14:8:2, 48:11:16:2) are plotted against the particle surface potential. A possible relationship may exist between Lp2A-I surface potential and the ability of the particles to accept CE from LDL.

Lagrost measured the effect of HDL_3 surface charge on CETP activity by isolating HDL_3 subfractions of different composition and electric charge properties by anion exchange chromatography [65]. The rate of CE transfer from HDL_3 subfractions to LDL was progressively enhanced as the negative charge density of the HDL_3 particles increased. This is in direct agreement with the results obtained in this study.

4.4 Effect of LDL charge on CETA

In incubations of CETP, native HDL and native LDL, a small amount of CE mass is transferred from LDL into HDL. The same experiment done with a reconstituted LpB shows that CE mass transfer occurs in the opposite direction. This difference in mass transfer between HDL and native LDL or LpB may be due to the difference in composition of the two apoB-containing lipoprotein particles. Addition of PI to the LpB particle almost completely inhibits CE mass transfer from HDL to LpB in the presence of CETP (Figure 19). This change in direction appears to be due to an increased negative charge density on LpB. The increase in net negative charge of the LpB particle also affects TG mass transfer. Inclusion of PI into the LpB particle appeared to reverse the direction of TG transfer that occurred (Figure 20) and promoted the transfer of TG out of LpB and into HDL. These studies show that an increase in the net negative charge of LDL promoted an increased CE and TG mass transfer into HDL. Other studies have found similar results with chemically modified LDL. Nishida *et al* used chemical modification of LDL to modify the lipoproteins' net negative surface charge [63]. They showed that a small increase in the negative charge on LDL promoted CE transfer from discoidal HDL into LDL, while greater increases eventually completely inhibited CE transfer into LDL.

Our data further shows that LDL charge can also affect the net mass transfer of CE between LDL and HDL particles.

4.5 Effect of anionic lipids on CETA in plasma

Incorporation of PI into lipoproteins in plasma *in vitro* also had a significant effect on lipid mass transfers. The addition of PI increased the net mass transfer of neutral lipid and free cholesterol out of the d<1.063 g/ml lipoproteins and into HDL. This is shown in Figure 22, where it is evident that the mass of all three lipids in LDL is reduced after incubation with PI. Phosphatidylinositol was previously shown to spontaneously incorporate into lipoproteins after *in vitro* incubation with them (Figure 21). Incubation of PI with plasma may increase the net negative charge of the plasma lipoproteins, in a similar manner to what occurs when a specific lipoprotein is incubated with PI. This increase in negative charge may regulate CETP and stimulate CETP-mediated neutral lipid and free cholesterol transfer to HDL.

Changes in lipoprotein composition and physical properties appears to control the direction of CETP-mediated lipid transfers in the plasma. Several studies indicate that abnormal plasma CETA in various dyslipidemic states may be related to alterations in the composition of plasma lipoproteins, rather than to changes in CETP mass. Sparks and coworkers demonstrated that in dysbetalipoproteinemia and hypoalphalipoproteinemia, CE transfer rates were increased towards VLDL and LDL, but decreased towards HDL [5, 118]. These observations may reflect alterations in lipoprotein composition. If non-esterified fatty acid (NEFA) metabolism and lipoprotein distribution is altered in dyslipidemic patients, this may partially explain/contribute to the increased CETP-mediated

transfer of CE into LDL/VLDL. NEFA can accumulate at the surface of lipoprotein particles and can enhance the CETP-mediated transfer of CE from HDL into VLDL and LDL [64, 74]. Although NEFA have a small effect on lipoprotein charge, it is thought that the increase in CETA into VLDL and LDL by NEFA incorporation is primarily due to their effect on lipid fluidity at the lipoprotein surface. Therefore, NEFA and PI may have unique and very different effects on CETP due, in part, to their very different effects on lipoprotein structure. Incorporation of PI into lipoproteins may be a novel way to affect lipoprotein surface charge and direct CETP-mediated neutral lipid transfer into HDL.

4.6 Effect of HDL stability on CETA

In this study, the free energy of denaturation of apoA-I on both Lp2A-I particle series were measured and are shown in Table 4. Figure 25 compares the CE transfer activities for both particle series to their thermodynamic stability. A relationship appears to exist between particle stability and CE transfer activity. As particle stability decreases, CE transfer into HDL increases. The POPC series particle with the least amount of POPC was shown to have the greatest ability to receive CE. There appears to be a stronger relationship between particle stability and CETA for POPC varying Lp2A-I particles than for particle charge and CETA. More experiments are necessary to form a complete picture of the effect of PI on particle stability and CE transfer activity, however it is apparent that a decrease in particle stability as PI content increases is concurrent with an increase in CE transfer activity. In general, PI decreases particle stability and increases the ability of the Lp2A-I to act as a substrate for CETP.

