THE INTERACTION OF CYCLOSPORIN A WITH LIPOPROTEINS

by

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Abstract

The success of transplantation has been largely attributed to the introduction of the immunosuppressant, Cyclosporin A (CsA). However, patients receiving CsA frequently become dyslipidemic and this is thought to augment the nephrotoxic and hepatotoxic effects by interfering with the distribution and pharmacokinetics of CsA in plasma. In addition, CsA alone can cause hypercholesterolemia, which is believed to contribute to the pathogenesis of post-transplant atherosclerosis and coronary artery disease. The mechanism of CsA-induced hypercholesterolemia is unknown but it has been suggested that CsA affects the uptake of LDL at the level of the LDL-receptor.

The objectives of this thesis were two-fold: 1) to test the hypothesis that CsA contributes to dyslipidemia by decreasing the cellular uptake of LDL via the LDL-receptor and 2) to determine how lipoproteins affect the binding and distribution of CsA in human plasma.

To investigate the effect(s) of CsA on LDL uptake via the LDL-receptor, the binding, internalization, and degradation of ^{125}I-LDL *In vitro* was measured in human skin fibroblasts. The results show that CsA does not decrease LDL binding. Further, CsA did not decrease the B_{max} or K_d of the LDL for its receptor, nor did it decrease LDL-receptor mRNA levels. Contrary to my expectations, CsA significantly increased LDL degradation. To determine whether the association of CsA with LDL had any effect on the binding of LDL to its receptor, CsA was equilibrated with ^{125}I-LDL prior to its incubation with human skin fibroblasts. These results demonstrate that the association of CsA with LDL did not affect the binding or K_d of LDL to its receptor. Collectively, these data show that CsA does

not reduce LDL uptake by decreasing the binding, internalization, or degradation of LDL and this suggests that decreasing LDL uptake via the LDL-receptor is not a mechanism by which CsA contributes to hyperlipidemia in patients receiving this drug.

To investigate CsA's distribution in dyslipidemic plasma, CsA was added to plasma from the following groups: normolipidemic, hypercholesterolemic, hypertriglyceridemic, hypoalphalipoproteinemic, and a combination of hypercholesterolemic and hypertriglyceridemic. By using the phosphotungstic acid precipitation method to fractionate plasma, it was shown that the distribution of CsA in all of the dyslipidemic groups was significantly different from the normolipidemic control. In addition, the amount of CsA associated with the VLDL/LDL and HDL fractions was quite variable between the groups but the amount with the LPDP fraction remained relatively constant. These data suggest that factors other than the amount of lipid, such as the composition of the lipoprotein, play a role in the distribution of CsA.

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List of Abbreviations

ACAT	acyl CoA: acyl transferase
ANOVA	analysis of variance
ATP	adenosine triphosphate
AUC	area under the curve
B_{max}	concentration of binding sites
BSA	bovine serum albumin
CAD	coronary artery disease
cDNA	complementary deoxyribonucleic acid
CM	chylomicrons
CMV	cytomegalovirus
CsA	cyclosporin A, cyclosporine, ciclosporin, (Sandimmune®)
CPM	counts per minute
Ci	curie
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanidine triphosphate
DEPC	diethyl pyrocarbonate
DIFP	di-isopropyl phosphoflurodate
DMEM	Dulbecco's minimal essential media
DNA	deoxyribonucleic acid
DPM	disintegrations per minute

dTTP	deoxythymidine triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetate
FCR	fractional catabolic rate
FCS	fetal calf serum
g	gram(s)
HDL	high density lipoprotein
HDL_2	high density lipoprotein subfraction 2
HDL_3	high density lipoprotein subfraction 3
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HLA-DR	human leukocyte antigen-DR
HMG-CoA	3-hydroxy-3-methylglutaric coenzymeA
hr.	hours
IDL	intermediate density lipoprotein
INF-γ	gamma-interferon
IL-2	interleukin 2
kb	kilobase
K_d	dissociation equilibrium constant (affinity constant)
LCAT	lecithin:cholesterol acyl transferase
LDL	low density lipoprotein
Lp(a)	lipoprotein a
LPDP	lipoprotein-deficient plasma

LPDFCS	lipoprotein-deficient fetal calf serum
LPDS	lipoprotein-deficient serum
min	minute
MOPS	morpholinopropanesulfonic acid
mm	millimetre
mM	millimolar
mg	milligram
mmol	millimole
ml	millilitre
mRNA	messenger ribonucleic acid
NF-AT	nuclear factor of activated T-cells
ng	nanogram
nm	nanometre
NMR	nuclear magnetic resonance
OD	optical density
p	statistical probability
PBS	phosphate buffered saline
$pd(N)_6$	polydeoxynucleotide consisting of 6 bases
PEG	polyethylene glycol
PTxA	post-transplant atherosclerosis
PTA	phosphotungstic acid
r	correlation coefficient

r^2	coefficient of determination
RIA	radioimmunoassay
RNA	ribonucleic acid
SEM	standard error of the mean
SD	standard deviation
SDS	sodium dodecyl sulphate
Tris	tris(hydroxymethyl)aminomethane
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
μ	micro
μg	microgram
μl	microlitre
UV	ultraviolet
V	volts
VRC	vanadyl ribonucleoside complexes
VLDL	very low density lipoprotein
v/v	volume per volume
w/v	weight per volume
°C	degrees centigrade

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Dedication

This thesis is dedicated to my husband, Alan, my son, Daniel, and to the memory of Dr. Phil Reid.

Chapter 1 - General Introduction

1.1 The Impact of Cyclosporin A (CsA) on Transplantation

The introduction of the immunosuppressant Cyclosporin A (CsA) virtually revolutionized the field of organ transplantation by dramatically reducing the incidence of acute rejection and subsequently increasing graft and patient survival (1,2). As a result, transplantation has become the accepted mode of therapy for end-stage heart, kidney, and liver disease. In Canada, the 1-year *patient* survival rates for these procedures are 95% for renal[1] transplant recipients and 85% for both liver[2] and heart[3] recipients (3). Furthermore, CsA has proven useful in experimental studies of pancreas, heart-lung, and small bowel transplants and has contributed to their recognition as accepted modes of therapy (4). Currently, the limiting factor to the number of transplants performed yearly is the availability of donor organs (5). In addition to its use in transplantation, CsA has also proven to be an effective drug in the treatment of certain autoimmune disorders including psoriasis and uveitis (6,7).

Despite its effectiveness, CsA is associated with a number of side effects, the most significant being nephrotoxicity (6,8). This fact is distressing to all transplant recipients, particularly those with renal transplants, since it complicates their medical management. The nephrotoxic effects are minimized by decreasing the dose of CsA suggesting that the effect is dose-dependent. This has placed greater emphasis on the importance of monitoring blood

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[1] Statistics for first cadaveric graft and transplants performed 1990-1993 (3).

[2] Statistics for first graft, patients of all ages, and transplants performed 1991-1993 (3).

[3] Statistics for first graft and transplants performed 1991-1993 (3).

levels of CsA. However, the concentration of CsA in blood or plasma (9.10) correlates poorly with either the immunosuppressive or toxic effects of the drug. Part of this discrepancy is thought to reside with the well-recognized lipophilic properties of this drug. Since patients receiving CsA frequently are, or will become, hyperlipidemic (11,12), variable plasma lipid levels affect the drug's efficacy by modifying CsA's binding and distribution, and hence, its pharmacokinetics (13,14,15,). This has prompted some transplant centres to adopt elaborate pharmacokinetic studies on patients prior to their transplant surgery such that CsA dosing can be tailored to individual needs (16). However, complicating these matters is a more recently described side-effect of CsA; the drug itself increases plasma lipid levels (17,18,). More specifically, patients receiving CsA monotherapy for disorders unrelated to organ transplantation have increased total and low density lipoprotein (LDL) -cholesterol levels (18,19). The mechanism(s) by which CsA increases cholesterol levels is unknown.

There are two important consequences associated with CsA-induced hyperlipidemia. First, hyperlipidemia may contribute to enhanced toxic and/or reduced efficacy of CsA due to changes in the binding and distribution of the drug in plasma. Second, hyperlipidemia has been implicated as a risk factor in the pathogenesis of post-transplant atherosclerosis and cardiovascular disease - the leading causes of morbidity and mortality in long-term transplant survivors (19,20). So, while the beneficial effects of CsA on graft and patient survival in the short-term are unquestionable, the effects of CsA on long-term survival are less certain.

1.2 CsA and Transplantation

CsA was isolated from a strain of fungi, *Tolypocladium inflatum Gams*, in 1969 (21). Jean Borel and his associates at Sandoz Pharma Ltd. (Basle), discovered its

immunosuppressive effects and, after realizing its potential, CsA was purified and further characterized (21). By 1976, studies conducted worldwide employing a variety of techniques and models confirmed the immunosuppressive effects of the drug and the first clinical trials were performed in 1978. The clinical trials conducted with CsA have consistently demonstrated that CsA therapy, when compared to other immunosuppressants such as azathioprine, or corticosteroids, resulted in superior graft and patient survival in heart, renal, and liver recipients (1,2,22,23). Graft recipients receiving CsA have also been shown to have a significant reduction in the incidence of serious or fatal infections by bacteria (24,25) or other pathogens (26,27). Furthermore, CsA is neither myelotoxic nor mutagenic (28).

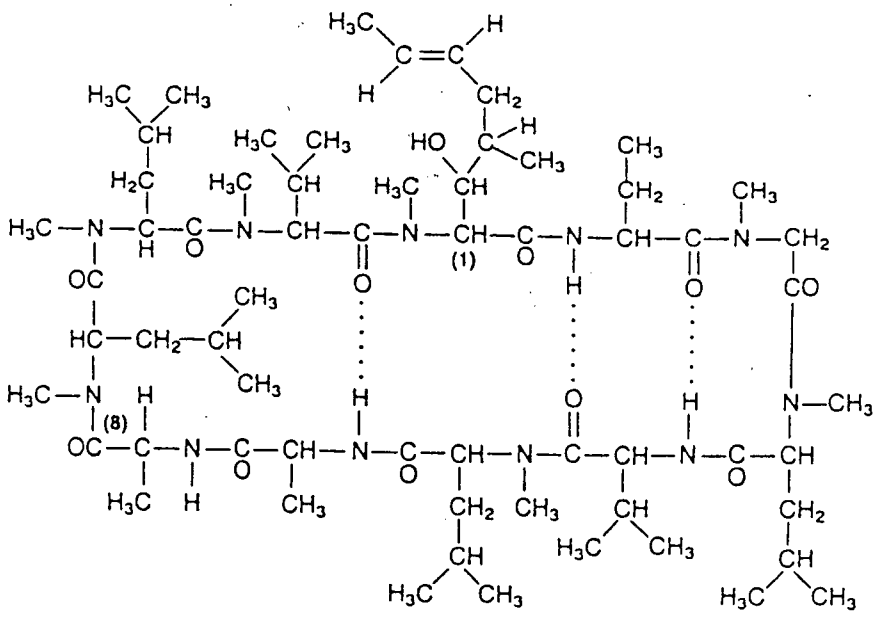


Figure 1. The molecular structure of CsA.

The use of CsA has broadened the spectrum of eligible patients for transplantation to include the very young and elderly, as well as pre-sensitized, diabetic, retransplant, and other high-risk patients (4,29,30). CsA therapy leads to a better quality of life (6), and is cost-effective (31,32). As a result of these findings, CsA is considered superior to other immunosuppressive agents (8) and has become the cornerstone immunosuppressant used in solid organ transplantation (6).

1.2.1 Chemistry of CsA

CsA is a cyclic polypeptide, consisting of 11-amino acids, with a molecular weight of 1202.64 (Figure 1). CsA is neutral and highly n-methylated and, as a result, is somewhat insoluble in water but soluble in organic solvents (8,9). The novel, 9-carbon, amino acid at position 1 ((4R)-4((E)-2-butenyl)-4,N-diethyl-L-threonine) was first described in CsA (8,9). The structure and conformation of CsA was elucidated from a series of experiments utilizing X-ray crystallography, NMR (nuclear magnetic resonance), and immunochemical reagents. The immunosuppressive activity of CsA resides in a cluster of amino acids on the surface of the molecule that includes amino acids 1, 2, 3, 10, and 11 (8,9).

1.2.2 Mode of Action of CsA

Current data suggest that the predominant effect of CsA is to reversibly inhibit the proliferation of T-helper cells while sparing T-suppressor cells. CsA decreases the production and release of IL-2 (interleukin-2) and other lymphokines (interferon-γ (INF-γ), B-cell growth factor) responsible for the proliferation and differentiation of T-lymphocytes and other immunocytes (8). The mechanism(s) by which CsA inhibits cell-mediated immunity is not well understood. A cytosolic binding protein for CsA, cyclophilin, has been identified

and cloned. Its sequence is highly conserved and is found in most eukaryote and prokaryote cells (33,34). The immunosuppressive effect of CsA is thought to be mediated through its binding to cyclophilin, and the subsequent binding of this drug-receptor complex to calcineurin, thereby inactivating calcineurin. Calcineurin is a Ca^{++}/calmodulin-dependent serine/threonine phosphatase. It is thought that in the T-cell receptor signal transduction pathway, calcineurin is responsible for the dephosphorylation, either directly or indirectly, of NF-AT (nuclear factor of activated T-cells) which results in the nuclear transport of this factor. NF-AT is one of a group of proteins that bind to the IL-2 enhancer and are thought to function co-operatively to activate and regulate transcription of the IL-2 gene (reviewed in 35-37).

1.2.3 Pharmacokinetics of CsA

Although CsA is absorbed from the upper small bowel, there is a large inter- and intrapatient variability in its absorption due to a number of factors including the presence of food, intestinal disease, diarrhea, liver function, and concurrent therapy with other drugs (8,9). Recently, the introduction of a microemulsion formulation of CsA, whose absorption is independent of bile and pancreatic juice, has greatly improved the oral bioavailability of this drug (38). CsA is widely distributed throughout the body with the highest concentrations in fat, adrenals, pancreas, and liver (9,39). In blood, approximately 40-60% is found with the red cells, 10-20% with leukocytes, and 30-40% in plasma (39,40). The high binding capacity of the red cells is apparently due to cyclophilin, the CsA-binding protein (41). In plasma, approximately 80% is bound to lipoproteins, 10-20% to other plasma proteins and the remainder is free (approximately 2%, although levels ranging from 5-20% have been

reported (39,40, rev. in 42). CsA undergoes extensive hepatic metabolism by the cytochrome P450 3A microsomal enzyme system to more than 20 metabolites by the hydroxylation, demethylation, carboxylation, and the cyclization at amino acid 1. There is also evidence to suggest that some metabolism of CsA occurs in the kidney and small intestine (8,9). CsA is excreted mainly in the bile (>90%), but approximately 6% is excreted in the urine (8,9). The half-life is reported to range from 15-27 hours in patients with normal renal function (8,9).

1.3 Plasma Lipoproteins

Plasma lipoproteins are a heterogenous population of particles which consist of a lipid core comprised of cholesteryl ester and triacylglycerol that is surrounded by a layer of unesterified cholesterol, proteins (termed apoproteins), and phospholipids. The apoproteins serve to solubilize the lipids for transport and are co-factors for many of the chemical interactions between the lipoprotein classes and with cell surfaces. There are four major classes, and several subclasses, of lipoproteins, as defined by their hydrated densities: chylomicrons (CM), very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). Each class differs in composition and size as outlined in Table 1 (43). Generally, the size of each class of lipoprotein is inversely related to its hydrated density. Lipoproteins undergo a series of complex metabolic processes in which their components are continually changed or exchanged within or between other lipoproteins. The role of the lipoproteins is not restricted to the transport of lipids, they also play an important part in cholesterol and triglyceride metabolism.

1.3.1 Chylomicrons (CM)

The CM and CM-remnants are the largest lipoproteins. Nascent chylomicrons are composed of cholesterol, triglycerides, and apo B-48. The fats are of dietary origin and the apo B-48 is synthesized by enterocytes. Upon synthesis, assembly, and secretion into the lymphatics by the intestinal mucosa, the chylomicrons enter the systemic circulation and acquire cholesterol, apo E and apo C from HDL. In the plasma, CM lose much of their triglycerides due to hydrolysis by lipoprotein lipase, with apo C functioning as a cofactor in

Table 1. Classification Properties and Composition of Human Serum Lipoproteins (from reference 43)

Parameter	CM	VLDL	LDL	HDL
Hydrated density (g/ml)	0.93	0.97	1.034	1.121
Solvent density for isolation (g/ml)	<1.006	<1.006	1.019-1.063	1.063-1.21
Molecular weight (X 10^6)	400-3000	5-10	2.75	0.36-.175
Diameter (nm)	>70	25-70	19.6-22.7	4-10
Cholesterol, unesterified (% by weight)	2	7	8	5
Cholesterol, esterified (% by weight)	3	12	42	13
Protein (% by weight)	2	8	22	40-50
Phospholipid (% by weight)	7	18	22	33-35
Triglyceride (% by weight)	86	55	6	3

CM; chylomicrons, VLDL; very low density lipoprotein, LDL; low density lipoprotein, HDL; high density lipoprotein

this process. This results in the formation of CM-remnants which are rapidly taken up by the

liver via an apo E-receptor mediated pathway (44).

1.3.2 VLDL and LDL

VLDL is synthesized, assembled, and secreted by hepatocytes. Nascent VLDL is a triglyceride-rich particle containing apo B-100 apo C and small amounts of apo E. Upon entering the circulation the VLDL particles acquire additional apo E, a considerable quantity of apo C, and cholesterol esters from HDL. Similar to chylomicrons, the triglycerides in the core of VLDL are hydrolyzed by lipoprotein lipase, with apo C functioning as a cofactor. This process is accompanied by the loss of some apo C and apo E, which are transferred back to HDL and results in the formation of VLDL-remnants (intermediate density lipoproteins (IDL)). The remnant particles are either taken up by the liver via the LDL-receptor pathway or converted to LDL by the continual removal of triglycerides, phospholipids, apo C, and apo E. The resulting LDL is a cholesterol-rich particle (approximately 60% cholesterol) with apo B-100 as its only apoprotein. There is also evidence to suggest that the liver can synthesize and secrete LDL directly (44).

LDL is the major carrier of cholesterol and cholesteryl esters. Approximately 60-70% (44) of LDL is removed via the LDL-receptor pathway, the remainder catabolized by LDL-receptor-independent pathways (44). The liver is responsible for degrading the majority of LDL and this is largely due to its high LDL-receptor activity (44,45).

1.2.3 The LDL-receptor

The LDL-receptor is the major regulator of intracellular cholesterol (44,45). The LDL-receptor pathway consists of an ordered sequence of events in which LDL is first bound to a high-affinity receptor on the cell surface, endocytosed via clathrin coated pits, and

delivered to lysosomes. Inside the lysosomes, cholesteryl esters are hydrolyzed and free cholesterol is made available to the cells while the receptor is recycled to the cell surface. The free cholesterol released by lysosomal hydrolysis has three well-defined effects on cellular cholesterol metabolism; it decreases 3-hydroxy-3-methylglutaric coenzyme A (HMG-CoA) reductase activity and thus suppresses cholesterol synthesis, it stimulates acyl-CoA:cholesterol acyltransferase (ACAT) activity, thus promoting its own esterification and, it decreases the synthesis of new LDL-receptors (44,45).

Brown and Goldstein first demonstrated the kinetics of LDL-receptor function *in vitro* by measuring the binding of ^{125}I-LDL in human skin fibroblasts in which the receptors had been upregulated by preincubating the cells in lipoprotein-deficient serum. At 37°C, binding equilibrium is reached in approximately 15 minutes and the rate at which new LDL particles are bound is balanced by the rate of internalization. The amount of ^{125}I-LDL within the cells increases and reaches a plateau where the rate of internalization is balanced with the rate of degradation. After an initial lag period, degraded ^{125}I-iodotyrosine appears in the medium, the amount of which increases at a constant rate until approximately 30 hours (44-47). There are approximately 7,500 to 15,000 receptors/cell (at 4°C) and this number varies depending on the rate of cell division, the age of the cells, and the cell's requirements for cholesterol. (44-47). The binding of ^{125}I-LDL is competitively inhibited by VLDL, a lipoprotein that shares the major apoprotein of LDL, apo B-100.

LDL-receptors have been detected in many human and animal tissues, however their activity differs widely from one cell type to another. Cultured cells are assumed to express LDL-receptor activity if they bind LDL with the same characteristics as those exhibited by

LDL binding in cultured human skin fibroblasts (44).

1.3.4 HDL

High density lipoproteins are a very heterogeneous group of lipoproteins and are comprised of 50% protein (mainly apo A-I and apo A-II) and 50% lipid (phospholipid, free and esterified cholesterol, and triglycerides). HDL is produced by the liver and intestine. The nascent, discoidal HDL acquires lipids and apoproteins (apo C and apo E) from the lipolysis of the triglyceride-rich lipoproteins, chylomicrons and VLDL. Mature, spherical HDL is thought to result from the generation of cholesteryl esters by lecithin:cholesterol acyl transferase (LCAT). The primary role of HDL is hypothesized to be the removal of cholesterol from peripheral tissue and its subsequent transport to the liver through a process known as reverse cholesterol transport (44,48). HDL is believed to receive unesterified cholesterol from the surface of other lipoproteins or from cellular membranes. The esterification of HDL-cholesterol by LCAT is thought to maintain the concentration gradient of cholesterol. The cholesteryl ester is then transported to the liver directly or transferred to other apo B-containing lipoproteins which are cleared via hepatic uptake (48).

1.4 The Hyperlipidemic Effect(s) of CsA

1.4.1 Post-transplant Hyperlipidemia

In the early 1970's it was reported (49), and later confirmed (50) that kidney transplantation did not remedy the hyperlipidemia associated with abnormal renal function, as it was hoped it would (49,50). Since the number and types of transplants has increased so has the incidence of post-transplant hyperlipidemia. Now there is an abundance of reports in the literature detailing significant increases in cholesterol (total and/or LDL-cholesterol), and

triglyceride levels in transplant patients, regardless of the type of transplant. The prevalence of hyperlipidemia in transplant patients approaches 75% (11,51,52) and hyperlipidemia has been documented in pediatric transplant recipients as well (52-55). Furthermore, researchers have reported that hyperlipidemia in transplant recipients is of a sufficient nature to necessitate medical intervention; either dietary modification or lipid-lowering drugs (56). Sharma *et al.* (55) found in their study of pediatric renal recipients that 32% of their patients warranted dietary intervention for their hyperlipidemia and 10% were candidates for lipid-lowering drugs according to the guidelines established by the National Cholesterol Education Program. In a separate pediatric cardiac transplant study, 86% of the children had cholesterol levels greater than the 90^{th} percentile, despite being on low fat diets (52). Most disturbing is that both pediatric and adult patients exhibit an "atherogenic lipid profile" (57). By that, it is meant that there are other plasma lipid factors present which are strong and independent predictors of coronary artery disease (CAD) in addition to an increase in total cholesterol (58). These include an increase in the cholesterol/HDL-cholesterol ratio, the LDL-cholesterol/HDL-cholesterol ratio, or a decrease in HDL-cholesterol levels (58). Additionally, there have been some reports of increased HDL_2 subfractions in cardiac transplant patients (59,60), and increased Lp(a) (lipoprotein (a)) levels in renal transplant recipients (61), both considered strong predictors of coronary artery disease (CAD). However, these data have not been substantiated in other studies of HDL_2 and Lp(a) levels in renal transplant recipients (59,62). Furthermore, several investigators have shown that the composition of various lipoproteins is altered in transplant patients and they are considered to be potentially atherogenic (63-65).

While the prevalence of post-transplant hyperlipidemia is well-known, the etiology is not. The causes of post-transplant hyperlipidemia are likely multifactorial (66,64) and have been reported to include poor graft function, pre-existing disease (*i.e.* diabetes), and drug therapy (*i.e.* diuretics, β-blockers, steroids) (66). In the past, high steroid doses were implicated as **the** major cause of the hyperlipidemia in renal transplant recipients (66). However, with the introduction of CsA, lower steroid doses were used and, in some cases, were withdrawn altogether (67), yet the hyperlipidemia persisted (66,67). Furthermore, investigators noticed the hyperlipidemic profiles in renal recipients had changed from primarily a hypertriglyceridemia, with or without hypercholesterolemia prior to transplantation, to a dominant hypercholesterolemia (66) (elevated LDL-cholesterol levels) following transplantation (12,68,69). In addition, the withdrawal of CsA from renal transplant patients, in an attempt to reduce chronic nephrotoxicity, was accompanied by a decrease in plasma cholesterol levels (56). This indirect evidence of CsA's hyperlipidemic effects prompted several researchers to investigate the role of CsA as an independent contributor to post-transplant hyperlipidemia. Several researchers (17,52,53,66,70,71) have investigated whether or not a correlation exists between the dose of CsA and the patients' lipid parameters. Some have found correlations between total and LDL-cholesterol, and CsA dosage or blood levels (66,71,72), however, many have not (52,53,70). Some of these findings suffered from the fact that they were conducted retrospectively on patients undergoing transplantation with various immunosuppressive and nutritional regimes. Nonetheless, recent studies have provided direct evidence that CsA induces hyperlipidemia in non-transplant patients (18,73). In one report, a prospective, double-blinded, placebo-

controlled, randomized trial of CsA monotherapy in patients with amyotrophic lateral sclerosis, Ballantyne *et al.* found that CsA significantly increased total and LDL-cholesterol levels (18). Furthermore, in a doubled-blinded, randomized trial of CsA monotherapy in patients with psoriasis, investigators found significant elevations in total cholesterol levels (73). LDL-cholesterol levels were not measured in this study. Since these reports, several others have now implicated CsA in the elevation of total and LDL-cholesterol (66), apolipoprotein B levels and cholesterol/HDL-cholesterol ratios (66) in transplant recipients. But the mechanism by which CsA elevates cholesterol levels, specifically LDL-cholesterol, remains poorly understood.

1.4.2 The Role of CsA in the Development of Hyperlipidemia

Generally, there are a number of mechanisms by which CsA may elevate plasma LDL-cholesterol levels; by increasing production of LDL, decreasing its clearance or, a combination of both.

CsA may contribute to an increased production of LDL by increasing the production of its precursor lipoprotein, VLDL (a triglyceride-rich particle). VLDL, in turn, would be catabolized to IDL (intermediate density lipoprotein) then LDL via the action of lipoprotein lipase. However, Lopez-Miranda *et al.* reported that in rats receiving CsA, the production rate of LDL was significantly decreased in comparison to untreated rats (74). However, rats are not a suitable animal model of human lipoprotein metabolism. In rats, there are decreased levels of LDL (in comparison to humans) and this is thought to be a result of the efficient clearance by the liver of CM and VLDL remnants (75). In addition, the rat lacks CETP and this absence results in the accumulation of cholesteryl esters in HDL and a relative paucity in

other lipoproteins (75). Kaptein *et al.* showed that the *in vitro* production rate of apo B-100 (a protein of VLDL and the sole protein of LDL) in $HepG_2$ cells was decreased in the presence of CsA (76). Recent studies in transplant recipients have demonstrated, by stepwise regression analysis, that there is no independent correlation between CsA and triglyceride levels (66). Collectively, these data imply that it is unlikely that CsA contributes to increased LDL-cholesterol levels by increasing the production of VLDL.

Another broad mechanism by which CsA may contribute to increased plasma LDL-cholesterol levels is by decreasing the clearance of LDL. Lopez-Miranda *et al.* found that the fractional catabolic rate (FCR) of LDL was lower in rats receiving CsA than in those who were not (74). They suggested that one of the mechanisms by which CsA decreases the FCR is by decreasing the blood flow to the liver (74). Although it has been shown that CsA decreases hepatic arterial blood flow (77), it is unlikely that this alone is responsible for the decreased clearance of LDL since the majority of the blood supplied to the liver is from the portal vein.

Since the liver is central to the metabolism of LDL, and is the primary organ responsible for its clearance, Lopez-Miranda *et al.* (74) and others (11,78,79) have suggested that the well-known, hepatotoxic effects of CsA, may be another mechanism by which CsA interferes with the clearance of LDL.

Clinically, CsA-induced hepatotoxicity is not as prevalent CsA-induced nephrotoxicity. The hepatotoxic effects are manifested by alterations in protein synthesis and bile acid metabolism (uptake, transport, and synthesis) (77,80). Usually, the elevations in plasma levels of bilirubin, alkaline phosphatase and transaminases (indicators of liver

function) are mild, implying that hepatocellular necrosis is not prevalent (77,80). Total protein, albumin, cholinesterase, and clotting factors V, VII, XII, and protein C have all been reported to be decreased in patients receiving CsA (80). These clinical observations have been supported by both *in vivo* and *in vitro* animal models of CsA-induced hepatotoxicity (80-82). Moreover, these effects were dose-dependent and were due to the parent drug alone, since increasing the production of CsA metabolites with enzyme inducers decreased the drug's toxicity (70,81). This effect is not limited to hepatocytes since there are a number of reports detailing dose-dependent decreases in protein synthesis in renal tubular cells, pancreatic exocrine and islet cells, and lymphocytes (80,81,83,84).

In addition to reducing hepatic protein synthesis, CsA has also been shown to inhibit both DNA and RNA synthesis as measured by the incorporation of ^{3}H-thymidine and ^{3}H-uridine respectively (80). This effect of CsA has also been described in activated lymphocytes, pancreatic, and kidney cells (80-84). Indeed, one mechanism by which CsA exerts its immunosuppressive effect is through the inhibition of mRNA synthesis for IL-2, INF-γ, and the IL-2 receptor (8,85). CsA has also been shown to affect the angiotensin type I receptor function by decreasing the number of binding sites, either at the level of mRNA (85) or protein (86) synthesis.

Since the liver metabolizes CsA, it has one of the highest concentrations of the drug (39,87,88). Moreover, the liver is also central to the metabolism of LDL-cholesterol as it is the primary organ responsible for the clearance of LDL particles via the LDL-receptor (89,90). Although it remains to be determined, it has been suggested that CsA may impair LDL-receptor function at the level of mRNA or protein synthesis (57,74). This would result

in a decreased number of binding sites, and therefore, an overall reduction in LDL binding. Alternatively, because CsA decreases hepatocellular uptake of a number of macromolecules including bile salts (taurocholate), ouabain (91) and albumin-bound bilirubin (80). The uptake of LDL, in addition to some other macromolecules, is dependent, not only on its binding to its specific receptor, but also on the endocytosis (internalization) of the receptor and ligand. Should CsA interfere with this process, then the uptake of LDL would be reduced.

One other mechanism by which CsA has been suggested to interfere with the clearance of LDL relates to the inherent hydrophobic properties of the drug and its subsequent binding to LDL. It has been proposed (92,93) that the binding of CsA to the LDL particle affects the uptake of LDL via the LDL-receptor, possibly by affecting the physical nature of the LDL particle (76,92,93). CsA has been demonstrated to bind to phospholipid vesicles (phosphatidyl choline) by partitioning into the hydrophobic bilayer (93). Once bound, CsA disrupts the membrane fluidity, as shown by a decrease in the transition temperature of the vesicle, thereby affecting the physical nature of the lipid bilayer (93). For this reason, it has been suggested that CsA, once bound to LDL, affects the physical properties of the lipid in a similar manner.

Modifying the physical nature of lipid bilayers in intact cellular membranes and reconstituted membranes can alter receptor affinity for its ligand, receptor number, and receptor-coupled functions. These phenomena have been demonstrated in the platelet thrombin, acetylcholine, and γ-aminobutyric acid receptors (94-97). To explain these observations, it has been proposed that membrane fluidity may contribute to changes in receptor conformation in the cell membrane (98,99). Additionally, mathematical analyses

have implied that a direct relationship exists between receptor turnover and membrane fluidity (98,99). However, the exact mechanism by which membrane fluidity results in these changes is unknown. Since CsA binds extensively to LDL, a cholesterol-rich phospholipid particle, and CsA alters the membrane fluidity in phospholipid vesicles, it has been hypothesized that CsA, once bound to LDL, alters the fluidity of the LDL particle (76,93,99). Since modifying membrane fluidity in other receptor systems has resulted in changes to receptor affinity, it has also been postulated that CsA bound to LDL modifies the affinity of LDL to its receptor, thus resulting in decreased LDL binding. This would ultimately result in a decreased clearance of LDL from the plasma.

1.4.3 The Consequences of Hyperlipidemia in Transplant Recipients

Regardless of the mechanism(s) for CsA's hyperlipidemic effects, the proposed consequences of increased plasma lipid levels are two-fold. First, hyperlipidemia has been implicated in the pathogenesis of post-transplant atherosclerosis (PTxA) and has been suggested to be a risk factor for the development of cardiovascular disease in transplant recipients. Collectively, these are the leading causes of morbidity and mortality in long-term transplant survivors (11,56). Second, due to the lipophilic nature of CsA, the drug is bound to lipoproteins in plasma. In cases of dyslipidemia, the binding of CsA to plasma lipoproteins is thought to interfere with the efficacy of the drug by modifying its distribution and hence, its pharmacokinetics (101-103).

1.5 Post-transplant Atherosclerosis

Post-transplant atherosclerosis (PTxA)(accelerated graft atherosclerosis) is a significant cause of late graft failure, morbidity and mortality in transplant recipients

(11,56,100). Despite the one-year *graft* survival in kidney recipients of 95% (3), only 20% are functional at 10 years (100). Furthermore, PTxA is the leading cause of retransplantation in liver recipients (104). In heart (105,106) and heart-lung (106) recipients the prevalence of PTxA is reported to be 50-60% at 5 years and is the major cause of morbidity and mortality (105,106).

The pathogenesis of PTxA is unknown. Although one may speculate that it is analogous to native CAD (coronary artery disease) seen in the general population (having no transplants), there are four major dissimilarities. First, the atherosclerotic lesion itself is different (20,104,107-109). It is characterized by a diffuse, concentric narrowing of the vessel lumen, whereas in CAD the lesion is focal and segmental (104). In CAD the lesions are confined to the epicardial arteries however, in PTxA they occur in the distal, secondary, and tertiary vessels. Ultra-structurally, both lesions exhibit marked intimal smooth muscle proliferation, however, in PTxA there is an accumulation of macrophages, T-cells, and foam cells in the intima. The amount of lipid accumulation in foam cells in PTxA is still the subject of discussion. Some investigators have reported that the lesion contains more lipoprotein, specifically LDL, than is present in native CAD (20), others have described lesions where variable amounts of lipid are seen (107,110). McManus *et al.* found that the coronary tissue level of total, free, and esterified cholesterol and phospholipid were greater than the 90th percentile of donor age-comparable, site-matched coronary tissue controls even though the pre- and post-transplant serum lipid values were not markedly elevated (111). However, most agree that calcium deposits are usually not present (20,108). One other distinction is that the endothelium in CAD is patchy and irregular but in PTxA the

endothelium usually remains intact (112). With some minor differences, the common features of the lesion of PTxA in cardiac recipients, namely perivascular inflammation, intimal thickening due to smooth muscle proliferation and the presence of inflammatory cells, are similar in kidney and liver recipients (104).

Second, PTxA progresses faster than native CAD and is present in both adult and pediatric graft recipients. The lesion has been described in cardiac transplant recipients in as little as 3 months post-transplant (113) but usually, within 3 years following transplantation, PTxA is prevalent (19,20,114).

A third and very notable distinction is that age (of the recipient) is not an independent risk factor for PTxA as in CAD (57).

Fourth, although the initiating event of PTxA has been hypothesized to be Type II vascular injury leading to the sequelae of platelet deposition, mitogen release, smooth muscle cell proliferation, and lipid and macrophage infiltration, the source of the vascular injury is debatable (115). Some investigators have suggested that viral injury, due to cytomegalovirus (CMV), is one possibility since the incidence of systemic CMV infections in transplant recipients is high (116).Two independent studies in cardiac recipients found a relationship between CMV infection and PTxA (117,118). Furthermore, the presence of CMV in human heart allografts has been shown using molecular techniques (118). Hruban *et al.* (118) and Wu *et al.* (120) found in their studies of coronary arteries in 9 allograft hearts with PTxA and 10 allograft hearts without PTxA, that the presence of CMV was higher in the PTxA allograft hearts. However, this result did not reach statistical significance (119,120). Arbusin *et al.* studied 45 endomyocardial biopsies from heart transplant recipients with CMV

infection and found CMV in 6 of 45 samples, using *in situ* hybridization, histological signs of viral infection, and immunohistochemistry of viral antigens (121). However, other researchers have suggested that CMV may not play a role in the pathogenesis of PTxA. Pahl *et al.* showed, in their study of 21 pediatric allograft recipients, that there was no correlation between the incidence of CMV infection shown serologically and the development of PTxA (122). Nadas *et al.* (123) investigated the presence of CMV genome and proteins in the blood vessels of kidney allografts. In the 24 biopsy specimens they studied, they found that none of the samples contained CMV despite the fact that PTxA was prevalent (123). This data is supported by the work of Gulizia *et al.* who investigated the presence of CMV in 41 human heart allografts and 22 donor age- and gender- comparable, and coronary site- matched controls (124). They found that there was no statistical difference between the prevalence of the CMV genome in the allograft or control groups, using both polymerase chain reaction and *in situ* hybridization techniques. In addition, there was no more evidence of allograft rejection in those grafts with CMV than in those grafts without. Furthermore, serological evidence of CMV infection in the allograft group and the presence of CMV DNA in allografts had no bearing on the duration of the graft. The authors suggested that their data argued against a direct role for CMV in PTxA (124).

Other sources for vascular injury have been suggested to arise from the procurement, preservation, and reperfusion of the donor organ (115). It is thought the vascular endothelium is damaged by the current methods of organ preservation and storage (116). But the most frequently proposed mechanism for the initiation of vascular injury is immune-mediated damage (19,114). In animal models of PTxA, the incidence and severity of the lesions are

decreased if the animal (rat) is treated with the immunosuppressants CsA and FK506 (125,126,127). In both animal models of PTxA (128) and human graft recipients (19,114,129), it has been demonstrated that activated T-cells, macrophages, and B cells constitute the major portion of the cells in the atherosclerotic lesions. Further, Uretsky *et al.* (68) reported that PTxA in cardiac transplant recipients was associated with 2 or more episodes of acute rejection. In addition, the presence of cytotoxic B-cell antibodies, thought to be directed at the HLA-DR antigen (human leukocyte antigen-DR) on vascular endothelial cells, was found to be a predictor of early myocardial infarction and sudden death in cardiac transplant recipients (130). Further, Winters *et al.* demonstrated that the relationship between the total number of rejection episodes and the percent luminal narrowing of the coronary arteries was highly significant (131). However, there is evidence to suggest that immune-mediated injury may not be necessary for the initiation of the vascular injury. Investigators have found that no correlation exists between the incidence of PTxA in cardiac graft recipients and the number of HLA mismatches or the number of rejection episodes (57). Furthermore, several centres have shown that with the introduction of improved immunosuppressive agents (CsA) and protocols, neither the incidence nor the severity of the disease has been reduced (68,132-134). In fact, the reverse may be true. In a review from Stanford, it was shown that with the introduction of CsA, the rate of cardiac vessel stenosis doubled (134).

While there are major dissimilarities between the pathogenesis of PTxA and native CAD, some aspects of both diseases are similar. Some of the suggested risk factors for PTxA (*i.e.* hypertension, hyperlipidemia, obesity) are analogous to those of native CAD

(19,20,114,131). In addition, Winters *et al.* showed, in their study of 15 failed human heart allografts, that the single, most predictive risk factor for the development of PTxA was post-transplant body mass index (131). It has also been suggested that hyperlipidemia may itself be a source of vascular injury in PTxA (135) and its role in the progression of the disease has been established in both animal models and humans. In one study, utilizing a rabbit model of cardiac transplant atherosclerosis, the authors demonstrated that immune-mediated injury, in the presence of a high cholesterol diet, accelerated the production of cholesterol-filled plaques (136). Further, in a rat model of cardiac transplant atherosclerosis, intimal thickening with lipid deposition was significantly increased in rats fed a combined diet that induced both hypercholesterolemia and hypertriglyceridemia in comparison to rats fed a normal diet (104). In a study of human heart transplant recipients, researchers concluded that increased levels of cholesterol and cytotoxic B-cell antibodies resulted in graft atherosclerosis (130). Moreover, these investigators demonstrated that the presence of an increased cholesterol level alone was a risk factor for the development of late (>3 years) PTxA (130). Since hyperlipidemia is a frequent occurrence following heart (11,12,51-53,59,137,138), liver (12,53), renal (54,63,70,71,139-144), and bone marrow transplantation (145), its role in the pathogenesis of PTxA is under currently under investigation.

