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**Functional and Immunochemical Characterization of Advanced  
Glycation End-Product (AGE)-Modified Low-Density Lipoproteins  
(AGE-LDL)**

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Submitted to the School of Graduate Studies and Research in Partial Fulfillment of the  
Requirements for the Degree of Masters of Science

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## Abstract

Low-density lipoproteins (LDL), the major cholesterol carriers of human plasma contain a single copy of apolipoprotein B (apoB). As a ligand for the cell surface LDL receptor, apoB has an important role in cholesterol metabolism. Biochemical modification of apoB, such as modification with advanced glycation end-products (AGEs) can dramatically affect the functional integrity of LDL. Diabetic or renally impaired patients exhibit elevated concentrations of AGE-modified LDL (AGE-LDL). A dyslipidemia characterized by hypertriglyceridemia, increased levels of small-dense LDL, and low plasma concentration of HDL occurs commonly in such patients and future coronary artery disease risk is increased 2 to 5 fold. Lipoproteins isolated from the plasma of diabetic or renal insufficient patients have been shown to exhibit important abnormalities, including an impaired capacity to be taken up by cell surface LDL receptors. The objective of this study was first to provide a more precise molecular basis for the reduced LDL receptor-binding activities of AGE-LDL, and then to compare the structural and functional properties of LDL that have experimentally been AGE-modified *in vitro* with LDL modified *in vivo* under pathophysiological conditions of diabetes or renal insufficiency. In this study I report a significant simplification in studying the molecular basis of LDL glycation. I first show that incubation of LDL with 0.145 mM fructose 6-phosphate for 4 days leads to AGE modification of the LDL that is equivalent to that of LDL that had been incubated with 200 mM glucose for 16 days. LDL modified by F6P and LDL modified by glucose are similar in terms of apoB epitope expression and LDL receptor-binding activity. The precise molecular basis for the uptake

abnormality of AGE-LDL has been of considerable interest. I have utilized a chemical modification strategy to reversibly protect arginine or lysine residues of apoB from exposure to reducing sugar, to determine their respective roles in the defective binding of AGE-LDL to the LDL receptor. I demonstrate that the AGE-modification of lysine, and not arginine, residues of apoB may be largely responsible for the defective binding of AGE-LDL to the LDL receptor. Finally, I compared the LDL isolated from 3 healthy normal subjects, 3 diabetic patients, and 3 renal insufficient patients to LDL that had been AGE-modified by *in vitro* glycation. I observed no convincing evidence that LDL isolated from either the diabetic or renal insufficient patients were AGE-modified. Moreover, these LDL did not appear to be defective in terms of their ability to bind to the LDL receptor. Nevertheless, differences between LDL isolated from patients and from normal subjects are seen in terms of the expression of certain apoB epitopes. It is also apparent that experimental AGE-modification *in vitro* does not accurately reflect the *in vivo* process.

# Table of Contents

Abstract.....	i
Table of Contents.....	iii
List of Tables.....	vi
List of Figures.....	vii
Dedication.....	ix
Acknowledgements.....	x
Abbreviations.....	xii
<b>Chapter I: Introduction</b>	
Section I: Plasma Lipoproteins.....	1
Section II: Low Density-Lipoproteins .....	2
Section III: The Low Density Lipoprotein Receptor.....	4
Section IV: Apolipoprotein B .....	8
Section V: Modifications of LDL that Increases Its Atherogenicity.....	12
Section VI: Maillard Reaction.....	14
Section VII: Advanced Glycation End-products and Disease.....	20
Section VIII: Receptors for Advanced Glycation End-products.....	21
Section IX: Lipid Advanced Glycosylation and Oxidative Modification.....	22
Section X: Low Density Lipoprotein Advanced Glycation.....	24
Section XI: Advanced Glycation End-products Modify The Basic Residues (Lysine, and Arginine) of Apolipoprotein B.....	25
Section XII: Rationale.....	28

## **Chapter II: Material and Methods**

Isolation of LDL.....	30
Measurement of Protein Concentration.....	30
Preparation of AGE-BSA, AGE-Peptide, AGE-LDL, and Preformed-AGE-LDL.....	30
Preparation of Lysine-protected-AGE-LDL .....	31
Preparation of Arginine-protected-AGE-LDL.....	31
Iodination of Immunoglobulin and LDL.....	32
LDL Receptor-binding Assay.....	32
Competitive Radioimmunoassay (RIA).....	33
AGE Measurements Using Competitive ELISA.....	34
Measurement of Relative LDL Surface Charge, Capillary Transfer Western blotting.....	34
Determination of Lipid Composition.....	34

## **Chapter III: Results**

AGE-LDL formation by F6P versus glucose.....	37
Modification of arginine residues of apoB .....	43
Modification of lysine residues of apoB.....	49
AGE-peptide modified-LDL.....	59
Characterization of LDL from patients with diabetes and renal insufficiency.....	59

## **Chapter IV: Discussion.....72**

## **Chapter V: Conclusions and Future Work.....81**

**Chapter VI: References.....84**

**Curriculum Vitae**

## List of Tables

Table 2-1: Physical and biochemical profile of the normal, diabetic, and renal insufficient subjects.....	61
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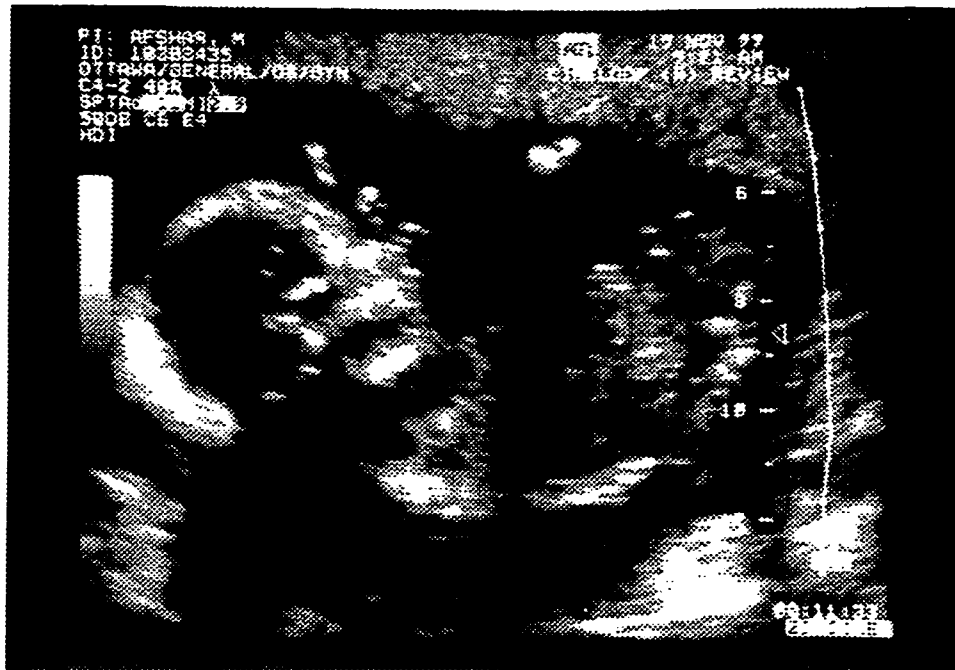
## List of Figures

Figure 1-1:	The classification, composition and hydrated density of normal human plasma lipoproteins.....	2
Figure 1-2:	Structure of a LDL particle.....	3
Figure 1-3:	Receptor-mediated clearance of LDL.....	5
Figure 1-4:	Structure of the LDL receptor.....	6
Figure 1-5:	Consensus sequence for the binding sites of LDL receptor and its ligand, apoB and apoE.....	8
Figure 1-6:	Secondary structure of apolipoprotein B in LDL predicted by computer modelling.....	9
Figure 1-7:	General Scheme of the Maillard Reaction.....	15
Figure 1-8:	The sodium salt of Actyr-N-Lys-Gly-NH <sub>2</sub> .....	18
Figure 1-9:	Chemical structures of various AGEs.....	19
Figure 1-10:	Scheme for the formation of AGEs on phospholipid head groups, followed by AGE-initiated oxidative damage on fatty acid side chains.....	23
Figure 1-11:	Structure of a LDL particle and potential sites of AGE modification on apoB-100.....	27
Figure 2-1:	Electrophoretic mobility of LDL as a function of time of glycation and concentration of reducing sugar.....	38
Figure 2-2:	Epitope map of human LDL.....	39
Figure 2-3:	The immunoreactivity of glycated LDL.....	40
Figure 2-4:	The immunoreactivity of glycated LDL.....	41
Figure 2-5:	The ability of glycated LDL to bind to the LDL receptor.....	42
Figure 2-6:	Reversible modification of arginine and lysine residues of apoB.....	44
Figure 2-7:	Electrophoretic mobility of LDL as a function of 1,2-	

	cyclohexanedione modification, and regeneration.....	45
Figure 2-8:	Electrophoretic mobility of LDL as a function of glycation, and/or 1,2-cyclohexanedione modification, and regeneration.....	47
Figure 2-9:	Arginine-protected AGE-LDL is unable to bind to the LDL receptor.....	48
Figure 2-10:	Electrophoretic mobility of LDL as a function of diketene modification.....	50
Figure 2-11:	Electrophoretic mobility of LDL as a function of glycation, and/or diketene modification, and regeneration.....	53
Figure 2-12:	Electrophoretic mobility of LDL as a function of arginine and lysine residues modification.....	54
Figure 2-13:	Immunoreactivity of AGE-LDL and lysine-protected AGE LDL.....	55
Figure 2-14:	The immunoreactivity of modified and control LDL.....	57
Figure 2-15:	Lysine-protected AGE-LDL is able to bind to the LDL receptor.....	58
Figure 2-16:	Non denaturing polyacrylamide gradient gel electrophoresis to determine the size of LDL samples .....	62
Figure 2-17:	Lipid composition of LDL isolated from three control, diabetic, and renal insufficient subjects .....	63
Figure 2-18:	Electrophoretic mobility of LDL isolated from three control, diabetic, and renal insufficient subjects .....	66
Figure 2-19:	AGE measurements using competitive ELISA.....	67
Figure 2-20:	Binding of LDL to the LDL receptor .....	68
Figure 2-21:	The immunoreactivity of control subjects' LDL .....	70
Figure 2-22:	The immunoreactivity of diabetic and renal insufficient subjects' LDL .....	71

# Dedications

This Thesis is dedicated to my parents, family: Shami, Shiro, Mory, Omid, and my  
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## Abbreviations

AGEs:	advanced glycation end-products
AGE-LDL:	AGE-modified LDL
apo:	apolipoprotein
B/Bo:	B is radioactivity bound in the presence of competitor and Bo is radioactivity bound in the absence of competitor
BHT:	butylated hydroxytoluene
BSA:	bovine serum albumin
CHD:	1,2-cyclohexanedione
ED <sub>50</sub> :	concentration of competitor required to reduce 50% of maximum binding
EDTA:	ethylen-diaminetetra-acetic acid
DKT:	diketene
FC:	free cholesterol
F6P:	fructose 6-phosphate
FBS:	fetal bovine serum
FDB:	familial defective apoB <sup>3500arg→gln</sup>
HDL:	high-density lipoproteins
HDA:	hydroxylamine
IDL:	intermediate-density lipoproteins
LDL:	low-density lipoproteins
mAb:	monoclonal antibody
NaCl-Tween:	0.15 molar sodium chloride containing 0.025% (v/v) Tween-20