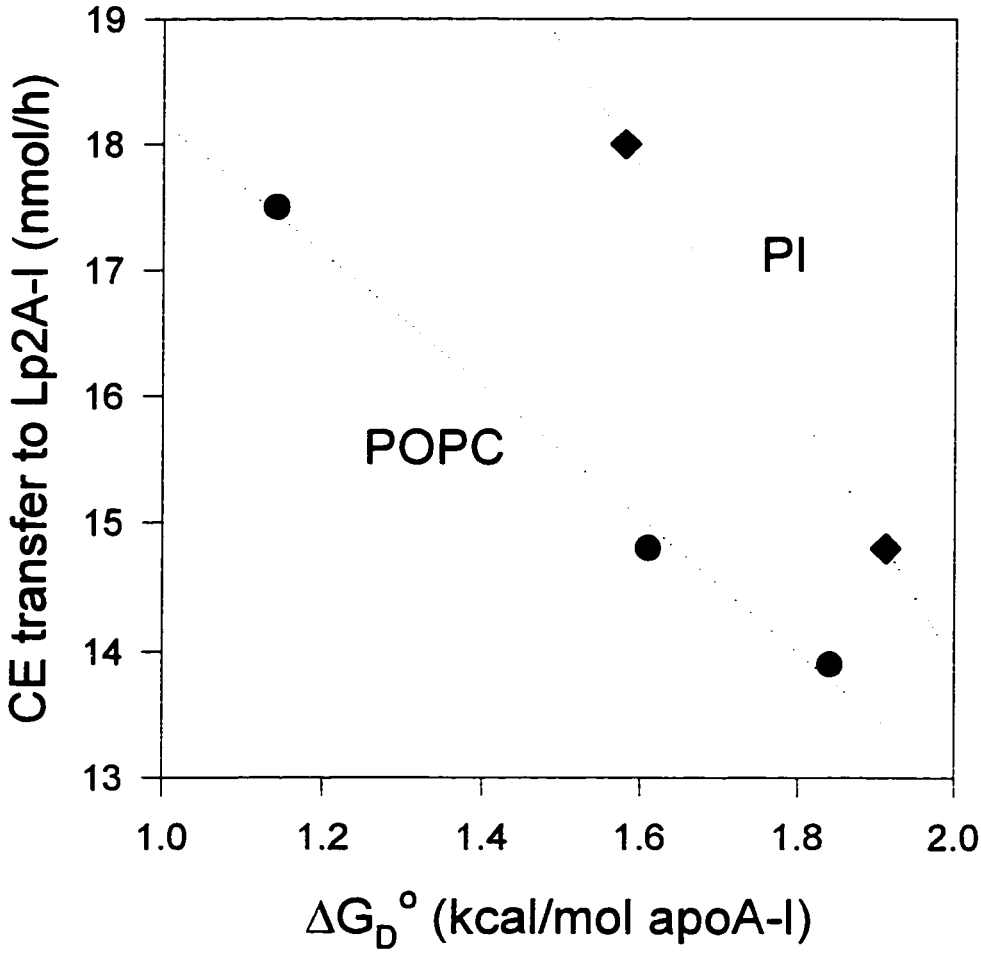


Figure 25. Effect of LpA-I Stability on CE Transfer

The rates of CE transfer from LDL into acceptor Lp2A-I varying in POPC content (46:14:2, 54:14:2, 82:16:2) and PI content (61:14:8:2, 48:11:16:2) are plotted against the particle stability (ΔG_D°). A possible inverse relationship is demonstrated between Lp2A-I stability and the ability of the particles to accept CE from LDL.