1.6 The Binding and Distribution of CsA in Plasma

The significance of CsA-induced nephrotoxicity has placed a great emphasis on the importance of monitoring CsA blood levels. However, the concentration of CsA in blood, plasma (9,10), or in lipoprotein fractions (10,146) correlates poorly with either the immunosuppressive or toxic effects of this drug. Additionally, the drug's effects cannot be

predicted by the free drug concentration, usually a more reliable indicator of the drug's efficacy (147,148). Whole blood, plasma, and free CsA levels show a high degree of inter- and intra-patient variability (147,149) with traditional pharmacokinetic parameters such as AUC (area under the curve), clearance, and volume of distribution, being highly variable between patients receiving CsA (9). Part of this discrepancy is thought to reside with the well-recognized, lipophilic properties of this drug. In renal (13,150,151) and bone marrow (10,152) transplant patients, rabbits (14) and hyperlipidemic rats (153), changes in the pharmacokinetics of CsA were attributed to changes in the lipid values. One investigator (13) concluded that the variable lipid levels did not affect the metabolism of the drug, but rather affected the pharmacokinetics by changing the binding and distribution of CsA (13).

The distribution of CsA in whole blood is highly variable but has been reported to be: red cells (40-60%), leukocytes (10-20%), and plasma (30-40%) (39,40,154,155). This distribution profile changes with temperature at which the assay was performed, CsA concentration, and hematocrit (39,40,155). At lower temperatures (21°C), more CsA is associated with red cells, but this effect is reversible (39,100). Decreasing the hematocrit from 45% to 25% results in a 50% decrease of CsA in the red cells (39). In addition, the ratio of CsA in blood to that in plasma decreases with a corresponding increase in CsA concentration (40). The binding of CsA to red cells is saturable at levels approximating 500-800 ng/ml (39,156). In plasma, the majority of CsA (70-85%) is associated with the lipoproteins (39,40). The nature of this association is unclear but it is non-specific, of low affinity and high capacity suggesting that the CsA is physically dissolved in the lipid (157). Of the remaining drug in plasma, 10-20% is bound to plasma proteins and approximately 2%

is unbound (30,40,155). This distribution profile is also temperature dependant with more CsA being protein-bound at lower temperatures (39,40). Plasma fractionation studies have indicated that approximately 70-80% of the lipoprotein-associated CsA is associated with the LDL and HDL fractions; 7-10% is found with VLDL and $<1\%$ with the CM fraction (39,40). This distribution remains constant over a wide range of CsA concentrations (0.5 μg/l-38 μg/l) and, unlike red cells, lipoproteins are thought to be unsaturable even at very high concentrations of the drug (155).

Even though it is generally assumed that dyslipidemia affects the distribution of CsA, relatively few studies have been conducted on this subject. In addition, their conclusions are conflicting and some do not support this general hypothesis (154-157). For example, Zaghloul *et al.* demonstrated that there was no significant difference in the distribution of CsA in plasma between patients receiving CsA, normolipidemic serum (with CsA added to the sample), and non-fasted, hyperlipidemic subjects, although the lipid levels of these groups were not disclosed (156). Gurecki *et al.*, showed that the distribution of CsA in the lipoprotein fractions in heart and liver transplant patients, with "considerable variations" in their lipid levels, was similar to that of a "healthy" volunteer who was presumably normolipidemic (155). Although neither of these studies supported the general assumption that dyslipidemia affects CsA distribution, the hyperlipidemic populations they studied were poorly defined. In a case report of a patient with Type V Hyperlipoproteinemia, (increased triglycerides, VLDL, and CM) researchers showed a significant redistribution of the CsA such that the lipoproteins, mainly the CM and VLDL, carried up to 60% of the drug and the red cells only 40%, even though the hematocrit was normal (159). This resulted in

significant changes to the clearance and volume of distribution of the drug (159). In hypertriglyceridemic rats (due to bile duct ligation) receiving CsA, more CsA was associated with the VLDL and LDL fractions in comparison to rats having only sham surgery (160). Since most of these studies have been conducted in patients and animals which are hypertriglyceridemic (155,157,159,160), but the majority of lipoprotein-bound CsA is associated with LDL and HDL (cholesterol-rich particles), the effects of increased or decreased levels of HDL or LDL on the distribution of CsA are of great interest.

In addition to these studies being conducted with mostly hypertriglyceridemic plasma, or with ill-defined dyslipidemic plasma, these studies also suffer from the poor choice of techniques chosen to investigate the distribution of CsA. Without exception, all were done using the technique of ultracentrifugation (39,40,154,155,160). Ultracentrifugation separates plasma lipoproteins according to their hydrated densities (161). Besides being time consuming and expensive, some ultracentrifugation methods may not fully resolve all of the lipoprotein classes. Neither are these methods innocuous. By definition, ultra-centrifugation subjects samples to high g forces to achieve separation. Since these high g forces have been shown to erode HDL particles (166), they may affect the binding and subsequent distribution of CsA in plasma. Further, the high salt content of the density solutions, which are used to facilitate separation, may interfere with the binding of CsA to lipoproteins and other proteins (162). Since these experiments are usually conducted in density solutions to which no free drug is added, and are done over extended periods, (usually >18 hours) the possibility that the drug-protein complex dissociates is high (168). Some investigators have tried to overcome this problem by performing these experiments at lower temperatures (4°C) to slow

the dissociation rate (39,40), but because CsA binding is highly temperature dependant, this may have affected the results (162). However, those studies which were performed at 37°C to ensure correct temperature-dependant binding, failed to take steps to inhibit endogenous metabolism of the lipoprotein particles during the long ultracentrifugation periods (39,40). Since ultracentrifugation methods were also used to investigate the distribution of CsA in plasma from normal individuals, it is not surprising that those results are so variable as well (39,40,100,154,155).

Because the results have been conflicting and confusing, it is necessary to determine whether well-defined dyslipidemia affects the distribution of CsA in plasma. Furthermore, these studies should be conducted with a technique other than ultracentrifugation, due to its inherent limitations. These studies are required for a number of reasons. First, the incidence of dyslipidemia is high in transplant patients and other patients receiving CsA (11,12,66,137). Second, the consequences of CsA binding to lipoproteins are not well understood.

1.6.1 Biological Significance of the Binding of CsA to Lipoproteins

One of the proposed consequences of CsA binding to lipoproteins is that binding interferes with the pharmacological effects of the drug. But the mechanism(s) by which this occurs is unknown. Traditional pharmacokinetics predicts that only the free or unbound fraction of the drug can leave the blood stream, distribute in the extravascular space, and exert pharmacological activity (163. If this were the case, an excess of binding protein(s) would decrease the unbound fraction of the drug and hence, reduce the pharmacological effects. In contrast, if there is fewer binding protein(s), the unbound fraction of the drug

would increase and the pharmacological effects would be augmented; sometimes to the extent that toxicity occurs. This has been suggested as one of the mechanisms by which lipoproteins affect CsA's pharmacology. While there are some reports in the literature that demonstrate decreased pharmacological effects of CsA with hyperlipidemia (specifically hypertriglyceridemia) (101,102), and increased toxic effects of CsA with hypolipidemia (specifically hypocholesterolemia) (103), none of these investigators showed that the free fraction of the drug actually changed (101-103).

Other mechanisms by which lipoproteins affect CsA's pharmacology have also been proposed. It has been suggested by several investigators that protein-bound drug, in addition to unbound drug, may be available to the effector tissues, depending upon which protein the drug is associated (164,165). There are, however, two distinct processes by which this is thought to occur. LeMaire *et al.*(166), suggested that protein-bound CsA enters tissue according to the "free-intermediate model" as first proposed by Partridge *et al.* (164). Following their studies with other drugs (167), steroid and thyroid hormones (168,169), and free fatty acids (170), Partridge *et al.* found that the free ligand concentration (drug or hormone) measured *in vitro* was significantly different from that measured *in vivo*. Specifically, the *in vitro* concentrations underestimated the *in vivo* concentrations of the drug or hormone (171). For this reason, they suggested an alternate mechanism by which drug(s) are available to enter tissues. Their model hypothesized that protein-bound ligand is transported to tissues where the drug dissociates from the protein, and diffuses across the cellular membrane. Their model is dependent upon 1) a relatively slow capillary transit time, 2) a fast ligand dissociation rate and, 3) increased membrane permeability or ligand diffusion

(164). LeMaire *et al.* suggested that CsA's high hepatic extraction rate supported this hypothesis (166).

Another mechanism by which protein-bound CsA enters tissues has been proposed by de Groen (172). He suggested that the binding of CsA to LDL facilitates the transfer of CsA into cells or effector tissues via the LDL-receptor (172). (This hypothesis has been extended by others to include HDL and the HDL-receptor as well (173)). de Groen supported his hypothesis from evidence gathered from the literature. He argued 1) that the tissue distribution of CsA was similar to the distribution of the LDL-receptor, rather than to the distribution of CsA's intracellular binding protein, cyclophilin (39), 2) that all of the tissues that require cholesterol for the production of steroid hormones, presumably supplied by LDL, show susceptibility to the toxic effects of CsA (174), 3) that when less CsA is associated with the LDL fraction, in the case of hypertriglyceridemia or when CsA is incorporated into liposomes (175,176), the toxic effects of the drug are diminished and, 4) that CsA has a very high hepatic extraction rate, and that the liver has large number of LDL-receptors (172). This hypothesis is not without merit since LDL has been used experimentally as a drug delivery vehicle for lipophilic drugs for a number of years (177,178).

There is additional evidence to support the suggestion that protein-bound CsA may be able to enter cells. First, the unbound-CsA concentration is not a good predictor of either the drug's efficacy or toxicity (147,147,155). In fact, one researcher concluded, after analyzing more than 1800 unbound-CsA concentrations in patients, that unbound-CsA levels gave no additional clinical information compared to those of the total plasma concentration (148).

Second, in studies with hyperlipidemic rats and their lean litter mates, which were both treated with CsA, Luke demonstrated that the immunosuppressive effects of CsA on stimulated T-lymphocytes were similar in both groups despite the fact that the hyperlipidemic rats had increased binding of the drug in the vascular space (179). He suggested that the lipoprotein-bound CsA was pharmacologically active in the hyperlipidemic rats since less free CsA may have been available due to increased vascular binding (179). Third, and most convincingly, Rodl *et al.* demonstrated that lipoprotein-bound CsA was pharmacologically active *in vitro* (180). In addition, they showed an enhanced anti-proliferative effect on stimulated lymphocytes with LDL-bound CsA in comparison to free CsA (at the same concentration of CsA), LDL alone, or when CsA and LDL were added separately to the cells (180). This being the case, their experiments also support the suggestion of Partridge *et al.* (140) that the drugs's availability to tissue, and hence, its pharmacological (or toxic) effects, also depend upon the protein to which the drug is bound, since this enhanced antiproliferative effect that was seen with LDL-bound CsA was not evident with either VLDL or HDL (169).

1.7 Summary and Specific Aims

The success of organ transplantation has been largely attributed to the introduction of the immunosuppressant CsA. However, CsA therapy is not without side effects, the most significant being nephrotoxicity. Hyperlipidemia, which is common in transplant recipients, is thought to augment the toxic and/or pharmacological effects of this drug by interfering with the distribution of CsA in plasma. However, it has not been demonstrated how hyperlipidemia affects CsA distribution.

In addition to its effects on the distribution of CsA, hyperlipidemia in transplant

recipients is thought to play a role in the pathogenesis of post-transplant atherosclerosis and cardiovascular disorders; the leading causes of morbidity and mortality in long-term transplant survivors.

Recently, CsA has been shown to increase plasma lipid levels by an unknown mechanism. The hyperlipidemia is predominantly a hypercholesterolemia, specifically increased LDL-cholesterol levels. Further, studies in CsA-treated rats showed the fractional catabolic rate (FCR) and production rate of LDL was decreased (74). Collectively, these data suggest that CsA affects LDL clearance rather than the production of its precursor lipoprotein, VLDL. Since the LDL receptor is the primary mediator for the cellular uptake and subsequent clearance of LDL, it has been postulated that CsA affects the uptake of LDL at the level of the LDL-receptor. The proposed mechanisms for CsA's effects may be that the drug affects LDL-binding, by decreasing the number of binding sites, perhaps by decreasing LDL-receptor mRNA levels, or by decreasing the internalization and/or degradation of LDL. One other postulated mechanism for CsA's effects reside with the drug's inherent hydrophobic properties. CsA circulates in the plasma bound mainly to LDL and HDL and it has been suggested that the binding of CsA to LDL may result in an altered affinity of LDL for its receptor (see Figure 2).

The **working hypothesis** for this thesis is that CsA decreases the uptake of LDL via the LDL-receptor, thus contributing to dyslipidemia in patients receiving this drug. In addition, this dyslipidemia affects the distribution of CsA in plasma.

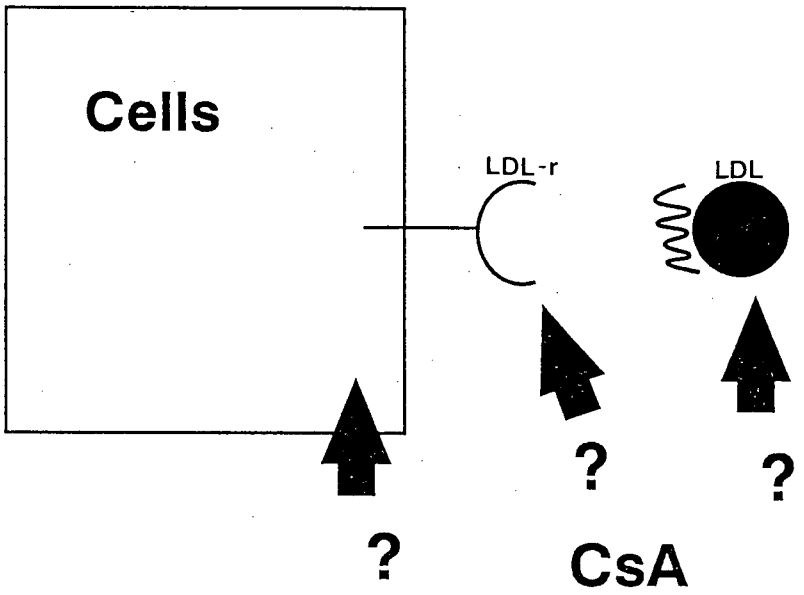


Figure 2. Proposed mechanisms for the hyperlipidemic effects of CsA.

The Specific Aims of this thesis are:

Part 1. The Effect of CsA on the Uptake of LDL via the LDL-receptor.

1) Determine whether the treatment of human skin fibroblasts with CsA *in vitro* affects their ability to bind, internalize, or degrade ^{125}I-LDL.

a) Measure the effects of CsA on the kinetic parameters of ^{125}I-LDL binding (B_{max}, K_d)

b) Determine the effects of CsA on ^{125}I-LDL binding, internalization, degradation.

c) Determine the effects of CsA on LDL-receptor mRNA levels

2) Determine whether or not the association of CsA with ^{125}I-LDL affects the affinity (K_d) of LDL for its receptor.

Part 2. The Binding and Distribution of CsA in plasma.

1) To establish a method for studying the distribution of CsA in plasma that meets the following criteria:

a) able to separate all major classes of lipoproteins (VLDL, LDL, and HDL) without affecting the distribution of CsA

b) is temperature insensitive

c) is practical and cost-effective

2) To compare the distribution of CsA in normolipidemic plasma to that of hypercholesterolemic, hypertriglyceridemic, hypoalphalipoproteinemic, and a mixed dyslipidemic plasma, using the method chosen above.

3) To determine the binding parameters of the individual lipoprotein classes with CsA

Chapter 2
The Effect of CsA on the Uptake of LDL via the LDL-receptor

2.1 Materials and Methods[4]

2.1.1 The Effect of CsA on the Binding, Internalization, and Degradation of ^{125}I-LDL via the LDL-receptor

2.1.1.1 Isolation of LDL

LDL (d=1.019-1.063 g/ml) was isolated from the serum of normolipidemic, healthy, fasted donors by sequential ultracentrifugation according to Havel *et al.* (181). Briefly, blood was collected by venipuncture into sterile tubes devoid of anticoagulant (Vacutainer, Becton Dickenson, NJ). Serum was separated from the cells by centrifugation at 1500 X g for 15 minutes at 4°C. The serum was then adjusted to a density of 1.019 g/ml by the addition of solid NaBr according to the Radding-Steinberg formula, $(N=(V(d_f-d_i))/(1-\nu \text{ X } d_f))$, where N=grams of sodium bromide, d_i=initial density, d_f=final density, V=volume of plasma in millilitres and ν is the partial specific volume of sodium bromide at 15°C (161). After density adjustment, the serum was centrifuged at 125,000 X g (Beckman L8-70, Beckman Instruments, Fullerton, CA) for 24 hours at 15°C. Following centrifugation, the top fraction removed by suction after removing the top of the tube by means of a tube slicer. The bottom fraction was resuspended and adjusted to a final density of 1.063 g/ml by the addition of solid NaBr as described above. Following this, the serum was centrifuged as described previously, and the top fraction, containing the LDL was removed as described. The LDL was recentrifuged at d=1.063 g/ml for an additional 24 hours. This latter centrifugation

[4] Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Company, St. Louis, MO.

served to clear the LDL preparation of any contaminating proteins as well as concentrating the LDL (47). The top fraction from this preparation was removed and dialyzed for 48 hours at 4°C with three changes of 150 mM NaCl, 0.24 mM EDTA, pH 7.4. The LDL was centrifuged at 10,000 rpm for 30 minutes at 4°C (Beckman J2-21, Fullerton, CA) to remove any aggregated LDL particles. Following this, the purity of the LDL preparation was confirmed by migration of a homogenous peak on agarose gel lipoprotein electrophoresis (182). The protein content was determined by the method described by Lowry *et al.* using bovine serum albumin as the standard (183).

2.1.1.2 Radioiodination of LDL

LDL was radioiodinated by the iodine monochloride method described by MacFarlane (184). LDL (2 mg of protein) was equilibrated with glycine, pH 10, by passing it through a Sephadex G25 column (Pharmacia, Dorval, Que). The LDL fractions, (recognized by their colour) were pooled, mixed with ICl (0.33 mM), $Na^{125}I$ (2 μCi) (Amersham, Oakville, Ont.) and glycine buffer (approximately 100 μl). The unincorporated label was separated from the incorporated label by exclusion chromatography through a Sephadex G25 column equilibrated with glycine, pH 10. The void fraction, containing the iodinated LDL, was equilibrated with HEPES, pH 7.4 by column chromatography through a Sephadex G25 column (Pharmacia, Dorval, Que.). The iodinated LDL was then characterized with respect to: a) the extent of the radiolabelling of the lipid fraction, b) the extent of radiolabelling of the protein fraction and, c) the specific activity. The radiolabelled LDL was stored at 4°C and used within 2 weeks (47).

2.1.1.3 Characterization of the Iodinated LDL

The extent of radiolabelling of the lipid in LDL was measured by mixing 10 μl of diluted, (1/100) radiolabelled LDL and 10 μl of unlabelled LDL (1 mg/ml) with 200 μl of methanol, 300 μl of $CHCl_3$, and 1 ml of diethyl ether. The contents were placed at -20°C for 10 minutes then centrifuged at 1500 x g for 30 minutes. The supernatant was removed and the radioactivity in the pellet and supernatant determined. The amount of protein-bound radioactivity was determined by precipitating an aliquot (100 μl of 1/100 dilution) of the radiolabelled LDL and unlabelled LDL (200 μl of 1 mg/ml) with isopropanol (300 μl). After a thorough mixing, the precipitate was collected by centrifugation for 10 minutes at 1500 X g. The amount of radioactivity in both the supernatant and precipitate was determined (LKB Clinigamma, Turku, Finland). The iodinated LDL met the following criteria: a) >98% of the ^{125}I-LDL was associated with the protein fraction, b) <5% of the ^{125}I was associated with the lipid, and c) the specific activity was in the range of 200-600 cpm/ng (159). A working stock solution of the ^{125}I-LDL ligand was prepared which corresponded to a specific activity of 50 cpm/ng protein and a protein concentration of 0.5 mg/ml (47).

2.1.1.4 Cell Culture of Human Skin Fibroblasts

On day 0, human skin fibroblasts (GM3348C,Human Genetic Mutant Cell Repository, Coriel Institute for Medical Research, Camden, NJ), between passages 10-20 (47), were plated on 60 X 15 mm culture dishes at a density of 0.5 x 10^5 cells/dish and grown in medium (DMEM (Dulbecco's modified essential medium), Gibco, Burlington Ont., 1% (v/v) Hybri-Max Antibiotic/Antimycotic solution) containing 20% (v/v) fetal calf serum (FCS) (Gibco, Burlington, Ont.) at 37°C and 5% CO_2 for 2 days, with or without the addition of

CsA (Sandoz Canada, Dorval, Que.). On day 3, the medium was replaced with fresh medium as described with 10% (v/v) FCS, with or without the addition of CsA and incubated for 3 days under the same conditions as described above. For the final 48 hours, the cells were grown in medium as above containing 10% (v/v) lipoprotein-deficient fetal calf serum (LPDFCS). The LPDFCS served to upregulate the number of LDL receptors (47). The determination of LDL-receptor activity was performed on day 7 when the cells were approximately 80% confluent.

For the cells grown in CsA at concentrations ranging from 5-15 μg/ml, the appropriate amount of CsA was added to warmed culture media (37°C) which contained either FCS or LPDFCS, incubated for 0.5 hour at 37°C, then added to the cells. The CsA was added on day 0 and each time the culture media was replaced. The CsA was dissolved in 99% ethanol to concentrations of 5 and 10 mg/ml of CsA and these concentrations were confirmed by RIA. Although the CsA was dissolved in 99% ethanol, at no point did the amount of ethanol in the cell cultures exceed 0.2% (v/v). This concentration of ethanol has been used by others to study the effects of CsA and they found that it does not effect cell growth or DNA synthesis in rabbit mesangial or endothelial cells (185). In addition, concentrations of < 10 mM ethanol, have no effect on ^{3}H-thymidine uptake or the stimulatory effects of insulin or insulin-like growth factor on DNA synthesis in NIH 3T3 fibroblasts (186). Further, ethanol at < 10 mM has no effect on the mitogenic effects of platelet-derived growth factor and fibroblast growth factor (186).

2.1.1.5 Determination of ^{125}I-LDL Binding, Internalization, and Degradation

The binding, internalization, and degradation of ^{125}I-LDL was performed according to

the methods of Goldstein *et al.* (47). The growth medium was removed and the cells were washed briefly in warm PBS. One millilitre of warm (37°C) medium (DMEM) containing 1% (v/v) LPDFCS and ^{125}I-LDL, in concentrations ranging from 2.5-50 μg/ml, in the presence or absence of 25-fold excess LDL (unlabelled) was added to the cells. The cells were incubated at 37°C for 2 hours after which the medium was removed for the determination of degraded ^{125}I-LDL (described below) and all further manipulations were carried out in a cold room at 4°C. The cells were washed quickly with 3 changes of an ice-cold buffer of 150 mM NaCl, 50 mM Tris-HCl, and 2 mg/ml of BSA. The cell monolayers were then incubated twice for 10 minutes in this buffer. The cells were then incubated in a buffered dextran sulphate solution (50 mM NaCl, 10 mM HEPES, pH 7.4, 4 mg/ml dextran sulphate) on a rotary shaker for 1 hour at 4°C. The buffered dextran sulphate solution was collected and the amount of radioactivity displaced by the dextran sulphate determined. The amount of ^{125}I-LDL displaced by the dextran sulphate corresponded to the surface-bound ^{125}I-LDL. The cells were then lysed by the addition of 1.0 ml of 0.1 N NaOH. An aliquot of the cell lysate was used for the determination of cellular protein (172) and the amount of radioactivity associated with the cells. The amount of cell-associated radioactivity corresponded to the amount of ^{125}I-LDL internalized.

To determine the degradation of ^{125}I-LDL to acid soluble material, the medium removed from the cells (1 ml) was added to 0.5 ml of 50% (w/v) trichloroacetic acid and incubated for 30 minutes at 4°C. The precipitate formed was removed by centrifugation at 800 X g for 15 minutes. A 1 ml aliquot of the supernatant was removed and 10 μl of 40% (w/v) KI and 40 μl of 30% H_2O_2 were added, and the mixture was incubated at room

temperature for 10 minutes. Following this, 2 ml of $CHCl_3$ was added, mixed thoroughly and the mixture was centrifuged at 800 X g to separate the phases. An aliquot of the upper, aqueous phase was removed to determine the amount of radioactivity. The radioactive counts were corrected for blank values, which were obtained in parallel experiments in the absence of fibroblasts (47).

The amount of bound or internalized ^{125}I-LDL was expressed as ng of LDL protein/mg cell protein and the amount of degraded ^{125}I-LDL was expressed as ng of LDL protein/mg cell protein/2 hr. With the exception of the cells treated with 15 μg/ml CsA, the amount of protein per dish was similar to that reported in other studies of LDL-receptor activity (180-250 μg/dish) (47,187).

At each concentration of LDL used to determine the binding, internalization, and degradation of ^{125}I-LDL, triplicate determinations were performed and controls for each were performed in duplicate and in parallel. In addition, at each concentration of CsA used, triplicate determinations of experimental group (CsA-treated) and controls were performed.

2.2.2 The Effect of LDL-associated CsA on the Binding of LDL to its Receptor

To prepare ^{125}I-LDL-CsA for binding experiments, iodinated LDL was incubated with CsA for 1 hour at 37°C. The binding of CsA to plasma lipoproteins *in vitro* reaches equilibrium within 30 minutes at 37°C (40). The ^{125}I-LDL-CsA was then chromatographed on a Sephadex G25 column (Pharmacia, Dorval, Que.) in a buffer of 0.15 M NaCl, 1 mM EDTA, and 0.03% (w/v) sodium azide to separate the bound CsA from the unbound fraction. Sufficient CsA was added to the iodinated LDL to achieve a concentration of either 0.03 μg or 0.3 μg CsA/μg LDL protein. The binding experiments were performed as

described in section 2.2.1.5. with the exception that those experiments performed with ^{125}I-LDL-CsA, the cells were incubated in absence or presence of 25-fold excess of LDL-CsA (unlabelled).

2.2.3 Effect of CsA on LDL-receptor mRNA Levels in Human Skin Fibroblasts

2.2.3.1 Cell Culture of Human Skin Fibroblasts

Human skin fibroblasts were plated and grown exactly as described above with the exceptions that they were plated at a density of 1.25 x 10^5 cells/dish in 150 X 15 mm culture dishes (Falcon, Lincoln Park, NJ). CsA, prepared as described above, was added to the cells to achieve concentrations ranging from 0.5-10 μg/ml. On day 7, when the cells were approximately 80% confluent, the cells were harvested and total cellular RNA was isolated by the method described below.

2.2.3.2 Isolation of Total RNA from Human Skin Fibroblasts

To control ribonuclease activity, all of the solutions described below were made with DEPC-treated (diethyl pyrocarbonate), sterile water. In addition, all glassware used in the preparation of these reagents was baked at 180°C for 8 hours.

The culture dishes were placed on a bed of ice and the cell monolayers were washed 3 times with ice-cold PBS. One millilitre of RNA extraction buffer containing 0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.6), 0.5% Nonidet-P40, 1mM dithiothreitol (DTT), 20 mM vanadyl ribonucleoside complexes (VRC) (Gibco, Burlington, Ont.) was added to each dish and allowed to spread evenly across the dish. Following this, 1.0 ml of proteinase digestion buffer (0.2 M Tris (pH 8.0), 25 mM EDTA, 0.3 M NaCl, 2% (w/v) SDS) was added and the mixture was allowed to spread evenly across each dish. The viscous lysate was

drawn up into a syringe fitted with a 21 gauge needle and forcefully ejected into a sterile polypropylene tube. This procedure was repeated 3 additional times to shear the cellular DNA. Proteinase K (Gibco, Burlington, Ont.) was then added to a final concentration of 200 μg/ml and the mixture incubated at 37°C for 30 minutes. Following incubation, the cellular protein was removed by extracting once with an equal volume of phenol:$CHCl_3$ (1:1). The aqueous and organic phases were separated by centrifugation for 20 minutes at 1500 X g and the aqueous phase transferred to a clean, sterile tube. Two and a half volumes of ice-cold ethanol (95%) were added to each tube, mixed thoroughly, and the mixtures placed at -20°C for 1 hour to precipitate the nucleotides. The precipitate was recovered by centrifugation at 1500 x g for 25 minutes. The supernatant was discarded and the pellet washed with 70% ethanol. The pellet was redissolved in 300 μl of 50 mM Tris (pH 7.8) and 1 mM EDTA. Magnesium chloride, DTT, and VCR were added to final concentrations of 10 mM, 0.1 mM, and 10 mM, respectively. Following this, RNase-free pancreatic DNase I (Pharmacia, Dorval, Que.) was added to a final concentration of 2 μg/ml and the mixture incubated for 1 hour at 37°C. Sodium dodecyl sulphate (SDS) and EDTA were added to a final concentration of 0.2% (w/v) and 10 mM, respectively. The mixture was extracted with an equal volume of phenol:chloroform (1:1). The organic and aqueous phases were separated as described above and the aqueous phase transferred to a sterile tube. Sodium acetate (pH 5.2) was added to a final concentration of 0.3 M and 2.5 volumes of ice-cold ethanol (95%) were added to each tube. The tubes were mixed thoroughly and placed at -20°C overnight to precipitate the RNA. The RNA was pelleted by centrifugation at 12,000 X g for 15 minutes and dissolved in 200 μl of TE (10 mM Tris, pH 8.0, 1 mM EDTA). To this, 0.5 ml of ethanol was added

and the RNA stored at -70°C. The RNA was recovered following the addition of sodium acetate (pH 5.2) to a final concentration of 0.3 M and 2.5 volumes of ethanol by centrifugation at 12,000 X g for 15 minutes. The RNA pellet was then dissolved in TE (10 mM Tris, pH 8.0, 1 mM EDTA) and an aliquot removed to determine the concentration by measuring OD_{260} (1 OD_{260}unit = 40 μg/ml of RNA) (188).

2.2.3.3 Denaturing Gel Electrophoresis of RNA

Electrophoresis of RNA was performed as described by Maniatis *et al.* (188). A 1% (w/v) agarose gel was prepared by melting agarose in DEPC-treated water. The gel was cooled briefly and 5 X gel running buffer (0.1 M MOPS (morpholinopropanesulfonic acid) pH 7.0, 40 mM sodium acetate, 5 mM EDTA (pH 8.0) and pre-warmed formaldehyde were added to give final concentrations of 1 X and 2.2 M, respectively. The agarose solution was cooled further, the gel was then cast and allowed to set for at least 30 minutes at room temperature. The RNA samples were prepared by mixing 0.5 μl of ethidium bromide, 2.5 μl of 5 X MOPS, 2 μl formaldehyde, and 5 μl of formamide with 20 μg RNA to make a final volume of between 18-22 μl. The samples were heated at 65°C for 15 minutes and placed on ice for 5 minutes. Two microlitres of loading buffer (50% (v/v) glycerol, 1 mM EDTA, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol) were added to the samples. The gel was electrophoresed for 2 hours in 1 X MOPS at 70 V after which the gels were photographed under UV light.

2.2.3.4 Transfer of RNA to Nylon Membranes

Northern transfer of the RNA was achieved by the method described by Maniatis *et al.* (188). Following electrophoresis, the gel was transferred to a dish and washed extensively

in DEPC-treated water to remove the formaldehyde. The gel was then placed on a support, the surface of which was covered with filter paper (Whatman 3 MM), in a plastic dish. The gel was placed on the filter paper in an inverted position and a wetted (1 X SSC) nylon membrane (Hybond, Amersham, Oakville, Ont.) cut to the same size as the gel, was placed on top of the gel, making sure that no air bubbles existed between the membrane and the gel. The gel was then carefully surrounded with saran wrap. Three pieces of wetted (1 X SSC) filter paper (Whatman 3MM), cut to the same size of the gel, were then placed on top of the nylon membrane and any bubbles between them were removed. A stack of paper towels were placed on top of the filter papers and weighted down. The plastic dish was then filled to approximately 3/4 full with 1 X SSC and the transfer was allowed to proceed overnight. Following the transfer, the nylon membrane was exposed to UV radiation for at least 5 minutes. The membrane was then hybridized to a radiolabelled cDNA probe for the human LDL receptor (189).

2.2.3.5 Preparation of LDL-receptor cDNA

A single bacterial colony containing the pLDLR-2 (ATCC, Rockville, MD) was transferred into 1.7 ml of LB broth containing 6.8 μl ampicillin (50 mg/ml) and incubated at 37°C for 8 hours with vigorous shaking. Following this, the culture was transferred to 250 ml LB broth with 25 mg ampicillin and incubated overnight at 37°C with vigorous shaking. After the overnight incubation, the bacterial cells were pelleted by centrifugation at 1500 X g for 20 minutes. The supernatant was discarded and the pellet was resuspended in 5 ml of a solution of 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 15% (w/v) sucrose, and incubated on ice for 20 minutes. Ten millilitres of a solution of 0.2 M NaOH and 1% (w/v) SDS were

added and the mixture further incubated for 10 minutes. Following this, 7.5 ml of a solution of 5 M potassium acetate, pH 4.8 was added and the mixture incubated on ice for 20 minutes. The mixture was centrifuged at 10,000 rpm (Beckman, J2-21, Fullerton, CA) for 20 minutes and the supernatant transferred to a clean, sterile tube. To this, 0.05 mg of ribonuclease (Pharmacia, Dorval, Que.) was added and the mixture incubated at 37°C for 20 minutes. The mixture was extracted twice with an equal volume of phenol:chloroform (1:1) and the upper, aqueous phase transferred to a clean, sterile tube. The DNA was precipitated by the addition of 2 volumes of ice-cold ethanol (95%) followed by incubation at -20°C for 30 minutes. The DNA was recovered by centrifugation at 1500 X g for 15 minutes at 4°C and resuspended in 1.6 ml of sterile water and 0.4 ml of 5 M NaCl and 2.0 ml 13% (w/v) PEG-8000 was added. This mixture was incubated on ice for 1 hour after which the precipitate was recovered by centrifugation at 12,000 rpm (Beckman, J2-21, Fullerton CA) for 15 minutes. The DNA pellet was washed with 70% ethanol, allowed to dry briefly, then resuspended in 250 μl of TE (10 mM Tris, pH 8.0, 1 mM EDTA) (175). An aliquot was removed and the concentration determined by UV absorbance at 260 nm. In addition, the absorbance ratio at 260:280 nm was also determined and was always greater than 1.8 (Beckman 3B UV/Vis Spectrophotometer, Fullerton, CA).

A 1 kb fragment of the LDL-receptor cDNA was then cut from the plasmid DNA, which contained the full length LDL-receptor cDNA, by restriction digest and agarose gel electrophoresis. Briefly, 1.125 μg of DNA was incubated with 5 μl of 10 X NEB buffer, (New England Biolabs, Inc., Arlington Heights, IL) 1.5 μl of each Bgl II and Eco RI (New England Biolabs,Inc., Arlington Heights, IL) 22 μl of sterile, distilled water, for 3 hours at

37°C. The digested DNA, molecular weight marker, and uncut plasmid DNA, were mixed (separately) with 2 μl of loading buffer (50% (w/v) glycerol, 1 mM EDTA, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol) then electrophoresed in a 0.8% (w/v) agarose gel in 1 X TAE (0.4 M Tris-acetate, 1 mM EDTA), containing 25 μl of ethidium bromide, at 90 V for 1 hour after which the 1 kb fragment was removed from the gel following visualization with a UV radiation. The 1 kb fragment was purified from the gel by the use of GeneClean II (BIO 101, Inc., La Jolla, CA.) kit with reagents and instructions provided by the manufacturer. The LDL-receptor cDNA probe was then stored at -20°C until needed.

2.2.3.6 Radiolabelling of the cDNA Probe for the LDL-receptor

One hundred and twenty-five nanograms of LDL receptor cDNA was placed in a sterile tube and enough sterile water added to give a final volume of 130 μl. The cDNA was boiled for 5 minutes then placed on ice for 5 minutes. Added to the cDNA was: 8 μl BSA (10 mg/ml), 20 μl ^{32}P-dATP (Amersham, Oakville, Ont.), 2 μl Klenow fragment (Pharmacia, Dorval, Que.) and 40 μl of 5 X OLB buffer. (OLB buffer consists of a mixture of three solutions, A, B, and C, in a ratio of 100:250:150. Solution A was comprised of 1 ml Solution O, 18 μl 2-mercaptoethanol, 5 μl of 100 mM of each dCTP, dTTP, dGTP. Solution O was 1.25 M Tris pH 8.0, 0.125 M $MgCl_2$. Solution B was 2 M HEPES, pH 6.6 and Solution C consisted of 50 OD units $pd(N)_6$ in TE (10 mM Tris, pH 8.0, 1 mM EDTA) at 90 OD units/μl.) The mixture was allowed to incubate for 4 hours at room temperature (175). The labelled cDNA probe was then boiled for 5 minutes and placed on ice for an additional 5 minutes before adding to the hybridization buffer.

2.2.3.7 Northern Hybridization

The 6 nylon membranes were placed in 3 heat-sealable plastic bags such that the top surface of each membrane faced outwards. The membranes were wetted with 10 ml of 2 X SSC for 10 minutes. The SSC was replaced with 7 ml of hybridization buffer consisting of 1% (w/v) BSA, 7% (w/v) SDS, 1 mM EDTA, 250 mM Na_2HPO_4, and 250 mM NaH_2PO_4 and the membrane was prehybridized at 65°C for 30 minutes. Following this, 67 μl of the radiolabelled cDNA probe, which had been previously boiled then quenched on ice, was added to each of the 3 bags. The bags were sealed and hybridized overnight at 65°C. Following hybridization the membranes were washed in 0.1 X SSC, 0.5% (w/v) SDS for 15 minutes at room temperature then a second wash was performed in the same solution but at 65°C for 15 minutes. The filter was briefly rinsed in 0.1 X SSC and blotted with paper towels. The membrane was wrapped in saran wrap and exposed to X-ray film (OMAT-AR, Eastman Kodak Co., Rochester, NY) with intensifying screens at -70°C for 4 hours to locate the radioactive bands (175). The bands were cut from the membrane, using the autoradiogram as a guide, and the amount of radioactivity associated with each band determined (Beckman LS-9000 Liquid Scintillation Counter, Beckman, Fullerton, CA).

2.2.4 Analysis of Binding Data

For the analysis of the binding data I assumed, as have others (190), that the interaction of LDL with the LDL-receptor could be described by a single-site model according to the equation:

$$[\![L]\!] + [\![R]\!] \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [\![LR]\!]$$

where $[\![L]\!]$ is the free LDL (ligand), $[\![R]\!]$ the unoccupied receptor and, $[\![LR]\!]$ the LDL-

receptor complex. At equilibrium, when the forward and reverse reactions are equal, the law of mass action states:

$$K_d = [\![L]\!] [\![R]\!] / [\![LR]\!]$$

where $K_d = k_{-1}/k_1$. If the total receptor concentration is B_{max} ($B_{max} = [\![R]\!] + [\![RL]\!]$) then:

$$K_d = [\![L]\!](B_{max} - [\![LR]\!]) / [\![LR]\!]$$

and

$$[\![LR]\!] = B_{max} [\![L]\!] / K_d + [\![LR]\!]$$

and this latter equation is commonly referred to as the Langmuir binding isotherm (191). The experimental data were fitted to this equation using a non-linear regression routine from the InPlot computer program (GraphPad, San Diego, CA) and the K_d and B_{max} were calculated. The algorithm used to adjust the variables, to quantitate goodness-of-fit, and to determine non-convergence is based on the method of Marquardt (192), with the modification of Tabato and Ito (193). Inplot defines convergence as "the point at which an iteration changes the value of each variable by $<0.01\%$ or changes the residual sum of the squares by $<0.01\%$." (194). Inplot is a recommended computer program for the analysis of binding data (182).