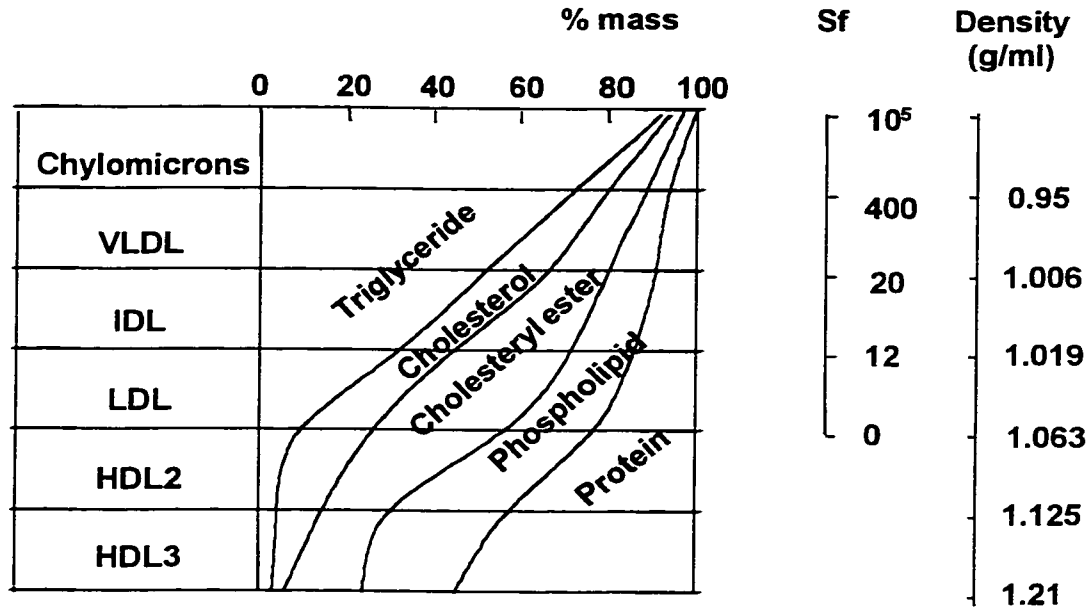
<b>PBS:</b>	phosphate buffered saline
<b>PBS-BSA:</b>	phosphate buffered saline containing 1% (w/v) bovine serum albumin
<b>PL:</b>	phospholipids
<b>RIA:</b>	radioimmunoassay
<b>Sf:</b>	Svedberg floatation
<b>TC:</b>	total cholesterol
<b>VLDL:</b>	very low-density lipoproteins

# Chapter I

## Introduction

### Section I : Plasma Lipoproteins

Lipoproteins are molecular complexes that contain both lipids and proteins. The protein components of lipoproteins are referred to as apolipoproteins. In 1941, Blix and colleagues, (Blix *et al*, 1941) reported that the major part of serum lipids migrated in an electric field with either an  $\alpha_1$  or a  $\beta$ -globulin mobility. For this reason, plasma lipoproteins were first classified on the basis of their electrophoretic mobilities. Further analysis of serum lipoproteins using zonal electrophoresis, revealed the presence of other bands at the origin and in the  $\alpha_2$ -(pre- $\beta$ ), prealbumin and  $\gamma$ -positions (Kunkel *et al*, 1952; Dangerfield *et al* 1955). However with the use of ultracentrifugation, an alternative classification of plasma lipoproteins was possible based on particle size and buoyant density (Gofman *et al*, 1949; DeLalla and Gofman, 1954). Lipoproteins can be separated, by fractional ultracentrifugal flotation in salt solutions of successively increasing densities, into four major classes designated as chylomicrons ( $d < 0.94$  g / ml), very low-density lipoproteins (VLDL,  $d = 0.94$ - $1.006$  g / ml), low-density lipoproteins (LDL,  $d = 1.006$ - $1.063$  g / ml), and high-density lipoproteins (HDL,  $d = 1.063$ - $1.021$  g / ml) (Figure 1-1).

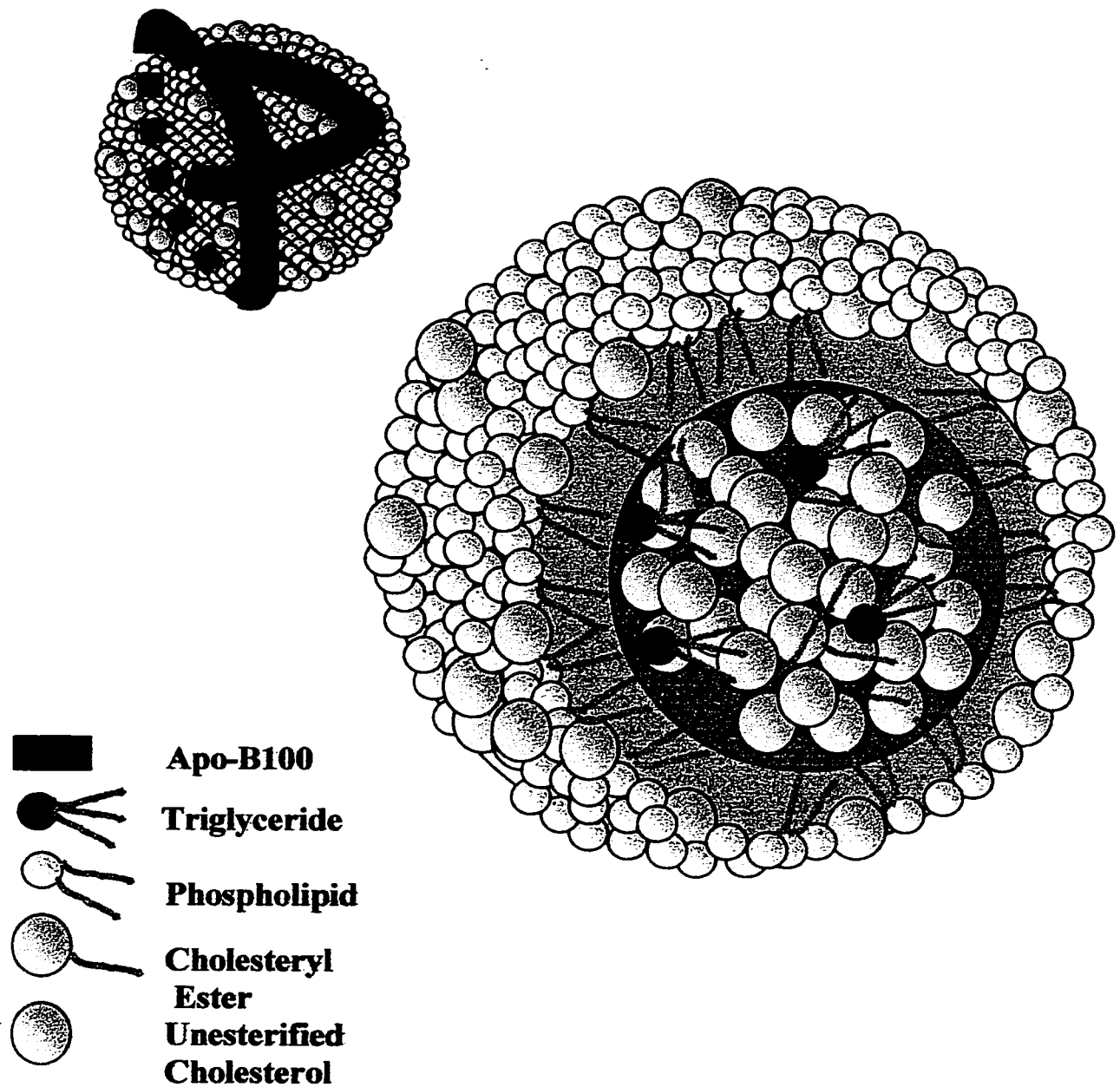


**Figure 1-1. The classification, composition and hydrated density of normal human plasma lipoproteins.**

With the increase in the protein content, the lipoprotein particles become denser mainly due to the loss of triglycerides. Sf, Svedberg floatation units. (Adapted from Mackner and Durrington, in Lipoprotein Analysis. Ed. Converse, C.A., and Skinner, C.A. 1992. P92)

## Section II : Low-Density Lipoproteins

The major cholesterol-carrying lipoproteins of plasma are the LDL. LDL consist of a lipid core composed primarily of cholesteryl esters. The surface coat of LDL contains unesterified cholesterol and phospholipids, together with a single apolipoprotein, apolipoprotein B-100 (apoB-100) (Beisegel *et al*, 1992) (Figure 1-2). The diameter of the LDL particles range from 20 to 25 nm. LDL is the product of extensive lipolysis. The precursor of LDL in the bloodstream are intermediate-density lipoproteins (IDL), which in turn originate from VLDL. VLDL are synthesized in the liver by hepatocytes and are secreted into the bloodstream, where they are hydrolyzed by lipoprotein lipase. Upon hydrolysis of triglycerides, VLDL become smaller and denser as they are converted to IDL. Under the combined actions of lipoprotein lipase and hepatic lipase, IDL undergo



**Figure 1-2. Structure of a LDL particle**

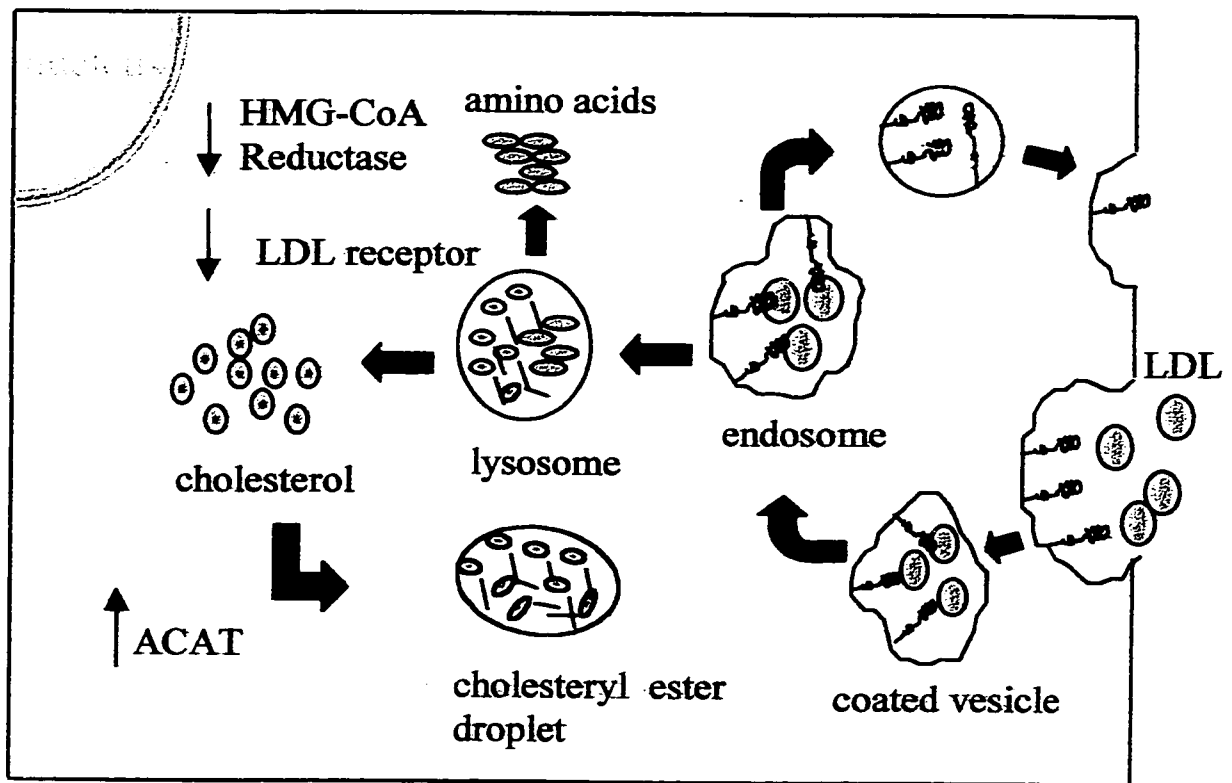
Low-density lipoproteins are spherical particles with a triglyceride and cholesteryl ester core and a phospholipid and unesterified cholesterol rich surface that is wrapped by apolipoprotein B-100.

further lipolysis and are converted to LDL. LDL is removed from the bloodstream by LDL receptors situated on the surface of hepatocytes and cells in peripheral tissues.