4.7 Mechanism of CETP

The purpose of this study was to identify some of the compositional and physical parameters that regulate the activity of CETP. The addition of PI to Lp2A-I particles increased the net negative charge of these particles and increases their ability to donate and receive CE. Altering POPC content also affected CETP mediated CE transfer, with transfer rates increasing as POPC content decreased. While PI had major effects on particle charge, POPC content had little effect on Lp2A-I electrostatic properties. Both series of particles, however, were shown to have an increase in CETP mediated CE transfer activity that was related to a decrease in particle stability. Foucher *et al* recently postulated that CETP activity is affected by lipoprotein fluidity [119]. Non-esterified fatty acids were observed to increase lipoprotein fluidity and CETP activity in reactions using these lipoproteins as substrates. It is possible that decreasing the particle stability may also affect CETP activity, by increasing the particle fluidity. These modifications of lipoprotein surface properties may affect one of the rate-limiting steps of the lipid transfer reaction, the availability of neutral lipids at the lipoprotein surface. Morton and Steinbrunner showed that the substrates for CETP may be the small amounts of neutral lipids which are localized near the phospholipid coat of lipoprotein particles [70]. Figure 26 presents the two rate limiting events of the lipid transfer reaction.

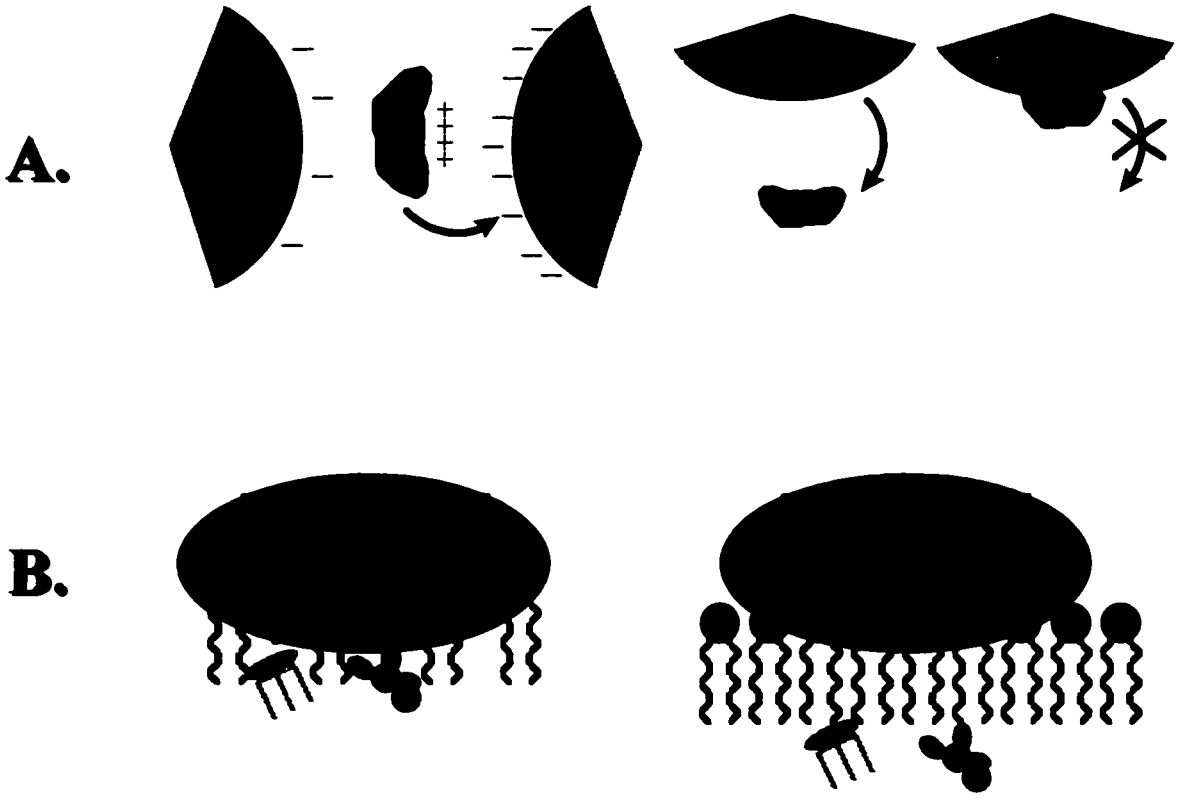


Figure 26. Rate limiting events of the CETP-mediated lipid transfer reaction.

A. Binding of CETP to lipoprotein substrates involves the presence of negative charges at the lipoprotein surface which interact with the positively charged CETP binding site. CETP binding too tightly may affect CETP lipid transfer activity.

B. Available neutral lipids localized near the phospholipid coat of lipoprotein particles act as CETP substrates. Increasing lipoprotein fluidity or decreasing particle stability may allow substrates to become more accessible.