2.2.5 Statistical Analyses

2.2.5.1 Statistical Analysis of LDL-receptor Activity

A classical 3-way analysis of variance (ANOVA) was performed for each of the three parameters of LDL-receptor activity; binding, internalization, and degradation. The three factors included as main effects in the analysis were concentration of LDL, date the analysis was performed, and experimental group (control or CsA-treated cells). Two important assumptions for parametric statistical analyses are: 1) normally distributed residuals and, 2)

homogeneity of variance (196). In this study, the plots of residuals *vs.* predicted values indicated the residuals were normally distributed however there was heterogeneity in the variance. Because the standard deviations were proportional to the means, the data were log-transformed to equalize the variance of residuals prior to analysis as recommended by Zar (183). The 3-way ANOVA was performed using SAS (Statistical Analysis System, SAS Institute Inc., Cary, NC) computer program and differences were considered statistically significant at $p < 0.05$.

2.2.5.2 Statistical Analysis of Protein Levels

A 2-way ANOVA was performed for the analysis of protein levels per culture dish. The two factors included as main effects in the analysis were the experimental group (control or CsA-treated cells) and the date of the analysis. The 2-way ANOVA was performed using SigmaStat (Jandel Corporation, Sausalito, CA) computer program and the differences were considered statistically significant at $p < 0.05$.

2.2.5.3 Statistical Analyses of K_d and B_{max}, and LDL-receptor mRNA Levels

The K_d and B_{max} were calculated as described previously and the goodness-of-fit was assessed by examining the r^2 (coefficient of determination), the graph of the curve superimposed on the data points, and a plot of the residuals as recommended by Motulsky and Ransnas (197). The differences between the experimental groups (control and CsA-treated cells or ^{125}I-LDL (control) and ^{125}I-LDL-CsA) were compared using a paired Student's t-test as recommended by Zar (196) and Motulsky and Ransnas (197). An unpaired Student's t-test was used to compare the differences between the control and CsA-treated fibroblasts for the analysis of LDL-receptor mRNA levels. Differences were considered statistically

significant at $p < 0.05$ where p represents the probability of a two-tailed test.

2.3 Results

2.3.1. The Analysis of LDL-receptor Activity

Representative graphs of the binding, internalization, and degradation of ^{125}I-LDL in human skin fibroblasts are shown in Figures 3, 4, and 5, respectively. Specific binding, internalization, and degradation were calculated as the difference between the total and non-specific component. In addition, in the calculation of ^{125}I-LDL degradation, the radioactive counts were corrected for blank values which were obtained in parallel experiments in the absence of cells. These counts did not exceed 0.02% of the total counts.

The total binding curve (Figure 3) is typically comprised of two components; a high affinity component where the amount of LDL bound rises sharply and linearly with an increasing LDL concentration, and a low affinity component where the binding of LDL is still linear, but the slope of the line is less steep (46). In contrast, the total degradation curve (Figure 5) is comprised of only a linear component (46). The binding, internalization, and degradation curves are typical of that seen with fibroblasts that were incubated in LPDFCS for $>$24 hours prior to the assay being performed. In contrast, cells that were not incubated in LPDFCS result in binding and internalization curves like those depicted in Figures 6 and 7. The curves are comprised of only one component which is linear and has a slope that is not steep. The specific binding and internalization at 10 μg/ml of LDL (Figures 3 and 4) are similar to that reported by Goldstein and Brown (47). However, the specific degradation at this concentration of LDL is approximately 2-fold higher (47).

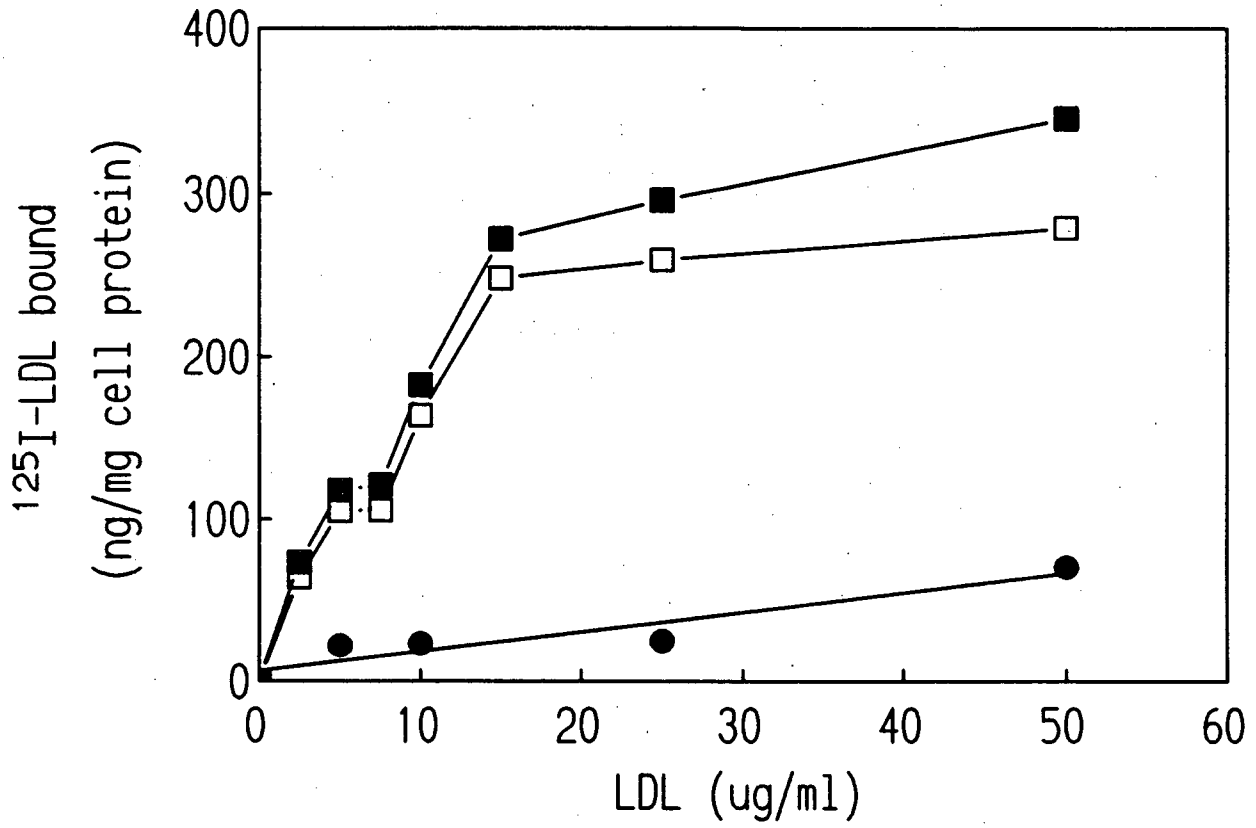


Figure 3. Representative graph of total, specific, and non-specific binding of ^{125}I-LDL to human skin fibroblasts as a function of LDL concentration. Cell monolayers were prepared as described in Materials and Methods. Prior to each binding experiment, the medium was replaced with the indicated concentrations of ^{125}I-LDL in the presence or absence of 25-fold excess of LDL (unlabelled). After incubation for 2 hours at 37°C the cells were washed as described in Materials and Methods then incubated at 4°C in a medium containing dextran sulphate to release the surface-bound LDL. The amount of surface-bound ^{125}I-LDL released was then determined. The cells were lysed by the addition of 0.1N NaOH and an aliquot removed for the determination of protein. The specific binding (□), was calculated from the difference between total (■), and non-specific (●), binding. Each value represents the average of triplicate determinations.

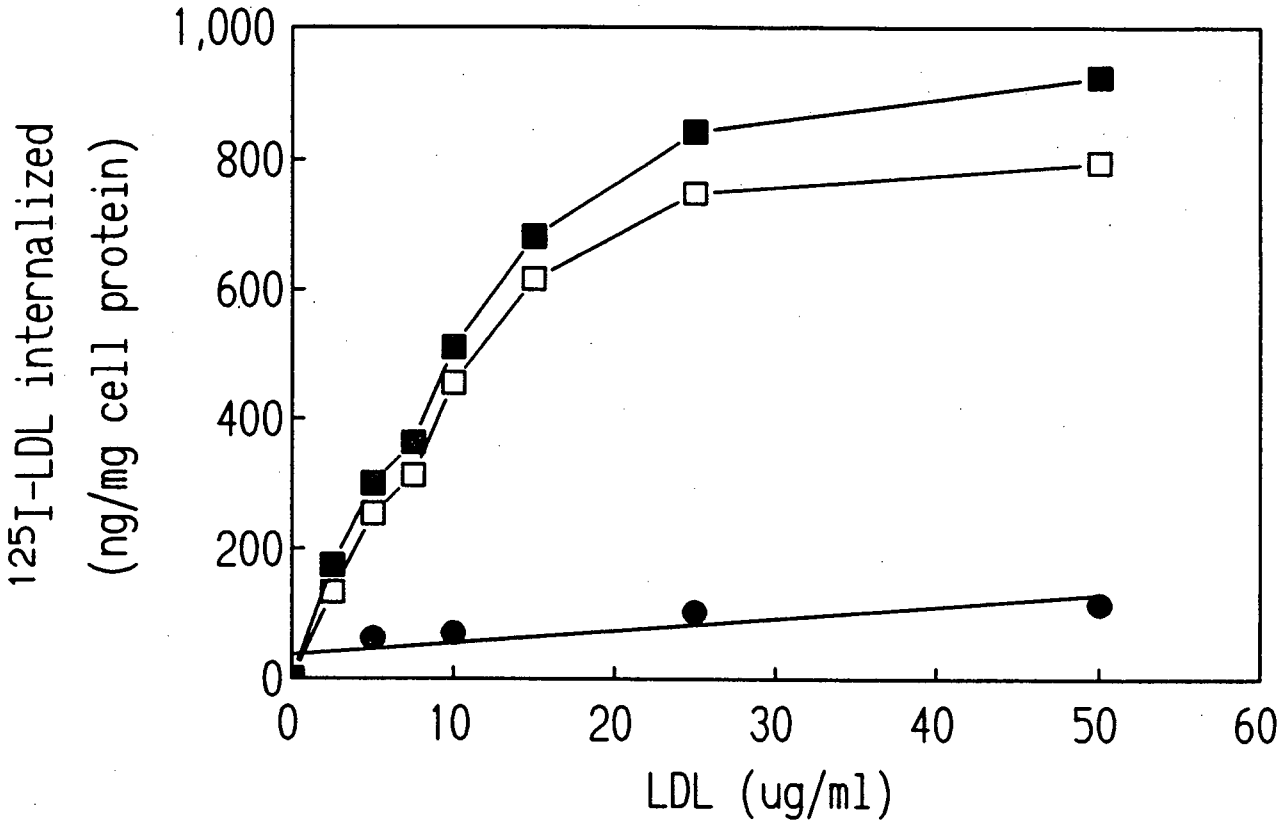


Figure 4. Representative graph of total, specific, and non-specific internalization of ^{125}I-LDL in human skin fibroblasts as a function of LDL concentration. Cell monolayers were grown as in Figure 3. Prior to each experiment the medium was replaced with the indicated concentrations of ^{125}I-LDL and the cells were incubated for 2 hours at 37°C. The medium was removed, the cells washed, and the surface-bound LDL removed as in Figure 3. The cells were lysed by the addition of 0.1 N NaOH and the amount of radioactivity associated with the cell lysates and the protein content determined. Specific internalization (□), was calculated from the difference between total (■), and non-specific (●), internalization. Each value represents the average of 3 determinations.

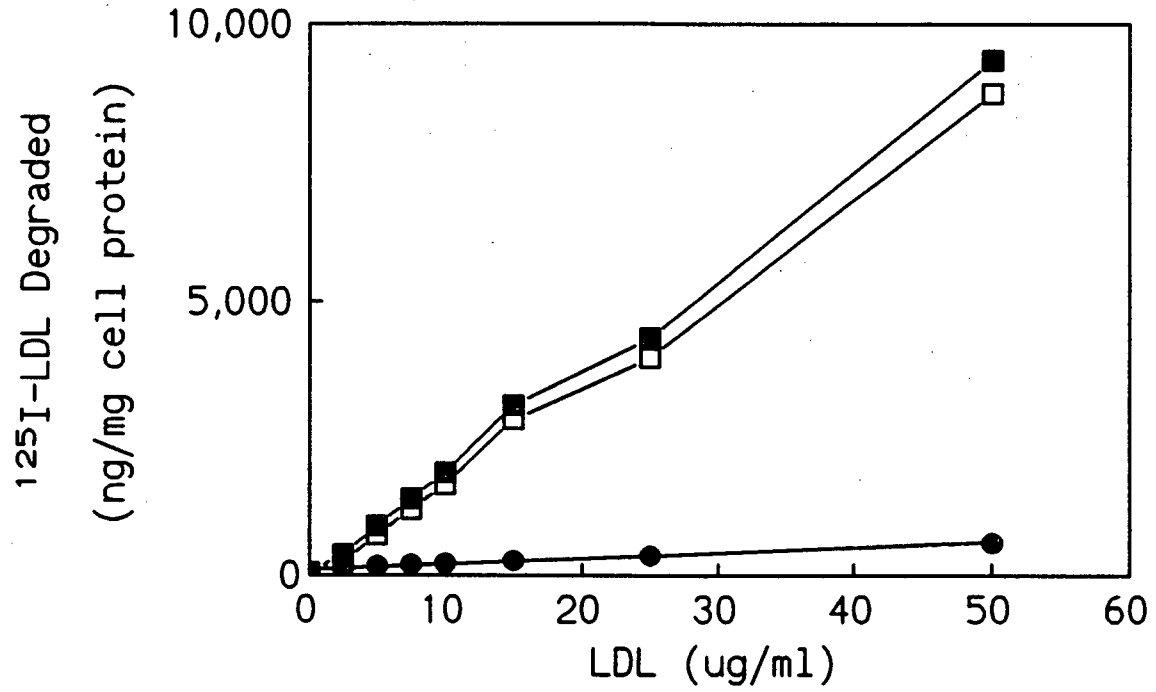


Figure 5. Representative graph of total, specific, and non-specific degradation of ^{125}I-LDL in human skin fibroblasts as a function of LDL concentration. Cell monolayers were grown as in Figure 3. Prior to each experiment the medium was replaced with the indicated concentrations of ^{125}I-LDL and the cells were incubated for 2 hours at 37°C. The medium was removed from the cells and added to trichloroacteic acid and incubated for 30 minutes at 4°C. The precipitate formed was removed by centrifugation at 800 X g for 15 minutes. Potassium iodide (10 μl of a 40% (w/v) solution) and H_2O_2 (40 μl of a 30% solution) were added to a 1 ml aliquot of the supernatant. To this, choloroform (2 ml) was added, mixed, and an aliquot of the upper, aqueous phase was removed for the determination of total radioactivity. The cells were lysed by the addition of 0.1 N NaOH and the protein content determined as described in Materials and Methods. Specific degradation (□), was calculated from the difference between total (■), and non-specific (●), internalization. In addition, the radioactive counts were corrected for blank values which were obtained in parallel experiments in the absence of fibroblasts. Each value represents the average of 3 determinations.

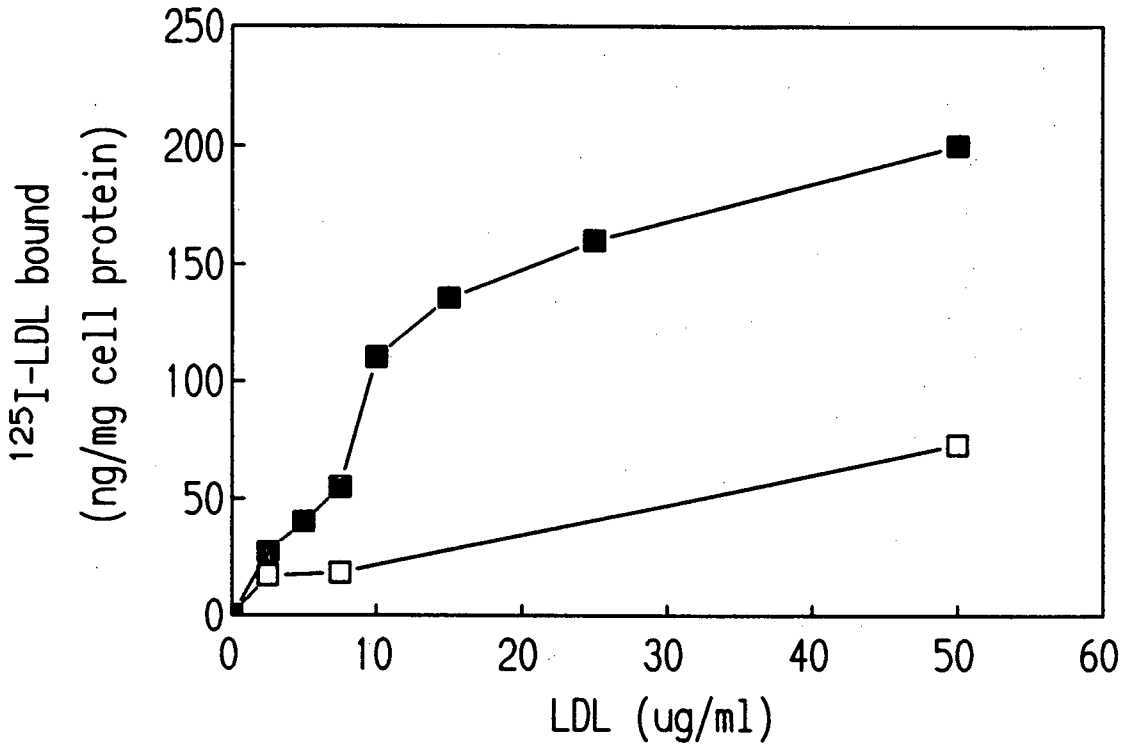


Figure 6. Comparison of specific binding of ^{125}I-LDL in upregulated and non-upregulated human skin fibroblasts. Upregulated (■), and non-upregulated (□) cell monolayers were grown as in Figure 3 except that the non-upregulated cells were grown in 10% FCS/DMEM rather than LPDFCS/DMEM for the final 48 hours of growth. The binding experiments were conducted and the specific binding was calculated as in Figure 3. Each value represents the average of triplicate determinations.

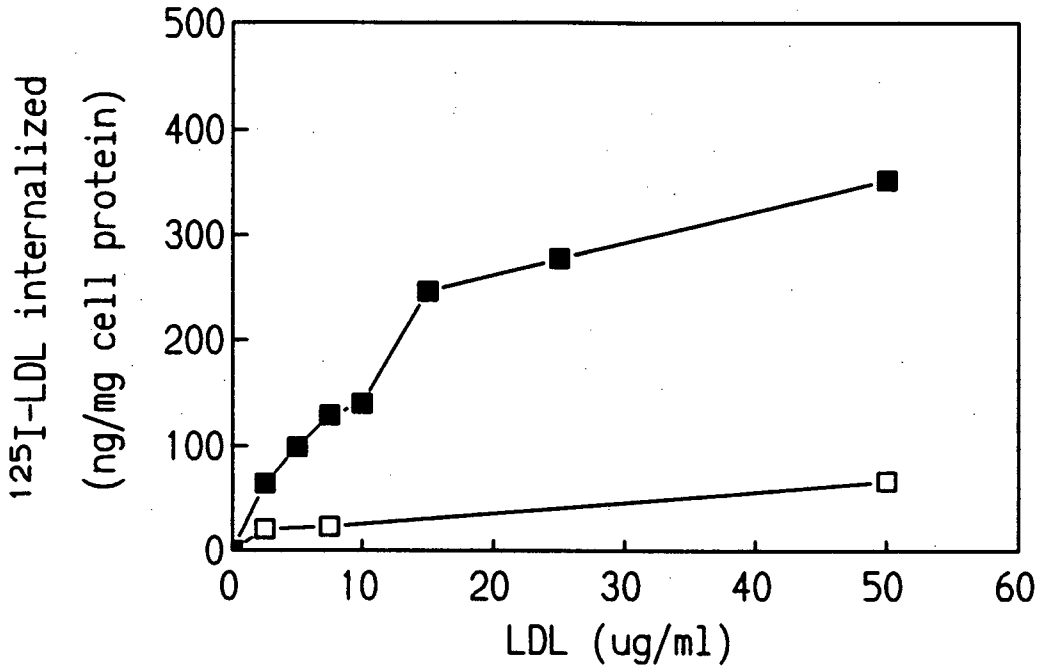


Figure 7. Comparison of specific internalization of ^{125}I-LDL in upregulated (■), and non-upregulated (□), human skin fibroblasts. Upregulated and non-upregulated cell monolayers were grown as in Figure 3 except that the non-upregulated cells were incubated in 10% FCS/DMEM rather than LPDFCS/DMEM for the final 48 hours of growth. The internalization experiments were performed and the specific internalization calculated as in Figure 4. Each value is the average of triplicate determinations.

2.3.2 The Effect of CsA on the Binding, Internalization, and Degradation of ^{125}I-LDL in Human Skin Fibroblasts

The effects of CsA at 5, 10, and 15 μg/ml on LDL binding, internalization, and degradation are depicted in representative graphs in Figures 8, 9 and 10, respectively. In normal cells the activity of the LDL receptor varies 10-fold depending on the rate of cell growth and the degree of cholesterol deprivation (47). For this reason, when two cell experiments are compared growth conditions are standardized to allow for meaningful comparisons (47). Even when such precautions are taken LDL-receptor activity varies by 2-3 fold in the same cell strain on different days or when using different batches of LPDFCS (47). This variability is shown in Figures 11, 12 and 13 where the results collected on three different days, for each of the parameters of LDL-receptor activity (binding, internalization, and degradation) are pooled and displayed graphically. Due to this day to day variability, the effect of CsA on LDL-receptor activity was analyzed using a 3-way ANOVA in which one of the main effects was the date of which the experiment was performed.

The results of the 3-way ANOVA show that ^{125}I-LDL binding was significantly increased in the presence of 15 μg/ml CsA ($p=0.0136$) (Figure 11). There was no difference in LDL binding in the cells treated with 5 or 10 μg/ml of CsA in comparison to their controls (Figure 11). LDL-receptor activity at 10 μg/ml of LDL is frequently cited (47,198) because at this concentration the non-specific component is usually less than 5-10% of the total activity in normal cells and thus any differences between the normal and experimental group are thought to reflect differences in the activity of the receptor (47). However, in the present study, the binding at 10 μg/ml LDL is not significantly different from the controls at

Figure 8. Representative graphs of the effect of CsA on the specific binding of ^{125}I-LDL to human skin fibroblasts as a function of LDL concentration. Cell monolayers were grown, with or without the addition of CsA at the concentrations indicated, as in Figure 3. LDL binding experiments were performed and specific binding determined as in Figure 3. CsA treated cells (□), untreated (control) cells (■). Determinations for each concentration of LDL were performed in triplicate.

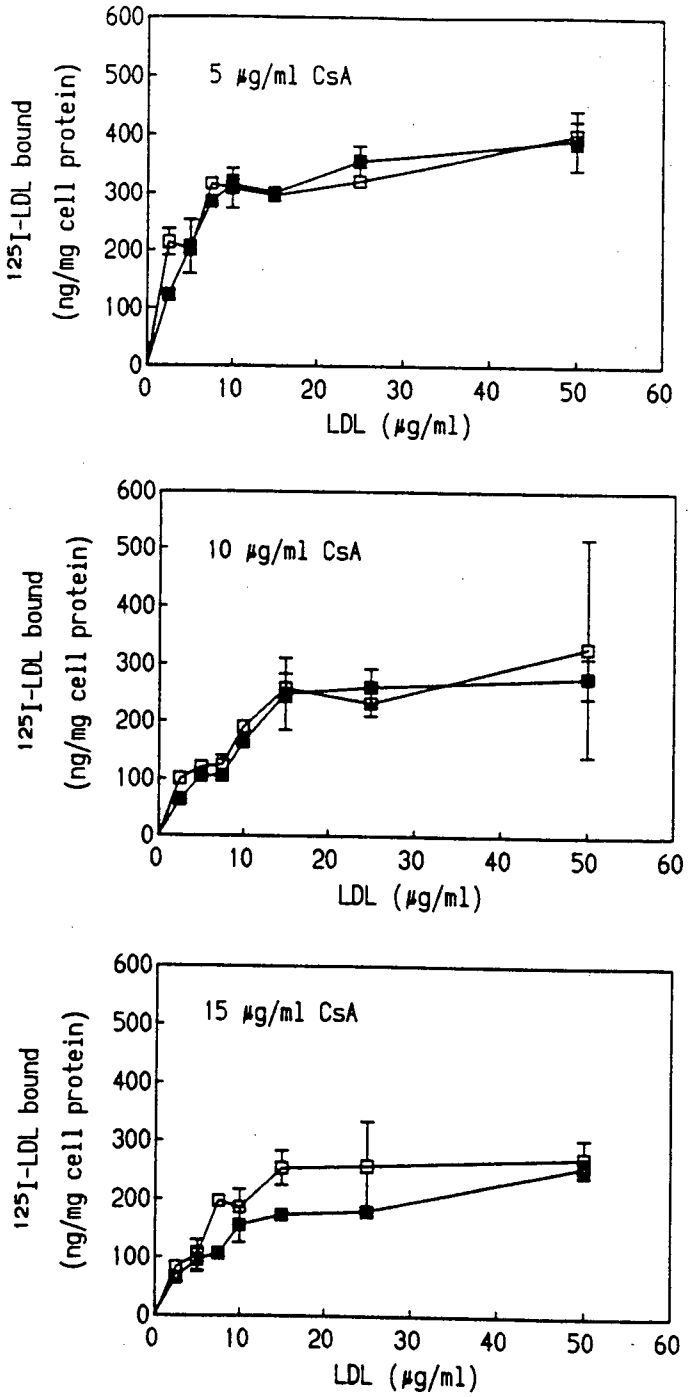
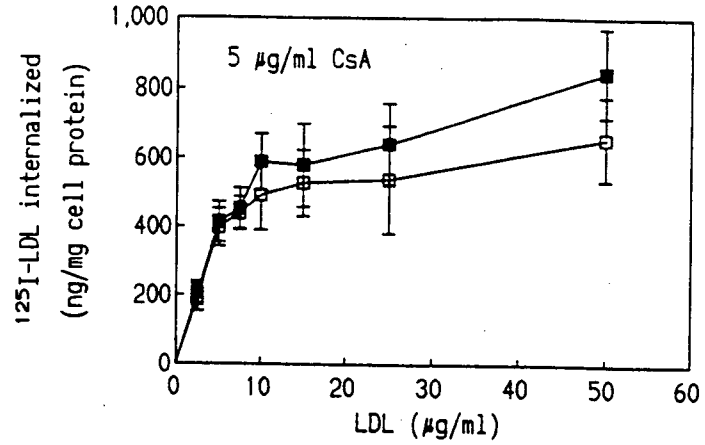
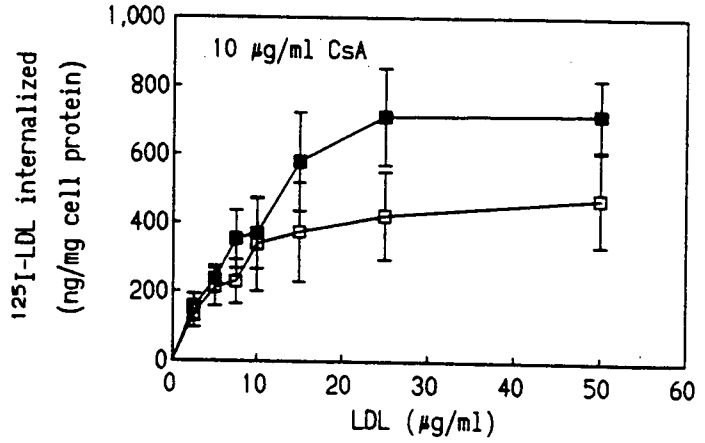

5 μg/ml CsA
10 μg/ml CsA
15 μg/ml CsA
125I-LDL bound
(ng/mg cell protein)
LDL (μg/ml)
0
100
200
300
400
500
600
10
20
30
40
50
60

Figure 9. Representative graphs of the effect of CsA on the specific internalization of ^{125}I-LDL in human skin fibroblasts as a function of LDL concentration. Cell monolayers were grown, with or without the addition of CsA at the concentrations indicated, as in Figure 3. The specific internalization of LDL was determined as in Figure 4. CsA treated cells (□), untreated (control) cells (■). Determinations for each concentration of LDL were performed in triplicate.


1,000
800
600
400
200
0
5 μg/ml CsA
125I-LDL internalized
(ng/mg cell protein)
0
10
20
30
40
50
60
LDL (μg/ml)


1,000
800
600
400
200
0
10 μg/ml CsA
125I-LDL internalized
(ng/mg cell protein)
0
10
20
30
40
50
60
LDL (μg/ml)

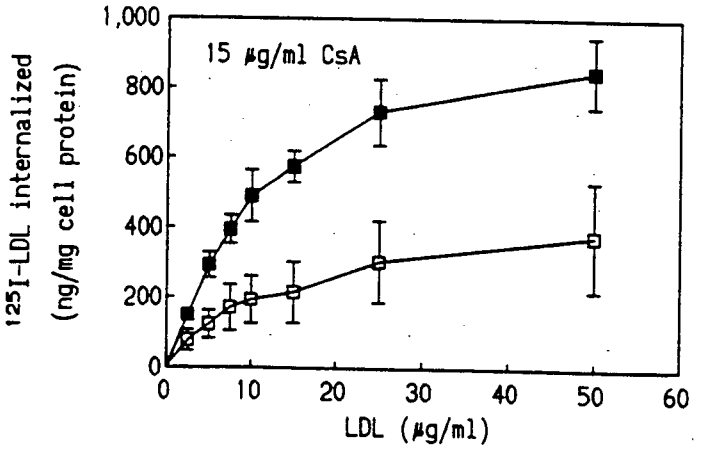
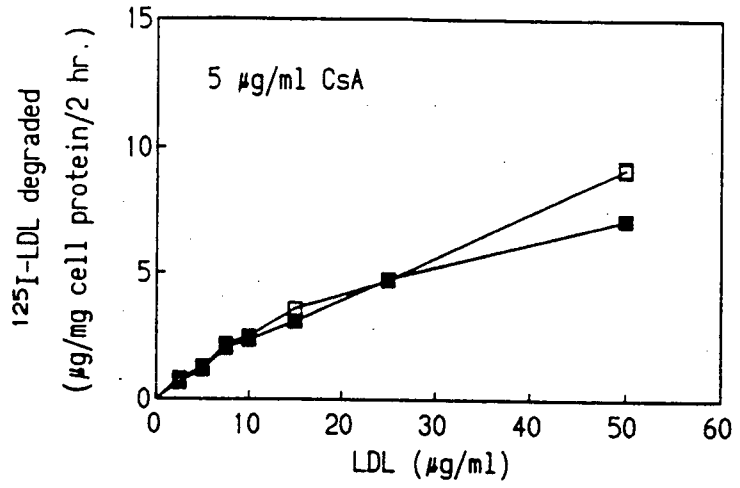
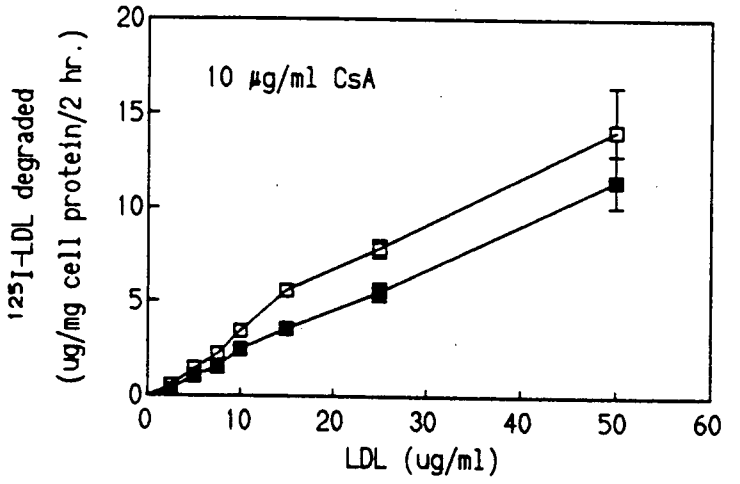

1,000
800
600
400
200
0
15 μg/ml CsA
125I-LDL internalized
(ng/mg cell protein)
0
10
20
30
40
50
60
LDL (μg/ml)

Figure 10. Representative graphs of the effect of CsA on the specific degradation of ^{125}I-LDL in human skin fibroblasts as a function of LDL concentration. Cell monolayers were grown, with or without the addition of CsA at the concentrations indicated, as in Figure 3. The specific degradation of LDL was determined as in Figure 5. CsA treated cells (□), untreated (control) cells (■). Determinations for each concentration of LDL were performed in triplicate.


5 µg/ml CsA
^{125}I-LDL degraded
(µg/mg cell protein/2 hr.)
LDL (µg/ml)
0
5
10
15
0
10
20
30
40
50
60


10 µg/ml CsA
^{125}I-LDL degraded
(ug/mg cell protein/2 hr.)
LDL (ug/ml)
0
5
10
15
20
0
10
20
30
40
50
60

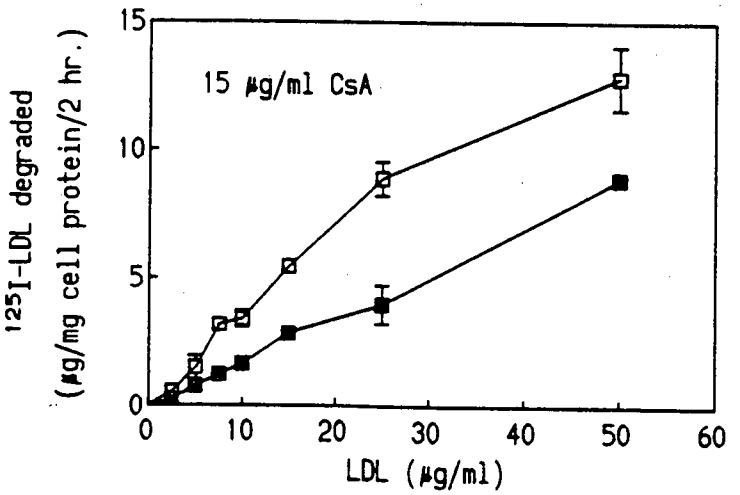
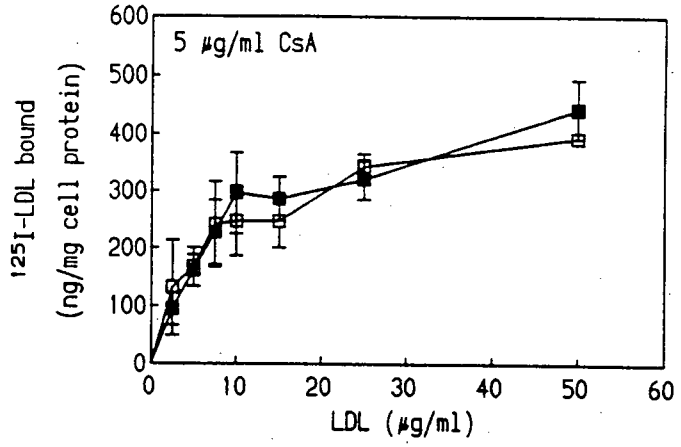
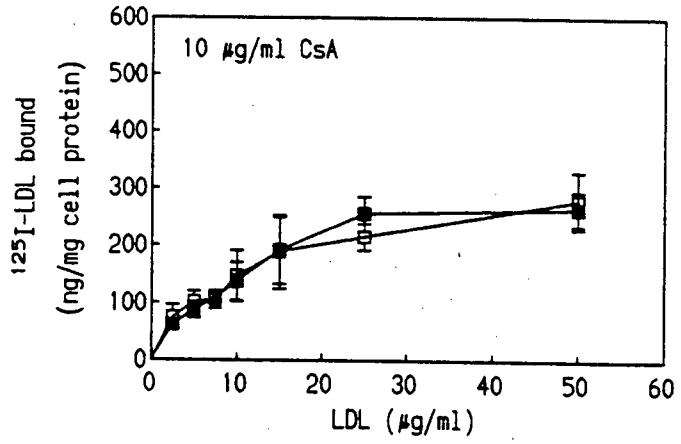

15 µg/ml CsA
^{125}I-LDL degraded
(µg/mg cell protein/2 hr.)
LDL (µg/ml)
0
5
10
15
0
10
20
30
40
50
60

Figure 11. The effect of CsA on the specific binding of ^{125}I-LDL to human skin fibroblasts as a function of LDL concentration. Cell monolayers were grown, with or without the addition of CsA at the concentrations indicated, as in Figure 3. The specific binding of LDL was determined as in Figure 3. CsA treated cells (□), untreated (control) cells (■). Determinations for each concentration of LDL were performed in triplicate and the experiment was repeated 3 times. Therefore, each value is the average of 9 determinations ± SD.


5 µg/ml CsA
^{125}I-LDL bound
(ng/mg cell protein)
LDL (µg/ml)
0
100
200
300
400
500
600
0
10
20
30
40
50
60


10 µg/ml CsA
^{125}I-LDL bound
(ng/mg cell protein)
LDL (µg/ml)
0
100
200
300
400
500
600
0
10
20
30
40
50
60

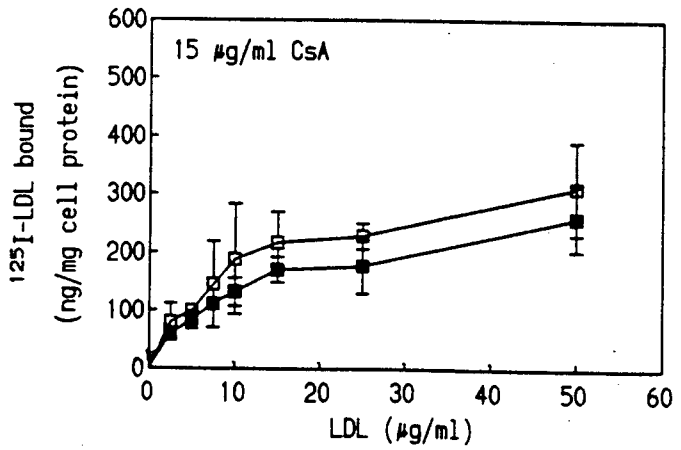
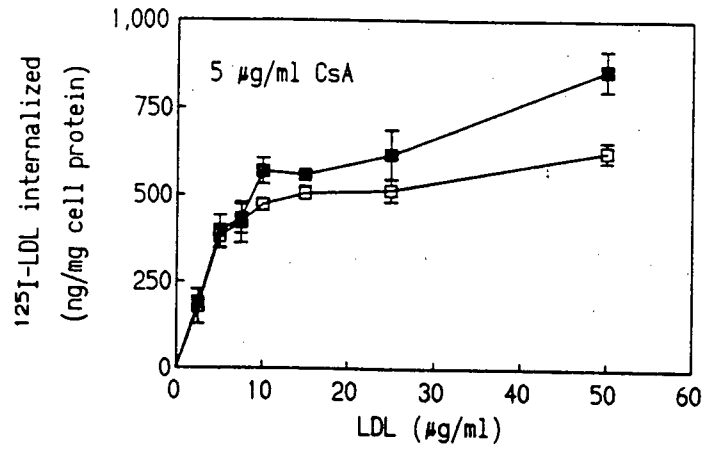
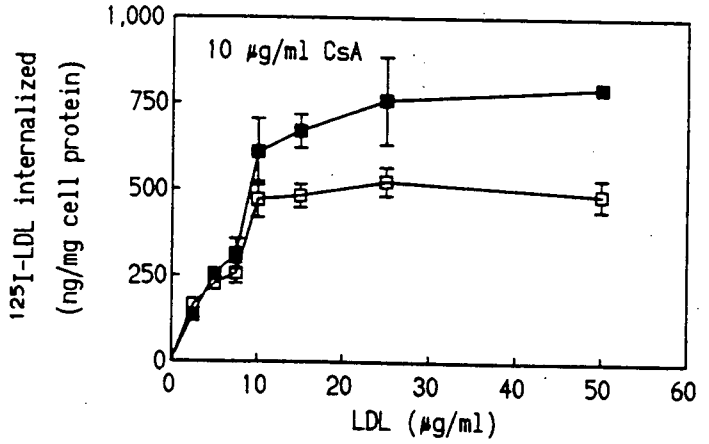

15 µg/ml CsA
^{125}I-LDL bound
(ng/mg cell protein)
LDL (µg/ml)
0
100
200
300
400
500
600
0
10
20
30
40
50
60

Figure 12. The effect of CsA on the specific internalization of ^{125}I-LDL in human skin fibroblasts as a function of LDL concentration. Cell monolayers were grown, with or without the addition of CsA at the concentrations indicated, as in Figure 3. The specific internalization of LDL was determined as in Figure 4. CsA treated cells (□), untreated (control) cells (■). Determinations for each concentration of LDL were performed in triplicate and the experiment was repeated 3 times. Therefore, each value is the average of 9 determinations ± SD.