LDL exhibit heterogeneity in terms of lipid composition, immunoreactivity, size, charge, and buoyant density in both normal and dyslipidemic individuals. For example, in normolipidemic individuals, small-dense LDL, with a diameter of about 19 nm, are a minor subclass of LDL compared to the major LDL sub-fraction that has a diameter of about 24 nm (Galeano *et al*, 1994). However, small-dense LDL represent the predominant LDL subclass in patients with even moderate hypertriglyceridemia. LDL particle diameter is associated with risk of myocardial infarction (Stampfer *et al*, 1996). Small-dense LDL is suggested to be atherogenic due to an increased susceptibility to oxidation (De Graaf *et al*, 1991), a higher affinity for arterial proteoglycans (Hurt-Camejo *et al*, 1990), a decreased affinity for the LDL receptor (Galeano *et al*, 1994) and an increased catabolism by a non-LDL receptor-mediated pathway (Shepperd *et al*, 1985).

### **Section III : The Low-Density Lipoprotein Receptor**

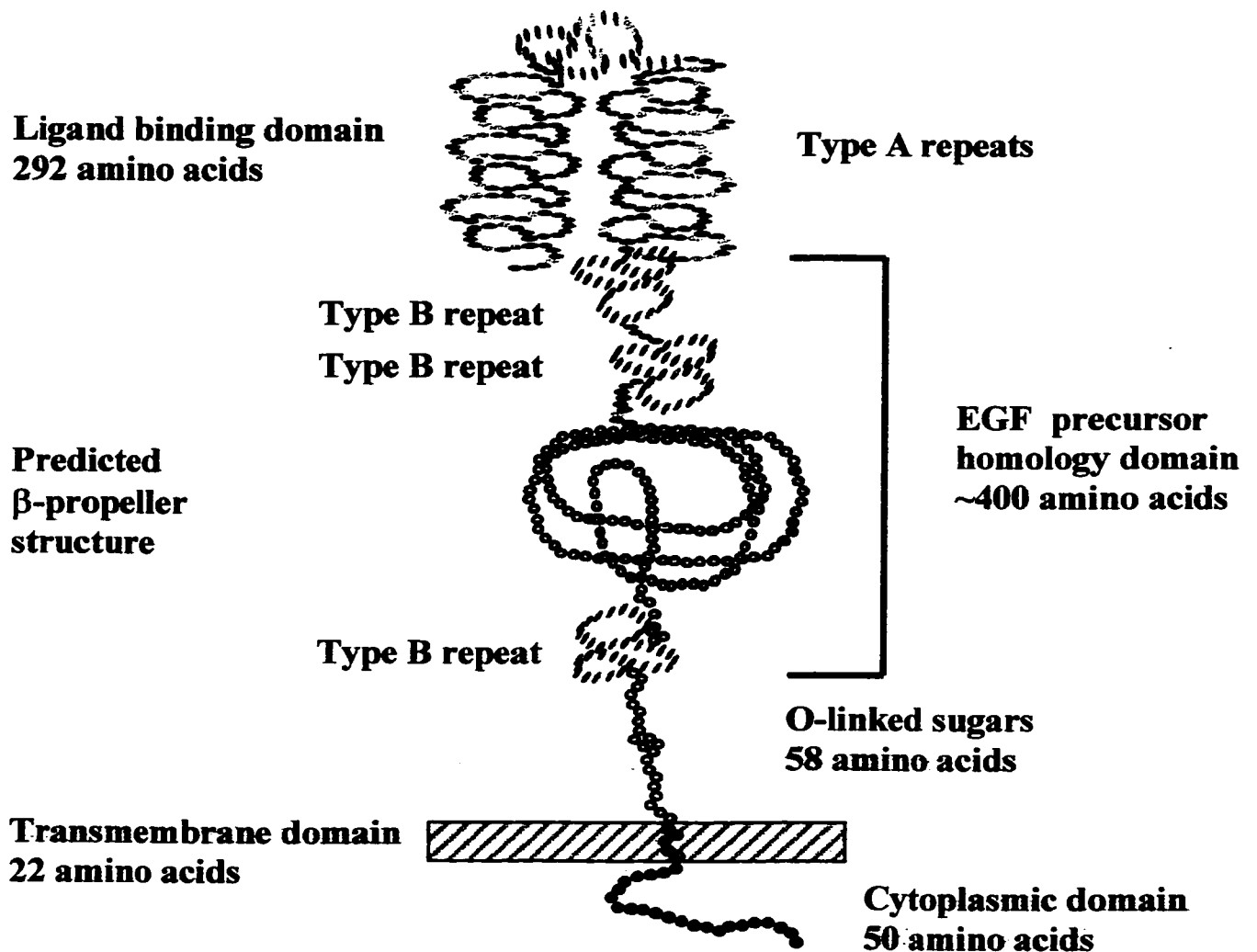
Uptake of LDL by either the liver or extrahepatic tissues can occur by both receptor and nonreceptor pathways. Two-thirds to three-fourths of the circulating pool of LDL is cleared by receptor pathways. LDL receptors are localized in clathrin-coated pits and function to internalize LDL into cells by receptor-mediated endocytosis (Goldstein *et al*, 1985b) (Figure 1-3).



**Figure 1-3 : Receptor-mediated clearance of LDL**

LDL receptors are transported to the surface of cells; where they migrate to clathrin-coated pits. When the LDL receptors bind to circulating LDL, the receptor-ligand complexes are internalized into endosomes. Following acidification of the vesicle, receptors dissociate from LDL and the former are recycled to the surface of the cell whereas the LDL are directed to lysosomes. The cholesterol esters of LDL are hydrolyzed to unesterified cholesterol, and apoB-100 is degraded to amino acids. The amount of cholesterol entering the cell regulates the activity of 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMG-CoA reductase) and the rate of synthesis of LDL receptors (Modified from Brown and Goldstein, 1985)

The LDL receptor is a membrane glycoprotein consisting of 839 amino acids. It is expressed on the surface of all cell types, and has a high affinity for apoB-containing LDL as well as for apoE-enriched VLDL and chylomicron remnants (Figure 1-4) (Brown and Goldstein, 1986).

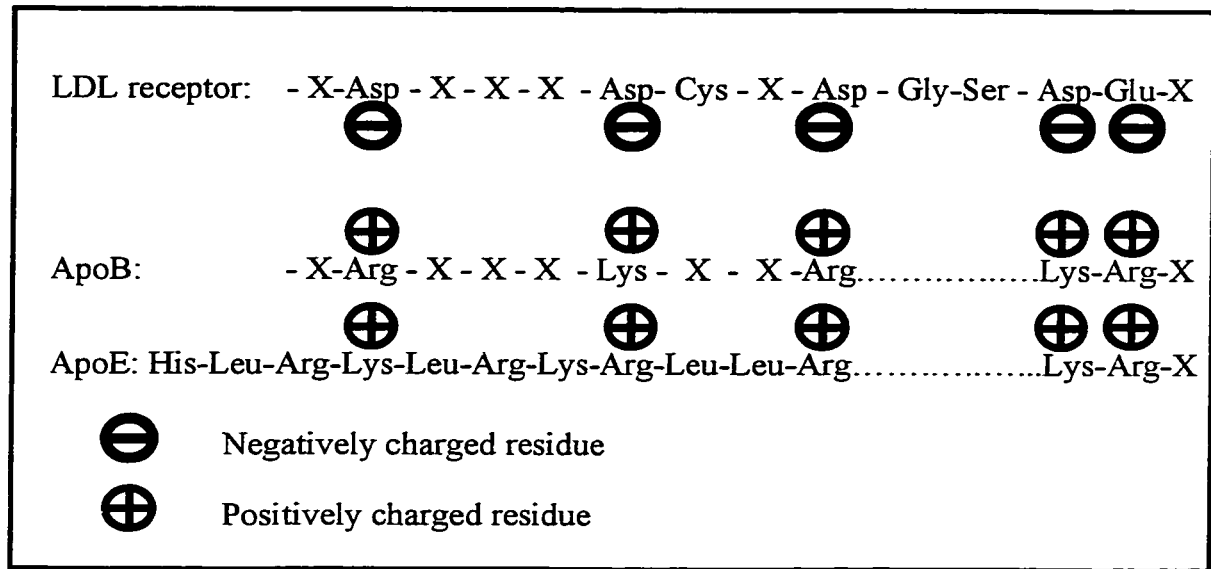


**Figure 1-4: Structure of the LDL receptor**

The LDL receptor is composed of distinct structural domains. The role of the individual domains in LDL receptor-mediated endocytosis has been elucidated from functional analysis of natural LDL receptor variants and LDL receptor variants generated by site-directed mutagenesis. See text for details.

The LDL receptor is composed of the ligand-binding domain, the EGF precursor homology domain, the O-linked glycosylation domain, a trans-membrane domain, and the cytoplasmic domain (Brown and Goldstein, 1985). The ligand-binding domain is made up of seven imperfect cysteine-rich sequences, responsible for ligand binding. The EGF precursor homology domain is essential for receptor recycling and appears to indirectly modulate ligand binding (Brown and Goldstein, 1985). The O-linked sugar domain has no clear function, but may serve as a spacer to increase accessibility of the receptor on the surface of the cell or to increase the stability of LDL receptor. The cytoplasmic domain has been shown to be responsible for clustering of the receptor within the clathrin-coated pits and to be necessary for the internalization and recycling of the receptor (Brown and Goldstein, 1985).

Each of the seven repeats in the ligand binding domain of LDL receptor contains a conserved motif that is characterized by a cluster of negatively-charged amino acids, Asp-X-Ser-Asp-Glu (DxSDE), located near the carboxy-terminus of each repeat. It was originally thought that these clusters of negatively-charged residues interact with clusters of positively charged residues on apoB or apoE (Figure 1-5) (Knott *et al*, 1985). More recently, however, it has been proposed that the DxSDE motif forms a calcium-binding site, that is essential for the proper folding of the repeat (Blacklow and Kim, 1996). As a consequence, most of the negatively-charged residues within this motif are coordinated with  $\text{Ca}^{++}$  and are, therefore, unavailable for ligand-binding. It was speculated that, during binding of ligands to the LDL receptor,  $\text{Ca}^{++}$  is released which would free negatively-charged residues to interact with positively-charged residues in apoB or apoE.



**Figure 1-5: Consensus sequence for the binding sites of LDL receptor and its ligand, apoB and apoE**

Sequence alignment of the LDL receptor, apoB, and apoE binding sites. The negatively-charged residues in the LDL receptor are thought to interact with positively-charged residues on apoB or apoE. The LDL receptor consensus sequence within the cysteine-rich repeats constitute the ligand binding domain. (Modified from Beisiegel, U. in the *Structure and function of apolipoproteins* Rosseneu, M. (Edt CRC p275)

## Section IV : Apolipoprotein B

Full-length apoB is called apoB-100, and is synthesized in human liver. ApoB is also synthesized in the intestine, but due to a stop codon it is only composed of the amino-terminal 2152 amino acids (48%) of the full-length protein (i.e. apoB-100). This post-translational editing of apoB mRNA that is responsible for the generation of apoB-48 causes the conversion of codon 2135 from CAA, that encodes Gln, to UAA, a translational stop codon (Powell et al, 1987; Chen et al, 1987). ApoB-100, as synthesized in the human liver, consists of 4536 amino acids with a molecular weight of 550 kDa. It is predicted that apoB-100 is composed of five domains that differ in their relative

affinities for lipid (Segrest *et al*, 1994, Yang *et al*, 1989). The amino terminal 1000 amino acids, the first domain, has relatively low affinity for lipids, whereas the third domain (residues 1700-3000), and the carboxy-terminal domain (residues 4100-4536) appear to have a high affinity for lipids. Residues 58-476 of the first domain are predicted to be enriched with  $\alpha$ -helices (Figure 1-6). Segrest and colleagues (Segrest *et al*, 1994) have also proposed a pentapartite model for apoB secondary structure based on a sophisticated computer-assisted analysis of apoB primary sequence (Figure 1-6). In this model, three amphipathic  $\alpha$ -helices are separated by two amphipathic  $\beta$ -sheets. The first  $\alpha$ -helix is predicted to fold as a globular structure with low affinity for lipids. Two  $\beta$ -sheet-enriched regions would bind to lipid irreversibly whereas the  $\alpha$ -2 and  $\alpha$ -3 domains would exhibit reversible lipid-binding.