In this study, the observations made may result from a combination of these rate-limiting steps being affected. Kato *et al* described the CETP reaction as being a delicate balance between electrostatic and hydrophobic forces [114]. It may be that the components of the CETP lipid transfer reaction that are affected are different for the two particle series. Addition of PI may have its affect on increasing the CETP reaction by increasing the net negative surface charge of the lipoprotein substrate. The 48:11:16:2 particle has the same thermodynamic stability as the 54:14:0:2, but a larger net negative

surface charge and increased CE transfer activity in CETP-mediated transfer reactions. Decreasing the stability of the particles in the POPC series may affect CETP and increase the lipid transfer reactions by making the neutral lipid substrates more accessible.

A recent study has shown CETP to modulate free cholesterol transfer as well as neutral lipid transfer [120]. The observations in this study have found that variation in POPC content had no effect on free cholesterol transfer, while the addition of PI into 54:14:0:2 particle increased free cholesterol mass transfer into the particles (Figure 18). This observation appears to support the hypothesis that POPC content of Lp2A-I modulates CETP activity by affecting lipoprotein surface fluidity and accessibility of substrates, whereas PI content modulates CETP mediated lipid transport by increasing the potential for binding. Since free cholesterol is predominantly found on the surface of lipoproteins, increasing the fluidity of the lipoprotein surface should not have an affect on free cholesterol transport. The increase in free cholesterol transfer by addition of PI, and lack of variation of free cholesterol transfer in the POPC particles may indicate that cholesterol transfer is primarily affected by particle charge in PI containing particles, rather than stability.

4.8 Physiological significance

The conclusion of this study is that CETP activity and substrate specificity are regulated by the physical properties of lipoprotein particles. The composition of the particles appears to dictate the direction of CETP-mediated neutral lipid transfer. Figure 27 summarizes the effect of charge on the direction of CE transfer between LDL and HDL.

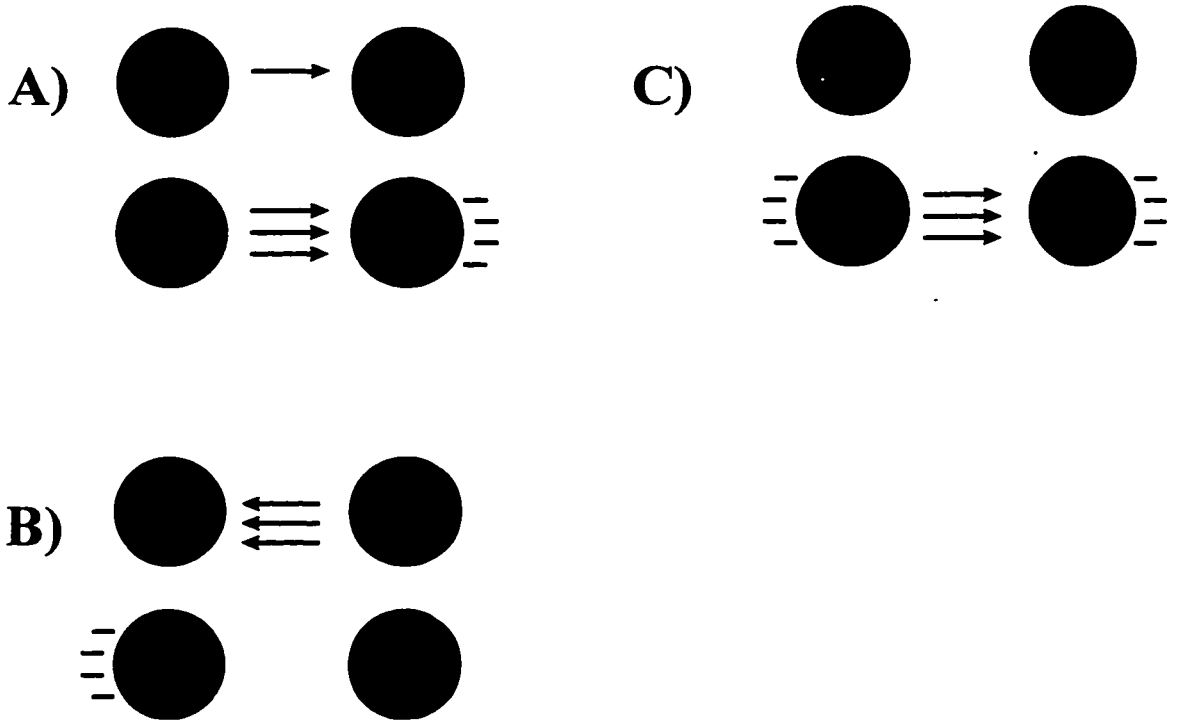


Figure 27. Effect of substrate charge on the direction and magnitude of CE transfer. A. CE transfer between LDL and Lp2A-I varying in PI content, B. HDL and LpB ± PI, C. plasma HDL and LDL, ± PI.