5 µg/ml CsA
^{125}I-LDL internalized
(ng/mg cell protein)
1,000
750
500
250
0
0
10
20
30
40
50
60
LDL (µg/ml)


10 µg/ml CsA
^{125}I-LDL internalized
(ng/mg cell protein)
1,000
750
500
250
0
0
10
20
30
40
50
60
LDL (µg/ml)

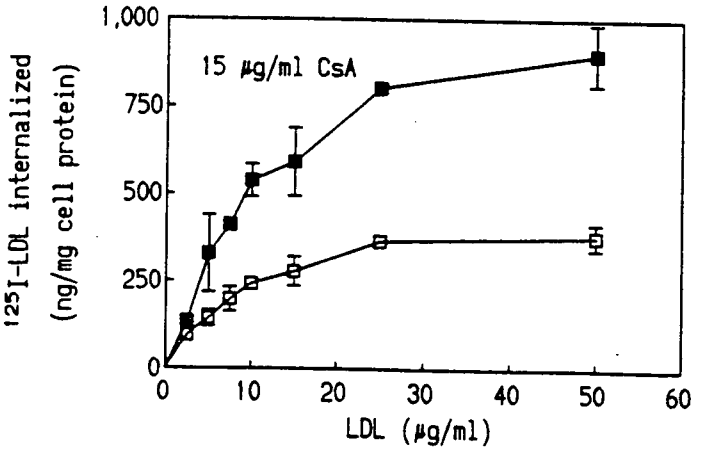
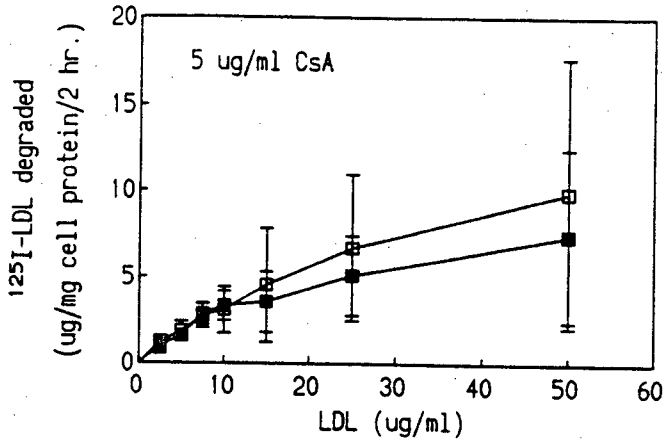
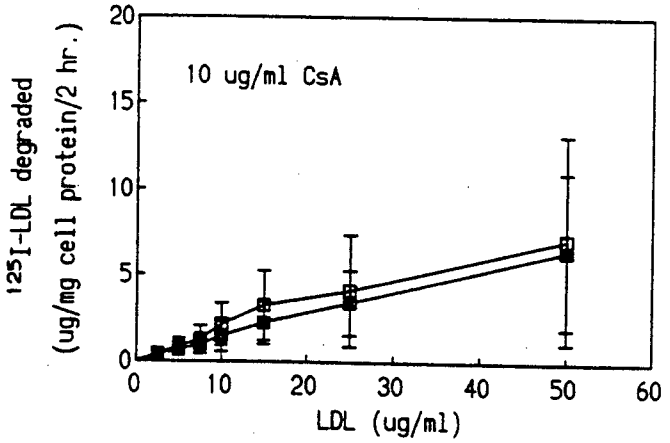
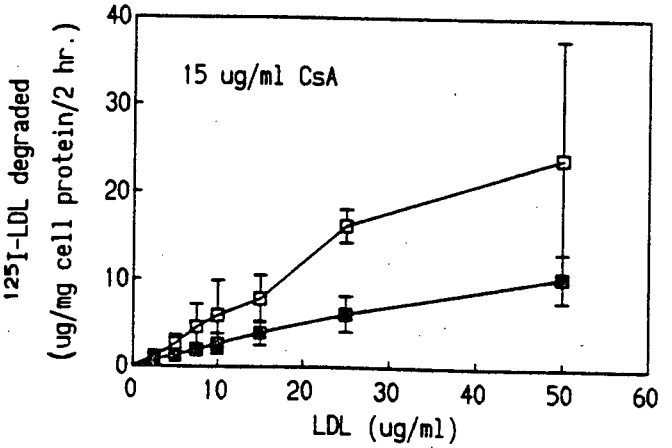

15 µg/ml CsA
^{125}I-LDL internalized
(ng/mg cell protein)
1,000
750
500
250
0
0
10
20
30
40
50
60
LDL (µg/ml)

Figure 13. The effect of CsA on the specific degradation of ^{125}I-LDL in human skin fibroblasts as a function of LDL concentration. Cell monolayers were grown, with or without the addition of CsA at the concentrations indicated, as in Figure 3. The specific degradation of LDL was determined as in Figure 5. CsA treated cells (□), untreated (control) cells (■). Determinations for each concentration of LDL were performed in triplicate and the experiment was repeated 3 times. Therefore, each value is the average of 9 determinations ± SD.


5 ug/ml CsA
^{125}I-LDL degraded
(ug/mg cell protein/2 hr.)
LDL (ug/ml)
0
5
10
15
20
0
10
20
30
40
50
60


10 ug/ml CsA
^{125}I-LDL degraded
(ug/mg cell protein/2 hr.)
LDL (ug/ml)
0
5
10
15
20
0
10
20
30
40
50
60


15 ug/ml CsA
^{125}I-LDL degraded
(ug/mg cell protein/2 hr.)
LDL (ug/ml)
0
10
20
30
40
0
10
20
30
40
50
60

any of the concentrations of CsA investigated (Table 2).

Table 2. Effect of CsA on specific binding of ^{125}I-LDL in human skin fibroblasts

CsA (μg/ml)	^{125}I-LDL Binding* (ng LDL protein/mg cell protein)	
	control	CsA
5	297 $\pm$ 71	248 $\pm$ 61 (NS)
10	137 $\pm$ 33	147 $\pm$ 44 (NS)
15	132 $\pm$ 24	189 $\pm$ 94 (NS)

Data are the means of 3 experiments $\pm$ SD
* ^{125}I-LDL binding at 10 μg/ml of LDL
NS; not significant

Table 3. Effect of CsA on specific internalization of ^{125}I-LDL in human skin fibroblasts

CsA (μg/ml)	^{125}I-LDL Internalized+ (ng LDL protein/mg cell protein)	
	control	CsA
5	588 $\pm$ 82	490 $\pm$ 100
10	518 $\pm$ 84	374 $\pm$ 104*
15	490 $\pm$ 74	194 $\pm$ 68**

Data are the means of 3 experiments $\pm$ SD
* $p < 0.05$, ** $p < 0.005$
+ ^{125}I-LDL internalized at 10 μg/ml of LDL

^{125}I-LDL internalization was significantly decreased by CsA concentrations of 10 and 15 μg/ml ($p=0.0001$ for each concentration of CsA) (Figure 12). Furthermore, internalization at 10 μg/ml LDL is significantly reduced at 10 and 15 μg/ml CsA (Table 3) ($p<0.05$ and 0.005, respectively). Although the overall effect of 5 μg/ml CsA was not

statistically significant ($p=0.0594$), there was a tendency for decreased ^{125}I-LDL internalization at LDL concentrations of ≥ 15 μg/ml (Figure 12).

CsA at concentrations of 10 and 15 μg/ml caused a significant increase in ^{125}I-LDL degradation ($p=0.0426$ and $p=0.0001$, respectively)(Figure 13). The effects of interactions between the factors were not included in the ANOVA for any of the parameters of LDL-receptor activity since the r^2 from plots of the data *vs.* predicted model was always greater than 0.85, with the exception of LDL-degradation at 5 μg/ml CsA. At this concentration of CsA, if the effects of the interactions between the factors are included in the ANOVA (date X concentration of LDL, date X experimental group, and concentration of LDL X experimental group) then ^{125}I-LDL degradation is significantly increased ($p=0.0321$) in comparison to its control (Figure 13).

CsA had no effect on the non-specific components of LDL-receptor activity (results not shown), regardless of any of the concentrations used..

2.3.3 The Effect of CsA on the B_{max} and K_d of the LDL-receptor in Human Skin Fibroblasts

The binding isotherms for LDL to fibroblasts grown in the presence or absence of CsA shown in Figure 11. The B_{max} and K_d for these binding isotherms were calculated from non-linear regression as described in section 2.2.4 (Tables 4 and 5). The r^2 values for these non-linear regression curves were >0.9. A representative non-linear regression of one binding isotherm is shown in Figure 14 and the Scatchard plot (bound/free *vs.* bound) of the data (Figure 15) is included for comparison. There was no difference in the K_d or B_{max} for the CsA-treated fibroblasts, at concentrations ranging from 5 to 15 μg/ml CsA, compared to their respective controls. As with the experiments described above, CsA had no effect on

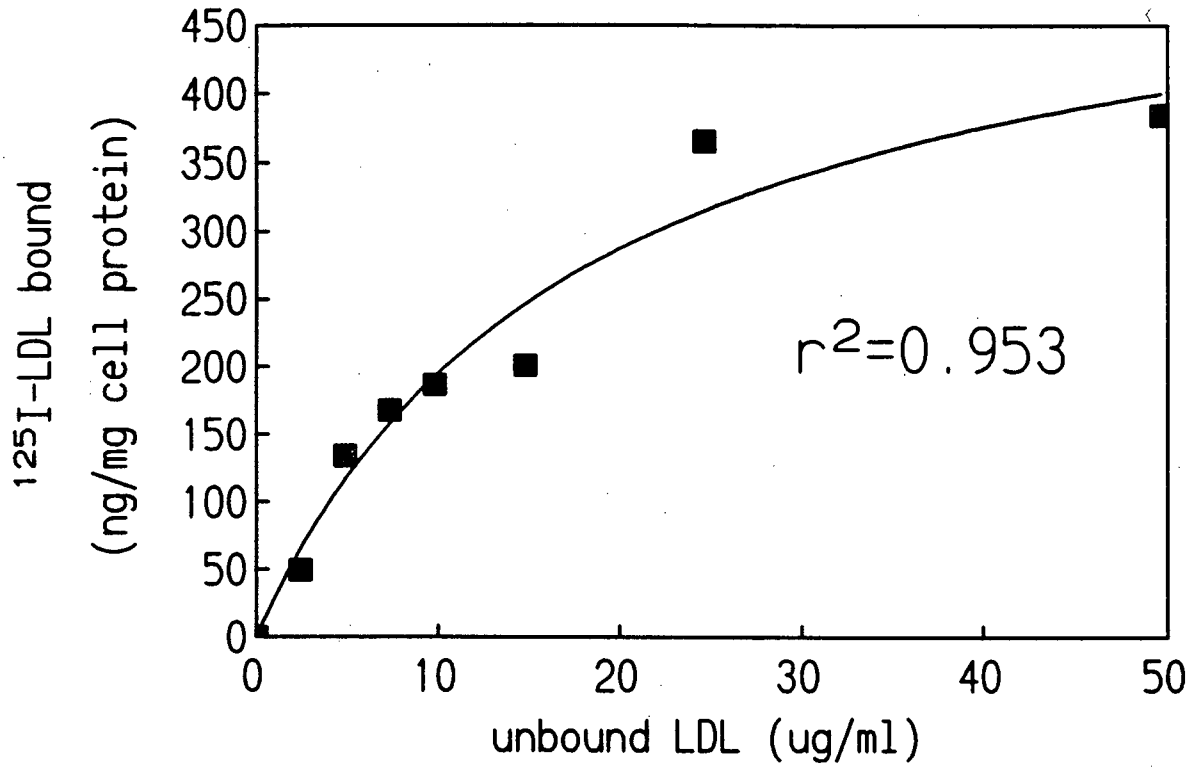


Figure 14. Representative non-linear regression of the binding of ^{125}I-LDL to human skin fibroblasts. Cell momolayers were grown and the binding experiment performed as in Figure 3. The bound ^{125}I-LDL was determined as the difference between total and non-specific binding as in Figure 3.

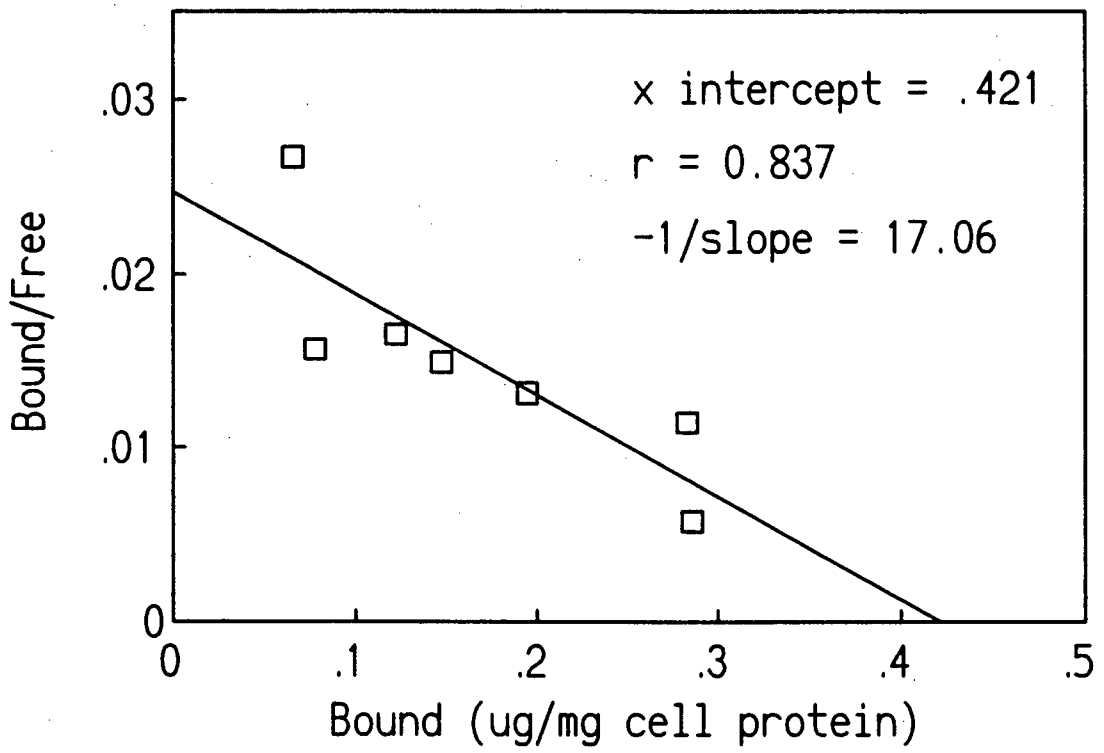


Figure 15. Representative Scatchard analysis of the binding of ^{125}I-LDL to human skin fibroblasts. Cell monolayers were grown and the binding experiments performed as in Figure 3. The bound ^{125}I-LDL was determined as the difference between total and non-specific binding as in Figure 3.

non-specific binding (results not shown).

Table 4. Effect of CsA on LDL-receptor affinity in human skin fibroblasts

CsA (μg/ml)	K_d (μg LDL protein/ml)	
	control	CsA
5	11.9 ± 6	10.0 ± 5.9 (NS)
10	14.3 ± 2.3	13.6 ± 2.9 (NS)
15	13.1 ± 3.1	11.9 ± 3.5 (NS)

Data are the means of 3 experiments ± SD
NS; not significant

2.2.4 The Effects of CsA on Cultured Human Skin Fibroblasts

The addition of CsA to the cultured fibroblasts inhibited cell growth and altered cell morphology. Microscopically, the fibroblasts treated with 15 μg/ml of CsA were vacuolated and appeared more elongated in comparison to their controls (results not shown). In addition, the proliferation of fibroblasts was decreased. This was reflected in a significant reduction in the amount of cellular protein per culture dish at 15 μg/ml of CsA (Figure 16).

Table 5. Effect of CsA on the number of LDL binding sites (B_{max}) in human skin fibroblasts

CsA (μg/ml)	B_{max} (ng/mg cell protein)	
	control	CsA
5	533 ± 162	461 ± 115 (NS)
10	350 ± 46	345 ± 33 (NS)
15	312 ± 94	381 ± 101 (NS)

Data are the means of 3 experiments ± SD
NS; not significant

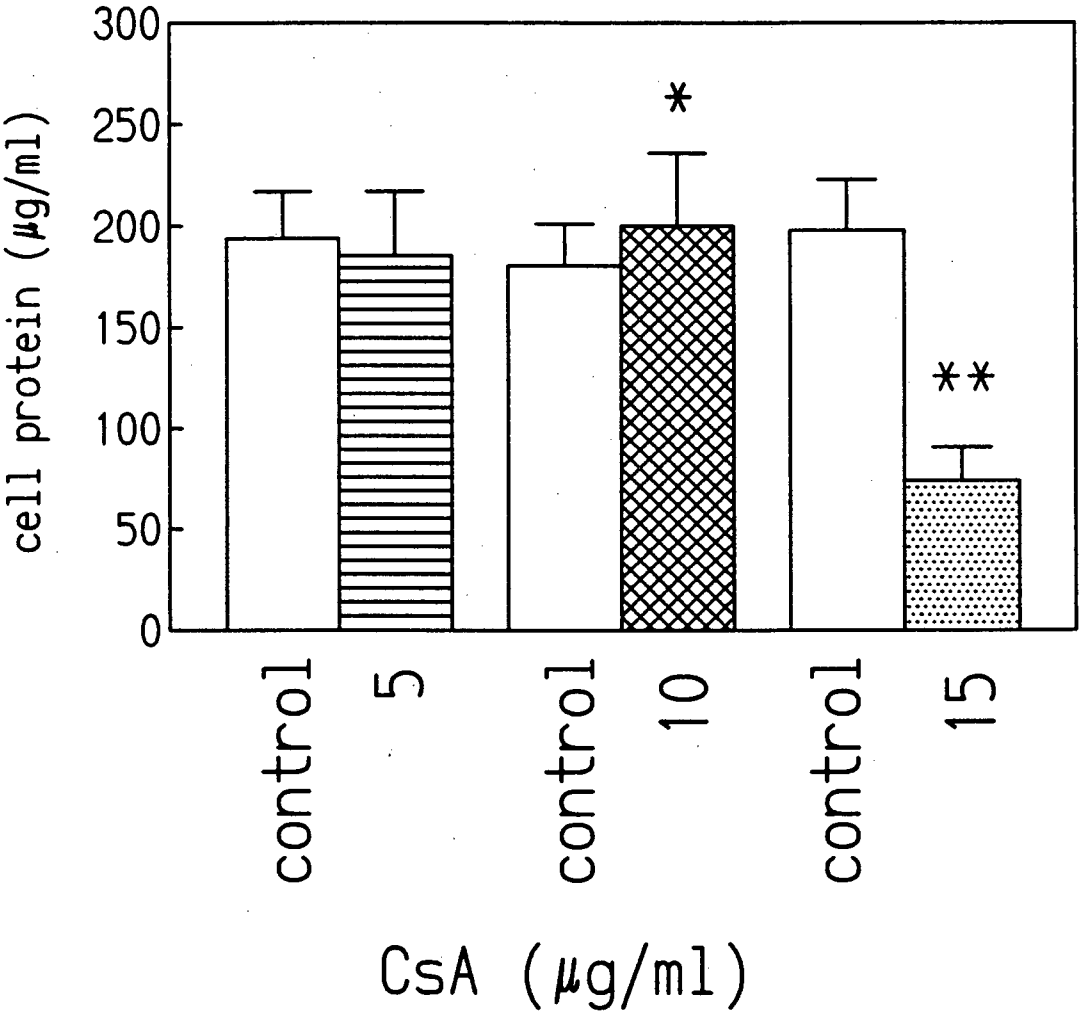


Figure 16. The effect of CsA on protein levels of human skin fibroblasts *in vitro*. Cell monolayers were grown as in Figure 3, with or without the addition of CsA at the concentrations indicated. The cells were lysed by the addition of 0.1 N NaOH and an aliquot removed for the determination of protein. Each bar is the average of 36 determinations $\pm$ 2 SD. controls (□) *$p < 0.01$, **$p < 0.001$

2.3.5 The Effect of CsA on LDL-receptor mRNA levels in Human Skin Fibroblasts

Due to the anti-proliferative effects of CsA on fibroblasts, the concentrations chosen to investigate the effect of CsA on LDL-receptor mRNA levels did not exceed 10 μg/ml.

A representative photograph of total RNA, isolated from CsA-treated and non-treated (control) fibroblasts and electrophoresed in a 1% denaturing agarose gel, is shown in Figure 17a. The picture clearly shows that the lanes are evenly loaded and the RNA is not degraded. The gel was transferred to a nylon filter then hybridized to the radioactive cDNA probe for the LDL-receptor mRNA (Figure 17b). The autoradiogram shows a single band following hybridization.

CsA had no effect on the total amount of RNA isolated from the treated cells in comparison to their controls at any of the concentrations chosen to study the effect of the drug (0.5-10 μg/ml)(Table 6). In addition, the relative amounts of LDL-receptor mRNA levels (per 20 μg total RNA) in cells treated with CsA at concentrations from 0.5-5 μg/ml were similar to their controls (Table 7). However, at a concentration of 10 μg/ml of CsA, the LDL-receptor mRNA levels significantly increase, rather than decrease, in comparison to their controls (Table 7).

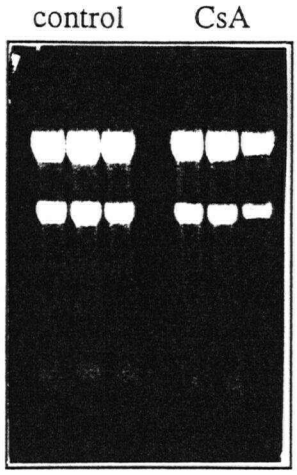


Figure 17a. Representative photograph of formaldehyde gel electrophoresis of RNA isolated from untreated (control; n=3) and CsA-treated (2.5 μg/ml; n=3) human skin fibroblasts. RNA was isolated, electrophoresed in a formaldehyde gel, and stained with ethidium bromide as described in Materials and Methods. This photograph shows that the lanes are evenly loaded and the RNA is not degraded.

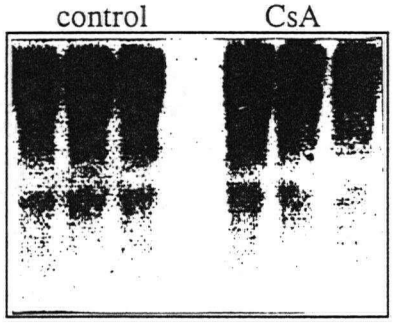


Plate 17b. Autoradiogram of RNA from untreated (control) and CsA-treated (2μg/ml n=3) human skin fibroblasts from Figure 17a, following hybridization to an LDL-receptor cDNA probe. The RNA was isolated, electrophoresed, capillary transferred and hybridized to an LDL-receptor cDNA probe as described in Materials and Methods.

Table 6. Effect of CsA on Total RNA levels in human skin fibroblasts

CsA (μg/ml)	Total RNA (μg)	
	control	CsA
0.5	65.1 $\pm$ 0.7	64.6 $\pm$ 1.4
1.0	66.1 $\pm$ 7.5	73.5 $\pm$ 10.5
2.5	59.0 $\pm$ 6.3	53.2 $\pm$ 10.4
5	66.9 $\pm$ 6.7	57.0 $\pm$ 6.0
10	88.9 $\pm$ 6.2	85.3 $\pm$ 5.5

Data are the means of 3 samples $\pm$ SD

Table 7. Effect of CsA on LDL-receptor mRNA levels in human skin fibroblasts

CsA (μg/ml)	CPM/20 μg RNA	
	control	CsA
0.5	730 $\pm$ 169	617 $\pm$ 193
1.0	1334 $\pm$ 70	1454 $\pm$ 155
2.5	1865 $\pm$ 171	1576 $\pm$ 119
5.0	1589 $\pm$ 171	1392 $\pm$ 44
10.0	907 $\pm$ 59	1358 $\pm$ 30*

Data are the means of 3 samples $\pm$ SD, CPM; counts per minute,*$p < 0.003$

2.3.6. The Effect of LDL-associated CsA on the binding of LDL to its receptor

The association of CsA with ^{125}I-LDL was verified by gel filtration chromatography. The column was calibrated with ^{125}I-LDL. The elution profile for LDL-^{3}H-CsA corresponded to that of iodinated LDL indicating that the CsA was associated with the LDL (Figure 18). The free ^{3}H-CsA, which accounted for $<10\%$ of the radioactive counts, absorbed to the column and eluted after repeated washings with 20% ethanol. Dual label (^{125}I-LDL-^{3}H-CsA) studies were not possible due to because of the technical limitations of the scintillation counter.

There was no difference in the binding affinity for LDL-^{3}H-CsA to the LDL-receptor at 0.03 or 0.3 μg CsA/μg LDL protein in comparison to native, iodinated LDL (Table 8). The specific binding was calculated from the difference between the total and non-specific binding as described previously and the specific binding affinities were calculated by non-linear regression as described above. The r^2 values for all of these regressions were >0.9.

Table 8. Effect of LDL-associated CsA on the affinity (K_d) of LDL for the LDL-receptor in human skin fibroblasts

	K_d (μg LDL protein/ml)	
CsA (μg CsA/μg LDL protein)	**control**	**CsA**
0.03	**15.7 $\pm$ 3.9**	**25.5 $\pm$ 7.4 (NS)**
0.3	**33 $\pm$ 5.1**	**36.4 $\pm$ 16.1 (NS)**

Data are the means of 3 experiments $\pm$ SD
NS; not significant

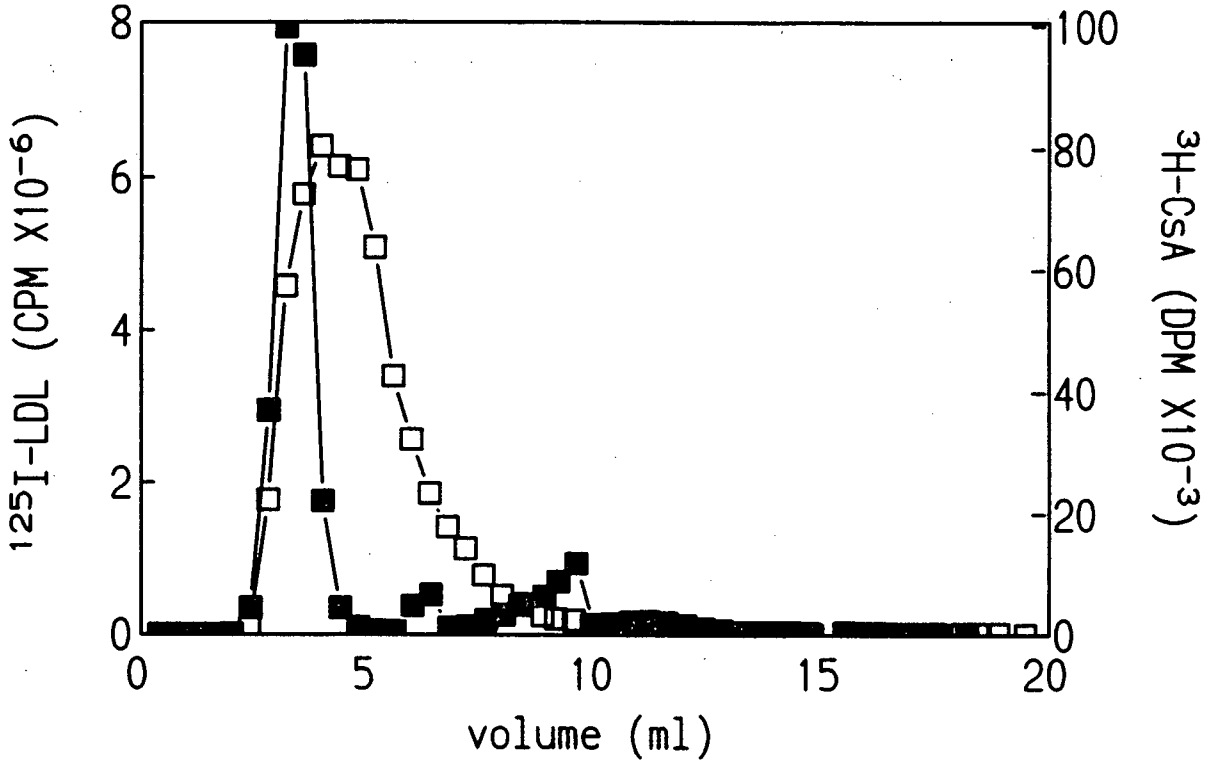


Figure 18. Elution profiles of ^{125}I-LDL and LDL-^{3}H-CsA from a G25 gel filtration column. ^{125}I-LDL and LDL- ^{3}H-CsA were applied separately to a gel filtration column and eluted in a buffer of 0.15 M NaCl, 10 mM EDTA, and 0.03% azide. The elution of the standards was monitored by the amount of radioactivity present. ^{125}I-LDL (CPM) (■), LDL-^{3}H-CsA (DPM) (□).

2.4. Discussion

2.4.1 The Effect of CsA on the Binding, Internalization, and Degradation of LDL via the LDL-receptor and on LDL-receptor mRNA levels in Human Skin Fibroblasts

The objectives of this part of the thesis were to determine if CsA decreased the uptake of LDL via the LDL-receptor, thereby providing, in part, a mechanism to account for the increased LDL-cholesterol levels seen in patients receiving CsA. To investigate the effect(s) of CsA a number of experiments were designed. First, I determined whether the treatment of human skin fibroblasts with CsA *in vitro* affected their ability to bind, internalize, or degrade ^{125}I-LDL. Specifically, I measured the effects of CsA on kinetic parameters of ^{125}I-LDL binding (B_{max}, K_d), in addition to ^{125}I-LDL internalization, degradation, and LDL-receptor mRNA levels. Second, I determined whether or not the association of CsA with ^{125}I-LDL affected the affinity (K_d) of the ligand for its receptor.

The results of this study demonstrate that CsA significantly increases ^{125}I-LDL degradation. This result is supported by the data which show that when CsA is added *in vitro* to cultured fibroblasts, the degradation of LDL is significantly increased at $\geq$ 5 μg/ml CsA. Moreover, internalization (*i.e.*, the amount of LDL contained in the cells) is significantly decreased in the presence of CsA. However, CsA had no effect on LDL-binding at 5 or 10 μg/ml but at 15 μg/ml, CsA significantly increased LDL-binding. Given that LDL-receptor activity in fibroblast (and hepatocyte) cultures is a function of cell density, with activity inversely proportional to cell density (44,199), and that CsA at 15 μg/ml significantly decreased cellular protein levels, the increased binding is a reflection of this fact rather than a true effect of the drug.

LDL that is degraded must first be bound then internalized by the cell. For this reason, when assessing the overall effect of CsA on LDL-receptor activity, the effect on binding, internalization, and degradation must be evaluated together. CsA increases degradation, and this increased degradation seems to exceed the ability of the cell to internalize LDL since the amount internalized is significantly decreased. Although the combined amounts of internalized and degraded LDL tend to increase in the CsA-treated cells, this result does not reach statistical significance and suggests that the rate of internalization is not affected by CsA. Alternatively, this interpretation is limited by the small sample size (n=3) and in order to determine whether if the internalization rate is affected, larger sample sizes are required.

My results are in agreement with those of Lopez-Miranda *et al.*, although they interpret their results differently (74). They found LDL degradation in human skin fibroblasts *in vitro*, was increased two-fold when the LDL used in the assay was isolated from CsA-treated rats, in comparison to LDL from rats treated with Cremaphore EL (CsA-vehicle). They suggested that the increased degradation was a result of the binding of CsA to the LDL particle, imparting a net charge or a conformational change to the LDL that increased the binding affinity of LDL (74). However, my results do not support this suggestion. My data show that the association of CsA with LDL does not affect the affinity of LDL for the LDL-receptor and this result is discussed in the next section (2.4.2). Another explanation of their results is the CsA, which was bound to the LDL then taken up by the cell, increased the degradative rate of LDL. This is not an unreasonable suggestion since the cells were exposed to CsA for 5 hours and the amount to which they were exposed was not reported (74).

Further, results from other experiments they performed support this suggestion. They found that when they treated fibroblasts with CsA, with concentrations ranging from 0.2-2 μg/ml, and repeated the experiments using LDL isolated from CsA-treated and untreated rats, there was no significant difference in the degradation rate between the two, although degradation was always higher in the experiments using LDL isolated from CsA-treated rats (74). This means that the CsA added to the cells had an effect; it increased the degradation of LDL. Further, that the effect of CsA on the fibroblasts was augmented when the experiments were performed with LDL that presumably contained CsA. They also injected CsA-treated and untreated rats with iodinated LDL, and found there was a significant increase in the amount of radioactivity in the liver of the CsA-treated rats (74). This could mean that the CsA increased the internalization of LDL.

I also determined that CsA (0.5-5 μg/ml) did not decrease LDL-receptor mRNA levels. This result is consistent with the data showing that there is no difference in the B_{max} or LDL binding in the CsA-treated cells in comparison to the controls. However, at 10 μg/ml of CsA there is a significant increase in LDL-receptor mRNA levels but this result does not translate to any increase in LDL binding, suggesting that there was no increase in LDL-receptor expression. The present finding of increased mRNA levels at this concentration of CsA is inconsistent with the accepted understanding of the regulation of LDL-receptor mRNA levels by free cholesterol (44,46,47). Once LDL is bound to its receptor, it is internalized and delivered to early endosomes, whereupon it dissociates from the receptor and the receptor recycles to the cell membrane. The endosome fuses with lysosomes and the LDL is degraded by lysosomal hydrolases. One of the products of this hydrolysis, free cholesterol,

regulates intracellular cholesterol metabolism: it decreases endogenous cholesterol metabolism by inhibiting HMG-CoA reductase, it stimulates cholesterol esterification via ACAT, and it inhibits the synthesis of LDL mRNA resulting in a decrease in the expression of new LDL-receptors (44). The present study shows that CsA (10 μg/ml) increases LDL degradation, which presumably results in the release of free cholesterol. However, no decrease in LDL-mRNA levels was observed. One explanation for this observation is that the regulatory mechanisms provided by LDL-derived, free cholesterol, responsible for decreasing the synthesis of LDL-receptors, may be absent. Further investigation of this might be shown by demonstrating increased HMG-CoA-reductase activity and decreased ACAT levels (44,47). An alternate explanation of the increased mRNA levels might be decreased mRNA degradation, which may or may not be due to CsA.

A possible criticism of these studies is that the level of drug used in these experiments is high and does not reflect the therapeutic blood levels of the drug. In my studies, the observed increase in LDL degradation occurred at 5 μg/ml of CsA and. This result is significant for a number of reasons: 1) a CsA concentration of 5 μg/ml is in keeping with reported tissue levels of CsA (86,200,201) and these are much higher than the blood levels. Fisher *et al.* found that the level of CsA in the epidermis of CsA-treated psoriatic patients was 1.0 ± 0.3 ng CsA/μg DNA and, given the wet weight of tissue, estimated that this corresponded to approximately 3 μg/ml CsA. Further, this level was similar to the peak blood concentrations of the drug and was approximately 10-fold the trough blood concentrations (200). This level of CsA in the epidermis is within the range reported for post-mortem samples of liver, heart, and kidney (201). Furthermore, Lensmeyer *et al.* found

that the sum concentrations of CsA and metabolites in post-mortem tissue samples ranged from 8-53 times the sum of the cyclosporins in the corresponding whole blood specimens (88).These results are in keeping with animal studies of tissue levels of CsA. Kumar *et al.*found that the levels of CsA in adipose tissue, liver, and kidney of CsA-treated rats (at 3 different doses of CsA) were approximately 20, 5, and 2-3 times that found in the blood, respectively (202). The ratio between the blood and tissue levels remained constant and was not affected by the dose or the length of administration (202). 2) Copeland and Yatscoff suggested that the tissue levels may be more relevant than blood levels when studying the effects of the drug (203). Kumar *et al.* found that the appearance and severity of renal, adipose, and hepatic lesions in CsA-treated rats corresponded better with the respective tissue levels rather than the blood levels (202). This fact is also reflected in patients that receive this drug since the blood levels of CsA are a poor indicator of the nephrotoxic effects (10). Furthermore, the hepatic and renal lesions in the rats were evident when only 59% of the animals had elevated liver enzymes and 69% of the rats had elevated serum creatinine levels (202). In summary, although the concentrations of CsA in this study are higher than blood levels of the drug, they are more indicative of the tissue levels and may be more relevant, and have ben used by a number of investigators to study the effects of CsA (200,201,205-207).

2.4.2. The Effect of LDL-associated CsA on the Binding of ^{125}I-LDL to its Receptor

The association of CsA with LDL does not appear to affect the strength of the binding of LDL to its receptor, suggesting that this is not a mechanism by which CsA affects the subsequent clearance of LDL. This conclusion is supported by the data which show that the

affinity (K_d) and binding at 10 μg/ml of LDL is similar in native LDL and LDL-CsA, at 0.03 and 0.3 μg CsA/μg LDL protein. These findings are in contrast to those of Princen *et al.* (208) and Lopez-Miranda *et al.* (74). Princen *et al.* reported that the affinity of LDL-CsA for the LDL-receptor in $HepG_2$ cells was four times that of LDL alone, but the concentration of CsA which resulted in this outcome was not stated. Unfortunately, further comparisons with their results are not possible since the data have only been published in abstract form (208). Lopez-Miranda *et al.* found that the *in vitro* degradation of LDL isolated from rats treated with CsA was twice that of LDL isolated from animals treated with Cremaphore EL (CsA vehicle) (74). They concluded that the association of CsA with LDL affected the affinity of LDL for its receptor thereby resulting in increased degradation. However, they did not determine the affinity of LDL-CsA, nor did they report the amount of CsA associated with LDL. Moreover, they did not investigate the possibility that CsA may have increased only the degradative processes and not the binding of LDL (74). My results, however, are supported by those of Sanghvi *et al.*(209) who, in their studies with ^{3}H-CsA-LDL in stimulated lymphocytes, found that 1) the K_d of ^{3}H-CsA associated with LDL (^{3}H-CsA-LDL), was similar to that of LDL rather than that of ^{3}H-CsA alone, 2) the uptake of ^{3}H-CsA-LDL appeared to reach saturation at a level of 50 μg/ml of LDL, which was similar to that for native LDL in this model and, 3) the uptake of ^{3}H-CsA-LDL was suppressed in the presence of unlabelled LDL (209). These findings imply that the uptake of CsA in stimulated lymphocytes may occur via the LDL-receptor. In addition, their results, in conjunction with the present study, suggest that the association of CsA with LDL does not appear to affect the overall binding or K_d of LDL.

My results of LDL-CsA binding are also in agreement with other studies investigating the association of lipophilic drugs with LDL, including dioleoyl floxuridine (210), ellipticine (211), benzophorphryin (198), and benzo(a)pyrene derivatives (212). A consistent finding in all of these studies is that the binding of the LDL-drug complex to the LDL-receptor was not affected by the presence of the drug (198,210-212). Further, one study reported that the association of ellipiticine derivatives with LDL did not affect the size or particle distribution of LDL and that LDL retained its immunological properties with anti-Apo B (211). Some of these findings have also been demonstrated with lipoprotein-associated CsA, including LDL. Lopez-Miranda *et al.* reported that the association of CsA with lipoproteins (VLDL, LDL, IDL, and HDL) did not affect the particle diameter or chemical composition of these lipoproteins, with respect to cholesterol, phospholipid, and triglyceride content (74). Similar results were reported by Rodl *et al.* who found that the association of CsA with VLDL, LDL, or HDL did not affect either their electrophoretic mobility or their mobility on thin layer chromatography (180).