**Figure 1-6: Secondary structure of apolipoprotein B in LDL predicted by computer modelling.**

The pentapartite model of apoB secondary structure is composed of the:  $\alpha_1$  58-476,  $\beta_1$  827-1961,  $\alpha_2$  2103-2560,  $\beta_2$  2611-3867, and  $\alpha_3$  4061-4338 domains (Segrest *et al*, 1994).

It now appears that both the B and E apoproteins contain a similar positively charged domain or structural sequence which confers LDL receptor-binding specificity on the lipoproteins. This led to the hypothesis that the basic amino acids in apoB may be participating in binding to the LDL receptor, through ionic interactions with acidic

residues within the ligand-binding domain of the receptor. It is now established that the arginine and lysine residues in apoB are functionally significant and that modification of these residues abolishes the ability of LDL to bind to the LDL receptor (Weisgraber *et al.* 1978; Mahley *et al.*, 1977). Once apoB was sequenced, several regions enriched in arginine and lysine residues became candidates for LDL receptor-binding sites, including Site A (residues 3,147-3,157) and Site B (residues 3,359-3,367) (Knott *et al.* 1985B).

A number of studies indicated that Site B (residues 3,359-3,367) is critical for receptor binding. Yang and coworkers (Yang *et al.*, 1986) demonstrated that a synthetic peptide that represents human apoB residues 3345-3381 can mediate lipoprotein binding to the LDL receptor. There is also experimental evidence that replacement of basic amino acids by neutral amino acids within Site B by mutagenesis eliminates apoB interaction with the LDL receptor (Boren *et al.*, 1998). Using immunochemical techniques Milne *et al.* have tested a panel of anti-apoB mAbs for their ability to block binding of LDL to the LDL receptor (Milne *et al.*, 1989). Antibodies that were specific for epitopes situated between apoB residues 3000-4000 block the binding of LDL to the LDL receptor, while mAbs whose epitopes are located outside of this region had no effect. In the genetic disorder, familial defective apoB-100 (FDB), high levels of LDL accumulate in the circulation because of a single site mutation (substitution of glutamine for the normally occurring arginine) at residue 3,500 of apoB-100. There is strong evidence to suggest that Arg 3,500 does not directly participate in the interaction of apoB with the LDL receptor but its replacement by glutamine provokes a conformational change in apoB that prevents its recognition by the LDL receptor (Boren *et al.*, 1998).

The topology of apoB on the lipoprotein surface is described by a ribbon and bow model (Chatterton *et al*, 1995). By immunoelectron microscopy of LDL, Chatterton and colleagues mapped the relative position of 11 anti-apoB monoclonal antibodies on the surface of human LDL. They proposed that the first 89% of apoB-100 wraps around the LDL particle (the ribbon). The C-terminal 11% of apoB stretches back and crosses the ribbon (the bow) (Figure 1-11). Since the bow crosses between residues 3000 to 3500 that includes the putative apoB LDL receptor-binding site, it has been proposed that it may function as a negative regulator of apoB-mediated binding to the LDL receptor (Chatterton *et al*, 1995; Boren *et al*, 1998). It is known that carboxy terminally-truncated apoB have increased affinity for the LDL receptor (Boren *et al*, 1998)

Several studies have attempted to determine the possible contribution of LDL size and composition to apoB conformation and function ( Kroon, 1994; Galeano *et al*, 1994). A number of studies have shown that the triglyceride content of the cholesteryl ester-rich core of LDL modulates the interaction of LDL with its receptor. Triglyceride-enriched LDL obtained from hypertriglyceridemic patients are taken up less efficiently by cultured cells; whereas LDL which has been depleted of triglyceride *in vitro* is taken up more efficiently (Avriam *et al*, 1990). However, the results from other investigators provide evidence that the triglyceride content alone is not a major determinant of the overall structure of apoB and suggest that the size of LDL plays a more important role in determining the conformation of the apoB molecule near its receptor recognition site and its affinity for the LDL receptor (Galeano *et al*, 1994; Krieger *et al*, 1979).

## **Section V : Modifications of LDL That Increases Its Atherogenicity**

It is now well established that elevated LDL cholesterol is an important cause of coronary heart disease (Tyroler, 1987; Russell, 1999). The majority of individuals with hypercholesterolemia have an increased number of LDL particles in their plasma. Clinical and experimental studies have shown that such elevated plasma concentrations of LDL are associated with accelerated atherogenesis (Tyroler, 1987; Goldstein *et al*, 1977; Steinberg, 1983). Because high plasma concentrations of cholesterol, in particular elevated LDL cholesterol levels, are one of the risk factors for atherosclerosis, the process of atherogenesis has been considered by many to result from the accumulation of cholesteryl ester within the artery wall.

The earliest type of atherosclerotic lesion, the fatty streak, is characterized by an accumulation of cholesteryl ester-laden cells in the subendothelium (Stary *et al*, 1994). These are called foam cells and are predominantly monocyte-derived macrophages. Exposure of macrophages to high concentrations of LDL leads to little intracellular cholesteryl ester accumulation due to the feedback mechanisms that are inherent in the LDL receptor pathway. In 1979, it was shown that macrophages, which were incubated in the presence of LDL that had been chemically modified by acetylation, accumulated cholesteryl esters and acquired the morphology of foam cells. While acetylation of LDL is considered to be non-physiological, it was subsequently demonstrated that other modifications to LDL that could potentially occur *in vivo* could also lead to lipid accumulation and transformation of macrophages into foam cells. These include LDL oxidation (Steinberg, 1997), glycation (Makita *et al*, 1991), aggregation (Khoo *et al*, 1988), association with proteoglycans, and incorporation into immune complexes

(Griendling *et al*, 1997). Oxidation of LDL, in particular, has been studied by many laboratories. Incubation of LDL with endothelial cells, smooth muscle cells and macrophages in culture induces the oxidation of polyunsaturated fatty acids on LDL. *In vivo*, this is thought to occur when LDL become trapped in the arterial wall. Oxidized LDL have been demonstrated to be present in human atherosclerotic lesions (Yla-Herttuala *et al*, 1989). The lipid peroxides that are formed during oxidation can fragment fatty acyl chains that attach covalently to apoB, rendering the modified LDL a ligand for several scavenger receptors that are found on the surface of macrophages, endothelial cells and vascular smooth muscle cells. The best characterized of these scavenger receptors is the scavenger receptor A (SR-A). Oxidized LDL that bind to the SR-A on macrophages are taken into the cell by endocytosis. As the SR-A is not down regulated by cholesterol, exposure of macrophages to oxidized LDL leads to the accumulation of cholesterol esters and foam cell formation. Other oxidized LDL receptors that may contribute to atherosclerosis are the scavenger receptor B-I (SRB-I), CD-36, and the lectin-like oxidized LDL receptor 1 (Kodama *et al*, 1990; Acton *et al*, 1994; Steinberg, 1997). Oxidized LDL have other pro-atherogenic properties that include being directly chemotactic for monocytes, being cytotoxic for endothelial cells, being mitogenic for macrophages, and having the ability to suppress nitric oxide production (Yla-Herttuala *et al*, 1989).

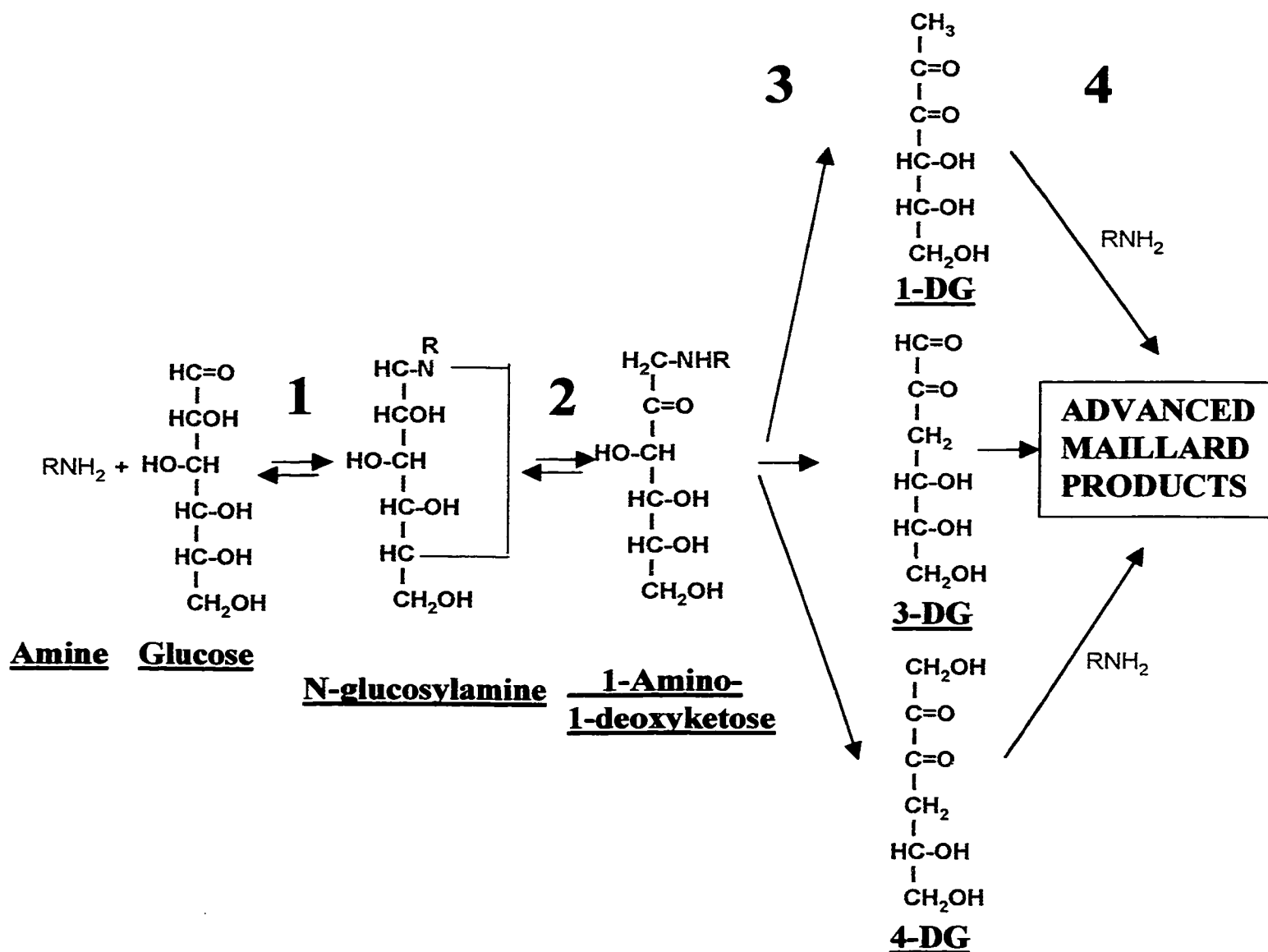
Atherosclerosis in diabetes is clearly multifactorial, but several potential mechanisms stand out and are in need of further focus. Foremost would be the unique effects of hyperglycemia mediated through the mechanisms of protein nonenzymatic glycation and glycooxidation. There has not been an adequate clinical trial of glucose

lowering and atherosclerosis outcomes. In a study by the UK Prospective Diabetes Study Group it was shown that intensive blood-glucose control by either sulphonylureas or insulin substantially decreases the risk of microvascular complications, but not macrovascular disease, in patients with type 2 diabetes (UKPDS Group, 1998). None of the individual drugs had an effect on cardiovascular outcomes. Intensive glucose control had little effect on CHD events where as a major impact was observed by lowering LDL cholesterol (4S, 1994). Another study by Scandinavian Simvastatin Survival Study Group shows that long-term treatment with a cholesterol lowering drug (simvastatin) improves survival in CHD patients.