Increasing the net negative charge of Lp2A-I particles by the addition of PI stimulated the net mass transfer of LDL CE into the Lp2A-I particles (Figure 27, Panel A). The addition of PI to the LpB particle almost completely inhibited CE mass transfer from HDL (Panel B). Incubation of PI with plasma increased the net mass transfer of neutral lipid and free cholesterol out of the d<1.063 g/ml lipoproteins and into HDL, evident by the reduction in mass of all three lipids in the d<1.063 g/ml lipoprotein fraction of plasma (Panel C). In each study within this thesis, increasing the net negative charge of either lipoprotein substrate, increases the propensity of CE to be transferred to HDL, and decreases the transfer of CE into LDL. Therefore, it appears that lipoprotein concentration and CETP mass modulates the rate of lipid transfers while lipoprotein composition controls the direction of lipid mass movement.

Because of the possible contribution of CETP to atherogenesis, inhibitors of the CETP transfer process have been considered potential therapeutic agents. Very few inhibitors of CETP have been found, and pharmaceutical companies have failed to formulate a drug to inhibit CETP as a treatment for CAD. Inhibitors of CETP that would act through modification of the lipoprotein surfaces are undesirable from a therapeutic standpoint since their action is not going to be limited to CETP activity alone. A more useful strategy would be to modulate CETP and the direction of CE transfer. Modification of the lipoproteins by restructuring the surface lipids may affect the exchange reaction. As shown in the PI and plasma studies, adding PI to plasma causes an increased transfer of CE, TG and FC from LDL to HDL. This could be of benefit to hyperlipidemic patients by transferring these lipids out of the pro-atherogenic LDL. PI might be able to be injected into plasma directly, or in lipid microemulsions, which would fuse with lipoprotein particles after injection. Since in this lab, PI has been shown to have very little effect on LCAT, infusion of PI probably would have little effect on the production of CE. These studies demonstrate that modulation of CETP-mediated neutral lipid exchange can be achieved.

5 Future Studies

The study of the effect of lipoprotein composition on CETP activity has been hampered by the lack of data concerning the binding of CETP to the donor and acceptor lipoproteins. To investigate the details of the binding of CETP involved in neutral lipid transport requires the use of well-defined, homogeneous substrates, as well as a method of measuring the affinity and avidity of CETP for these substrates. The work presented in this thesis successfully showed that homogeneous well defined Lp2A-I particles that vary in PI and POPC content could be prepared and studied. One major experimental observation that is absent in this thesis is how PI and POPC composition affect the physical interaction between CETP and different lipoprotein particles. The binding of CETP to lipoprotein surfaces is of fundamental importance when considering the mechanisms of lipid transfer. Future prospects for this study will entail the examination of the reaction of purified CETP and reconstituted particles by Biomolecular Interaction Analysis (BIA). In this method, CETP is immobilized onto the surface of a sensor chip through amine coupling to a carboxymethyl dextran coat on the chip. Samples containing the Lp2A-I are then injected over the surface in a controlled flow. Any change in surface concentration resulting from interaction is detected as a surface plasmon resonance (SPR) signal, expressed in resonance units. Past studies have measured the binding of protein to multilammellar vesicles by determining the separation of bound from unbound species by ultracentrifugation [121]. The heterogeneity of the multilammellar vesicles makes the separation incomplete. Methods involving the enhancement of intrinsic fluorescence of the protein on binding to lipid surfaces are not acceptable because binding may occur without

any change in the quantum yields of the tryptophan residues in the protein [122]. The SPR biosensor present a novel method of directly observing the binding of proteins to lipoprotein surfaces. Studies on CETP binding to lipid emulsions have already been performed using a SPR biosensor [123]. These studies suffer the same problems as others in that the lipid emulsions that are used are heterogeneous and compositionally different. By using an SPR biosensor and homogeneous, compositionally well-defined Lp2A-I or LpB particles, future studies will provide further insight into the nature of the interactions between CETP and lipoprotein substrates.