There are two potential sources of error associated with the assay that may result in an incorrect interpretation of the results. The first is that the binding assay may be too insensitive to detect subtle differences in the binding between the ^{125}I-LDL and the ^{125}I-LDL-CsA particles. Usually, the differences in affinities are studied using a competitive assay (213). This type of assay could not be performed in this study since the CsA would have probably redistributed so that there would have been no difference between the bound and competing ligands. Mendel reported that in general, competitive studies reliably detect two to three-fold differences in affinity and that smaller differences are much harder to detect (213).

Since this was not a competitive study, one might infer that determining subtle differences in affinities using a binding assay is even less reliable.

The second source of error may be that CsA dissociates from the LDL during the experimental procedure, resulting in no apparent difference between the two particles. In this study, the integrity of the LDL-CsA complex was validated prior to the binding experiments by gel filtration chromatography. Tritiated CsA, when incubated with LDL and applied to a gel filtration column, eluted from the column in the same fractions as ^{125}I-LDL, while free CsA bound to the column and eluted after repeated washings. Other investigators have verified the association of various drugs with LDL using this method in addition to ultracentrifugation (211,212), inclusion gel filtration chromatography (212), and precipitation with antibodies (211). Although the integrity of the ^{125}I-LDL-CsA complex was verified prior to the LDL-binding experiments, it cannot be assumed that the ^{125}I-LDL-drug complex remained intact throughout the experiment. Due to difficulties in performing dual-label experiments with ^{125}I-LDL and ^{3}H-CsA, the amount of bound or intracellular ^{3}H-CsA could not be determined by this method. Further, the level of CsA (unlabelled) following binding could not be determined by chemical methods either, since the assay for unlabelled CsA is a radioimmunoassay using iodinated CsA as the radioactive tracer. But the integrity of the LDL-CsA complex has been implied by the results of Sanghvi *et al.* who demonstrated that the *in vitro* uptake of ^{3}H-CsA associated with LDL in stimulated lymphocytes could be competed with LDL alone, and that the kinetics of the uptake of CsA were similar to that of LDL rather than CsA (209). However, they did not, nor did I, demonstrate that the ratio of the concentrations of CsA to LDL remained unchanged throughout the procedure.

It is tempting to speculate that the observed *in vitro* interactions between ^{125}I-LDL and the LDL-receptor mimic the *in vivo* setting. However, the conditions *in vivo* are decidedly different from that *in vitro*, making direct comparisons tenuous. In making comparisons, one major assumption is that the LDL-drug complex remains intact *in vivo*. This assumption, however, may not be valid. For example, de Smidt *et al.* found that even though the integrity of the LDL-floxuridine complex was validated prior to and during *in vitro* LDL-receptor binding experiments, the complex dissociated when similar experiments were conducted *in vivo* (210). The authors concluded that *in vivo*, the LDL-drug complex redistributes and re-equilibrates (210). One other explanation may be that since the drug is lipoprotein-bound, its distribution/redistribution may be affected by the normal metabolic processes of the lipoprotein, as is thought to be the case when benzo(*a*)pyrene is incorporated into chylomicrons (CM) (214). Investigators found that when benzo(*a*)pyrene is incorporated into CM, then injected intravenously into rats, within approximately thirty seconds, over 50% of the drug is bound to albumin, however, the rest is with VLDL, CM, and CM-remnants. Over time, most of the drug becomes associated with LDL and HDL, then after 60 minutes, the drug is found mainly within the lung, liver, and kidney (214). The authors concluded that not only does the drug redistribute, but that the CM-drug complex is also subject to normal metabolic processes of the CM *in vivo* (214). This argument may be relevant to the fate of the LDL-CsA complex *in vivo*.

Even though I found no difference in the affinities of the particles, had I determined that there was one, the biological significance of such a find is debatable. Several investigators have determined that receptor number rather than receptor affinity is more

significant in determining the uptake of the ligand, for a variety of ligands (213,94). In one study of LDL affinity in dyslipidemic patients[5], Mendel found that there was substantial variability in the affinities in the normal population (control) (213). Further, the mean and standard deviation of the affinity of LDL in dyslipidemic patients was nearly identical to the control (213). Mendel also showed that there was an inverse relationship with affinity and plasma LDL-cholesterol concentration and suggested that receptor number rather than affinity plays a more important role in determining plasma LDL-concentration (213). However, Mendel was studying patients with relatively small differences in LDL affinity. In reports of patients with a genetic defect of LDL (a glutamine-for-arginine substitution at amino acid 3500 of apo B-100), that markedly reduced their affinity for the LDL-receptor, the authors identified this defect as the cause of the elevated LDL-cholesterol plasma concentrations (215). Therefore, these data suggest that the significance of an altered affinity of LDL for its receptor may depend on the magnitude of the difference.

Rodl *et al.* determined that the amount of CsA with LDL in pooled plasma from CsA-treated patients (mean CsA plasma concentration of 210 $\pm$ 80 ng/ml) was 37 $\pm$ 12 ng CsA/ mg of LDL (154). Given this, then the levels of the drug used to determine the effect of CsA associated with LDL, on LDL binding to its receptor *in vitro*, are considerably higher than that found *in vivo*. However, the concentrations of CsA used in this study are similar to those used by Sanghvi *et al.* in their studies of LDL-CsA uptake via the LDL-

[5] The dyslipidemic subjects were randomly chosen from new patients at a Lipid Clinic. Mendel then excluded those patients from the study that: had a triglyceride level > 1000 mg/dl, were dysbetalipoproteinemic (E_2/E_2 apo E phenotype), were receiving lipid lowering drugs or, had secondary causes of hyperlipidemia (213).

receptor in stimulated peripheral blood lymphocytes (209).

In this thesis I hypothesized that CsA decreased the uptake of LDL via the LDL-receptor. This would provide, in part, a mechanism to account for the increased LDL-cholesterol levels seen in patients receiving this drug. Contrary to my expectations, I found that LDL was degraded faster in CsA-treated fibroblasts. In addition, I found that CsA does not decrease the binding of LDL to its receptor. These data imply that this is not a mechanism by which CsA therapy leads to increased plasma levels of LDL-cholesterol if these observations with fibroblasts are applicable to other cell types and transferable to the *in vivo* setting. Moreover, the question as to how CsA affects LDL-cholesterol levels remains unknown. These studies have focused on LDL-receptor activity and have not investigated any mechanisms that might increase the production of LDL.

In this thesis I investigated the effects of CsA on the uptake of LDL via the LDL-receptor. However, more than 20 metabolites of CsA have now been identified (9,10). In renal transplant recipients, the relative concentrations of metabolites AM1, AM9, and AM19 are 1.5, 0.75, and 0.7 times that of the parent drug, respectively (216-220). Although these metabolites have less than 10% of the immunosuppressive effect of CsA (220), they have been implicated in the pathogenesis of CsA-induced nephrotoxicity (203,204). The effects of CsA metabolites on LDL-receptor activity are unknown and because this work was beyond the scope of the thesis, they were not investigated.

LDL-receptor activity was initially demonstrated in cultured fibroblasts, however, they have been demonstrated in virtually all animal cells that grow in culture (221). I employed the well-established (44) fibroblast model in this study to investigate the effect of

CsA on LDL receptor activity. *In vitro*, the addition of saturating concentrations of LDL (approximately 15 μg/ml LDL) to fibroblasts results in a 10-15 fold reduction in the number of LDL-receptors (222,223), suggesting that most cells in the body are not in a state of LDL-receptor upregulation. This is supported by *in vivo* studies in rats and hamsters of LDL-receptor dependent that show that LDL uptake in tissues other than the liver, small intestine, endocrine organs, and spleen is very low (224). Further, that approximately 90% of all LDL-receptor activity was in the liver. These researchers also showed that the rate of LDL-receptor uptake in the liver is dependent on the concentration of LDL in plasma (224). They determined that the half-maximal rate of LDL transport in the liver was achieved at a plasma LDL concentration of 90 mg/dl (224). This is quite different from the situation in both cultured fibroblasts and hepatocytes where the half-maximal binding, internalization, and degradation are seen at approximately 3 mg/dl (225). Given the importance of the liver in the clearance of LDL, it remains to be determined whether hepatocytes also manifest these alterations.

2.4.3 The Effect of CsA on Human Skin Fibroblasts in Culture

The anti-proliferative effects of CsA *in vitro* are well-documented and are dependant on the cell type, concentration of CsA, the length of time the cells were exposed to the drug, and the presence or absence or serum in the growth medium (28,201,205-207). For example, in rabbit thoracic smooth muscle cells, cell proliferation (defined by cell number) was significantly inhibited at 1 μg/ml of CsA on the 4th day of culture whereas in aortic endothelial cells, at the same concentration of CsA, the reduction was not significant even by the 7th day of culture (206). Bjorkhem *et al.* reported that CsA had no effect on cultured

human macrophages at 30 μg/ml after 48 hours of culture (226). Dartsch and Schmid (227) suggested that fibroblasts are particularly resistant to the toxic effects of CsA *in vitro* since they showed that the level of CsA needed to decrease fibroblast proliferation (10 μg/ml) was approximately fifteen-fold greater than the amount needed to demonstrate the same effect with canine kidney cells. They also found that the anti-proliferative effects are reversible. Dartsch and Schmid also demonstrated that the anti-proliferative effects of the drug were not accompanied by any signs of cytotoxicity such as vacuolization, cell detachment, increased numbers of dead cells, or the presence of long cytoplasmic protrusions. Furthermore, the CsA-treated cells are still viable ($>90\%$ viable) as assessed by trypan blue (206) or neutral red bioassay (207). In contrast to the findings of Dartsh and Schmid (227), I observed vacuolization in the cultured fibroblasts at 15 μg/ml of CsA, rather than 10 μg/ml. In addition, cell proliferation, indirectly measured by the protein content of the culture dishes, was significantly reduced at 15 μg/ml of CsA. Further, the cellular protein content was significantly increased at 10 μg/ml CsA. However, these discrepancies might be explained by the differences in the length of the time the cells were exposed to the drug and the culture medium used. Dartsch and Schmid (227) grew the fibroblasts in CsA for only 4 days whereas, in the present study, the cells were exposed to CsA for 7 days and for the last 48 hours of growth, the cells were incubated in DMEM supplemented with lipoprotein-deficient serum (10% v/v). Fisher *et al.* (200) reported that when cells are cultured in serum-free medium, rather than when supplemented with fetal calf serum, the effects of CsA are more apparent at lower concentrations of the drug. The authors attributed these findings to the binding of CsA by the additional proteins and/or lipoproteins in the fetal calf serum since

there was approximately 2-3 times the amount of CsA associated with the cells in the absence of serum (200).

The fibroblasts were exposed to 5, 10, or 15 μg/ml CsA for 7 days. However, the amount of intracellular CsA was not determined. Since the contents of the incubation media changed during the course of the cells' growth, this may have affected the amount of CsA associated with the cells. When the fibroblasts were incubated DMEM/FCS (10% v/v) for the first 5 days of growth, less CsA may have be available to the cells. This might have been the result of 2 separate processes. First, it is thought that the proteins and/or lipoproteins in the FCS bind the drug (200) and, second, CsA binds to plastic. (The fibroblasts were cultured in plastic culture dishes.) However, because the CsA was pre-incubated with DMEM/FCS prior to it being added to the cells, this may have decreased the binding of CsA to the plastic. Nonetheless, the presence of the FCS may have resulted in a decreased amount of CsA available to the cells. When the cells were incubated with DMEM/LPDFCS (10% v/v) for the final 48 hours, more CsA may have been available to the cells due to the low concentration of lipoproteins. One way in which these problems may have been circumvented would have been to add the CsA only during the last day of growth. Not only would this have minimized the apparent fluctuations in the amount of CsA available to the cells, but the toxic effects of the drug would have been minimized. However, this step may have affected the results on LDL-receptor activity that were obtained in this study.

2.4.4 The Use of Cultured Fibroblasts *versus* Hepatocytes

Although LDL-receptor binding kinetics and activity were first described in human skin fibroblasts, this activity has also been demonstrated in other cell types including

hepatocytes. While there are many similarities between the two cell types there are a number of differences. First, LDL-receptors in hepatocytes are regulated differently than in fibroblasts; they are less responsive to feedback regulation. In fibroblasts, preincubation with LDL concentrations high enough to result in the down-regulation of LDL-receptor activity decreases LDL-receptor activity in hepatocytes by only 25-50% (44,). Further, HDL_3 has an increased stimulatory effect on LDL-receptor activity in hepatocytes in comparison to fibroblasts (44). Second, hepatocytes degrade LDL with lower efficiency than do fibroblasts (228) and there is a longer lag-time for the appearance of degradation products in the medium of hepatocyte cultures (30 minutes vs. 90 minutes) (228). Third, a low-affinity, non-saturable pathway for binding and degrading up to 25% of LDL has been identified in hepatocytes but is undetectable in fibroblasts (44). Fourth, the distribution of the receptor on the cell surface is different. On hepatocytes the receptor is thought to be evenly distributed over the cell surface whereas on fibroblasts the receptor is clustered in coated pits (44). However, this difference is somewhat controversial since there are some reports that show the receptor clustered in coated pits on hepatocytes (229).

Normal human hepatocytes are very difficult to obtain for experimental purposes. For this reason, many researchers use $HepG_2$ or Hep 3B cells which are derived from well-differentiated human hepatomas (190). For the most part, the regulation of LDL-receptor activity is similar to that seen in normal hepatocytes (190), although a few differences have been reported. $HepG_2$ cells have been reported to regulate LDL-receptor activity by cellular cholesterol levels as well as through the activation of second messengers (cAMP; cyclic adenosine monophosphate) by PMA (phorbol myrisitate acetate). Treatment of $HepG_2$ cells

with PMA resulted in nearly a 20-fold increase in LDL binding and a 9-fold increase in degradation (230). This effect was not apparent with normal human or rat liver cells (230). Other differences between normal human hepatocytes and $HepG_2$ cells is $HepG_2$ cells have defective bile acid synthesis and conjugation (231). Further, they secrete apo B-100 as a constituent of a lipoprotein with the density of LDL (76), whereas *in vivo*, apo B-100 is secreted predominantly as a constituent of VLDL (76).

In this thesis I chose to use fibroblasts in the preceding experiments rather than hepatocytes for the following reasons:

1) LDL-receptor activity was first described in fibroblasts and is very well-characterized in these cells. Cultured cells are assumed to express LDL-receptor activity if they bind LDL with the same characteristics as those exhibited by LDL binding in cultured human skin fibroblasts (44).

2) The binding and degradation of LDL in human fibroblasts is almost completely mediated via the LDL-receptor, unlike that in hepatocytes (44).

3) Since normal human hepatocytes were not available to me I would have had to use abnormal hepatocytes ($HepG_2$ or Hep 3B cells). Given that there have been differences noted in cholesterol metabolism between the abnormal and normal hepatocytes, had I found any CsA-induced effects on LDL-receptor activity, I would not have know if they were specific for these abnormal cells or indicative of normal hepatocytes.

4) Had I used hepatocytes, the effect of CsA on LDL degradation may not have been demonstrated given the incubation time (2 hours) chosen for the experiments and the fact that degradation in hepatocytes is slower and less efficient in comparison to fibroblasts.

In summary, the primary intent of this work was to study the effect of CsA on LDL via the LDL-receptor. Since fibroblasts are the best characterized cells with respect to LDL-receptor activity (44) and LDL uptake is almost completely mediated via the LDL-receptor, fibroblasts were used in this study (44).

2.4.5 Rationale for the use of Non-linear Regression in the Analysis of the Binding of ^{125}I-LDL to its Receptor

Typically, saturation binding isotherms, (Langmuir binding isotherm) are rectangular hyperbolas where the X-axis represents the radioligand concentration and the Y-axis represents specific binding (191). In the past, the determination of the K_d and B_{max} from these graphs was difficult so a number of linear transformations were applied so that these parameters could be determined. Scatchard analysis (Rosenthal-Scatchard Plot) is one form of linear transformation in which the specific binding is divided by the free radioligand concentration. A plot of bound/free (Y-axis) *versus* bound (X-axis) in a typical one-site model yields (hypothetically) a straight line with a negative slope. The K_d is the negative inverse of the slope and the B_{max} is the X-intercept (191). With all linear transformations, however, the weighting of errors is disproportionate across the range of ligand concentrations employed (191). Therefore, at low values of ligand concentration experimental errors are magnified and this effects the slope of the line much more than errors at high ligand concentrations. This distortion is more severe in transformations that combine x and y values as in Scatchard analysis (173,232). As a result, the binding data may be calculated incorrectly or misinterpreted (232). Furthermore, transforming the data and performing Scatchard analysis invalidates some of the assumptions of linear regression; that the

experimental error is Gaussian and uniform and that x is an independent variable (173,232). Therefore, Scatchard plots should not be used to analyze the results although they may still be useful for visualizing the data. In contrast to Scatchard analysis and other linear transformations, "the Langmuir binding isotherm does not produce inherent weighting errors. The common availability of appropriate computer programs makes nonlinear fitting to the Langmuir binding isotherm the most reliable means of evaluating experimental data for B_{max} and K_d." (191). Although initial reports by Brown and Goldstein on LDL-receptor binding were analyzed following linear transformation of the data, more recent papers by other authors have used non-linear regression (190). Therefore, in this thesis, the binding data were analyzed by non-linear regression. A Scatchard-Rosenthal plot is included for those readers more familiar with this method.

Chapter 3
The Distribution of CsA in Plasma

3.1 Introduction

The pharmacokinetics of CsA are variable (9) and one of the factors known to affect CsA's pharmacokinetics is the distribution of the drug in plasma (151). Since CsA is lipophilic it is bound to lipoproteins in plasma (39,40). The majority of the drug is associated with LDL and HDL, two cholesterol-rich particles (39,40). Although several investigators have demonstrated that hypertriglyceridemia (156,159) and hyperchylomicronemia (159) affected the distribution of CsA, studies by other researchers found that hyperlipidemia did not affect the drug's distribution (160), but the type of hyperlipidemia was not well-defined (160). Since the majority of CsA in plasma is bound to HDL and LDL, the effect of hypercholesterolemia and other well-defined dyslipidemic states on CsA's distribution merits investigation. Further, the effect of dyslipidemia should be studied by a technique other than ultracentrifugation. All of the previous studies were conducted using ultracentrifugation and this can affect CsA's binding and subsequent distribution in two ways. First, the high g forces used to achieve separation of plasma lipoproteins may erode the lipoprotein particles (43) and affect the binding equilibrium and distribution of the drug (233). Second, the high salt content of the density solutions used to facilitate separation may interfere with the hydrophobic bonds between the lipoproteins and CsA, as has been demonstrated with other lipophilic drugs (234).

The objectives of this part of the thesis are two-fold. First, investigate a suitable method with which to study the distribution of CsA in plasma. Second, using this method, determine the effects of dyslipidemia on the distribution of CsA in plasma. The methods

investigated were gel filtration chromatography, sequential ultracentrifugation, and phosphotungstic acid precipitation. These methods were compared to the results obtained by density gradient ultracentrifugation. The phosphotungstate method was selected as the best method and it was used to investigate the effects of dyslipidemia (hypercholesterolemia, hypertriglyceridemia, hypoalphalipoproteinemia, and a combination of hypercholesterolemia and hypertriglyceridemia) on the distribution of CsA in plasma.

3.2 Materials and Methods

3.2.1 Separation of Serum Lipoproteins by Gel Filtration Chromatography

3.2.1.1 Isolation of Total Serum Lipoproteins

Serum lipoproteins ($d < 1.25$ g/ml) were isolated from the serum of healthy donors by preparative ultracentrifugation (181). The serum was collected and the density adjusted to 1.25 g/ml as described in a previous section (2.1.1.1). The serum was centrifuged at 125,000 X g at 15°C or 37°C for 48 hours. For those samples centrifuged at 37°C, 1 mM DIFP (di-isopropyl phosphofluorodate) was added to the sample to inhibit endogenous enzymatic activity (235). Following centrifugation, the top fraction containing the lipoproteins was removed by suction after slicing off the top of the centrifuge tube using a tube slicer. The lipoproteins were dialyzed against three changes of 0.15 M NaCl, 1 mM EDTA and 0.03% (w/v) sodium azide at 4°C for 24 hours. Following dialysis, radiolabelled (^{3}H-CsA) (Amersham, Oakville, Ont.) or unlabelled CsA was added to a final concentration of 200-300 ng/ml, and allowed to equilibrate for 1 hour at 37°C. An aliquot was then removed for the determination of total radioactivity (Beckman LS-9000, Liquid Scintillation Counter, Beckman Instruments, Fullerton, CA).

3.2.1.2 Gel Filtration Chromatography

Serum lipoproteins were separated by gel filtration chromatography utilizing a Superose 6 column (1.6 cm X 50 cm) (Pharmacia, Dorval, Que.). One millilitre of total serum lipoproteins isolated as described above and containing CsA, was applied to the column and 1.0 ml fractions were eluted in a buffer of 0.15 M NaCl, 1 mM EDTA, and 0.03% (w/v) sodium azide at a flow rate of 1 ml/min at room temperature or at 37° C. To maintain the column at 37°C, the eluting buffer was kept in a water bath (37°C), the column was wrapped with a heating tape and the temperature of the column effluent monitored. The column was monitored by absorbance at 280 nm and the elution of ^{3}H-CsA was followed by determining the amount of radioactivity in each column fraction (Beckman LS-9000, Liquid Scintillation Counter, Beckman Instruments, Fullerton, CA). The column was standardized by with respect to elution times for VLDL, LDL, HDL (ultracentrifugally isolated), and human serum albumin. In addition, the column was characterized with respect to cholesterol, triglycerides, and phosphorous by the methods described below.

3.2.1.3 Characterization of the Superose 6 Column

3.2.1.3.1 Standardization of elution volumes for VLDL, LDL, HDL, and Albumin

VLDL ($d < 1.006$ g/ml), LDL ($d = 1.006$-1.063 g/ml), and HDL ($d = 1.063$-1.21g/ml) were isolated from the serum of heathy donors according to the method of Havel *et al.* (170). One millilitre of VLDL, LDL, HDL, or human serum albumin (70 g/l) was applied to the column and eluted as previously described. The column effluent was monitored by absorbance at 280 nm and the elution volumes for the individual lipoproteins (VLDL, LDL, HDL) and human serum albumin determined.

3.2.1.3.2 Determination of Total Cholesterol

Total cholesterol in the column fractions was determined by the method described by Heider and Boyett (236). The method is based on the enzymatic hydrolysis of cholesterol esters to free cholesterol, the oxidation of cholesterol with the liberation of hydrogen peroxide, and the reaction of this peroxide with a fluorogen to form a fluorescent product. An aliquot (30 μl), of the column fraction was added to 0.40 ml of a solution containing 0.05 M sodium phosphate buffer (pH 7.0), 0.08 U/ml cholesteryl ester hydrolase, 0.08 U/ml cholesterol oxidase, 30 U/ml horseradish peroxidase, 5 mM sodium taurocholate, 0.17 mM carbowax-6000 and 0.15 mg/ml *p*-hydroxyphenylacetic acid. After an incubation period of 20 minutes at 37°C, the fluorescent product was enhanced by the addition of 0.8 ml of 0.5 N NaOH. The amount of fluorescence was measured using an Aminco Bowman spectrofluorometer (American Instruments, Silversprings, MD) at an excitation wavelength of 325 nm and an emission wavelength of 425 nm. The cholesterol content of the column fractions was determined from a standard curve using cholesteryl oleate as the standard.

3.2.1.3.3 Determination of Phosphorous

One hundred microlitres of the column fraction was added to a tube containing 2 ml $CHCl_3$, 2 ml methanol (100%), 2 ml H_2O, 50 μl glacial acetic acid and 0.10 ml 5 M NaCl. The tube was mixed thoroughly then centrifuged at 1500 X g for 10 minutes. The bottom, organic layer, was transferred to a clean tube and 1.0 ml aliquot transferred to a clean tube then dried under N_2. Following this, 0.1 ml of H_2SO_4 was added and the tube was heated at 153°C for 10 minutes. The tube was then placed in an ice bath and 0.05 ml of 6% H_2O_2 added. The tubes were mixed thoroughly and returned to 153°C for 40 minutes. Following

this, the tubes were cooled and 2.5 ml H_2O, 0.5 ml 2.5% ammonium molybdate, 0.5 ml 10% ascorbic acid were added and the tubes thoroughly mixed. The tubes were then placed at 100°C for 7 minutes after which they were cooled completely and the absorbance at 797 nm was determined (Lambda 3B spectrophotometer, Perkin Elmer, Oak Brook, IL). The amount of phosphorous in the column fractions was determined from a standard curve of KH_2PO_4. This method is that described by Anderson and Davis (237).

3.2.1.3.4 Determination of Triglyceride

Triglyceride content in column fractions was determined by the method of Mendez *et al.* (238). This method relies on the enzymatic liberation of glycerol from triglycerides by lipase, its conversion by glycerol kinase to glycerol-3-phosphate, which then is oxidized by glycerol-3-phosphate oxidase, producing H_2O_2. The peroxide then forms a peroxidase-catalyzed fluorogen with *p*-hydroxyphenylacetic acid. An aliquot (0.1 ml) of the column fraction was mixed thoroughly with 1 ml of a solution containing 0.05 M potassium phosphate buffer, pH 8.5, 0.6 mM $MgCl_2$, 0.54 mM ATP, 0.01% triton X-100, 0.1 U/ml glycerol kinase, 1.5 U/ml glycerol-3-phosphate oxidase, 1.2 U/ml peroxidase, 0.8 mg/ml *p*-hydroxyphenyl acetic acid, and 1 U/ml lipase. The mixture was incubated at 37°C for 45 minutes after which the fluorescence was determined spectrofluorometrically (Aminco Bowman, American Instruments, Silver Springs, MD) at an excitation wavelength of 325 nm and an emission wavelength of 415 nm. The amount of triglyceride present in the column fractions was determined from a standard curve of triolein.

3.2.2 Separation of Serum Lipoproteins by Ultracentrifugation

3.2.2.1 Density Gradient Ultracentrifugation

Tritiated CsA (Amersham, Oakville, Ont.) (mebmt-β-^{3}H-CsA, 5-20 Ci/mol) (200 ng/ml) was added to 2.0 ml of serum pooled from healthy donors, allowed to equilibrate as described in section 3.2.1.1 and an aliquot removed to determine the amount of radioactivity present. The density of the serum was adjusted to 1.25 g/ml by the addition of solid NaBr as previously described. A density gradient of 1.006-1.25 g/ml of NaBr was made by means of a gradient mixer and the 1.25 g/ml serum containing the CsA was underlayed on this density gradient using a glass syringe. The sample was centrifuged for 48 hours at 15°C or 37°C for 48 hours at 125,000 X g. Following ultracentrifugation, the sample was removed from the tube by means of a pump and collected into 0.5 ml fractions. An aliquot of each fraction was removed to determine the amount of radioactivity as described. The density of each fraction was also determined by weighing a 0.2 ml aliquot.

3.2.2.2 Sequential Ultracentrifugation

VLDL ($d < 1.006$ g/ml), LDL ($d = 1.006$-1.063 g/ml), and HDL ($d = 1.063$-1.22 g/ml) were isolated from serum of healthy, fasted donors by preparative ultracentrifugation according to Havel *et al.* (181). Briefly, serum was separated from cells by centrifugation as described and a known amount of ^{3}H-CsA (Amersham, Oakville, Ont.) and unlabelled CsA were added to achieve a concentration of 200-300 ng/ml, mixed thoroughly, and allowed to equilibrate at 37°C for 1 hour. An aliquot was removed for the determination of total radioactivity as described. The serum was centrifuged for 18 hours at 15°C or 37°C after which the top layer, containing VLDL was removed and the amount of radioactivity

determined as described. The amount of radioactivity in the bottom layer was also determined after which the density was adjusted to 1.063 g/ml by the addition of solid NaBr as described previously. The serum was centrifuged for 24 hours at 15°C or 37°C. The top layer, containing LDL was removed, and the amount of radioactivity determined. The amount of radioactivity associated with the bottom layer was also determined, after which the density was adjusted to 1.22 g/ml by the addition of solid NaBr as described. The serum was then centrifuged for 48 hours at 15°C or 37°C. Following centrifugation, the top fraction containing HDL, was removed as was the bottom layer and the amount of radioactivity in both fractions determined.

3.2.3 Separation of Plasma Lipoproteins by Phosphotungstic Acid (PTA) Precipitation

3.2.3.1 Preliminary Investigation with PTA Precipitation

A preliminary study was undertaken to determine the feasibility of the PTA precipitation method for studying CsA distribution in plasma. Tritiated CsA was added to EDTA plasma of patients from the Shaughnessy Hospital Lipid Clinic or to the plasma of healthy donors to a concentration of 200 ng/ml, allowed to equilibrate as described in section 3.2.1.1, and an aliquot removed for the determination of radioactivity. Fifty microlitres of phosphotungstic acid reagent (PTA)(4% (w/v) tungstophosphoric-phosphoric acid) (BDH, Edmonton, Alta.) were added to 0.5 ml of plasma, mixed thoroughly and incubated for 2 minutes at room temperature. Following incubation, 12.5 μl of 2 M $MgCl_2$ was added, the sample mixed thoroughly, then incubated at 4°C for 30 minutes. The sample was centrifuged at 3,000 X g for 30 minutes at 4°C (239). An aliquot of the supernatant was removed to determine the amount of radioactivity present.

3.2.3.2 Effect of Temperature on PTA Precipitation

Tritiated CsA (200 ng/ml) was added to 0.5 ml normolipemic plasma, allowed to equilibrate as described above and an aliquot removed to determine the amount of radioactivity present. PTA reagent was added as described above and the samples incubated at room temperature. Following the addition of 12.5 μl of 2 M $MgCl_2$, the sample was mixed and incubated for 30 minutes at either 4°C, room temperature, or 37°C. The precipitate was collected as described and resuspended in 0.5 ml 0.5 M Na_2CO_3. An aliquot of the precipitate and supernatant were removed to determine the amount of radioactivity present. In addition, the amount of cholesterol present before and after precipitation at the various temperatures was determined as described below (section 3.2.5).

3.2.3.3 Sequential PTA Precipitation

Tritiated CsA (200 ng/ml) was added to the plasma of selected patients from the Shaughnessy Hospital Lipid Clinic or to plasma of healthy donors, allowed to equilibrate as described above, and an aliquot removed to determine the total amount of radioactivity present. To precipitate VLDL (239), 12.5 μl of PTA reagent was added to 0.5 ml of plasma. The sample was mixed thoroughly, then incubated for 2 minutes at room temperature. Following this, 12.5 μl of 2 M $MgCl_2$ was added, the sample mixed thoroughly, then incubated at 4°C for 30 minutes. Following incubation, the sample was centrifuged at 14,000 X g for 15 minutes. An aliquot of the supernatant was removed to determine the amount of radioactivity. To precipitate LDL and VLDL, the procedure was as described above except 50 μl of PTA reagent was added to the sample (239). The sample was incubated as described and the precipitate collected by centrifugation at 4°C at 3000 X g for 30 min. The

supernatant was removed and the precipitate resuspended in 0.5 ml of 0.5 M Na_2CO_3. An aliquot of each was removed to determine the amount of radioactivity present. To precipitate HDL, a 0.5 ml aliquot of the supernatant left after precipitating the VLDL and LDL was used. To this, 450 μl of PTA reagent was added, incubated for 2 minutes at room temperature after which 38 μl of 2 M $MgCl_2$ was added and the sample incubated for 30 minutes at 4°C. The precipitate formed was collected by centrifugation and resuspended as described above (239). The amount of radioactivity associated with each fraction was determined.

3.2.3.4 Distribution of CsA in Normolipidemic and Dyslipidemic Plasma using Sequential Phosphotungstic Acid Precipitation

Plasma was collected from healthy, normolipidemic volunteers and dyslipidemic patients. The following dyslipidemic groups were studied: hypercholesterolemic (total cholesterol = 6.5-7.5 mmol/l), hypertriglyceridemic (triglyceride = 4-6 mmol/l), hypoalphalipoproteinemic (HDL $<$ 0.9 mmol/l), and hypercholesterolemic and hypertriglyceridemic (cholesterol = 6.5-7.5 mmol/l and triglyceride = 4-6 mmol/l). The distribution of CsA in these plasma samples was determined by sequential PTA as described above.

3.2.4 Determination of CsA by Radioimmunoassay

CsA concentrations in serum, plasma, and column fractions were determined with a radioimmunoassay kit as outlined by the manufacturer (Cyclo-trac SP, Stillwater, MN).

3.2.5 Plasma Chemistries

Plasma cholesterol, HDL-cholesterol, and triglyceride measurements were performed

in the clinical laboratory of the University Hospital - Shaughnessy Site using a Technicon RA-500 by routine methods. LDL-cholesterol levels were calculated from the Friedwald formula (240) :

$$\text{LDL-c} = \text{TC} - \text{HDL-c} - (\text{triglyceride level}/2.22)^{6}$$

where LDL-c is LDL-cholesterol, TC is total cholesterol, and HDL-c is HDL-cholesterol.

3.2.6 Statistical Analyses

3.2.6.1 Distribution of ^{3}H-CsA in Serum using Density Gradient Ultracentrifugation

Statistical analyses were performed using the InStat statistical program (GraphPad, San Diego, CA). Differences between the means were compared using the Student's t- test for unpaired data. Differences were considered significant at $p < 0.05$, where p represents the probability for two-tailed tests.

3.2.6.2 The Effect of Temperature on PTA Precipitation

Statistical analysis was performed using the statistical program described above. Differences between the means of the dyslipidemic (experimental) groups were compared to the normolipidemic (control) group using a one-way ANOVA with a Dunnet's post-test. Differences were considered significant at $p < 0.05$.

3.2.6.3 Distribution of ^{3}H-CsA in Normolipidemic and Dyslipidemic Plasma using Sequential PTA Precipitation

Due to the heterogeneity of variances and the fact that the standard deviations were proportional to the means, the data from the normolipemic control group and all of the patient groups studied were log-transformed prior to statistical analysis as recommended by

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[6]This formula is valid only if the fasting triglyceride level is <4.52 mM (241).

Zar (196). The statistical tests used were a one-way ANOVA with multiple comparisons performed according to Bonferroni. Statistical analysis was performed using InStat (GraphPad, San Diego, CA) computer program and differences were considered significant at $p < 0.05$.

3.3 Results

3.3.1 Separation of Serum Lipoproteins by Gel Filtration Chromatography

3.3.1.1 Characterization of the Superose 6 Column

VLDL, LDL, and HDL were fully separated on the Superose 6 column (Figure 19). Their elution volumes did not change (within two fractions) when the flow rate was decreased to 0.5 ml/min, the pH was varied between 5.5-7.4, or when the concentration of NaCl in the eluting buffer was 10 times the original (results not shown). Unfortunately, the column did not resolve HDL and albumin (Figure 19). The elution profiles of cholesterol, triglyceride, and phosphorus (Figures 20 and 21) demonstrated that the majority of cholesterol eluted at the same time as LDL, the majority of triglyceride eluted with VLDL and, the majority of phosphorous eluted with HDL. This is consistent with the facts that LDL is a cholesterol-rich particle, VLDL is a triglyceride-rich particle and, HDL contains the majority of phospholipid, in normolipidemic serum. However, the elution profiles of cholesterol and triglyceride from a dyslipidemic patient (renal transplant recipient) are different (Figure 22). The ratio of the amount of cholesterol to triglyceride in the peak corresponding to VLDL appears greater in the dyslipidemic patient (Figure 22) in comparison to that of the normolipidemic patient (Figure 20). The recovery of cholesterol and triglyceride from the column approximated 70% and 80%, respectively.

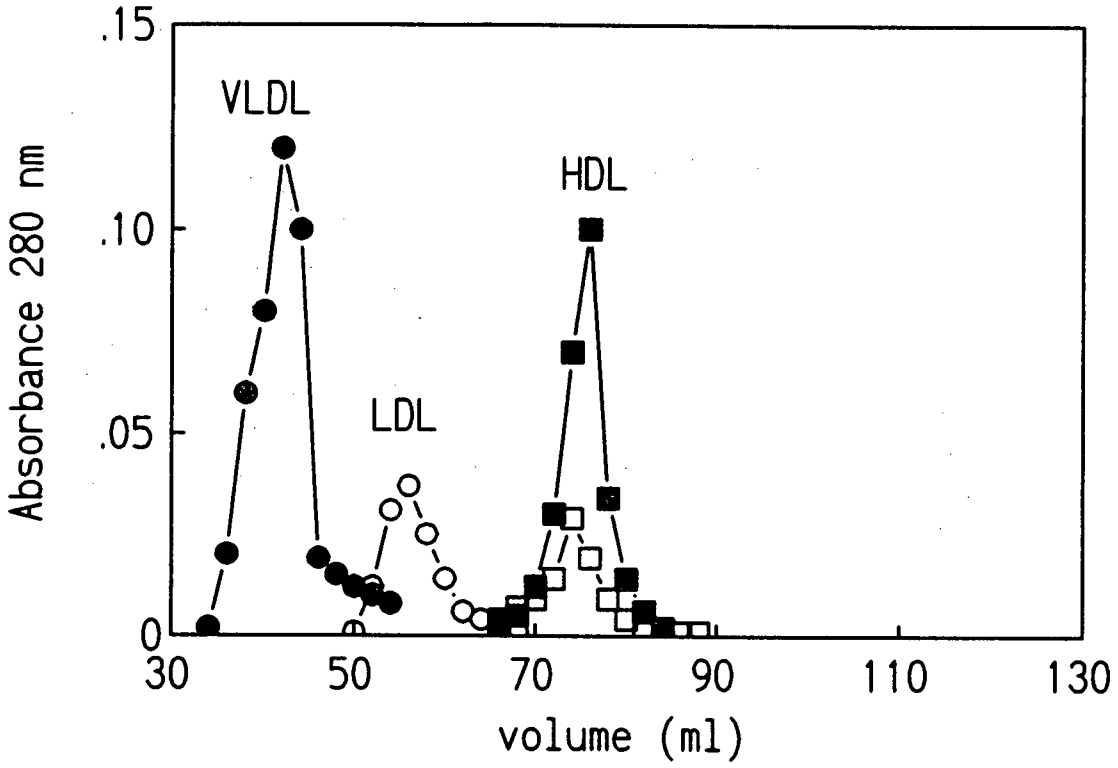

Figure 19. Elution profile of lipoprotein standards and human serum albumin from a Superose 6 gel filtration column. Ultracentrifugally isolated lipoprotein standards (VLDL (●), <1.006 g/ml, LDL (○), 1.006-1.063 g/ml, and HDL (■), 1.063-1.22 g/ml) and human serum albumin (□), were applied separately to the gel filtration column and eluted in a buffer of 0.15 M NaCl, 1 mM EDTA, and 0.03% azide, at 21°C at a flow rate of 1 ml/min. The elution of the lipoproteins and human serum albumin was monitored by Absorbance at 280 nm.