## **Section VI : Maillard Reaction**

Another form of modification, non-enzymatic glycation, also occurs *in vivo*. Glucose reacts non-enzymatically with protein amino groups to initiate glycation, the early stage of the Maillard reaction. The discovery of the amino-carbonyl reaction, in 1912, by Louis-Camille Maillard was made in the course of his studies on the synthesis of peptides under physiological conditions. The chemistry of the Maillard reaction is complex. The discovery that non-enzymatic reactions between carbonyl groups of carbohydrates and free amino group on proteins, peptides, amino acids and organic amines can occur at physiological conditions of temperature, pH and concentration has aroused considerable interest among biomedical researchers working in the field of diabetes and aging. However, although a substantial amount of work has been undertaken in order to understand the biology of this reaction, relatively little progress has been achieved towards understanding the chemistry of the Maillard reaction under

conditions. The Maillard reaction can be divided into four steps : (1) reversible formation of glycosylamine, (2) Amadori rearrangement of the glycosylamine to the ketose-amine, 1-amino-1-deoxyketose, (3) degradation and dehydration of the amino sugar, and (4) reaction of amino groups with intermediates formed in step 4 and subsequent rearrangements to form advanced Maillard products (Figure 1-7).



**Figure 1-7: General Scheme of the Maillard Reaction.** DG, deoxyglucose (George *et al.* 1989)

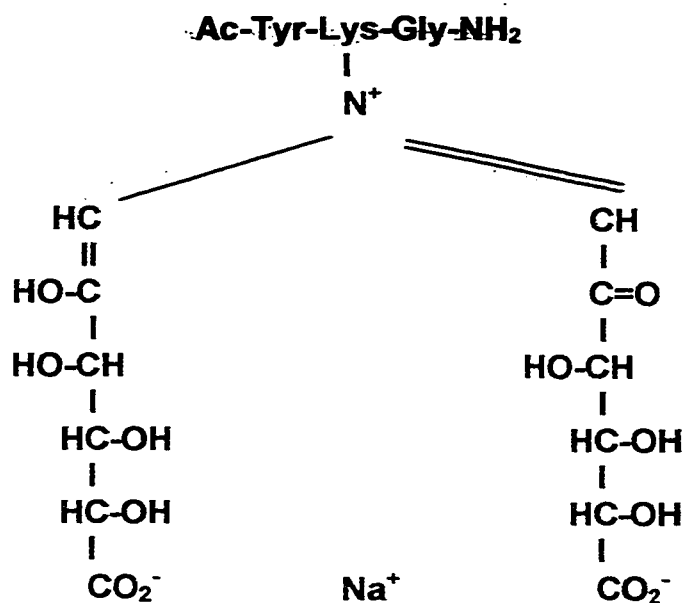
Reducing sugars react with primary and secondary amines in non-polar or polar solvents to give rise to N-substituted glycosylamine derivatives in which the glycosidic hydroxyl group is replaced by an amino group (Ellis, 1959). Under acidic conditions, glycosylamines either undergo Amadori rearrangement to 1-amino-1-deoxy-2-ketoses or they are hydrolysed back to their starting materials i.e. glucose and amine (Paulsen, 1980). It should be noted that the reactions in the initial stage of the Maillard reaction are favoured by acidic conditions, an apparent contradiction with the observation that neutral or alkaline conditions promote the glycation reactions.

One of the most extensively studied products resulting from non-enzymatic glycation is the ketoamine which is formed via Amadori rearrangement of glycosylamines. The interest in the study of this product stems from the fact that its presence has been confirmed in well characterized proteins such as hemoglobin. The level of glycated hemoglobin is commonly used as a measure of average glycemia over several weeks (Koenig *et al.*, 1976). Other proteins as well as lipoproteins are also modified by ketoamine.

Although the Amadori product is a rather stable compound, with time, it undergoes a number of transformations as shown in Figure 1-7. At higher pH, the 1-amino-deoxy-ketose enolizes in position 2-3 and eliminates the amine from C-1 to form 1-deoxyglucose (1-DG) (Anet, 1964). At lower pH, the ketoamine undergoes a 1-2 enolization leading to the formation of 3-deoxyglucosone (3-DG). Another diketone formed through this pathway is 4-deoxyglucosone (4-DG) (Figure 1-7). Of interest is that the liver contains enzymes that can metabolize 3-DG (Hata *et al.* 1988). This suggests the presence of a biological defense mechanism against the advanced Maillard reaction.

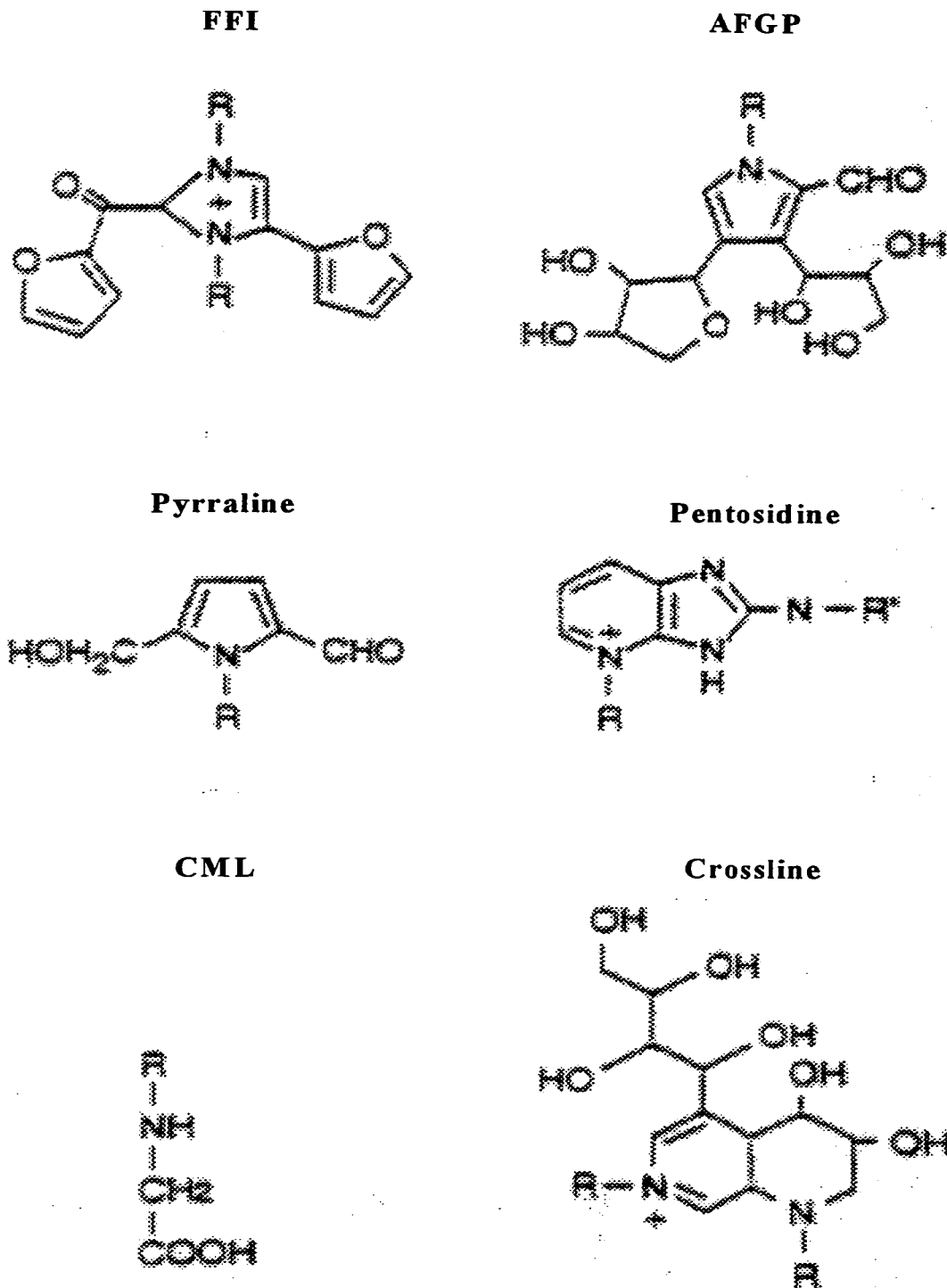
The three deoxy compounds described above, either degrade to form compounds such as furfuraldehyde (Taufel and Iwainsky, 1952), reductones (Hodge and Rist, 1952), pyranones (Mills *et al*, 1970), and pyrrolinone reductones (Ledl and Fritsch, 1984). These late products of Maillard reactions are detectable by UV light but are generally not coloured. On standing, they may react further with amines or other carbonyls to form brown, fluorescent compounds, the structures of which are poorly understood. Some of these coloured compounds are melanoidins, i.e. highly polymerized nitrogenous substances which are unlikely to be found *in vivo*. There is, at present, little evidence that the late stages of the Maillard reaction occur *in vivo* in most tissues. The Amadori products are relatively stable and it is unlikely that they will go on to form pigmented Maillard products except in the most long-lived proteins, such as the lens crystallins and collagen.

Because of its relevance to diabetic complications, the reaction between glucose, peptides and proteins was of great interest. Formation of di-hexosyl adducts was noted to occur between the  $\epsilon$ -amino group of the tripeptide Ac-Tyr-Lys-Gly-NH<sub>2</sub> acetate and sodium glucuronate under physiological conditions. The major product was the sodium salt of Actyr-N-(D-arabino-5-carboxy-5-carboxy-2,3,4,5, tetrahydro-1-pentenyl)-N-(D-arabino-5-carboxy-3,4,5-trihydroxy-2-oxopentylidene) Lys-Gly-NH<sub>2</sub> (Takeda, 1977) (Figure 1-8).



**Figure 1-8: The sodium salt of Actyr-N-(D-arabino-5-carboxy-5-carboxy-2,3,4,5, tetrahydro-1-pentenyl)-N-(D-arabino-5-carboxy-3,4,5-trihydroxy-2-oxopentylidene) Lys-Gly-NH<sub>2</sub>**

The structure was elucidated on the basis of <sup>1</sup>H and <sup>13</sup>C NMR, UV spectra, and pH titration. The structure of the compound shown in figure 1-8 reveals two sugars bound to an amino group via a conjugated enolketolimmonium structure. It is unknown whether the compound is fluorescent. Although numerous studies have been devoted to the chemistry and biology of protein-bound Amadori products, few studies have been carried out to elucidate the precise structure of protein-bound Maillard products. This may be attributable to the extraordinary difficulty associated with structure elucidation of adducts and crosslinks of non-enzymatically browned proteins. However, increasing evidence has implicated α-dicarbonyl structures such as 1-, 3-deoxyglucosones, protein-bound dideoxyones, and fragmentation products such as glyoxal and methylglyoxal in the covalent crosslinks produced by the advanced glycation reaction (Figure 1-9) (McLellan *et al*, 1994).



**Figure 1-9: Chemical structures of various AGEs.** “R” denotes the lysine, “R” the arginine, carbon backbone. FFI, pyrraline, AFGP, and crossline are synthetic products isolated from model, in vitro reactions. Pentosidine was isolated from human dura collagen (Bucala *et al*, 1997).

## Section VII: Advanced Glycation End-Products and Disease

Formation of Maillard products starts with the reaction of the amino groups of proteins, particularly the side chains of lysine, arginine, and histidine, with reducing sugars, including glucose, fructose, hexose-phosphates, trioses, and triose-phosphate. Protein-bound Amadori-products through subsequent rearrangements, dehydrations, and oxidations, form a heterogeneous group of fluorescent and brown products, the so called "advanced glycation end-products" (AGEs). Among the physiologically relevant sugars, glucose is the least reactive, and may, in part, be the reason for its selection by evolution as the main biological energy carrier.