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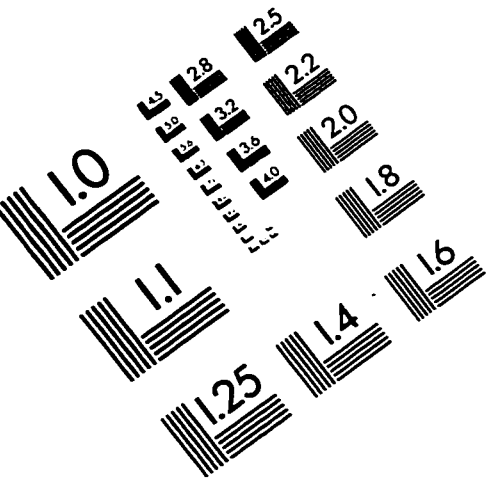

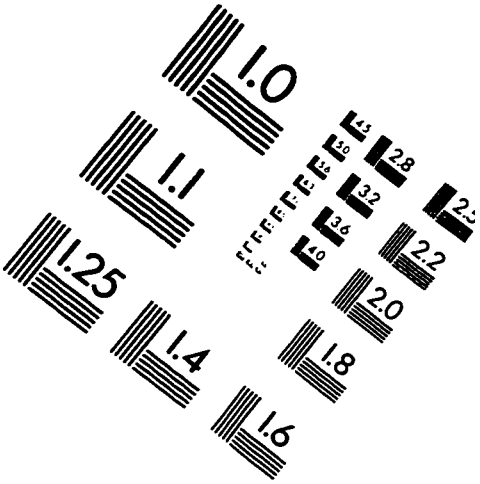

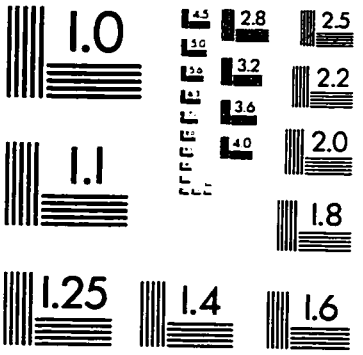

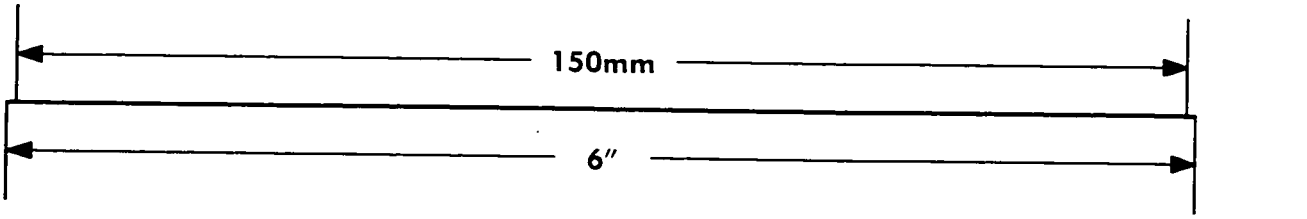

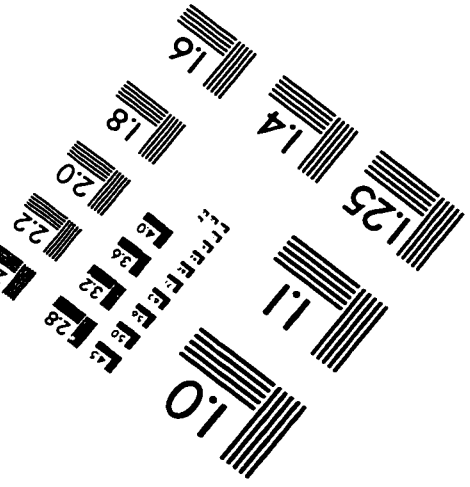

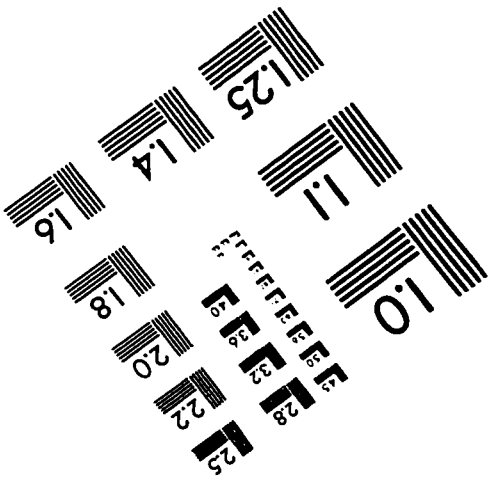

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