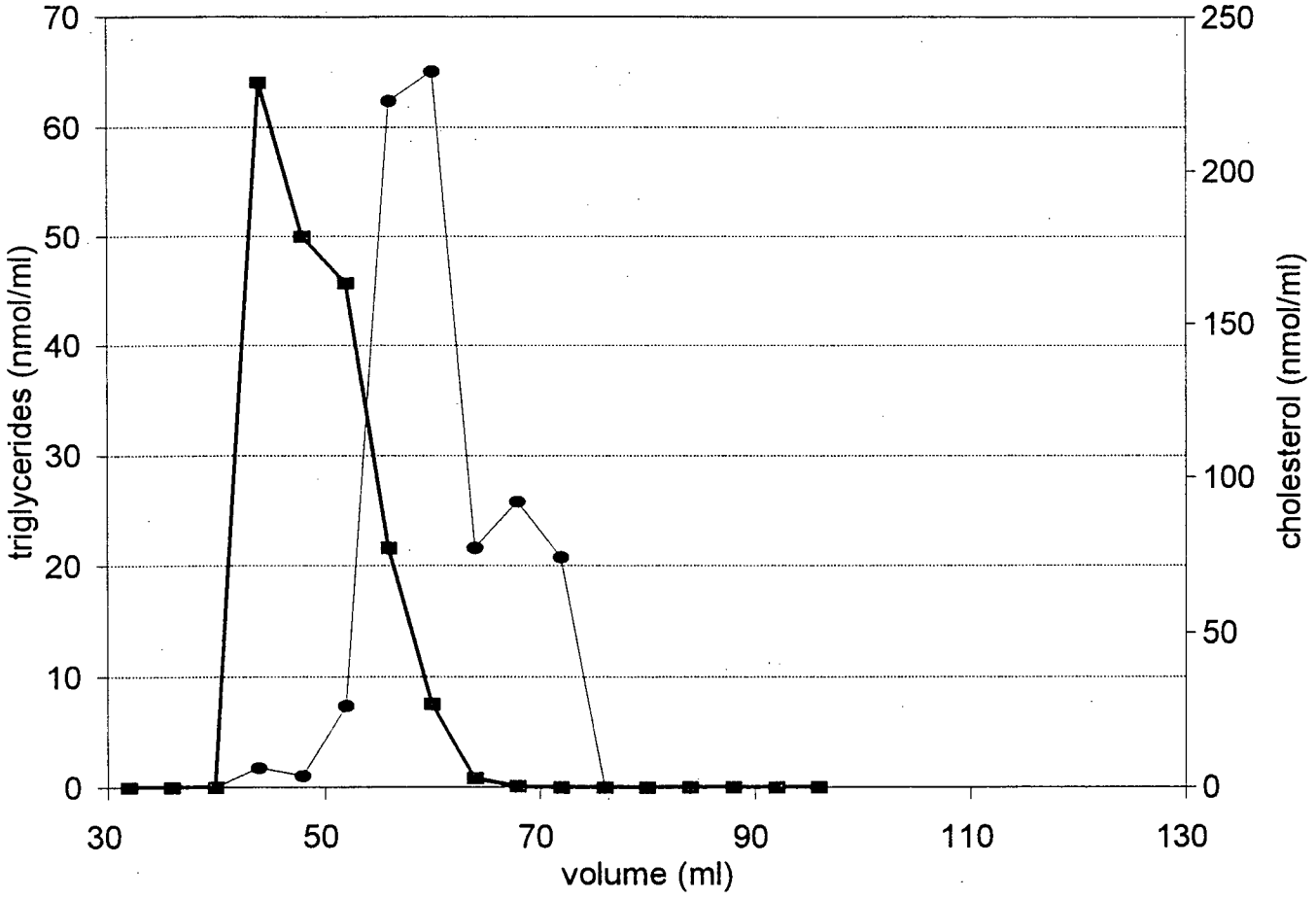

Figure 20. Elution profile of serum total lipoproteins from a Superose 6 gel filtration column when the column is monitored for cholesterol and triglyceride levels. Ultracentrifugally isolated total serum lipoproteins ($d < 1.25$ g/ml) were applied to a gel filtration column and eluted as in Figure 19. Their elution was monitored by triglyceride (■) , and cholesterol (●) levels.

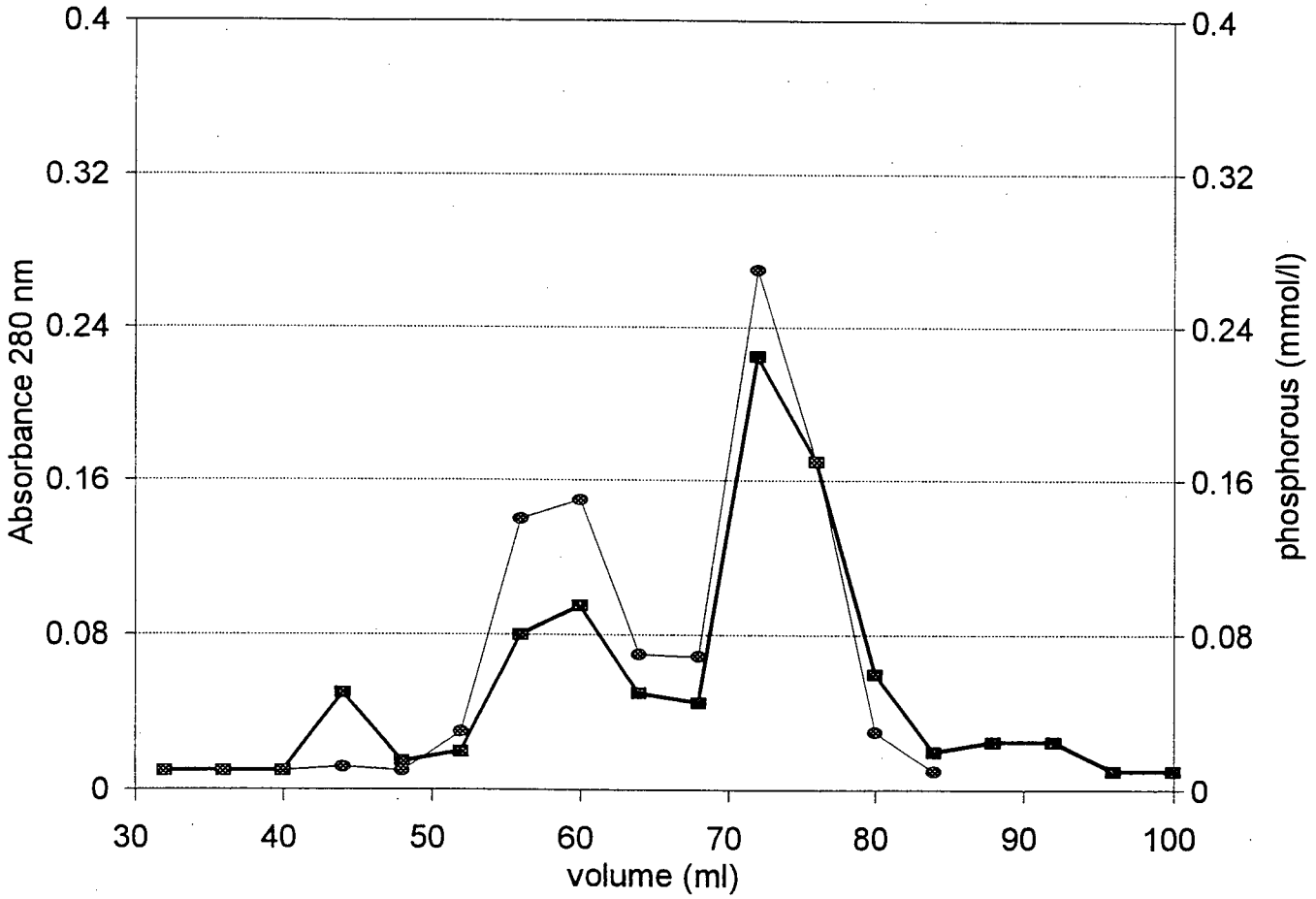

Figure 21. Elution profile of total serum lipoproteins from a Superose 6 gel filtration column when the column is monitored for phosphorus. Ultracentrifugally isolated total serum lipoproteins ($d < 1.25$ g/ml) were applied to a gel filtration column and eluted as in Figure 19. Elution was monitored by Absorbance at 280 nm. (■), and phosphorous (●).

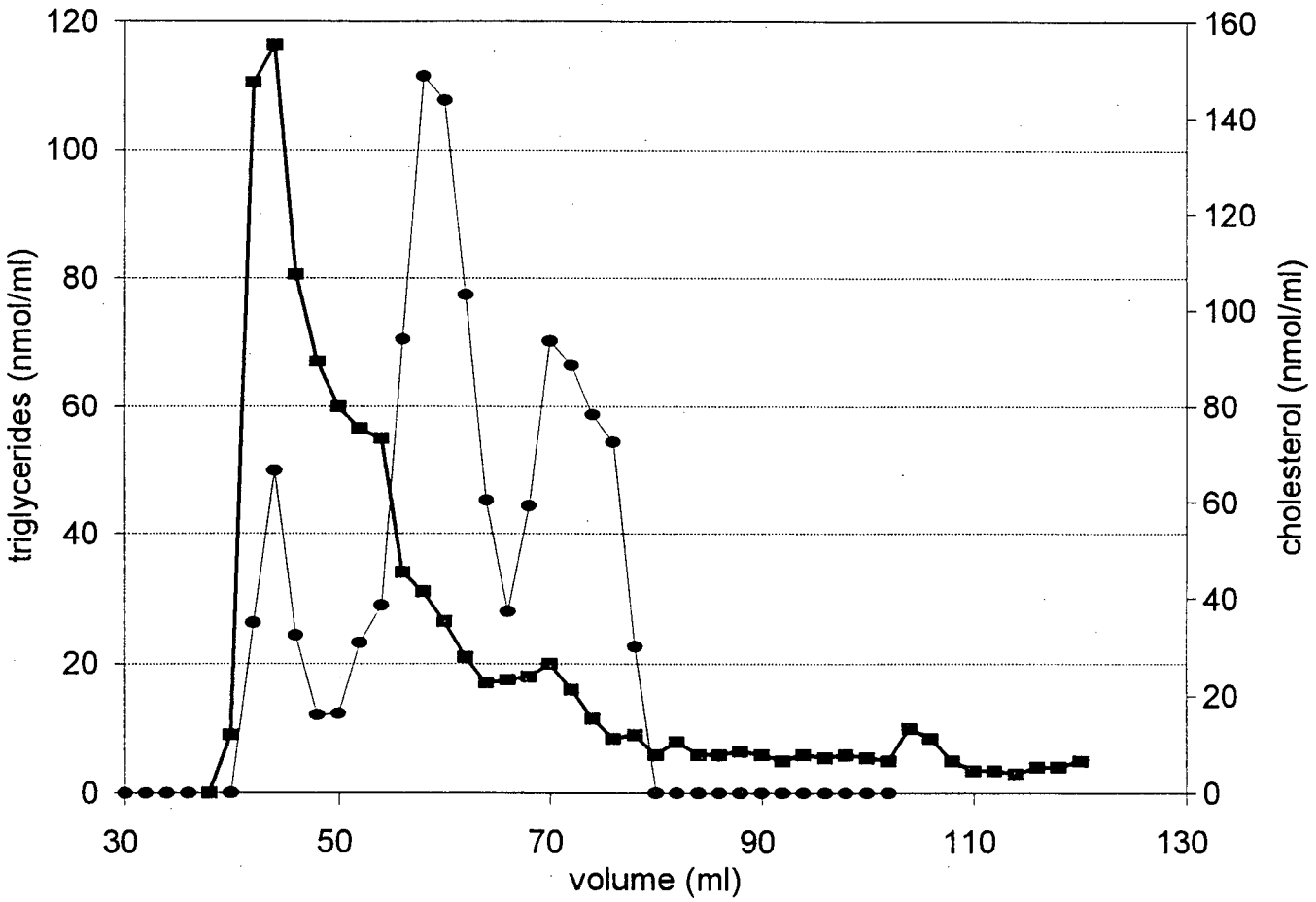

Figure 22. Elution profile of total serum lipoproteins isolated from a transplant patient from a Superose 6 gel filtration column. Ultracentrifugally isolated total serum lipoproteins ($d < 1.25$ g/ml) from a transplant patient were applied to a gel filtration column, eluted as in Figure 19, and their elution was monitored by triglyceride (■), and cholesterol (●), levels.

The elution profiles and recovery of ^{3}H-CsA and CsA as measured by RIA, were similar (Figure 23). The recovery of CsA by RIA was typically 75-83% of that recovered by monitoring ^{3}H-CsA. This being so, it was decided that with subsequent experiments, the amount of CsA in column fractions would be determined by estimating the amount of radioactivity only, since it was more efficient.

3.3.1.2 Distribution of ^{3}H-CsA in Normolipidemic Serum using Gel Filtration Chromatography following Ultracentrifugation

None of the ^{3}H-CsA applied to the column eluted with either VLDL or LDL at 21°C following gel filtration of total serum lipoproteins (Figure 24). In addition, only 22% of the ^{3}H-CsA was present with the fraction which corresponded to HDL and albumin. When the column temperature was increased to 37°C, even less ^{3}H-CsA (<9% of total radioactivity applied to column) was associated with the HDL and albumin fraction and, as before, none was present with either VLDL or LDL (Figure 25).

The recovery of ^{3}H-CsA following ultracentrifugation at 15°C was nearly complete (99%), with most (90%) being recovered in the lipoprotein fraction (Table 9). When the temperature of ultracentrifugation was increased to 37°C, the recovery was slightly decreased (87%) and less was associated with the lipoprotein fraction (79%) (Table 9). The recovery of CsA from the gel filtration column at both temperatures was poor (50% and 59%) (Table 9). The recovery of CsA did not appear to be dependant on the concentration of CsA since it was similar at 200 ng/ml and 2000 ng/ml (results not shown). Further, if the CsA was pre-equilibrated and chromatographed with whole serum instead of total lipoproteins, the recovery remained low (results not shown). Binding of the radioactive label to the column's

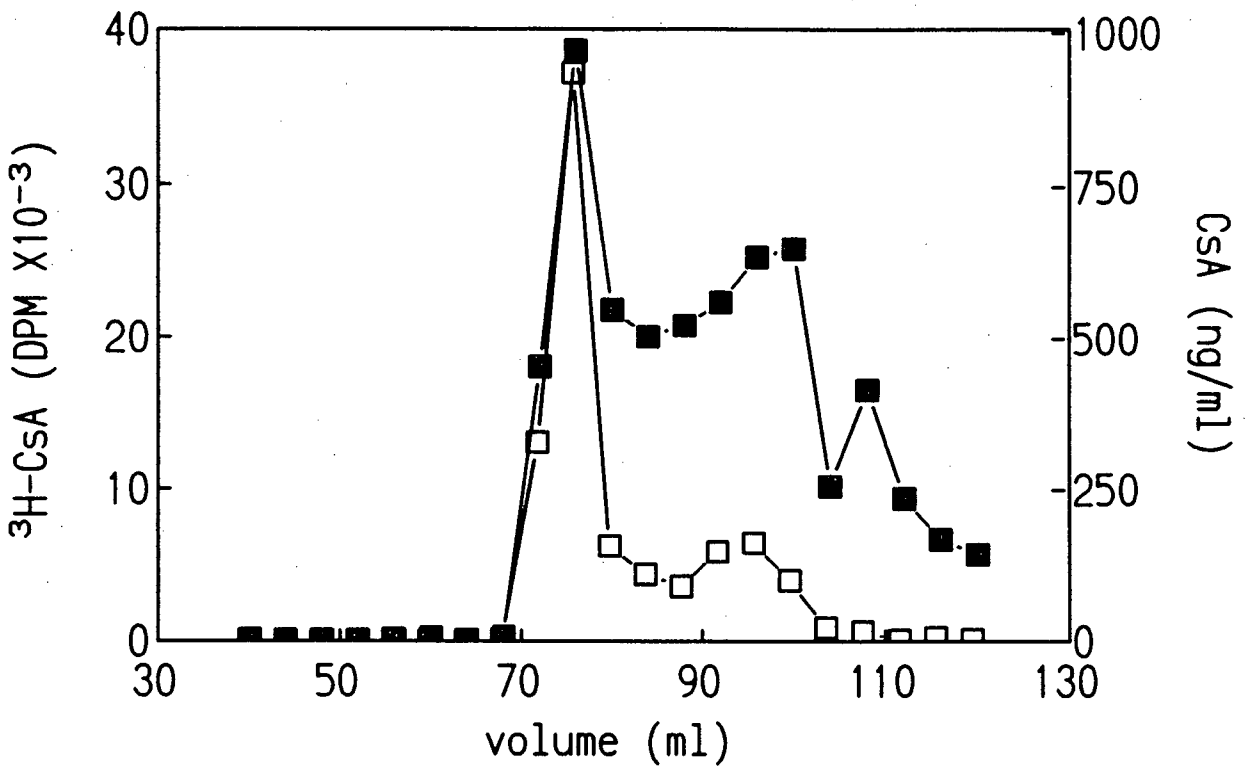

Figure 23. Elution profile of ^{3}H-CsA and CsA from a Superose 6 gel filtration column when the column is monitored for radioactivity and CsA levels. Labelled (^{3}H) and unlabelled CsA was pre-incubated with ultracentrifugally isolated total serum lipoproteins ($d < 1.25$ g/ml) for 1 hour at 37°C, applied to a gel filtration column and eluted as in Figure 19. The elution of CsA was monitored by radioactivity (DPM) (■) and CsA levels (□).

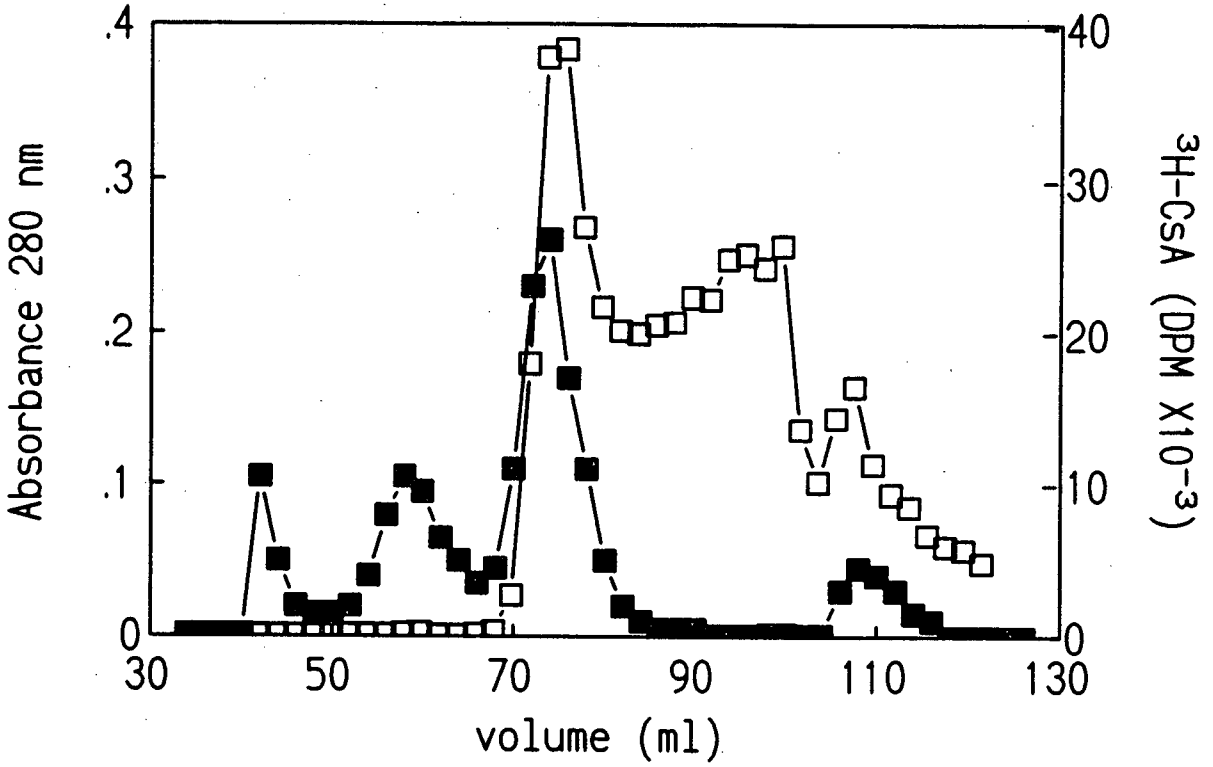

Figure 24. Elution profile of total serum lipoproteins and ^{3}H-CsA from a Superose 6 gel filtration column at 21°C. ^{3}H-CsA was pre-incubated with ultracentrifugally isolated total serum lipoproteins ($d < 1.25$ g/ml) as in Figure 23, applied to a gel filtration column and eluted as in Figure 19 at 21°C. The elution of lipoproteins was monitored by Absorbance 280 nm.(■), and ^{3}H-CsA by radioactivity (DPM) (□).

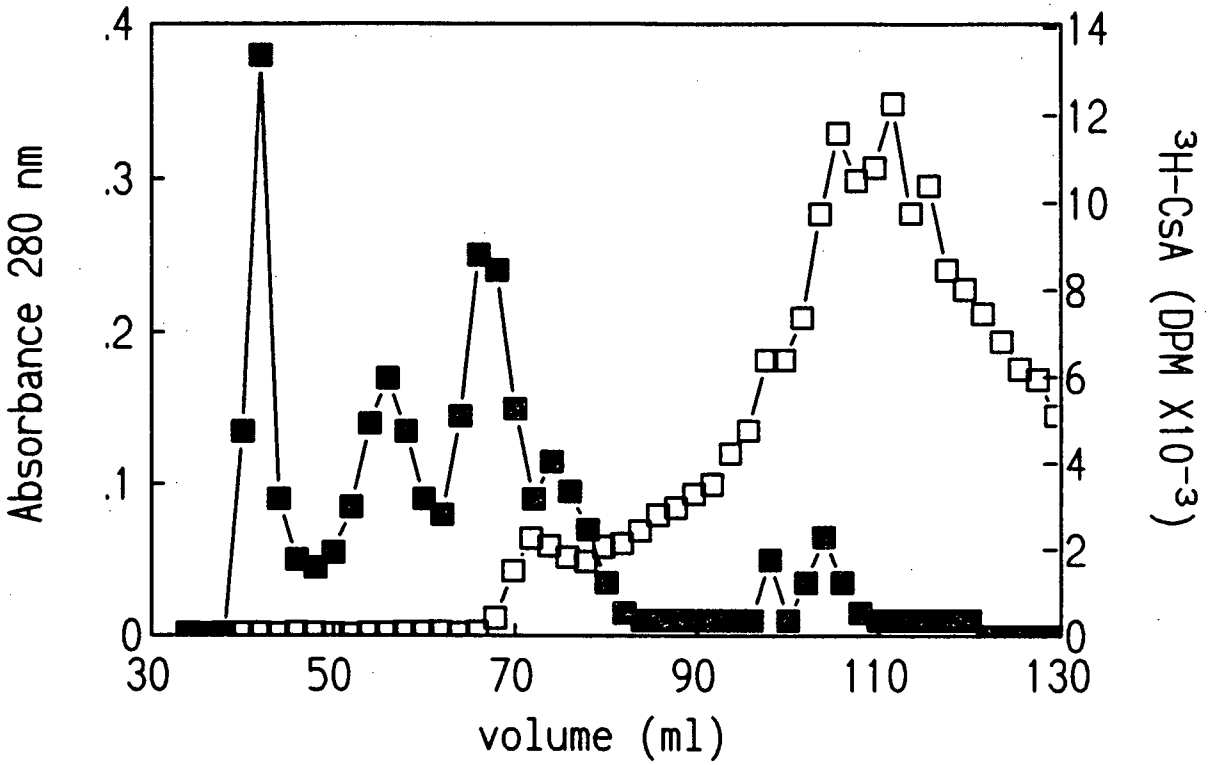

Figure 25. The effect of temperature on the elution profile of total serum lipoproteins and ^{3}H-CsA from a Superose 6 gel filtration column. ^{3}H-CsA was pre-incubated with total serum lipoproteins ($d < 1.25$ g/ml) as in Figure 23, applied to a gel filtration column and eluted in a buffer of 0.15 M NaCl, 1 mM EDTA, 0.03% azide at 37°C. The elution of lipoproteins was monitored by Absorbance at 280 nm. (■), and ^{3}H-CsA by radioactivity (DPM) (□).

pre-filter accounted for $<0.2\%$ of the losses.

Table 9. The effect of temperature on the recovery (%) of ^{3}H-CsA following ultracentrifugation and gel filtration chromatography

	Temperature	
Procedure	**15°C**	**37°C**
Total recovery following ultracentrifugation	**99%**	**87%**
Recovery in lipoproteins following ultracentrifugation	**90%**	**79%**
Recovery following gel filtration	**52%**	**59%**

Data are means of 2 experiments

When individual lipoproteins, LDL or HDL, were pre-equilibrated with ^{3}H-CsA and chromatographed, none of the CsA eluted with these fractions (Figures 26a, 27a). However, when these experiments were repeated using an eluting buffer with 10 times the original NaCl concentration, all of the recovered ^{3}H-CsA eluted with the lipoprotein (Figures 26b, 27b). In addition, the recovery of the radioactive label increased from 42% to 62% for LDL, and from 36% to 83% for HDL. Increased binding of ^{3}H-CsA to lipoproteins and better recovery of the radioactive label were also evident when total serum lipoproteins were chromatographed at incremental NaCl concentrations (Figure 28). At 10 times the original NaCl concentration, 13% and 78% of the recovered ^{3}H-CsA eluted with the LDL and HDL/albumin fractions, respectively (Table 10). Further, this increased recovery with a high-salt eluting buffer also occurred with samples from patients receiving CsA as part of their immunosuppressive therapy (results not shown).

3.3.2 Distribution of ^{3}H-CsA in Serum using Density Gradient Ultracentrifugation

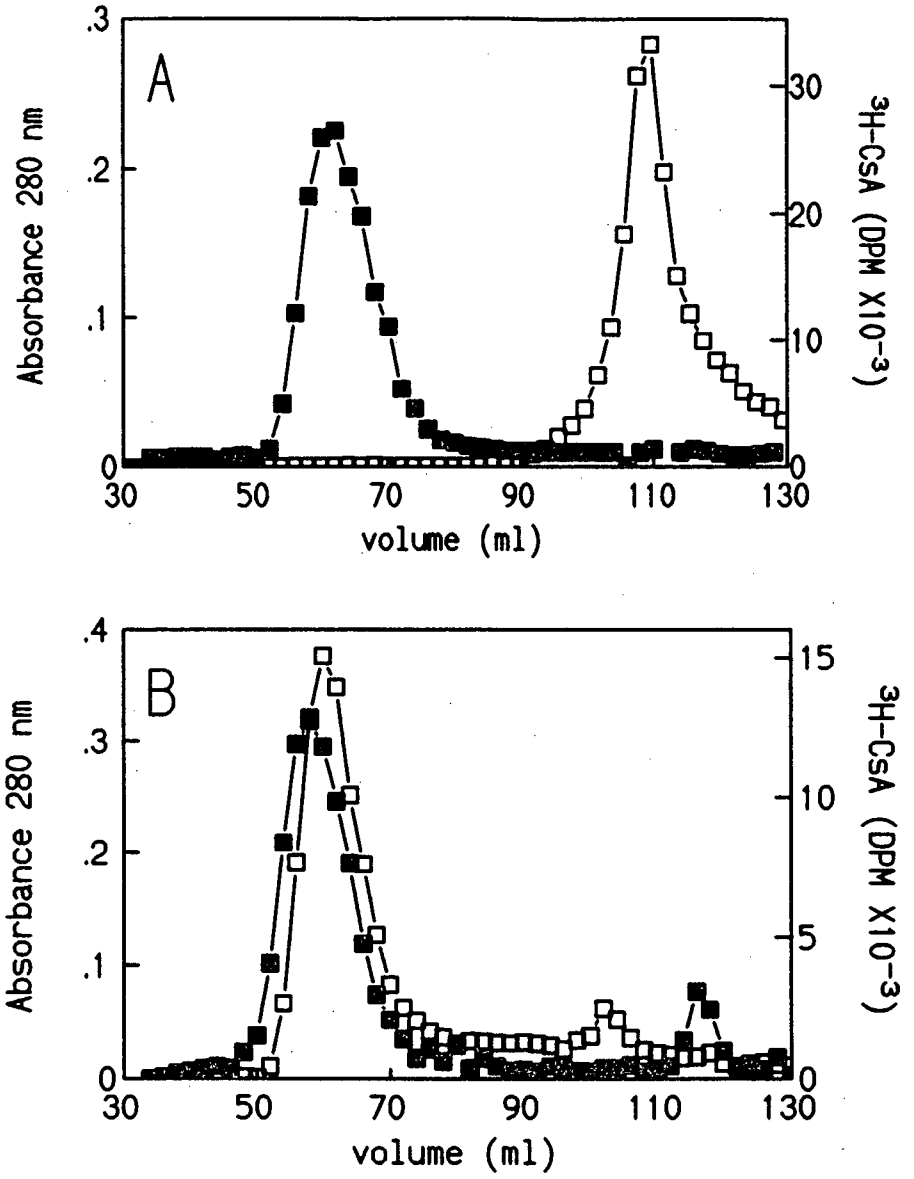

Figure 26. The effect of increasing the concentration of NaCl in the eluting buffer from 0.15 M to 1.5 M on the elution profile of LDL and ^{3}H-CsA from a Superose 6 gel filtration column. ^{3}H-CsA was pre-incubated with ultracentrifugally isolated LDL (d=1.006-1.063 g/ml) for 1 hour at 37°C, applied to a gel filtration column and eluted with a buffer containing 0.15 M (A) and 1.5 M (B) NaCl at 21°C. The elution of LDL was monitored by Absorbance at 280 nm. (■), and ^{3}H-CsA by radioactivity (DPM) (□).

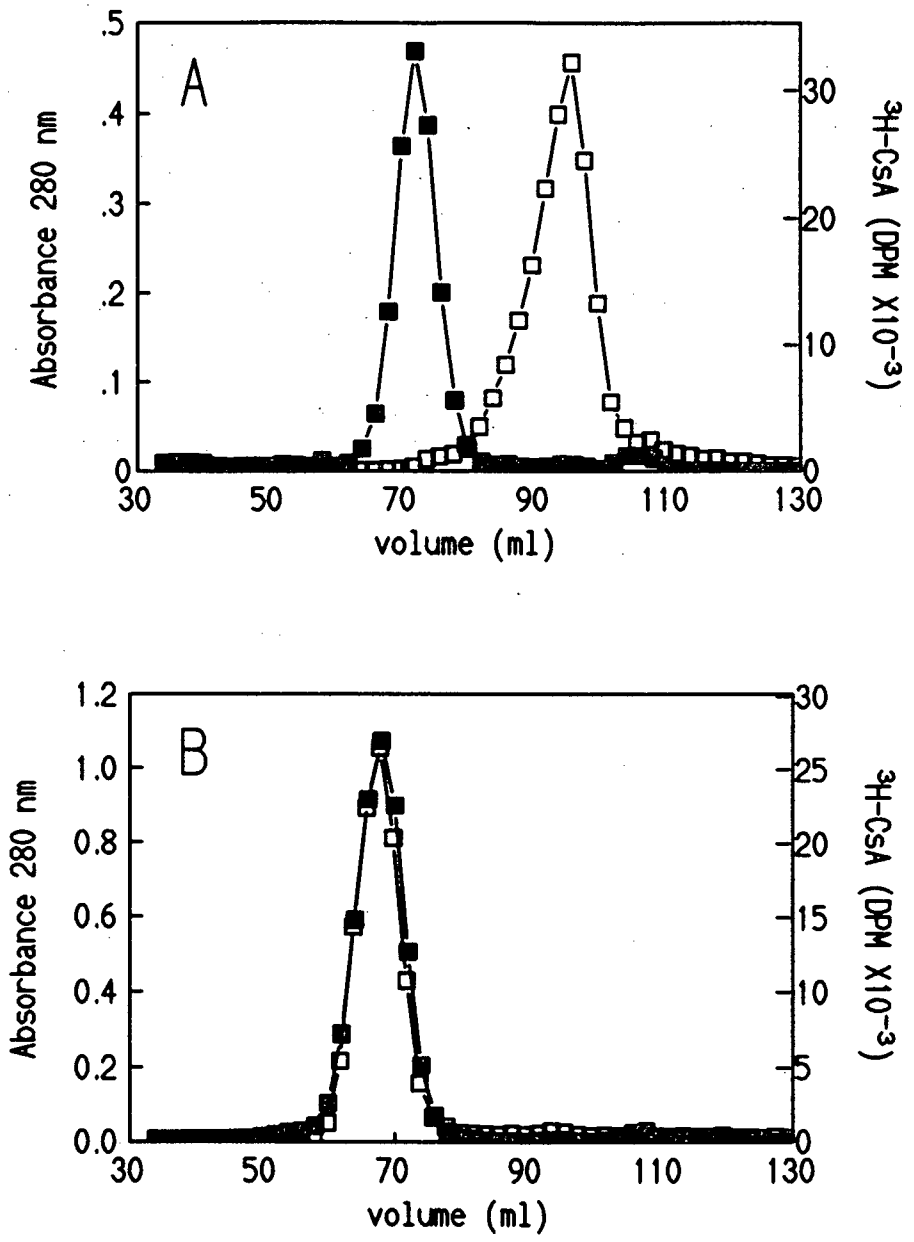

Figure 27. The effect of increasing the concentration of NaCl in the eluting buffer from 0.15 M to 1.5 M on the elution profile of HDL and ^{3}H-CsA from a Superose 6 gel filtration column. ^{3}H-CsA was pre-incubated with ultracentrifugally isolated HDL (d=1.063-1.22) for 1 hour at 37°C, applied to a gel filtration column, and eluted with a buffer containing 0.15 M (A) and 1.5 M (B) NaCl as in Figure 26. The elution of HDL was monitored by Absorbance at 280 nm. (■), and ^{3}H-CsA by radioactivity (DPM) (□).

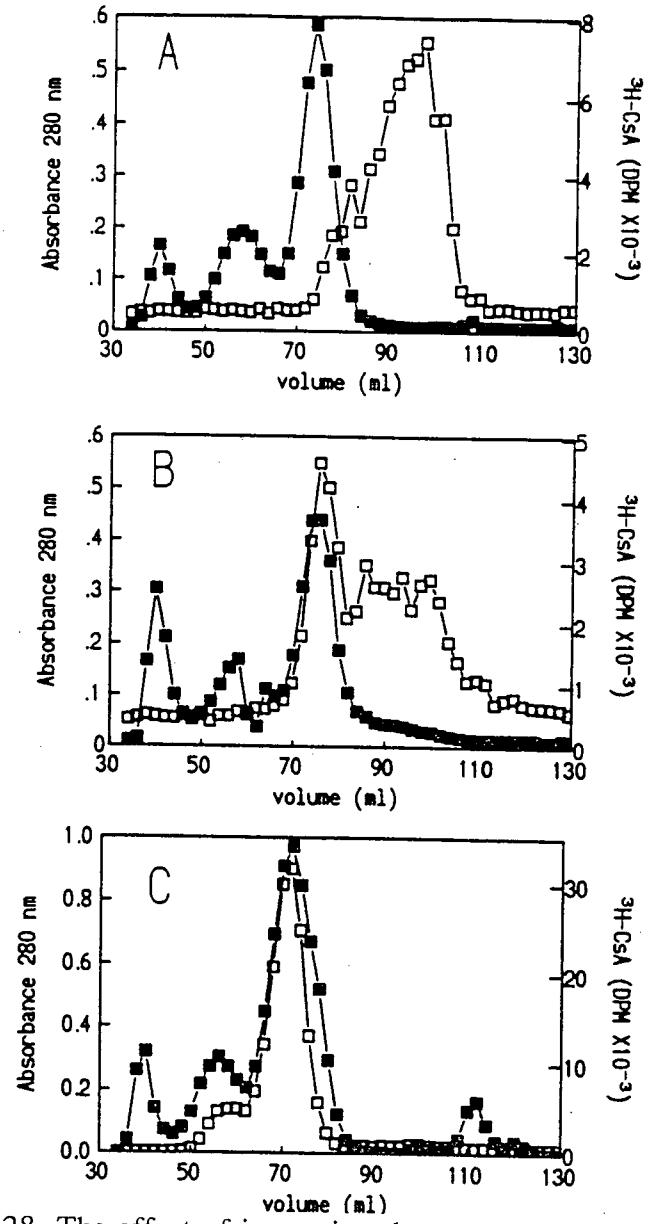

Figure 28. The effect of increasing the concentration of NaCl in the eluting buffer on the elution profile of total serum lipoproteins and ^{3}H-CsA from a Superose 6 gel filtration column. ^{3}H-CsA was pre-incubated with ultracentrifugally isolated total serum lipoproteins ($d < 1.25$ g/ml) for 1 hour at 37°C, applied to a gel filtration column and eluted with a buffer of 0.15 M NaCl (A), 0.75 M NaCl (B), and 1.5 M NaCl (C). The elution of serum lipoproteins was monitored by Absorbance at 280 nm. (■), and ^{3}H-CsA by radioactivity (DPM) (□).

Typical density gradients, as described in Materials and Methods, resulted in gradients which were linear over the greater part but suffered from curvature of the gradient at the top and bottom of the tube (Figure 29). As a result, VLDL and LDL were not well-resolved. Consequently, density fractions were grouped into $d > 1.22$ g/ml, $d = 1.063\text{-}1.22$ g/ml (HDL), and $d < 1.063$ g/ml (VLDL and LDL), to facilitate data analysis.

In normolipemic serum, the majority (50.1 $\pm$ 6.1 (SD) %) of ^{3}H-CsA was found with $d = 1.063\text{-}1.22$ g/ml (HDL), at 15°C. VLDL and LDL ($d < 1.063$ g/ml) contained less ^{3}H-CsA than did $d > 1.22$ g/ml (13.1 $\pm$ 1.8 and 16.9 $\pm$ 2.3 %, respectively). However, when the ultracentrifugation temperature was increased to 37°C, there was a significant increase in the amount of ^{3}H-CsA associated with VLDL and LDL (16.8 $\pm$ 1.2 %, $p = 0.014$) and a reduction of the amount with $d > 1.22$ g/ml to 7.5 $\pm$ 0.5%, $p = 0.0003$ (Table 11). The amount of ^{3}H-CsA with $d = 1.063\text{-}1.22$ g/ml remained relatively constant. This result is in keeping with others (41,99) who have shown that CsA binding is temperature dependent, with more CsA bound to the lipoprotein fraction and less to the protein fraction in plasma at 37°C.

Table 10. Total recovery (%) of ^{3}H-CsA following gel filtration chromatography of lipoproteins at various concentrations of NaCl

	% of total ^{3}H-CsA (DPM) recovered		
Lipoprotein Chromatographed	**0.15 M NaCl**	**0.75 M NaCl**	**1.5 M NaCl**
LDL	**42**	**ND**	**61**
HDL	**36**	**ND**	**83**
Total lipoproteins	**50**	**80**	**78**

Data are means of 2 experiments
ND; not determined, DPM; disintegrations per minute

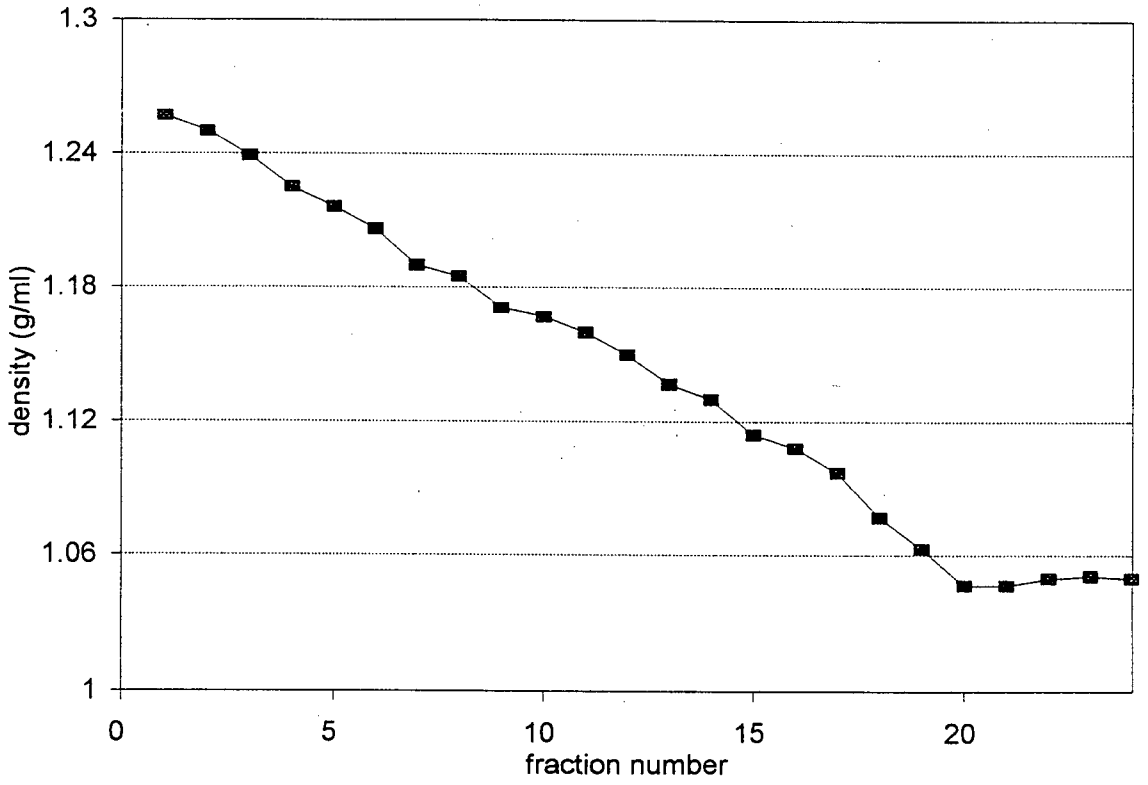

Figure 29. Relation between fraction density and fraction number following density gradient ultracentrifugation of ^{3}H-CsA and serum. ^{3}H-CsA was pre-incubated with serum at 37°C for 1 hour. The density of the serum was adjusted to 1.25 g/ml by the addition of solid NaBr, underlayed on a density gradient of 1.0-1.25 g/ml, and ultracentrifuged at 15 °C for 48 hours as described in Materials and Methods. Fractions corresponding to 0.5 ml were removed and the density of each fraction determined.