In diabetes, persistent hyperglycemia leads to an increase in the level of AGEs. Reactive AGEs and their intermediates form *in situ* on blood and tissue proteins. However, experimental studies also indicate that these products may arise by the entry into the plasma compartment of AGE-peptides produced by the normal catabolism of AGE-modified tissue proteins (Makita *et al*, 1991; 1994). High concentrations of circulating AGE-peptides therefore can occur even under non-hyperglycemic conditions if plasma filtration is impaired by renal failure. The reactive nature of AGE-peptides, together with their inefficient removal by standard hemodialysis regimens, has led to the concept that circulating AGE-peptides comprise an important component of the so-called uremic toxins or 'middle molecules' which accumulate during renal insufficiency and contribute to the morbidity and mortality of chronic renal failure (Makita *et al*, 1994). Dyslipidemia and severe atherosclerotic vascular disease occurs in patients with renal insufficiency irrespective of diabetes. Diabetic patients with endstage renal disease (ESRD), for example, suffer from a particularly poor long-term prognosis. Their two year

survival rate is <50% and cardiovascular complications account for the single most common cause of premature death (Friedman, 1992)

## **Section VIII : Receptors for Advanced Glycation End-Products**

Several years ago, quantitative considerations suggested that net AGE formation occurred faster *in vitro* than *in vivo* (Vlassara *et al*, 1986). This led to the hypothesis that important, AGE-specific turnover mechanisms may exist in the body to limit the accumulation and tissue toxicity of AGE-modified proteins. Experimental studies by Vlassara and co-workers have since established the existence of a specific AGE-receptor system (Vlassara *et al*, 1986). AGE-receptors also have been localized to the vascular endothelium (Esposito *et al*, 1989). Several distinct AGE-binding proteins have since been described that include an endothelial cell-derived, 35 kDa species called 'RAGE', (Schmidt *et al*, 1992), a 32 kDa macrophage component shown to be Galectin-3 and a 50-60 kDa membrane-bound protein identified to be a subunit of the oligosaccharyltransferase complex, OST-48 (Vlassara *et al*, 1995).

RAGE is an integral membrane protein and a new member of the immunoglobulin superfamily of cell surface molecules (Neeper *et al*. 1992). A second AGE binding protein was purified from tissue extracts which was closely related to the iron binding protein lactoferrin (LF), lactoferrin-like AGE binding protein or LF-L (Schmidt *et al*. 1992). Latter Schmidt and coworkers (Schmidt *et al*. 1994) showed that the endothelial cell surface binding site for AGEs consists of LF-L bound noncovalently to RAGE anchored in the cell membrane. RAGE mediates the binding of AGEs to endothelial cells and mononuclear phagocytes, interacts with LF-L, and appears to activate intracellular

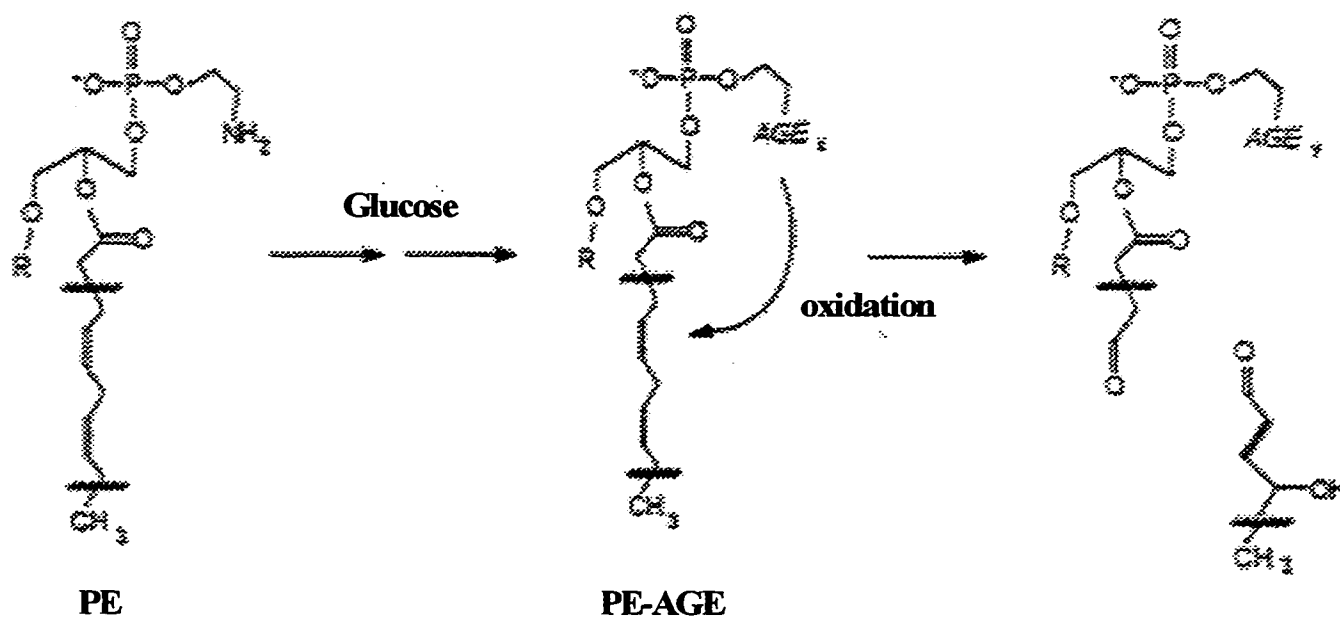
signal transduction mechanisms consequent to its interaction with the glycated ligand (Schmidt *et al.* 1994).

Under physiological conditions, amphoterin, a protein associated with basement membranes and abundant in the developing central nervous system (Rauvala *et al.*, 1987), was found to bind RAGE (Brett *et al.*, 1993) and is thought to be one of the physiological ligands. In pathological events such as, diabetes and renal insufficiency, RAGE has also been known to bind AGE-modified proteins. Interaction of RAGE with these ligands enhances receptor expression and initiates a positive feedback loop whereby receptor occupancy triggers increased RAGE expression (Schmidt *et al.* 1998). Sustained expression of RAGE by critical target cells sets the stage for chronic cellular activation and tissue damage. In a model of accelerated atherosclerosis associated with diabetes in genetically manipulated mice, blockade of cell surface RAGE by infusion of a soluble, truncated form of the receptor completely suppressed enhanced formation of vascular lesions (Wautier *et al.*, 1996). This suggests that interaction of cellular RAGE with AGEs could be a factor contributing to a range of chronic vascular dysfunction in diabetic vasculopathy and atherosclerosis. However, definite proof that RAGE-dependent mechanisms actually underlie the pathogenesis of human diseases must await more direct experiments in mice, in which expression of RAGE has been genetically manipulated, and in humans after the development of low molecular weight RAGE inhibitors.

## **Section IX : Lipid Advanced Glycosylation and Oxidative Modification**

Investigation into the advanced glycosylation of lipids and lipoproteins began several years ago after considering the possibility that phospholipids which contain

primary amino groups could react with glucose to form AGEs in much the same way that polypeptide amines form AGEs (Bucala *et al*, 1993). In model studies, buffered suspensions of phosphatidylethanolamine (PE) or phosphatidylcholine (PC) were incubated with glucose and the metal chelator EDTA at 37°C. PE but not PC (which contains a blocked, quaternary amine) was observed to react with glucose to form products with the same absorbance, fluorescence and immunoreactive properties as the AGEs that form on proteins (Bucala *et al*, 1993). It is hypothesized that intramolecular oxidation-reduction reactions, which are known to occur during advanced glycosylation, might act within the hydrophobic microenvironment of phospholipids to initiate fatty acid oxidation (Figure 1-10). AGE-modified phospholipids can modulate lipoprotein binding to RAGE (Ravandi *et al*, 1999)



**Figure 1-10: Scheme for the formation of AGEs on phospholipid head groups, followed by AGE-initiated oxidative damage on fatty acid side chains. PE: phosphatidylethanolamine, PE-AGE: AGE-modified phosphatidylethanolamine, R: unspecified fatty acid side chain (Bucala *et al*, 1993).**

The contribution of advanced glycosylation to the oxidative modification of LDL was first observed in an *in vitro* study. Purified human LDL was incubated with glucose (in presence of metal chelators) and analyzed for both advanced glycosylation and oxidative modification. Incubation of LDL with 200 mM glucose for 3 days resulted in the formation of readily measurable levels of AGEs on both lipid and apoprotein. These studies indicated that lipid-linked AGEs formed more rapidly than ApoB-AGEs, reaching a specific activity 100-fold greater than the ApoB-linked AGEs. Measurements of oxidative modification further showed that LDL was oxidized concomitantly with the formation of AGEs (Bucala *et al*, 1993).

## **Section X : Low-Density Lipoprotein Advanced Glycation**

Human LDL is glycated with about one glucose per two molecules of apoB in normal subjects and up to two glucose residues per molecule of protein in diabetic patients (Schleicher *et al*, 1981). This is a considerable amount of modification considering the fact that LDL have an average circulating half-life of only 36-48 hours. To better define the relationship between advanced glycosylation and LDL oxidation *in vivo*, Bucala and co-workers have analyzed LDL from both nondiabetic and diabetic individuals by an AGE-specific ELISA for the presence of phospholipid-AGEs and ApoB-AGEs (Bucala *et al*, 1993). LDL-advanced glycosylation was also examined in patients with diabetes and renal insufficiency. As discussed earlier, it had been determined that reactive AGEs circulate in high concentrations during renal insufficiency and that these AGEs can attach readily to plasma components such as LDL. There was a

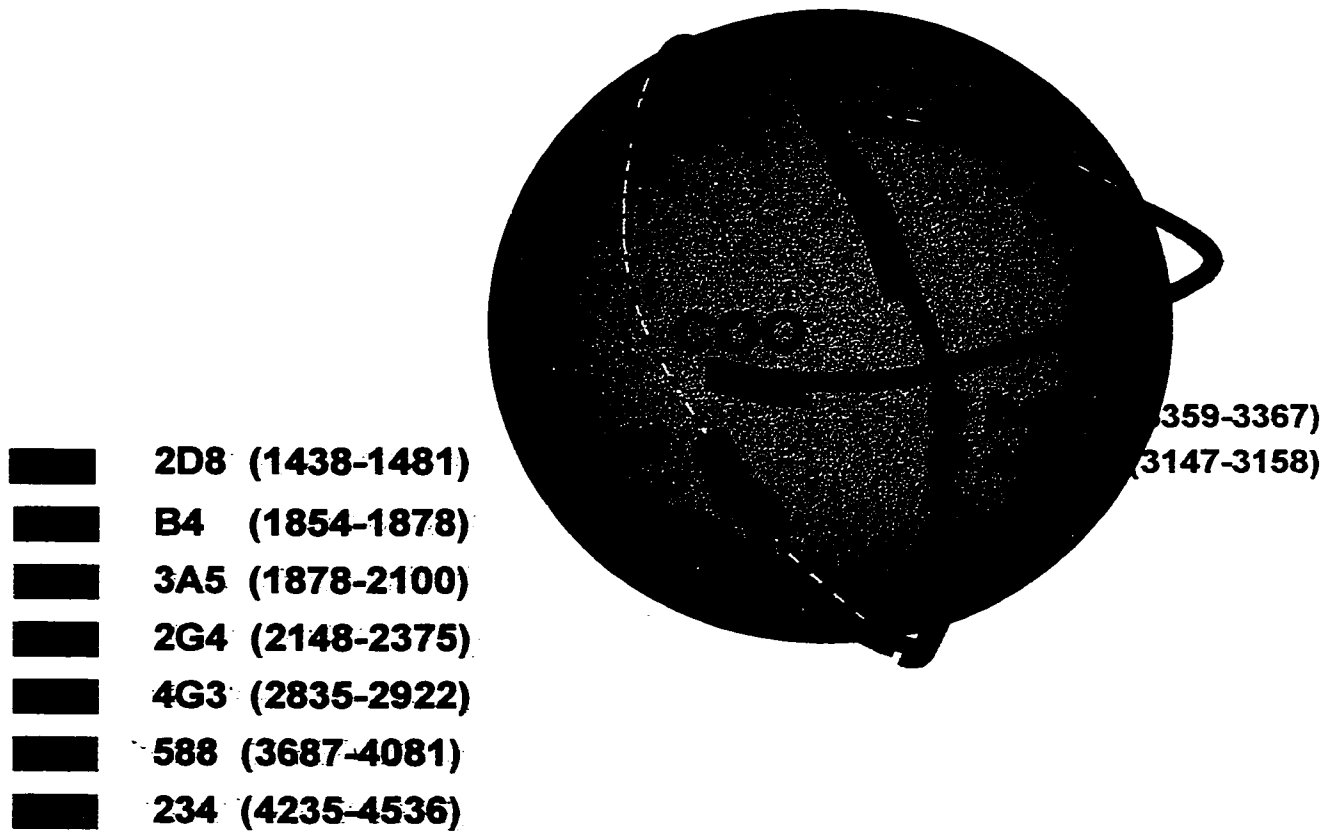
significant elevation in the levels of ApoB-AGE and lipid-AGE in LDL when each of the patient groups was compared to the control (nondiabetic / normal renal function) group. The highest levels of AGE-modification were observed in patients with renal insufficiency, pointing to the important role of circulating, reactive AGE-peptides in modifying proteins including the apoB moiety of LDL. AGE ELISA analysis of LDL specimens isolated from diabetic individuals revealed increased levels of both apoprotein- and lipid-linked AGEs in 12 out of the 16 diabetic patients when compared to that obtained from normal, nondiabetic controls (Bucala et al, 1993). LDL from diabetic or renal insufficient individuals also showed significantly greater oxidative modification, assessed by formation of thiobarbituric acid (TBA)-reactive substances, than the LDL from the control, nondiabetic individuals. Therefore, a significant correlation exists between the level of AGE modification and LDL oxidation.

## **Section XI: Advanced Glycation End-products Modify The Basic Residues (Lysine, and Arginine) of Apolipoprotein B**

Chemical modification of basic residues within the LDL-receptor binding domain of ApoB has been shown to interfere with the ability of LDL to undergo receptor-mediated uptake and degradation. Mahley and co-workers (Mahley *et al*, 1977) established that arginine is a functionally significant residue in or near the LDL receptor recognition sites on the B and E apoproteins, which determines specificity for binding to the cell surface receptors. Chemical modification of arginine residues abolishes the ability of these apolipoproteins to bind to the LDL receptor (Mahley *et al*, 1977). It was subsequently shown that chemical modification of lysine residues of apoB and apoE also

rendered these apolipoproteins incapable of mediating lipoprotein binding to the LDL receptor (Weisgraber *et al*, 1978). Chemical modification of only 15% of the lysine residues of LDL prevented it from competitively displacing modified LDL from the high affinity receptor sites or from binding directly to the receptor. As AGE modification is primarily targeted to lysine and arginine residues of proteins, it was reasoned that LDL modified by AGEs might exhibit delayed clearance kinetics *in vivo*. When the plasma clearance of AGE-LDL was examined in transgenic mice expressing the human LDL-receptor, markedly delayed clearance kinetics were observed when AGE-LDL was compared to control, native LDL (Bucala *et al*, 1994). *In vitro* AGE modification of LDL was also shown to decrease its binding affinity for the LDL receptor on cultured human fibroblasts (Bucala *et al*, 1995).

A major site of AGE modification within the apoB primary structure has recently been identified (Bucala *et al*, 1995). This AGE-immunoreactive site was mapped to a single 67 amino acid region and lies 1791 residues N-terminal to the putative LDL-receptor binding domain (Figure 1-11). It was proposed that modification at this site induces a conformational change in the apo B LDL receptor binding site. However, in another study using a panel of anti-apoB monoclonal antibodies, it was demonstrated that *in vitro* glycation of LDL resulted in altered expression of multiple apoB epitopes, including several that flank the putative apoB LDL receptor-binding site (Figure 1-11) (Wang *et al*, 1998).



**Figure 1-11. Structure of a LDL particle and potential sites of AGE modification on apoB-100.**

## Section XII : Rationale

Patients with diabetes or renal insufficiency experience a high incidence of atherosclerotic vascular disease, which has been attributed in part to abnormal lipoprotein metabolism. Diabetic or renally impaired patients also exhibit elevated concentrations of AGE-modified LDL (AGE-LDL) (Bucala *et al*, 1993). The AGE modification of LDL interferes significantly with its normal, receptor-mediated uptake, as shown by fractional clearance studies performed in transgenic mice expressing the human LDL receptor (Bucala *et al*, 1994). Thus, excessive formation of AGEs on LDL has been proposed to be an important mechanism for the dyslipidemia and accelerated atherogenesis that often is observed in patients with diabetes or renal insufficiency. The precise molecular basis for this uptake abnormality is still elusive.

Mahley and co-workers (Mahley *et al*, 1977; 1978) established that the chemical modification of lysine or arginine residues in apoB interferes with the ability of LDL to undergo receptor-mediated uptake and degradation. AGE modification of lysine and arginine residues in apoB, may be responsible for the markedly delayed clearance kinetics, of AGE-LDL *in vivo*. Although potential sites of apoB modification by *in vitro* glycation have been reported (Bucala *et al*, 1995; Wang *et al*, 1998), it is unclear whether the modification of lysine and/or arginine residues of apoB are responsible for the defective binding of glycated LDL to the LDL receptor. To address this question and to characterize AGE-modified LDL, we utilized a chemical modification strategy to reversibly protect arginine or lysine residues of apoB from exposure to reducing sugars. This technique allowed us to protect the amino groups of these residues from glycation

and to determine their possible role in defective binding of AGE-LDL to the LDL receptor.

To achieve this, prior to glycation of LDL, arginyl residues were modified with cyclohexanedione. Lysine residues were similarly protected using another selective reagent, diketene. Under normal circumstances these modifications are readily reversible by hydroxylamine; however, *in vitro* glycosylation requires a long incubation time which interferes with the subsequent deprotection of the residues. To reduce the glycation period we have substituted glucose with a more potent glycating agent, fructose 6-phosphate (F6P). We demonstrate that LDL modified by F6P and LDL modified with glucose have similar properties.

The observation that measurable quantities of AGEs occur on short-lived plasma proteins such as LDL prompted us to consider the hypothesis that these modifications may arise from reactive, circulating AGE-peptides in addition to direct modification by reducing sugar. This hypothesis was further supported by the finding that AGE-LDL levels are increased in patients with renal insufficiency independently of diabetes (Makita *et al*, 1991). An *in vitro* model was designed to compare LDL that had been modified by exposure to AGE-bound peptides with LDL that had been modified by reducing sugar. AGE-modified peptides (AGE-peptide) were prepared by proteolysis of F6P-mediated AGE-modified albumin (AGE-BSA). AGE-LDL was prepared by incubating native LDL with preformed AGE-peptide. Finally, we have compared LDL that had been modified *in vitro* with LDL that had been isolated from patients with diabetes or renal insufficiency.

We have used anti-apoB monoclonal antibodies (mAbs) to study the immunochemistry and function of AGE-LDL. Each anti-apoB mAb is specific for an

epitope that could represent either a discrete segment of apoB primary structure or a conformational structure composed of non-contiguous residues. Anti-apoB mAbs have previously been used to provide the first experimental evidence that a LDL particle contains a single molecule of apoB (Milne and Marcel, 1982), to identify the apoB LDL receptor-binding domain (Milne *et al*, 1989), and to determine the topography of apoB on native LDL (Chatterton *et al*, 1995).

## Chapter II

### Material and Methods

#### *Isolation of LDL*

EDTA plasma from healthy donors was collected and supplemented immediately with 1mM ethylene-diaminetetra-acetic acid (EDTA), 20  $\mu$ M butylated hydroxytoluene (BHT), 0.5 mM phenyl methanesulfonyl fluoride, and 0.02% sodium azide. LDL (density 1.019-1.063 g / ml) were isolated by sequential ultracentrifugation at 40,000 rpm for 40 hours (Havel *et al*, 1955). For the patient study, the frozen plasma was thawed and immediately supplemented with 1mM EDTA, 20  $\mu$ M butylated hydroxytoluene (BHT), 0.5 mM phenyl methanesulfonyl fluoride, and 0.02% sodium azide. LDL was isolated from plasma of one type I diabetic subject, two type II diabetic subjects, three normal subjects, and three renal insufficient subjects as described above.

#### *Measurement of Protein Concentration*

Protein concentration was determined by a modified Lowry method (Markwell *et al*, 1978). Bovine serum albumin (BSA) was used as the protein standard.

#### *Preparation of AGE-BSA, AGE-Peptide, AGE-LDL, and Preformed-AGE-LDL*

AGE-BSA was prepared by incubating 50 mg / ml BSA with 0.5 M F6P in PBS (145 mM NaCl / 10 mM sodium phosphate, pH 7.4) at 37°C for 5 days. Synthetic AGE-peptides were prepared by proteolysis of AGE-modified albumin (AGE-BSA) (Makita *et*

*al*, 1992). 5 ml of AGE-BSA [30 mg / ml, in PBS] was incubated with proteinase K (5 mg) overnight at 37 °C. Resulted peptides then were concentrated by centrifugation of the digested material through a Centriprep-10 membrane (10-kDa molecular mass cutoff) (Amicon). AGE-LDL were prepared by incubating LDL (3 mg / ml) with 0.145 mM F6P at 37 °C for up to 4 days in PBS containing 1 mM EDTA (Bucala *et al*, 1995). AGE-peptide-modified LDL were prepared by incubating LDL (3 mg / ml) with AGE-peptide (45 mg / ml) at 37 °C for up to 12 days in PBS (145 mM NaCl / 20 mM sodium phosphate, pH 7.4) containing 1 mM EDTA. Control LDL were incubated under the same conditions without F6P. After incubation, the LDL were dialyzed against PBS containing 1 mM EDTA and 0.02% NaN<sub>3</sub>.

#### *Preparation of Lysine-Protected-AGE-LDL*

LDL (3 mg / ml) were dialyzed against 0.1 M borate buffer, pH 8.5. The diketene reagent was made by adding a 25 µl aliquot of diketene to 1.0 ml of 0.1 M borate buffer, pH 8.5, in a glass tube and mixture was vortexed vigorously (Weisgraber *et al*, 1978). For an extensive modification, 180 µl of the diketene solution was added per mg of LDL protein (168 µmol / mg). The mixture was allowed to stand for 5 min at room temperature. The lysine-protected-LDL were then dialyzed for 16-24 hr against saline-EDTA (0.15 M NaCl, 0.01% EDTA, pH 7.4). In order to glycate the lysine-protected LDL, the modified LDL were incubated with 0.145 mM F6P at 37 °C for up to 4 days in PBS containing 1 mM EDTA. lysine-protected-AGE-LDL were then dialyzed against saline-EDTA overnight. The diketene adduct was removed by mixing the modified LDL with a solution (half the volume of the modified LDL) containing 4M hydroxylamine, 1.2

M mannitol, pH 7, and then incubating the mixture at 37 °C for 7-16 hrs. Finally, the regenerated lysine-protected-AGE-LDL were dialyzed for 24-48 hr against saline-EDTA before use in tissue culture experiments. Control LDL were incubated under the same conditions with or without F6P or diketene.