Table 11. Distribution (%) of ^{3}H-CsA in normolipidemic serum following density gradient ultracentrifugation

	% of ^{3}H-CsA added to sample	
Density (g/ml)	15°C	37°C
< 1.063	13.1 ± 1.8	16.8 ± 1.2*
1.063-1.22	50.1 ± 6.1	50.8 ± 1.3 NS
> 1.22	16.9 ± 2.3	7.5 ± 0.5**

Data are the means of 4 samples ± SD
*** p=0.0003, ** p=0.014, vs. values at 15°C**
NS; not significant

3.3.3 Distribution of ^{3}H-CsA in Serum using Sequential Ultracentrifugation

Most of the ^{3}H-CsA distributed into the HDL fraction (40%) followed by d>1.25 g/ml (12.8%), LDL (11.8%), and VLDL(3.6%), at 15°C in normolipemic serum. Approximately 32% of the radioactive CsA was unaccounted for. When ultracentrifugation was performed at 37°C, the amount of ^{3}H-CsA associated with HDL increased to 61%, whereas the amounts associated with both LDL and d>1.25 g/ml decreased to 5.3% and 8.1%, respectively. At this temperature only 23% of the label was unaccounted for. In addition, this method was very slow (4 days) and cumbersome. For these reasons, the procedure was not repeated to collect sufficient data to analyze statistically, nor was it used to analyze patient samples.

3.3.4 Distribution of ^{3}H-CsA in Plasma using PTA Precipitation

3.3.4.1 Preliminary Study of ^{3}H-CsA Distribution in Plasma using PTA Precipitation

The results of this preliminary investigation demonstrated that there was a strong, positive correlation between the amount ^{3}H-CsA in the supernate following precipitation and the samples' HDL-cholesterol level (r =0.610, p<0.0001) (Figure 30). There was a

negative correlation between the amount of ^{3}H-CsA in the supernatant and the LDL-cholesterol level ($r = -0.423$, $p < 0.0034$) and the non-HDL- cholesterol (total cholesterol - HDL-cholesterol) level ($r = -0.567$, $p < 0.0001$) (Figures 31, 32). There was a poor correlation between the amount of ^{3}H-CsA in the supernate and the amount of radioactive CsA added to the sample ($r = 0.257$, $p = 0.071$) (Figure 33).

This method, however, separated plasma into only two fractions, the precipitate which included VLDL and LDL, and the supernate, which included HDL and the remainder of the plasma proteins. As a result, amounts of ^{3}H-CsA associated with the discrete lipoprotein fractions (VLDL, LDL, and HDL) as well as the lipoprotein-deficient plasma could not be determined. To circumvent these problems, two new procedures were investigated. One procedure included the additional step of ultracentrifuging an aliquot of the plasma to isolate the VLDL fraction, then performing PTA precipitation on a separate aliquot as described in section 3.2.3.1., and the results are given in section 3.3.4.2. Although the percentage of CsA with VLDL could be determined, the amount of CsA distributed between HDL and remaining plasma proteins could not be resolved. The second procedure (section 3.2.3.3) was a sequential precipitation of VLDL, VLDL and LDL, then HDL, and the results are explained in section 3.3.4.4. In addition to investigations to better resolve the lipoprotein fractions using PTA precipitation, the effect of temperature on precipitation was also determined and the results are given in section 3.3.4.3.

3.3.4.2 Preliminary Study of ^{3}H-CsA Distribution in Plasma using Ultracentrifugation and PTA Precipitation

There was a strong correlation of ^{3}H-CsA in the VLDL following ultracentrifugation

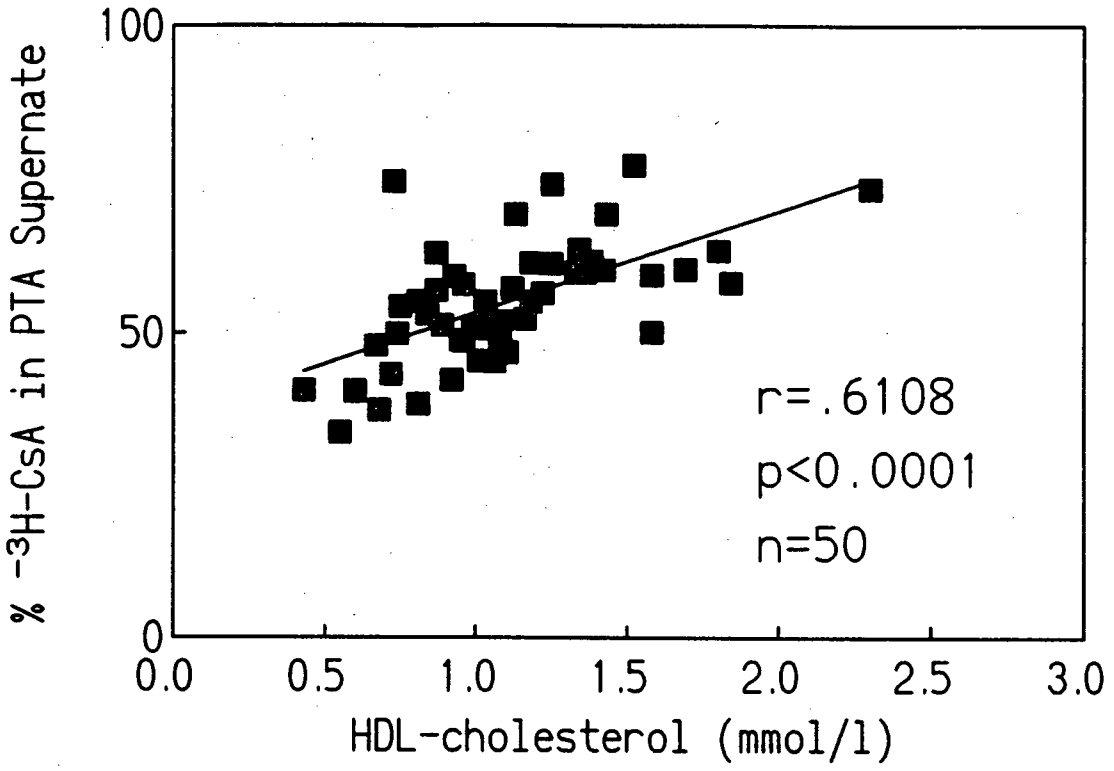

Figure 30. Correlation of HDL-cholesterol with ^{3}H-CsA (%) in supernate following PTA precipitation. ^{3}H-CsA was pre-incubated with patient plasma samples at 37°C for 1 hour and an aliquot was removed prior to the addition of ^{3}H-CsA for the determination of HDL-cholesterol. The precipitation of VLDL/LDL was conducted by the addition of phosphotungstic acid and $MgCl_2$ as described in Materials and Methods and an aliquot of the supernatant removed for the determination of radioactivity.

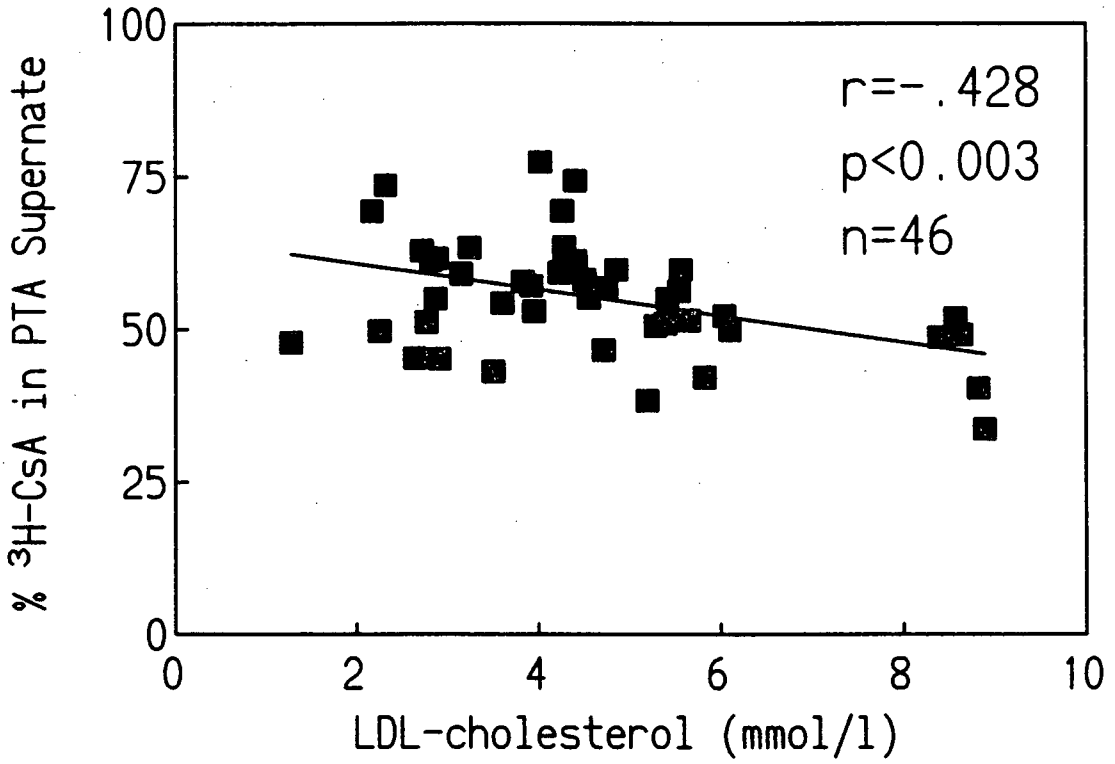

Figure 31. Correlation of LDL-cholesterol with ^{3}H-CsA (%) in supernate following PTA precipitation. ^{3}H-CsA was pre-incubated with patient plasma samples and PTA precipitation was conducted as in Figure 29. An aliquot of the sample was removed prior to the addition of ^{3}H-CsA for the determination of LDL-cholesterol. An aliquot of the supernatant was removed following precipitation for the determination of total radioactivity.

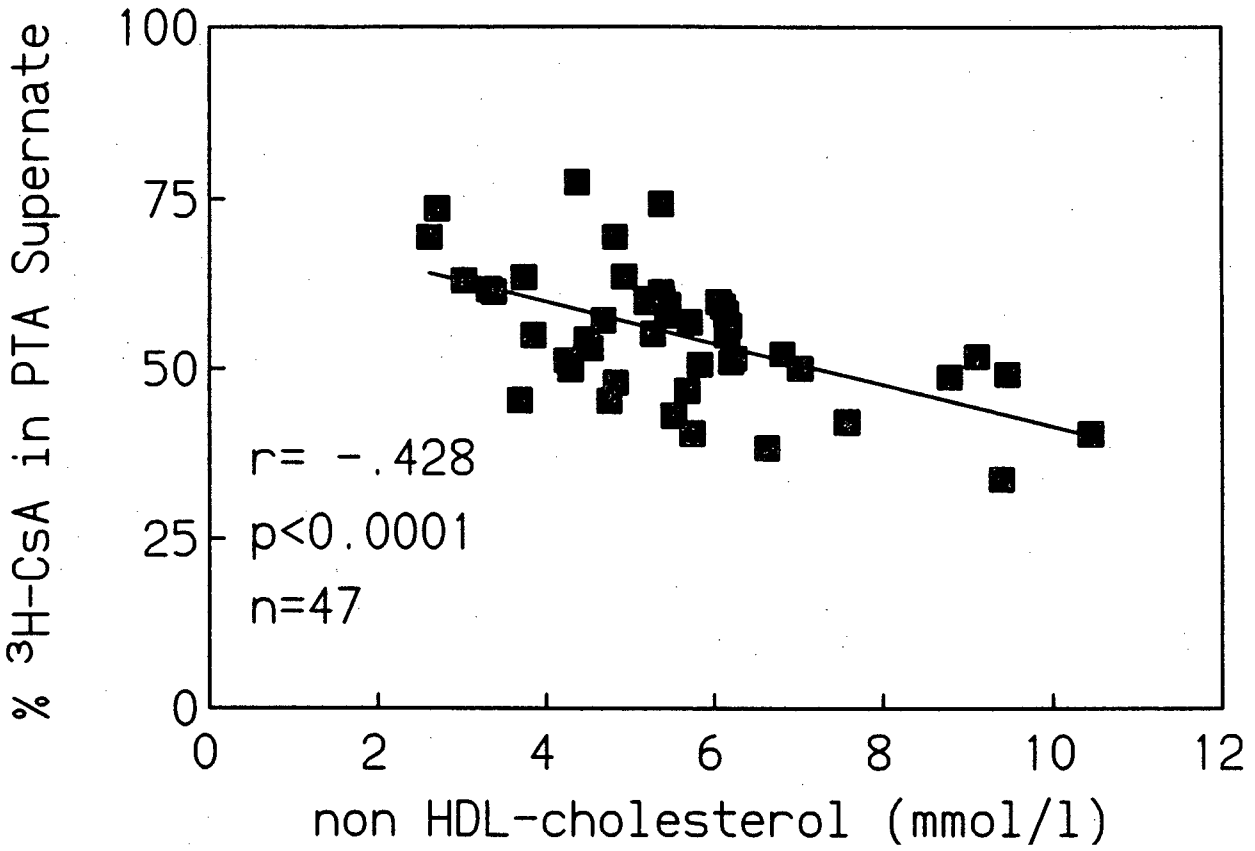

Figure 32. Correlation of non-HDL-cholesterol with ^{3}H-CsA (%) in supernate following PTA precipitation. ^{3}H-CsA was pre-incubated with patient plasma samples and PTA precipitation was conducted as in Figure 29. An aliquot of the sample was removed prior to the addition of ^{3}H-CsA for the determination of HDL- and total cholesterol levels. The non-HDL-cholesterol level was calculated by the difference between total and HDL-cholesterol levels. An aliquot of the supernatant was removed following precipitation for the determination of total radioactivity.

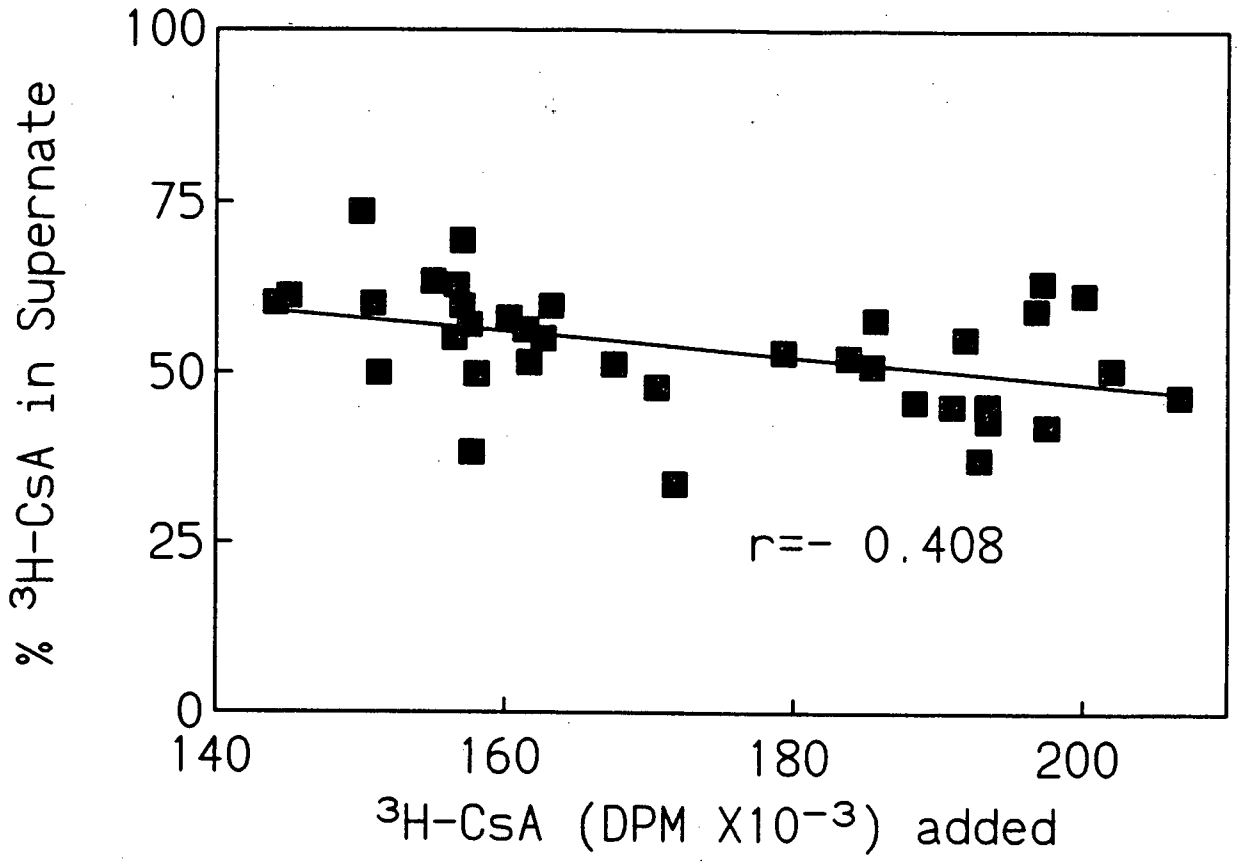

Figure 33. Correlation of ^{3}H-CsA added to the patients' plasma with ^{3}H-CsA(%) in supernate following PTA precipitation. ^{3}H-CsA pre-incubated with patient plasma samples and PTA precipitation was conducted as in Figure 29. An aliquot of the supernatant was removed following precipitation for the determination of total radioactivity.

to the triglyceride level of 15 normal and dyslipidemic samples ($r=0.812$, $p<0.0002$) (Figure 34). When this method was used to determine the distribution of ^{3}H-CsA in normolipemic samples, most of the radioactive label was found with the HDL and other proteins fraction (62.8 $\pm$ 4.6 (SD) %). The LDL contained 31.4 $\pm$ 2.7% and the VLDL 4.4 $\pm$ 1.3% (Table 12).

Table 12. Distribution (%) of ^{3}H-CsA in normolipidemic plasma following phosphotungstic acid (PTA) precipitation and ultracentrifugation

Lipoprotein	%^{3}H-CsA $\pm$ SD
VLDL	4.4 $\pm$ 1.3
LDL	31.4 $\pm$ 2.7
HDL and other plasma proteins	63.8 $\pm$ 4.6

Data are means $\pm$ SD for 10 samples

Table 13. Effect of temperature on HDL-cholesterol levels measured following phosphotungstic acid (PTA) precipitation

Incubation Temperature	HDL-cholesterol (mmol/l) $\pm$ SD
4°C	1.36 $\pm$ 0.02
21°C	1.41 $\pm$ 0.027*
37°C	1.45 $\pm$ 0.03*

Data are means $\pm$ SD for 10 determinations
* $p<0.001$ vs. 4°C.

3.3.4.3 The Effect of Temperature on ^{3}H-CsA Distribution in Plasma using PTA

When the PTA incubation temperature was increased, there was a significant increase in the HDL-cholesterol level ($p<0.001$) at both room temperature and at 37°C in comparison to control (4°C)(Table 13) indicating incomplete precipitation of the VLDL and/or LDL. There was a corresponding decrease in the amount of ^{3}H-CsA in the supernate

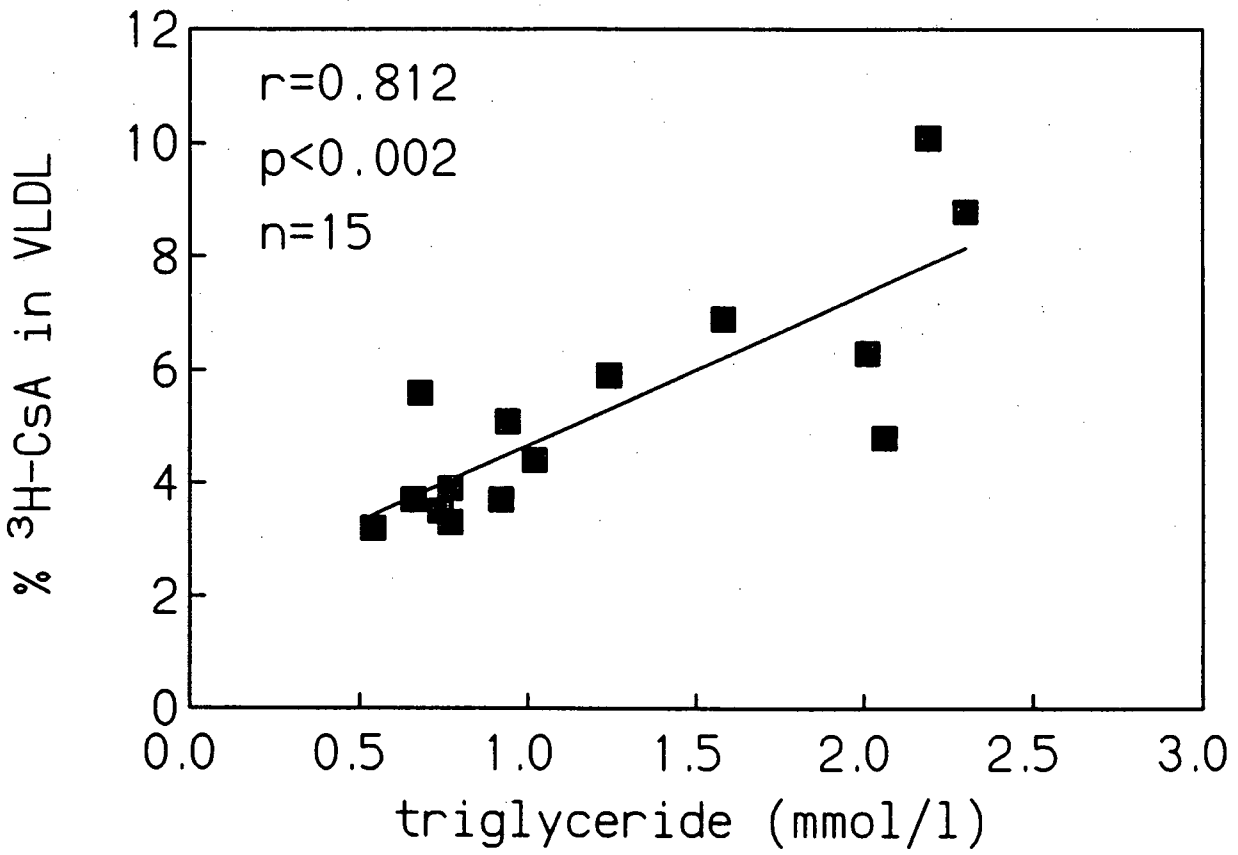

Figure 34. Correlation of triglyceride level with ^{3}H-CsA (%) in the VLDL fraction. ^{3}H-CsA was pre-incubated with plasma samples as in Figure 29 and the VLDL ($d < 1.006$ g/ml) isolated by ultracentrifugation for 16 hours at 15°C. An aliquot of the sample was removed prior to the addition of ^{3}H-CsA for the determination of triglyceride levels.

following precipitation at these temperatures ($p < 0.001$) (Table 14).

Table 14. Effect of temperature on the amount of ^{3}H-CsA in the supernate following PTA precipitation

Incubation Temperature	^{3}H-CsA (DPM) in supernate $\pm$ SD
4°C	1188 $\pm$ 37
21°C	1137 $\pm$ 30*
37°C	1101 $\pm$ 35**

Data are means $\pm$ SD for 10 determinations
***p < 0.01, ** p < 0.001 vs. values at 4°C.**

3.3.4.4 Distribution of ^{3}H-CsA in Normolipidemic and Dyslipidemic Plasma using Sequential PTA Precipitation

It was expected that this method would be able to separate all of the lipoprotein classes and the remaining plasma proteins so that the amount of ^{3}H-CsA associated with each fraction could be determined. This was not the case. When the precipitate that was formed with the VLDL was centrifuged it floated on top of the supernate and made sampling of the precipitate or supernate extremely difficult. As a result, the amount of ^{3}H-CsA with VLDL could not determined. Further, since the PTA method is affected by temperature, as shown above, the amount of CsA within each fraction at 37°C could not be determined.

In normolipidemic plasma, at 4°C., most of the ^{3}H-CsA is associated with the HDL fraction (44.4 $\pm$ 4.27 (SD) %). The VLDL/LDL fraction contained 31.9 $\pm$ 3.6% and 19.7 $\pm$ 3.1% was associated with lipoprotein-deficient plasma (LPDP). However, the distribution of CsA in the plasma of the dyslipidemic patient groups differed significantly from normolipemic controls (Table 15). As expected, there was an increased amount ^{3}H-CsA with the LDL/VLDL fraction in patients with hypercholesterolemia (46.3 $\pm$ 7.7%, $p < 0.05$),

hypertriglyceridemia (54.3 $\pm$ 9.2%, $p<0.01$), and combined hypertriglyceridemia and hypercholesterolemia (55.3 $\pm$ 9.2%, $p<0.01$) (Table 15) when compared to normolipidemic controls. Within all patient groups, there was a significant reduction in the amount of CsA associated with the HDL fraction as compared to the controls (Table 15). However, the only patient group that demonstrated a significant change in the amount of CsA associated with the LPDP (lipoprotein-deficient plasma) fraction, compared to the controls, were patients with combined hypertriglyceridemia and hypercholesterolemia (12.9 $\pm$ 5.0%, $p<0.05$) (Table 15). Because the PTA method is affected by temperature, the amount of CsA within each fraction at 37°C could not be investigated.

Table 15. Distribution (%) of ^{3}H-CsA in normolipidemic and dyslipidemic plasma using sequential PTA precipitation

Plasma Type	% of ^{3}H-CsA added to sample		
	VLDL/LDL (SD)	HDL (SD)	LPDP (SD)
normolipidemic	31.9 (3.6)	44.4 (4.2)	19.7 (3.1)
hypercholesterolemic	46.3* (7.7)	20.9*** (7.7)	20.9 (2.6)
hypoalphalipoproteinemic	42.7 (10.8)	15.7*** (3.9)	20.9 (3.6)
hypertriglyceridemic	54.3** (13.1)	20.0*** (4.6)	18.9 (7.3)
hypercholesterolemic + hypertriglyceridemic	55.3** (9.2)	20.1*** (3.7)	12.9* (5.0)

Data are the means of 6 samples, (SD)
* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. normolipidemic plasma
LPDP; lipoprotein-deficient plasma

3.4 Discussion

3.4.1 Methods used to Study the Distribution of ^{3}H-CsA in Serum and Plasma

The objectives of this part of the thesis were to investigate a suitable method with which to study the distribution of CsA in plasma then, using this method, determine the distribution of CsA in dyslipidemic plasma and compare it to that in normolipidemic controls.

Of the different methods investigated, PTA precipitation was chosen as the best method with which to study the distribution of CsA in plasma. (A summary of the results is given in Table 16.) The PTA method fulfilled two of the three criteria by which these isolation methods were judged. First, and most importantly, PTA precipitation separated the lipoproteins from other plasma proteins and, in doing so, did not appear to affect the distribution of CsA. PTA separated the plasma into three distinct fractions, VLDL/LDL, HDL, and lipoprotein-deficient plasma. However, it was not possible to separate VLDL and LDL by PTA precipitation as described by Burstein and Scholnick (239) but other investigators have also encountered this difficulty (43). Although PTA precipitation is reported to produce a VLDL/LDL fraction which is essentially free of HDL (43,239), there are small amounts of other plasma proteins which contaminate the precipitate at each precipitation step. The contamination is thought to arise from two sources; a mechanical transfer of proteins onto the precipitate and actual precipitation of small amounts of proteins other than lipoproteins (439,166). Since this contamination is negligible (43,239), it was disregarded. Similar to PTA precipitation, density gradient ultracentrifugation did not fully resolve VLDL and LDL. Although VLDL and LDL were separated using sequential

ultracentrifugation, it was a much more labour intensive technique and was not practical for the analysis of large numbers of samples required in this study (n=30). Finally, although gel filtration chromatography completely resolved all of the lipoprotein classes of interest (VLDL, LDL, and HDL), it could not be used to study the amount of CsA associated with lipoprotein-deficient plasma fraction since only the lipoprotein fraction was chromatographed.

PTA precipitation did not appear to affect the distribution of CsA in plasma as demonstrated by the preliminary investigation. Specifically, it showed that it was unlikely that the CsA was being precipitated independently of the lipoproteins since the amount of CsA in the supernate following precipitation did not correlate with the amount of CsA added to the sample yet did correlate with the cholesterol level, LDL-cholesterol level, and the non-HDL cholesterol level. This conclusion is consistent with the studies of Danon and Chen on the distribution of ^{3}H-reserpine (242) and ^{14}C-imipramine (243) in plasma. They found that the distribution of these two drugs was not affected by the precipitation technique since the amount of radioactivity associated with the VLDL/LDL precipitate was similar to that found with the VLDL/LDL peak following gel filtration chromatography (242,243). In addition, PTA precipitation has been used by other researchers to study the clearance of CsA from various lipoprotein particles (150).

Although the other techniques investigated also separate the lipoprotein classes, they did not adequately fulfil the requirement that the method separate the lipoproteins but not interfere with the distribution of CsA. Ultracentrifugation is not a technique favoured by those studying the binding of drugs to proteins since it is commonly believed that the binding equilibrium is altered during this long process (244). As a result, a free drug concentration

gradient is established, and sedimentation occurs (244). However, Gilbert and Jenkins (245) have shown that the equilibrium is not significantly affected if the drug is of low molecular weight and the sedimentation coefficient of the drug-protein complex is the same as the protein alone. However, drugs of low molecular weight have been shown to diffuse back, due to thermal agitation, resulting in an altered equilibrium and an overestimation of the free drug concentration (246). In addition, it has also been shown that the use of high-salt density solutions to facilitate the separation of lipoproteins, results in the underestimation of lipoprotein-associated drug (234). Since VLDL has a density low enough to float in plasma without the addition of NaBr, Brajtburg *et al.* demonstrated that when the binding of Amphotericin B to VLDL was determined in the presence and absence of NaBr, the amount of drug with VLDL was significantly decreased in the presence of NaBr salt (234). Because Br^- is one of a group of alkali anions which have the ability to decrease the polarity of the surrounding water (247), they concluded that NaBr interferes with the hydrophobic bonds between the lipoprotein and the drug (234). This phenomenon is also shown in the present study. In the studies using sequential and density gradient ultracentrifugation (at 15°C), where NaBr is used to facilitate separation, the amount of CsA associated with VLDL and LDL fractions approximated 13% and 16%, respectively (Table 11 and section 3.3.2). However, in the study which used both ultracentrifugation (without NaBr) coupled to PTA precipitation, the amount of CsA with VLDL and LDL approximated 35% (Table 12).

Gel filtration chromatography also affected the distribution of CsA. Following chromatography, very little of the CsA was found with the lipoproteins. A likely interpretation of this finding is that CsA dissociates from the lipoproteins during

chromatography, and some becomes incorporated into the column and elutes in the included peak and some remains bound to the column. The equilibrium gel filtration method, described by Hummel and Dreyer (247), was used to overcome this problem, however, due to technical difficulties (*i.e.* CsA in the eluting buffer precipitated at temperatures $\geq 21°C$ (249), this technique was unsuccessful. When the salt (NaCl) concentration of the eluting buffer during conventional chromatography was increased 10-fold to 1.5 M, the CsA eluted with the lipoproteins. This apparent discrepancy of increased binding of CsA to lipoproteins with an increased salt concentration, is likely explained by technical differences between Brajtburg and colleagues' study and this one. First, there is increased binding of CsA to lipoproteins at higher temperatures (40). In this study gel filtration was performed at 21°C whereas ultracentrifugation in both the present study and Brajtburg *et al's.* were done at 15°C (231). Second, the concentration of salt (NaBr, approximately 11 M) in the density solution was far greater and therefore not comparable to the salt concentration (NaCl, 1.5 M) in the eluting buffer. Third, the effect of Br^- on the polarity of water is greater than that of Cl^- (247).

The distribution of a drug is dependant on its binding. If something affects the binding then, subsequently, the distribution will be affected as well. The binding of a drug or ligand (L) to a single "receptor" (R) is described as

$$L + R \rightleftarrows RL$$

and binding implies a reversible and semi-stable reaction (191). Usually, binding is studied at equilibrium where there is no net change in the reactants and the products. Binding interactions are fast or slow depending on the time necessary for the for the system to reach

equilibrium. If the binding interaction is fast, then it is possible that during the separation procedure of bound and unbound ligand the equilibrium is disturbed, bound ligand dissociates and, as a result, the amount bound is underestimated. If the bound ligand remains in contact with the free ligand then this is not a problem since equilibrium can be maintained. The binding interaction rate of CsA to lipoproteins is unknown. However, the time needed for CsA to equilibrate in plasma at 37°C *in vitro* is 30 minutes (39,40). Further, both ultracentrifugation and gel filtration chromatography are relatively slow procedures, in comparison to PTA precipitation. Dissociation of the CsA-lipoprotein complex is evident during gel filtration and may occur during ultracentrifugation. In contrast, PTA precipitation is a relatively quick method of separation (approximately 40 minutes) and during the procedure unbound CsA remains in contact with the bound fractions. Therefore, it could be argued that PTA precipitation is a superior method with which to study the distribution of CsA in comparison to gel filtration and ultracentrifugation because during separation the equilibrium is maintained, and binding and distribution are unaffected.

PTA precipitation proved to be the most efficient method, thereby fulfilling the second criterion required. PTA precipitation was simple to perform, relatively inexpensive, had a high through-put and better recovery (>80%), compared to the other methods investigated. Both gel filtration and ultracentrifugation were time-consuming, required a much larger sample volume than PTA precipitation, and resulted in poorer recoveries. During ultracentrifugation, the label may have been lost on the walls of the centrifuge tubes, the tubing used to manipulate the sample, and in the sticky residue at the bottom of the tube. With sequential ultracentrifugation, these losses were compounded with each successive

centrifugation. However, both of the ultracentrifugation techniques had much better recoveries than gel filtration. The label could only be removed from the column following repeated washings in 20% ethanol and eluting buffer.

Table 16 Summary of methods used to study the distribution of CsA in plasma or serum

Method	Criteria used to Judge Methods		
	Separation of fractions without affecting distribution of CsA	Method insensitive to temperature	Method Efficient
PTA	-separated VLDL/LDL, HDL, and LPDP -did not affect distribution	NO	YES -better recovery (>80%) -fast, cost-effective
Gel filtration	-resolved VLDL, LDL, HDL but not LPDS -method affected distribution	YES	NO
Density gradient UC	-separated VLDL/LDL, HDL,LPDP -method affected distribution	YES	NO
Sequential UC	-separated VLDL, LDL, HDL, LPDP -method affected distribution	YES	NO

VLDL; very low density lipoprotein, LDL; low density lipoprotein, HDL; high density lipoprotein, LPDP: lipoprotein-deficient plasma, LPDS; lipoprotein-deficient serum, UC; ultra-centrifugation

Unfortunately, PTA precipitation did not fulfil the third criterion, the technique could not be used to study the effects of temperature on the distribution of CsA in plasma.

Following precipitation at room temperature and 37°C, the cholesterol levels in the supernate were significantly higher than those of the control (4°C). This is probably due to incomplete precipitation of the VLDL and LDL (239). With density gradient ultracentrifugation there is a significant increase in the amount of CsA associated with $d < 1.063$ g/ml (VLDL/LDL) and a decrease in the amount of drug with $d > 1.22$ g/ml (LPDS) at 37°C, which confirms the results of other investigators (100). Since 37°C is representative of the temperature *in vivo*, then it can be expected that in patients receiving this drug that most of the CsA in plasma will be lipoprotein-bound. However, these results should be interpreted with caution due to the inherent difficulties in adjusting densities for centrifugation at 37°C. Because density is a function of temperature, adjustments for both the changes in partial specific volume of NaBr and the differential expansion of the lipoproteins and solvent are made. The adjustments for differential thermal expansion are made from tables calculated from studies performed exclusively with LDL using NaCl as the density solvent (43). The adjustments for VLDL and HDL using NaBr as the density solvent are unknown. However, using the same values for VLDL and HDL in NaBr may be an acceptable approximation since the adjustments for LDL are small (less than 2.3 mg/ml for every 5°C difference in temperature) (43).

Performing gel filtration chromatography at 37°C is also fraught with difficulties. Although the eluting buffer is easily kept at 37°C, the column has to be wrapped with heating tape to ensure that the eluent is at the same temperature. This may result in "hot spots" along the column where the heating tape comes in direct contact with the column.

Finally, the nomenclature used for the isolated lipoprotein fractions in the preceding discussion must be addressed. Historically, the classification of the lipoproteins was derived

from the method with which they were separated. VLDL, LDL, and HDL fractions were described based on their limiting hydrated densities following ultracentrifugation (43). PTA precipitation, as performed in this study, separates plasma into fractions that approximate the α- and β- lipoproteins, described following electrophoresis, or the high ($d = 1.063$-1.20 g/ml) and low density ($d < 1.063$ g/ml) classes described following ultracentrifugation (43,239). The fractions obtained by precipitation differ slightly from those isolated by ultracentrifugation and are more analogous to those separated by electrophoresis. In practice, some investigators define these lipoproteins classes in analogous terms. Although these particles are very similar, they are not identical so, in principle, applying these terms is incorrect. They should be named β-lipoproteins (VLDL/LDL fraction) and α-lipoproteins (HDL fraction). However, to be consistent with the nomenclature in the current literature on CsA distribution, the terms VLDL/LDL and HDL have used to designate the lipoprotein fractions separated by precipitation. In this setting, these terms are acceptable.

3.4.2 The Distribution of ^{3}H-CsA in Dyslipidemic Plasma using PTA Precipitation

The results of this study demonstrate that dyslipidemia significantly affects the distribution of CsA in human plasma at 4°C *in vitro*. This study confirms previous investigations (159,179,173) and also clarifies the differential effects of the type and degree of dyslipidemia on the distribution of CsA. Specifically, more CsA associates with the VLDL/LDL fraction in patients with hypercholesterolemia, hypertriglyceridemia, or a combination of both in comparison to normolipidemic controls. The shift in distribution in all instances seems to be at the expense of the HDL fraction since significantly less CsA is associated with it. This is in keeping with the observations of others (173,250) who showed

that HDL-associated CsA readily transfers from HDL to both LDL and VLDL *in vitro,* but the reverse is not true (173).

Although both hypercholesterolemia and hypertriglyceridemia alone can influence the distribution of CsA, their effect seems to be augmented when combined. The results do not support either of the conclusions reached by Hughes *et al.* (173) and Legg *et al.* (149). Hughes *et al.* concluded that triglyceride was the major factor influencing the distribution of CsA in plasma (173). However, this conclusion was based on a sample population that, by definition was hypercholesterolemic as well as hypertriglyceridemic (251), thereby making their conclusions questionable. In contrast to Hughes *et al.*, Legg *et al.* concluded that cholesterol, rather than triglyceride, influenced the distribution of CsA in plasma and that the effect of cholesterol was four times that of triglyceride (149). Legg and colleagues' conclusions were based upon a mathematical model in which one of the assumptions made was that <u>all</u> of the triglyceride in the sample was in the VLDL fraction and that triglyceride was the only binding material in this fraction (149). However, this assumption is invalid since VLDL contains proteins (apo B, C, and E), phospholipid and small amounts of cholesterol in addition to triglyceride, all of which may be involved in binding CsA. Further, all of the major lipoprotein fractions (See Table 1) contain some amount of triglyceride (43).

The present study also shows that significantly less CsA is found with the HDL fraction in hypoalphalipoproteinemic plasma and more CsA is associated with the VLDL/LDL fraction, although this latter result does not reach statistical significance. The amount of CsA with the lipoprotein-deficient plasma fraction remains relatively constant compared to the control and this observation is in keeping with others in their studies with

hyperlipidemic patients (155,159) and rats (160). However, in the case of combined hypercholesterolemia and hypertriglyceridemia the amount of CsA with LPDP was significantly decreased. Perhaps this may be due to the combined effects of hypercholesterolemia and hypertriglyceridemia displacing CsA from LPDP, and this result may would have shown with hypercholesterolemic and hypertriglyceridemic patients if samples with higher levels of cholesterol and triglyceride were included in the study. This result suggests that LPDP has a higher affinity for CsA in comparison to lipoproteins. Further, there are reports in the literature that in hypocholesterolemic patients, the toxic effects of CsA are apparent at lower doses of the drug (103). The authors suggested that this was due to an increase in the free fraction of CsA (103). If this were the case, then one must assume that the binding capacity of LPDP for CsA was saturated. Therefore, these data suggest that CsA may occupy a classical binding site; one of high affinity, specificity, and saturability, with LPDP. Albumin is the most abundant protein in LPDP (and serum and plasma) and has the ability to bind many different types of drugs (acidic, basic, and lipophilic) (233). For these reasons, one might infer that CsA occupies a binding site on albumin.

The finding that dyslipidemia affects the distribution of CsA is significant because of the high prevalence of dyslipidemia, specifically hyperlipidemia, that is seen in transplant patients. Further, the lipid levels chosen to represent the dyslipidemias are found in these patients. Factors affecting the binding and distribution of a drug are of great importance with drugs that are highly bound, as is CsA (approximately 2% free) (233). If another drug competes for binding sites and displaces CsA or if the amount of binding protein decreases,

the level of the free drug increases. Conversely, if the amount of binding protein increases then the free fraction of the drug will decrease. Since only the free fraction of the drug is thought to exert any pharmacological effect, then a change in the free fraction will affect the efficacy and toxicity of the drug (233). This is one mechanism by which dyslipidemia may affect CsA's efficacy and toxicity. One other mechanism relates to the lipoprotein-bound drug rather than the free fraction. There is some evidence in the literature to suggest that protein-bound drug may also be available to the effector tissue, depending upon which protein the drug is associated (164). In the case of LDL-bound CsA, it has been suggested that bound CsA gains entrance into tissues via the LDL-receptor. Although there is no direct evidence to support this suggestion, it has been shown that LDL-bound CsA has an increased pharmacological effect in comparison to CsA alone or bound to VLDL or HDL *in vitro* (180). Should this be the case *in vivo*, when there are increased LDL levels, the drug will redistribute such that more will be associated with LDL, and the efficacy of the drug will be altered.

In this thesis I investigated the effects of dyslipidemia on the distribution of CsA in plasma. However, it must be pointed out that more than 30 metabolites of CsA have been identified. The immunosuppressive properties of these metabolites are $< 10\%$ of the parent drug (204), however, data suggest that some of the metabolites may contribute to the pathogenesis of CsA-induced nephrotoxicity (203,204). The metabolite AM1 (formerly M17) is the major metabolic in blood (216-219) and in renal transplant recipients its concentration exceeds that of CsA (219). In plasma, at 37°C, the major hydroxylated metabolites (AM1, AM9) are associated predominantly with the red cells and the demethylated metabolites are

associated predominantly with the plasma (92, 219). Although the distribution of AM1 is affected by both the temperature and hematocrit, this effect is not as significant as with CsA (92). Further, like CsA, the relative proportion of AM1 in plasma increases when the concentration in whole blood exceeds 500-1000 μg/l, but this effect is more pronounced with CsA (92). It is unknown whether these studies were conducted in normolipidemic plasma. Further, the effect of dyslipidemic plasma on the distribution of the metabolites is unknown. However, Lensmeyer *et al.* suggested that metabolites with modifications at amino acids 1 and 9 will likely display diverse temperature-dependent solubility (92). Should this be the case, then the distribution of these metabolites may be more affected by dyslipidemia than is CsA.

3.4.2 The Distribution of ^{3}H-CsA in Normolipidemic Plasma using PTA Precipitation

This study demonstrated that the distribution of CsA in normolipidemic controls, using PTA precipitation method, is similar to that as reported by other researchers despite being conducted at 4°C (155,157). However, it should not be concluded that the distribution of CsA is independent of temperature. Indeed, the preliminary experiments with PTA demonstrated that CsA binding to lipoproteins is temperature dependent because significantly more CsA is associated with the VLDL/LDL precipitate at higher temperatures despite less LDL and/or VLDL being precipitated. This suggests that at 37°C, even more CsA may be lipoprotein-bound than can be determined, given the limitations of this method. In addition, these data suggest that the other researchers, who conducted their experiments using ultracentrifugation at higher temperatures than employed in this study, may have also underestimated the amount of CsA which is lipoprotein-bound (234). For example, the high

ionic strength buffers used to facilitate separation during ultracentrifugation may have interfered with the hydrophobic bonds between CsA and lipoproteins (234,247). The present investigation confirms the findings of other researchers who showed that the HDL fraction contained the majority of CsA in normolipidemic plasma regardless of the method used (39,155). This suggests that factors other than the mass of lipid in plasma, such as the composition of the lipoprotein, affect CsA's distribution. However, given that CsA's distribution is temperature dependent, there may be another explanation of these results. At lower temperatures ($<37°C.$) more CsA is associated with plasma proteins than with lipoproteins (100). Since these experiments were conducted at a temperature lower than 37°C, more CsA is associated with HDL because it has a higher protein content than any of the other lipoprotein classes, with the exception of VHDL (very high density lipoprotein) (43). Previous investigators have proposed that the distribution of CsA is not only influenced by the mass of lipoproteins in plasma (39,40,155,252), but also by the temperature (39,40), and the composition of the lipoproteins (155). Neither of these assumptions were investigated in this study. The effects of temperature on CsA distribution could not be studied due to the limitations of the PTA precipitation technique. A second limitation of this method was that the amount of CsA in the VLDL fraction could not be determined since PTA precipitated both VLDL and LDL together (43). A third limitation precluded studies of patients with severe dyslipidemias because the plasma sample would have to be diluted to near-normal ranges to ensure that all of the lipoproteins were precipitated (43). I felt that this step might have affected the binding equilibrium and the subsequent distribution of CsA.

One of the assumptions made in this study is that CsA distributes in plasma in the

same manner when added *in vitro* as it does *in vivo*. However, other researchers have suggested that this assumption may be invalid (159,173). Several investigators have postulated that externally administered CsA may associate with the surface of the lipoprotein particle whereas CsA taken orally associates with the hydrophobic, lipid core of lipoprotein (159,173). For this reason, the distribution of the drug may be dependent on the route of administration. Although Verril *et al.* demonstrated that the distribution of CsA, when added *in vitro*, was different from that *in vivo*, this difference may have been due to the lower temperature of the plasma (22°C) to which CsA was added, as suggested by the authors (159). In addition, the experiments of Hughes and co-workers', one of the groups of researchers who favoured the hypothesis that externally administered CsA may associate with the surface of the lipoprotein, do not support this hypothesis (173). They showed that the CsA in LDL and VLDL fractions from patients with orally administered CsA, readily transfers to HDL *in vitro*. This observation was similar to that as shown by Mraz when CsA was added *in vitro* (250). Further, if the *in vitro* association of CsA with lipoproteins differs from that *in vivo*, then it may be reasonable to expect that the kinetics of this binding would also be different. But this is not the case. Legg and Rowland concluded (252), from their binding studies of CsA added *in vitro* to lipoproteins, that the binding of CsA was temperature dependant, and hydrophobic, and as such, was similar to that seen when a ligand is physically dissolved in the binding material (253,254). In conclusion, the majority of the data suggest that CsA is associated with the hydrophobic, inner lipid core of the lipoprotein particle when added to plasma *in vitro*.

In summary, PTA precipitation was chosen as the best method with which to study

the distribution of CsA in plasma because it separates the plasma lipoproteins without any apparent affect on the distribution of CsA. Further, PTA precipitation is extremely efficient in comparison to the other techniques investigated. By using the PTA method, it was shown that dyslipidemia significantly affects the distribution of CsA in plasma.

Chapter 4
The Binding of CsA to Lipoproteins

4.1 Introduction

The binding of drugs to proteins in plasma (or blood) influences the distribution, and hence the pharmacokinetics of the drug (233). The magnitude of this effect will depend on a number of factors including the physiochemical properties and concentration of the drug, the concentration of the protein(s) and, the nature and strength of the drug-protein interaction (233). Given the results presented in the previous chapter, that lipoproteins affect the distribution of CsA, and the conclusions of many investigators that CsA binds to lipoproteins, I wished to describe more fully the interaction between the individual lipoproteins classes (VLDL, LDL, and HDL) and CsA. To this end, I wanted to determine the affinity constants (K_d), which describe the strength of the interaction between the drug and binding protein (or receptor and ligand) and the number of binding sites (B_{max}) with each lipoprotein class, which would provide information on the magnitude of the binding. However, due to the lipophilic nature of CsA, the number of methods available to determine these parameters was limited. I chose two methods to investigate the binding of CsA to lipoproteins, equilibrium gel filtration chromatography, using the technique first described by Hummel and Dryer (245), and ultrafiltration.

4.2 Materials and Methods

4.2.1 Equilibrium Gel Filtration Chromatography

The binding of CsA to HDL was studied using the equilibrium gel filtration method described by Hummel and Dryer (248) and the external calibration modification of this method by Sun *et al.* (255). The Hummel and Dryer technique uses an eluent containing the

ligand of interest at a known concentration. A small amount of protein to which the ligand binds is applied to the column, eluted and the elution of the protein and ligand monitored. A typical elution profile is characterized by a positive peak corresponding to the ligand-protein complex and a negative peak (trough) at the ligand retention volume. The area of the trough (or peak, depending on the methods used to monitor the column) is proportional to the amount of drug bound. The external calibration of this method described by Sun *et al.* compares the trough area with those obtained when increasing amounts of ligand are applied to the column and eluted in the same aqueous buffer which is devoid of the ligand.

HDL ($d < 1.063$-1.22 g/ml) was isolated from the serum of normolipidemic, healthy volunteers by preparative ultracentrifugation according to Havel *et al.* (181). The blood was collected, the serum prepared, and HDL isolated as described in a previous section (section 2.1.1.1) with the exception that the serum was adjusted to a density of 1.063 g/ml for the first ultracentrifugation and 1.22 g/ml for the second ultracentrifugation. In addition, for the second ultracentrifugation, the serum was centrifuged for 48 hours at 15°C. The HDL was recentrifuged at 1.22 g/ml at 15°C for an additional 48 hours. The HDL was then dialyzed against three changes of 0.15 M NaCl, 1 mM EDTA and 0.03% sodium azide at 4°C for 24 hours. The purity of the HDL preparation was confirmed by migration of a homogenous peak on agarose gel electrophoresis (182). The cholesterol level and protein (183) content of the HDL was determined as described previously.

The gel filtration column (Superose 12, 1.6 cm X 50 cm, Pharmacia, Dorval, Que.) was equilibrated with 2 column volumes of buffer of 0.15 M NaCl, 1 mM EDTA, and 0.03% sodium azide containing various concentration of ^{3}H-CsA ranging from 25-350 ng/ml

at 4°C. The concentration of CSA was confirmed by RIA performed as described previously. These concentrations were chosen as they are within the range of therapeutic blood levels (64). HDL (0.20 ml) at a concentration of 5.0 mM cholesterol, was applied to the column and 0.5 ml fractions eluted in the same buffer used to equilibrate the column at a flow rate of 0.05 ml/min at 4°C. The column was monitored by absorbance at 280 nm and the elution of ^{3}H-CsA was followed by determining the amount of radioactivity in each column fraction (Beckman LS-9000, Liquid Scintillation Counter, Beckman Instruments, Fullerton, CA). The column was standardized with respect to elution times for HDL (ultracentrifugally isolated) and ^{3}H-CsA.

4.2.2 Ultrafiltration

HDL was isolated from the serum of normolipidemic volunteers as described above. The HDL was dialyzed as described and its purity confirmed by migration of a homogenous peak on agarose gel electrophoresis. The cholesterol level and protein content of the lipoprotein preparation were determined as described previously. The HDL was diluted in a buffer of 0.15 M NaCl, 1 mM EDTA, and 0.03% sodium azide, to a concentration of 1.0 mmol/l cholesterol and 1.025 mg of protein/ml.

Prior to any CsA-binding experiments being performed, the determination of free CsA concentration using ultrafiltration was validated. Radiolabelled (^{3}H-CsA, Amersham, Oakville, Ont.) and unlabelled CsA (Sandoz, Canada, Dorval, Que.) were added to HDL (1.0 mmol/l cholesterol) to achieve a concentration of 200 ng/ml and a specific activity of 2150 DPM/μl. The concentration of CsA was confirmed by RIA.The solution was mixed thoroughly and incubated at 37°C for 1 hour. Aliquots (1.0 ml) of this mixture were pipetted

into a series of sample (upper) reservoirs of an ultrafiltration device (Amicon MPS-1, Amicon Corporation, Danvers, MA,) fitted with a YM10 membrane (10,000 molecular weight cutoff) in accordance with the manufacturer's instructions. The sample was further incubated for 30 minutes at 37°C after which they were centrifuged at 1000 X g for 8-15 minutes at 37°C. The temperature during centrifugation was maintained at 37°C by placing the entire centrifuge in an incubator and the temperature kept constant at 37°C $\pm$ 2. Following centrifugation, the volumes in the upper and lower reservoirs (lower reservoir corresponds to the filtrate) were determined and an aliquot of each removed for the determination of radioactivity. In addition, an aliquot of the filtrate was removed for the determination of protein (161).

The results of these experiments were analyzed by a one-way ANOVA with multiple comparisons performed according to Bonferroni. Statistical analysis was performed using InStat (GraphPad, San Diego, CA) computer program.

4.3 Results

4.3.1 Equilibrium Gel Filtration Chromatography

Equilibrium gel filtration chromatography of HDL and CsA resulted in chromatograms as depicted in Figure 35. The elution profiles of the ligand (CsA) and binding protein (HDL) are similar to those described by Hummel and Dryer (248) and others (257) investigating different ligands and their binding proteins. Typically, there is a peak corresponding to the ligand-protein complex and a trough at the retention volume of the ligand.

In a series of experiments the binding of CsA to HDL at 4°C was determined and a

direct plot (bound *vs.* free) is shown in Figure 36. The binding is linear over the concentrations of CsA used and does not appear to reach saturation and, therefore, does not fit the typical rectangular hyperbola curve seen in the Langmuir isotherm (233). In addition, when the data is transformed for Scatchard analysis, it does not fit the typical curves seen for this type of data reduction either. Further, I determined that the free fraction of CsA seems to be independent of the concentration of the drug (Table 17).

I attempted to determine the binding of CsA to HDL at 21° and 37°C using this method, however at temperatures ≥21°C, the CsA in the eluent buffer precipitated and, therefore, these experiments could not be performed.

Table 17. Fraction of CsA free (%) as a function of CsA concentration

CsA (ng/ml)	CsA Fraction Free (%)
25	5.64 ± 1.0
50	4.78 ± 1.3
107	4.12 ± 0.7
129	3.64 ± 1.3
266	4.00 ± 1.2
338	5.03 ± 1.3

results are the mean of two experiments ± SD

4.3.2 Ultrafiltration

To validate the use of ultrafiltration as a means of determining free CsA concentrations I measured the retention of HDL by the YM10 membrane, determined the recovery of ^{3}H-CsA, and monitored the free concentration of CsA as a function of increasing filtrate volume. I found that the concentration of protein in the filtrate was very low (<25 μg/ml). The recovery of ^{3}H-CsA following ultrafiltration was low, but consistent, and ranged

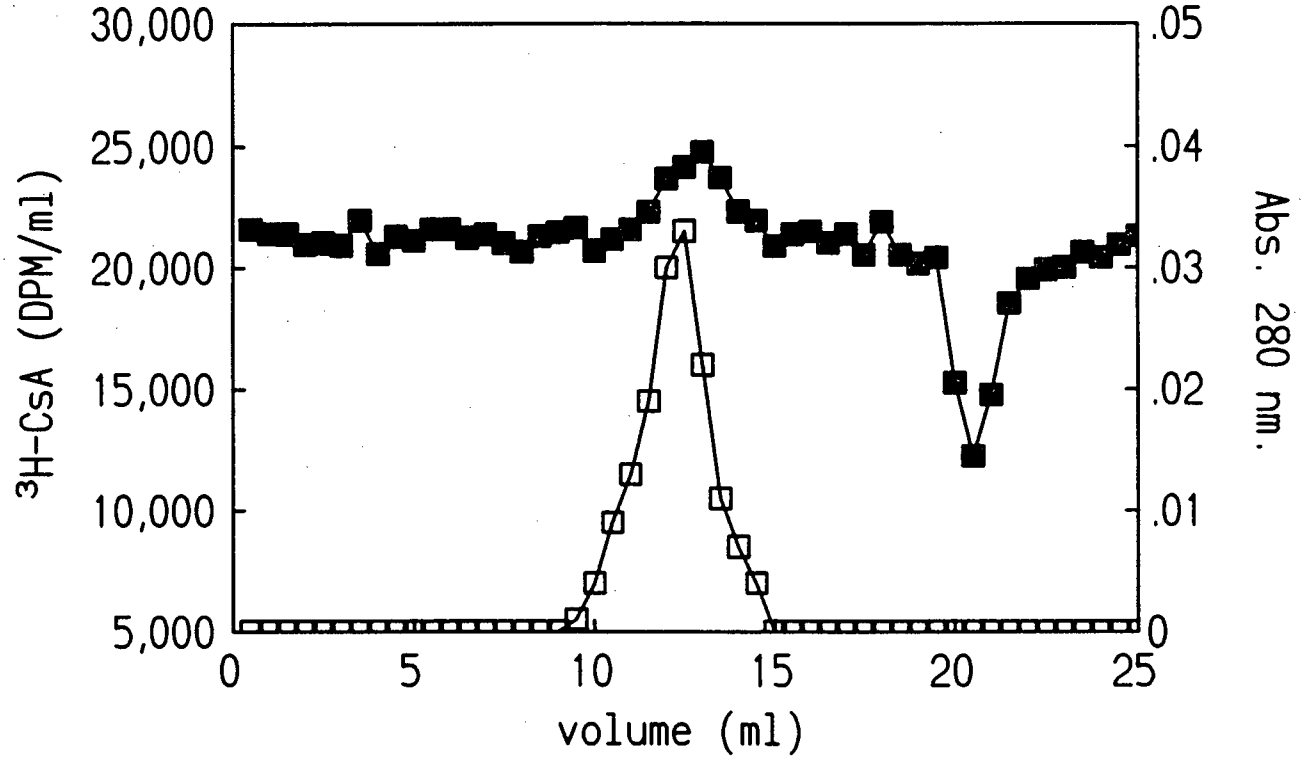

Figure 35. Representative graph of equilibrium gel filtration of CsA and HDL. HDL ($d < 1.22$ g/ml) and ^{3}H-CsA were pre-incubated at 37°C for 1 hour then applied to a Superose 12 gel filtration column and eluted in a buffer of 0.15 M NaCl, 10 mM EDTA, and 0.03% azide and 100 ng/ml CsA (labelled and unlabelled) at a flow rate of 0.05 ml/min. at 4°C.

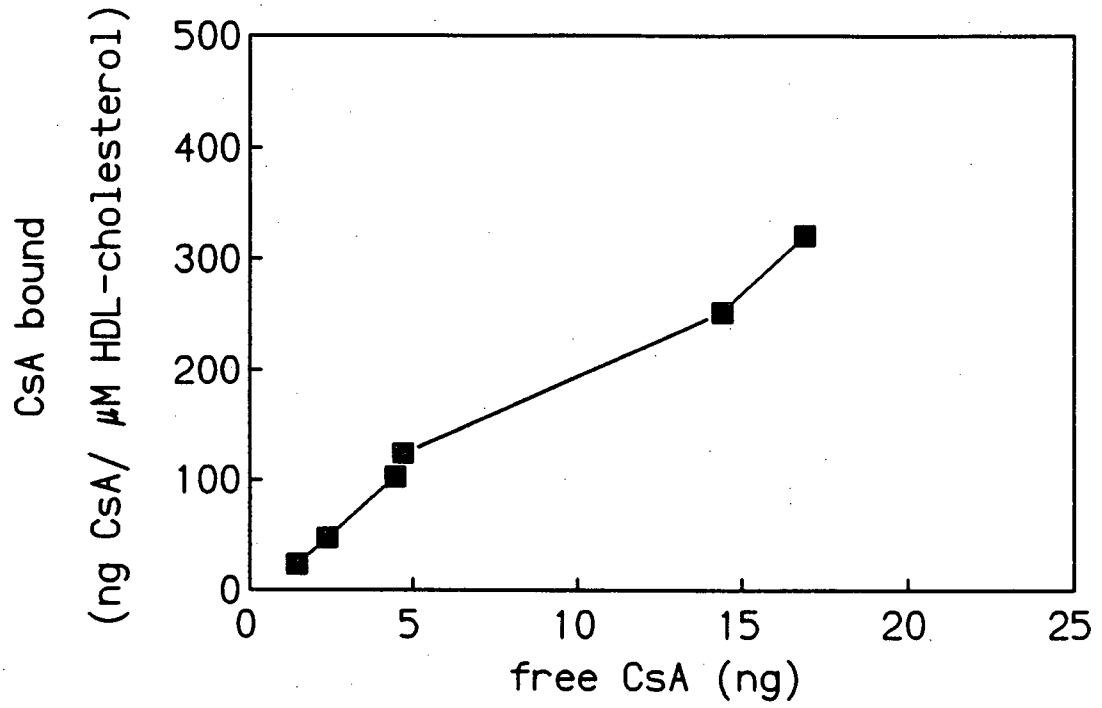

Figure 36. Graph of the amount of ^{3}H-CsA (DPM) in the filtrate as a function of filtrate volume. HDL was pre-incubated with ^{3}H-CsA at 37°C for 1 hour then applied to an ultrafiltration device and centrifuged at 37°C at 1000 X g for 8 to 15 minutes. The filtrate volume was determined and an aliquot removed for the determination of total radioactivity.

from 68.2 to 71.3% (Table 18). Further, I determined that the concentration of ^{3}H-CsA in the filtrate increased as the filtrate volume increased. This increase was significant at a filtrate volume of 410 μl (corresponding to a centrifugation time of 15 minutes) in comparison to that at 252.5 μl (corresponding to 8 minutes of centrifugation) ($p<0.05$. Figure 37).

Table 18. Total Recovery (%) of ^{3}H-CsA following Ultrafiltration

Time of Centrifugation	Volume of Filtrate (μl)	Recovery of ^{3}H-CsA (DPM/ml)
8 minutes	**252.5 $\pm$ 6.3**	**68.2 $\pm$ 2.0 %**
10 minutes	**272.0 $\pm$ 9.9**	**71.3 $\pm$ 0.8 %**
12 minutes	**342.0 $\pm$ 31**	**69.3 $\pm$ 2.9 %**
15 minutes	**410.5 $\pm$ 44**	**70.7 $\pm$ 6.0 %**

Data are the means of 2 experiments

4.4 Discussion

4.4.1 Ultrafiltration

The results of the experiments used to validate ultrafiltration as a means of determining the free concentration of CsA precluded its use. Although I showed that the membrane retained the binding protein (HDL), the recovery of ^{3}H-CsA was low (approximately 70%). Further, the concentration of CsA in the filtrate increased with increasing volume. Both of these results suggest that the drug was binding to the membrane or some other part of the ultrafiltration device. In addition, the concentration of CsA in the filtrate did not reach a plateau even though the volume filtered was >40% of the initial volume. Sebille *et al.* (257) recommended that the volume filtered during ultrafiltration

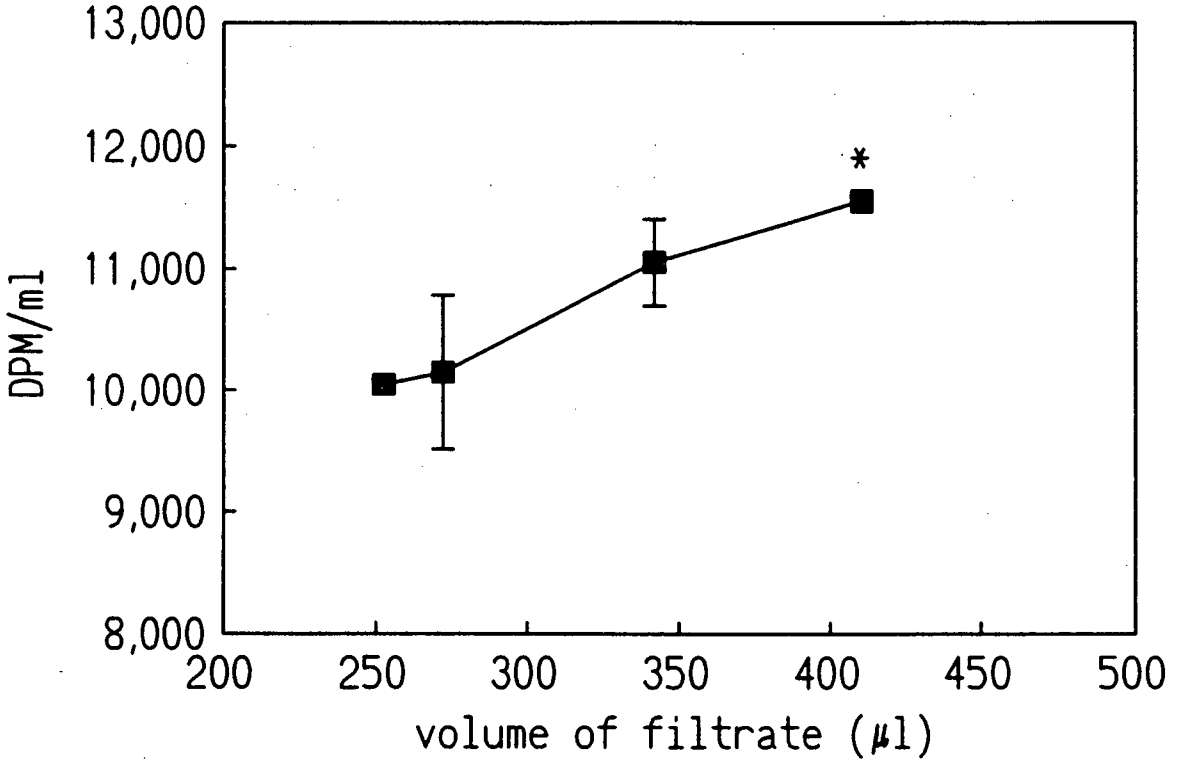

Figure 37. Graph of the amount of ^{3}H-CsA (DPM) in the filtrate as a function of filtrate volume. HDL was pre-incubated with ^{3}H-CsA at 37°C for 1 hour then applied to an ultrafiltration device and centrifuged at 37°C at 1000 X g for 8-15 minutes. The filtrate volume was determined and an aliquot removed for the determination of total radioactivity. (*$p < 0.05$)

should not exceed 40% of the initial volume since it has been shown that the free ligand concentration will remain constant provided that the ultrafiltrate does not exceed 40% of the initial volume (258). For these reasons, ultrafiltration was not used as a method with which to study the binding of CsA to lipoproteins. Awani and Sawchuk (14) and Legg and Rowland (252) also reported difficulties when they attempted to determine the free concentration of CsA in patient samples using ultrafiltration and therefore, did not pursue this method. Awani and Sawchuk (14) recovered only 38% of the drug when they performed their experiments using a protein-free filtrate of serum. They suggested that the low recovery was due to CsA binding to the ultrafiltration membrane (14). In contrast, Sgoutas *et al.*, in their binding experiments with CsA and lipoproteins, did not report any loss of the drug even though they used the same ultrafiltration device (157).

4.4.2 Equilibrium Gel Filtration

Equilibrium gel filtration chromatography was used to determine the binding of CsA to HDL at 4°C. The results of these experiments suggest that the binding of CsA to HDL is non-saturable or pseudo-non-saturable since binding is a linear function of increasing free drug concentration (233). These results are similar to those of other investigators who have shown, using ultrafiltration (157), equilibrium dialysis (148), ultracentrifugation (252), and partitioning (156), that the free fraction of CsA in plasma or serum is independent of the concentration of the drug. This has been demonstrated up to a CsA concentration of 5 μg/ml, which is approximately ten times the recommended therapeutic level of the drug. This phenomenon has also been shown at 4°, 21°, and 37°C. (252,156,39,40). Further, my results support those of Sgoutas *et al.* who, in their binding studies with HDL, LDL, and VLDL,

demonstrated non-saturable or pseudo-non-saturable binding to all three classes of lipoprotein (157). In addition, in their binding studies with HDL, they reported that the free fraction of CsA was approximately 5% at 200 ng/nl CsA using 1 mg of lipoprotein, which is similar to my results (157). Although neither the K_d nor B_{max} could be calculated due to the nature of the binding, they did suggest that CsA demonstrated a higher affinity for HDL in comparison to either VLDL or LDL (157).

In cases of non-saturable binding, saturation cannot be shown because either the solubility of the drug is limited or because the drug affinity is too low to measure accurately (233). If non-saturable binding data is transformed to a Scatchard-Rosenthal plot (bound/free *vs.* bound), the slope of the line would be zero or, in some cases, positive (233). Further, non-linear regression analysis of such data offers no advantage (233). For these reasons, the kinetics of the binding cannot be determined. Many investigators have described this type of interaction as adsorption rather than true binding since the kinetics of this binding show none of the characteristics of the binding of a small molecule to a fixed number of sites on a protein; high affinity, saturation, or specificity (191,233). In the case of non-saturable binding to lipoproteins, researchers have described the adsorption of the drug as dissolution of the drug in the lipid core of the lipoprotein (253,254). Indeed, it has been suggested by a number of authors (157,252) that CsA dissolves in or partitions into the lipid core of lipoproteins.

Chapter 5 - General Discussion and Future Aims

5.1 General Discussion

In very broad terms, the main objective of this thesis was to investigate the effect of CsA on plasma lipids and to investigate the effect of lipids on CsA.

The working hypothesis was that CsA decreases the uptake of LDL via the LDL-receptor thus contributing to dyslipidemia in transplant patients. Further, dyslipidemia affects the binding and distribution of CsA in plasma.

In the first part of this thesis, the effect of CsA on the uptake of LDL via the LDL-receptor in human skin fibroblasts was investigated. Since LDL uptake via the LDL-receptor is dependent upon the binding, internalization, and degradation of LDL, all of these aspects were studied, using the methods first described by Goldstein and Brown (46). This study demonstrated that CsA did not appear to affect LDL uptake by reducing binding. CsA did not decrease the number of binding sites or reduce LDL-receptor mRNA levels. Further, the physical association of CsA with the LDL particle did not appear to have any affect on LDL affinity. Contrary to my expectations, CsA significantly increased the *in vitro* degradation of LDL. These data imply that a decreased uptake of LDL via the LDL-receptor is not a mechanism by which CsA might increase LDL-cholesterol levels (Figure 38).

The second part of this thesis focused on the effect of dyslipidemia on the binding and distribution of CsA. The distribution of CsA in all of the dyslipidemic groups studied was significantly different from that of the normolipidemic controls. Further, the amount of CsA associated with the VLDL/LDL and HDL fractions was quite variable between the dyslipidemic groups, but the amount with the LPDP fraction remained relatively constant,

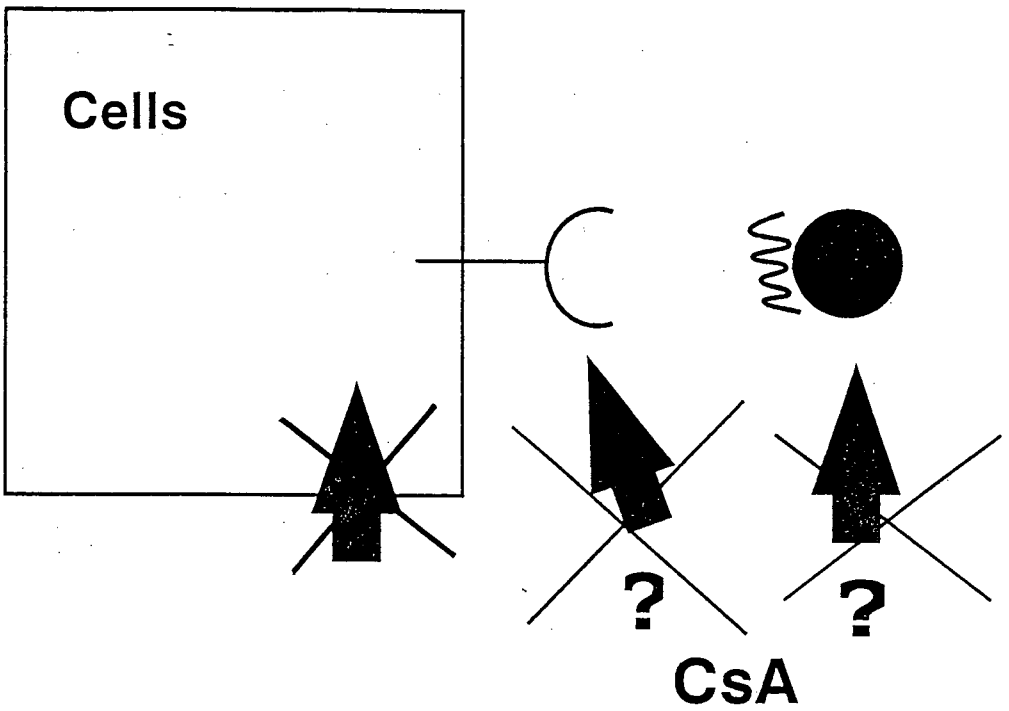

Figure 38. The effect of CsA on the uptake of LDL via the LDL-receptor.

with the exception of the combined hypercholesterolemic and hypertriglyceridemic group which showed a decrease in the amount with LPDP. In normolipidemic plasma, the HDL fraction contained the majority of the drug. Together, these data suggest that factors other than the mass of the lipid, such as composition of the lipoprotein, play a role in the distribution of CsA.

Because of the consequences of post-transplant hyperlipidemia many transplant centres have initiated lipid-lowering strategies for transplant recipients which include dietary and pharmacological interventions. Dietary modifications have been reported to improve lipid profiles of transplant recipients however, dietary changes alone may be inadequate and patient compliance is often poor (139). A number of cholesterol-reducing agents have been

utilized in transplant recipients, including bile acid resins and fibric acid derivatives, but to date, the most successful cholesterol-lowering drugs used are the statins (56). The statins are a group of drugs (lovastatin, pravastatin, and simvistatin) which are competitive inhibitors HMG-CoA reductase, the rate-limiting enzyme for cholesterol synthesis (56). Initial studies with lovastatin reported a high incidence of rhabdomyolysis which, in one case was severe enough to cause renal failure (259). However, several subsequent studies have demonstrated that by decreasing the dose, this side effect could be avoided. Further, the drug was still as effective in decreasing total and LDL-cholesterol levels at the lower dose (260). This apparent discrepancy was due to concomitant CsA therapy which inhibited the hepatic metabolism of lovastatin, resulting in increased lovastatin plasma levels (260,56). There have also been a number of studies on the effectiveness of simvistatin (261) and pravastatin (262) in transplant recipients and all have reported a decrease in total and LDL-cholesterol levels (approximately 20-25% and 35-45%, respectively). However, unlike that found with non-transplant patients receiving pravastatin, there was no corresponding increase in HDL-cholesterol levels (261,262). Although cholesterol-lowering agents have proven effective in decreasing total and LDL-cholesterol levels in transplant recipients, to date there have been no studies that have demonstrated that there is a decreased incidence of PTxA or CAD in these patients (56).

One other benefit in reducing post-transplant hyperlipidemia may be that the binding and distribution of CsA would not be so variable within and between patients. This may result in better efficacy of CsA therapy.

5.2 Future Areas of Research

The findings in this thesis have suggested several potential areas of future research pertaining to both the distribution of CsA and the role of the drug in CsA-induced hyperlipidemia. One research area would be to determine the role that the composition of the lipoprotein plays in affecting CsA distribution, since the composition of the lipoprotein classes is quite variable, depending on the type and cause of dyslipidemia. In addition, the effect of dyslipidemia on CsA's distribution still needs to be investigated with experiments conducted at 37°C. Given the benefits of precipitation techniques, perhaps a method that uses a precipitating reagent that is not affected by temperature, can be investigated.

In regard to the role of CsA in the pathogenesis of PTxA, one area of research would be to investigate some of the other suggested mechanisms of CsA-induced hyperlipidemia. One such avenue would be to determine the effect of CsA on VLDL production and clearance. These studies are important for a number of reasons. First, in some patients receiving CsA, elevated triglyceride levels, with or without elevated LDL levels, have been described (10). Second, there have been a few, yet somewhat conflicting, reports on the effects of CsA on hepatic and endothelial lipase (59,264). Third, even though Lopez-Miranda *et al.* (74) showed that the production rate of LDL was decreased in CsA-treated rats, these experiments merit repeating in humans since rats are not a suitable animal model of lipoprotein metabolism (75). Collectively, these data imply that the effect of CsA on VLDL metabolism warrants further investigation.

Given that CsA decreases bile acid synthesis uptake, and transcellular transport (78,91,208), a second avenue of research would be to determine if the affect of CsA on bile

acid metabolism contributes to the pathogenesis of CsA-induced hyperlipidemia. This research is particularly important since it has been suggested recently (226) that macrophages and endothelial cells utilize this pathway as an alternate means of dealing with intracellular cholesterol.

Finally, given that PTxA is thought to be, at the least, partially immune-mediated, one other avenue of research would be to re-examine the possibility that clinicians, in their efforts to decrease the side effects of CsA and other immunosuppressive drugs, may be under medicating their patients with respect to immunosuppressive therapy. Obviously, a crucial companion to these investigations would be continued research into the development of new immunosuppressants.

5.3 Significance of this Thesis

The finding that CsA's distribution changes with dyslipidemia is important since the majority of patients receiving CsA are, or will become, dyslipidemic. Moreover, changes in the drug's distribution will be realized in these patients since the cholesterol, triglyceride, and HDL-cholesterol levels used in the present study are representative and frequently encountered in these patients. More important, however, is the pharmacological role which lipoprotein-bound CsA may play. While some argue that only the free fraction of the drug has pharmacological activity and others suggest that the bound drug may also be active, in either case, the binding of CsA to lipoproteins will affect the drug's pharmacology and possibly augment its side effects.

Post-transplant atherosclerosis and CAD are the leading causes of morbidity and mortality in transplant patients surviving longer than 1 year. Because there is no effective

treatment for this disease, prevention is a priority. Evidence from animal and human transplantations has suggested that immune-mediated injury coupled with hyperlipidemia may play an important role in the pathogenesis of this disease. Although the causes of hyperlipidemia are considered to be multifactorial, several reports have suggested that CsA contributes to this process. For the first time, the findings presented in this thesis suggest that CsA does not contribute to increased plasma LDL-cholesterol levels by decreasing the uptake of LDL via the LDL-receptor. These findings provide new information to extend our understanding of the effects of CsA. Furthermore, these results offer the possibilities of new avenues of research which in themselves will lead to a better understanding of the role of CsA in post-transplant atherosclerosis.

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