#### *Preparation of Arginine-Protected-AGE-LDL*

LDL (3 mg / ml) in saline-EDTA were mixed with an aliquot (twice the LDL volume) of 1.0 M 1,2-cyclohexanedione in 0.1 M sodium borate buffer pH 8.1 and incubated at 37 °C for 2 hrs (Mahley *et al*, 1977). The arginine-protected LDL were then dialyzed for 20-40 hr against saline-EDTA. In order to glycate the arginine-protected LDL, the modified LDL were incubated with 0.145 mM F6P at 37 °C for up to 4 days in PBS containing 1 mM EDTA. Arginine-protected-AGE-LDL was then dialyzed against saline-EDTA overnight. The 1,2-cyclohexanedione adduct was removed using hydroxylamine as described above. Control LDL were incubated under the same conditions with or without F6P or 1,2-cyclohexanedione.

#### *Iodination of Immunoglobulin and LDL*

The goat anti-mouse IgG was labeled by the chloramine-T method (Milne *et al*, 1992). The specific activity of the labeled IgG was between 10,000-30,000 cpm / ng of IgG. For the LDL receptor binding assay, LDL were iodinated by the method of Bilheimer (Bilheimer *et al*, 1972). One mCi of sodium <sup>125</sup>Iodine was used to iodinate 1 mg of LDL in the presence of 40 nmoles of iodine monochloride and 0.5 M glycine. Free iodine was removed by gel filtration on Sephadex G-25 (Pharmacia) followed by an

extensive dialysis against PBS, pH 7.4. The specific activity ranged from 200-800 cpm / ng of apoB.

#### *Binding of LDL to the LDL Receptor on Cultured Human Fibroblasts*

For LDL receptor binding experiments, human fibroblasts were plated in 35-mm dishes with 2 ml of Hanks' Balanced Salt Solution (HBSS) (GibcoBrl) supplemented with 10% (v/v) fetal bovine serum (FBS). When cells reached about 70% confluence, they were rinsed with 1 ml of sterile HBSS supplemented with 5% (v/v) lipoprotein deficient serum (LPDS), and then were maintained in HBSS containing 10% LPDS for 24-48 hours. Prior to the experiments, cells were placed at 4°C for 30 min, the medium was then removed and replaced by 3 µg of <sup>125</sup>I-LDL, alone or in the presence of unlabeled competitor in HBSS containing 10% LPDS. Modified or control LDL were tested for their ability to compete with <sup>125</sup>I-native LDL for binding to the LDL receptor on the surface of the cultured cells (Arnold *et al*, 1992). After a 3.5 hr incubation at 4°C, surface bound <sup>125</sup>I-LDL was determined. Assays were performed in triplicate. Results are expressed as a ratio between radioactivity bound in the presence of competitor at different concentrations to that bound in the absence of competitor.

#### *Competitive ApoB Radioimmunoassay (RIA)*

The competitive apoB radioimmunoassay has been described previously (Marcel *et al*, 1984). Immulon II Removawells (Dynatech Laboratories, Chantilly, VA) were coated by an overnight incubation with 200 µl of reference LDL (30 µg / ml in phosphate-buffered saline (PBS), pH 7.2) at room temperature. Plates were washed with

a solution of 0.15 M NaCl containing 0.025% Tween 20 (NaCl-Tween) and then subsequently saturated by incubation for 1 hr with 250  $\mu$ l of 1% bovine serum albumin in PBS (PBS-BSA). Serial dilutions (125  $\mu$ l in PBS-BSA) of test and control LDL were prepared in separate microtitre plates to which was added 125  $\mu$ l of anti-apoB mAb appropriately diluted in PBS-BSA. After a 2 hr incubation, 200  $\mu$ l of the LDL-mAb mixture was transferred to the LDL-coated Removawells. The plates were incubated overnight and again washed with the NaCl-Tween solution. 200  $\mu$ l of  $^{125}$ I-goat anti-mouse IgG was diluted to 70 ng / ml in 1% PBS-BSA and added to each well and incubated overnight. The wells were then washed with the NaCl-Tween solution as above and counted for bound radioactivity.

#### *Measurement of AGE-LDL by Competitive ELISA*

Immulon II Removawells were coated by a 2 hr incubation with 100  $\mu$ l of a solution of AGE-BSA (10  $\mu$ g / ml, dissolved in PBS) at room temperature. Wells were washed three times with 150  $\mu$ l of a solution containing PBS, 0.05% Tween 20, and 1mM  $\text{NaN}_3$  (PBS-Tween). Wells were blocked by incubation for 1 hr with 100  $\mu$ l of PBS containing 0.1% BSA, and 1 mM  $\text{NaN}_3$ . After washing with PBS-Tween, several dilutions of LDL were added, followed by 50  $\mu$ l of anti-AGE mAb 4G9 (final dilution, 1:800). Plates were incubated for 3 hr at room temperature. Wells were then washed with PBS-Tween and 100  $\mu$ l of diluted anti-mouse horse radish peroxidase conjugate was added and incubated for 24 hr. After washing, 100  $\mu$ l of 3,3',5'-Tetramethylbenzidine (TMB) (sigma) colourimetric substrate was added to each well. After incubation for 20 min, absorbance at 415 nm was read using a microplate reader (BioRad). Results are

expressed as  $B / B_0$ , calculated as [ experimental OD – background OD (no antibody) ] / [ maximum OD (no competitor) – background OD ].

#### *Measurement of Relative LDL Surface Charge, and Capillary Transfer Western Blotting*

The relative LDL surface charge was assessed by agarose gel electrophoresis (Beckman, Palo Alto, CA). LDL samples were loaded onto a 0.5% agarose gel and placed in a 100 V electric field for 30 min. After electrophoresis, the lipoproteins were fixed and the gel was dried. The lipoprotein pattern was visualized by staining the film with a lipid-specific stain according to the manufacturer's recommendations. LDL samples that were subjected to electrophoresis were transferred onto a nitrocellulose membrane using a capillary transfer method. After saturation of the membranes with 5% milk powder in PBS, the transferred proteins were sequentially incubated with anti-AGE mAbs and horse radish peroxidase-conjugated anti-mouse IgG. Bound conjugate was visualized using an ECL kit (Amersham, UK).

#### *Determination of Lipid Composition*

Free cholesterol, total cholesterol, and phospholipid contents were determined enzymatically with Boehringer Mannheim (Indianapolis, IN) kits.

## Chapter III

### Results

*LDL modified by F6P and LDL modified by glucose are similar in terms of apoB epitope expression and LDL receptor-binding activity*-In previous experiments in this laboratory (Wang *et al*, 1998), we prepared AGE-modified LDL *in vitro* by incubating freshly isolated native LDL with 200 mM glucose at 37° C for 16 days, as a model for studying AGE-induced alterations in the structural and functional properties of LDL. This was also the protocol used by others (Bucala *et al*, 1995). In order to reduce the incubation time required for *in vitro* AGE modification of LDL, we have investigated glycation with fructose 6-phosphate (F6P), a more reactive reducing sugar than glucose. The incubation of LDL (3.0 mg / ml) with F6P (0.145 mM) for 4 days produced a level of LDL modification, as monitored by electrophoretic mobility, that is comparable to that of LDL that had been incubated with 200 mM glucose for 16 days (not shown). LDL incubated in the presence of F6P showed an increase in electrophoretic mobility as a function of time of incubation and concentration of the reducing sugar (Figure 2-1). The defective mobility observed with LDL incubated with 0.050 mM F6P is probably due to aggregation of LDL.

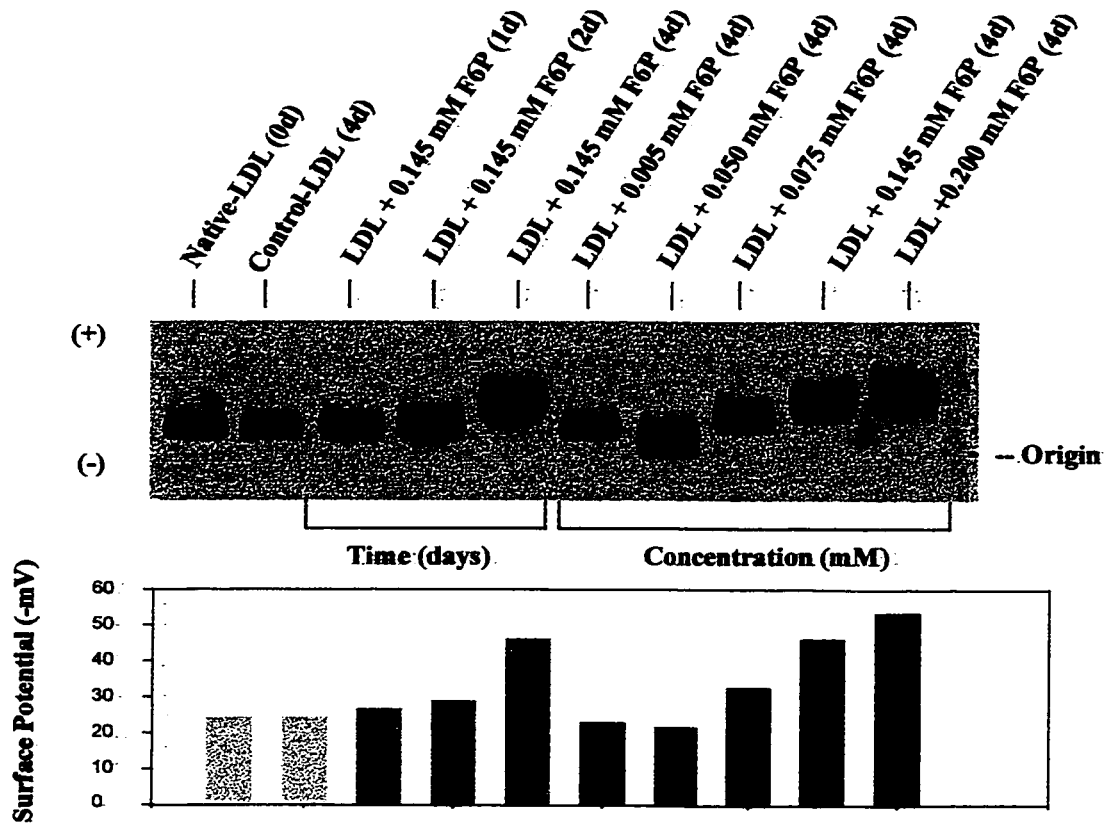
It has been shown that *in vitro* glycation of LDL, using glucose, results in modification at distinct sites in apoB-100 (Wang, 1998). To define the immunochemical changes of apoB that accompany LDL glycation, using F6P, we have tested LDL, that had been incubated with F6P, using a solid phase RIA with a panel of 18 anti-apoB

**Figure 2-1: Electrophoretic mobility of LDL as a function of time of glycation and concentration of Fructose 6-phosphate.**

LDL that had been incubated without (LDL) or with 0.145 mM fructose 6-phosphate (F6P) (LDL + F6P) for 0, 1, 2, or 4 days, and those incubated with 0.005, 0.050, 0.075, 0.145, and 0.200 mM F6P for 4 days were subjected to agarose (0.5%) gel electrophoresis. Samples were applied to a thin film agarose gel and were allowed to migrate for 30 min at 100V. The gel was stained for lipid according to the manufacturer's recommendations. Similar results were seen in other agarose gels. Electrokinetic theory has been applied to estimate colloidal particle net charge from the electrophoretic mobility (Abramson *et al.* 1942).

mAbs. The position of the epitopes within apoB primary structure recognized by the mAbs is shown in figure 2-2. Complete competition curves for 4 of the mAbs from one experiment are presented in figure 2-3 and the ED<sub>50</sub> values for all antibodies with LDL from two subjects are shown in figure 2-4, together with an apoB epitope map. Glycated LDL are as reactive as LDL that had been incubated in the absence of F6P with certain mAbs (e.g. 1D1), and are much less reactive than non-glycated LDL with other mAbs (e.g. 2D8, 4G3, B4). The apoB epitopes that are modified by F6P (mapped by mAbs 2D8, B4; 3A5, 4G3, 234) are the same as those previously reported to be modified in LDL that had been exposed to glucose (Wang, 1998).

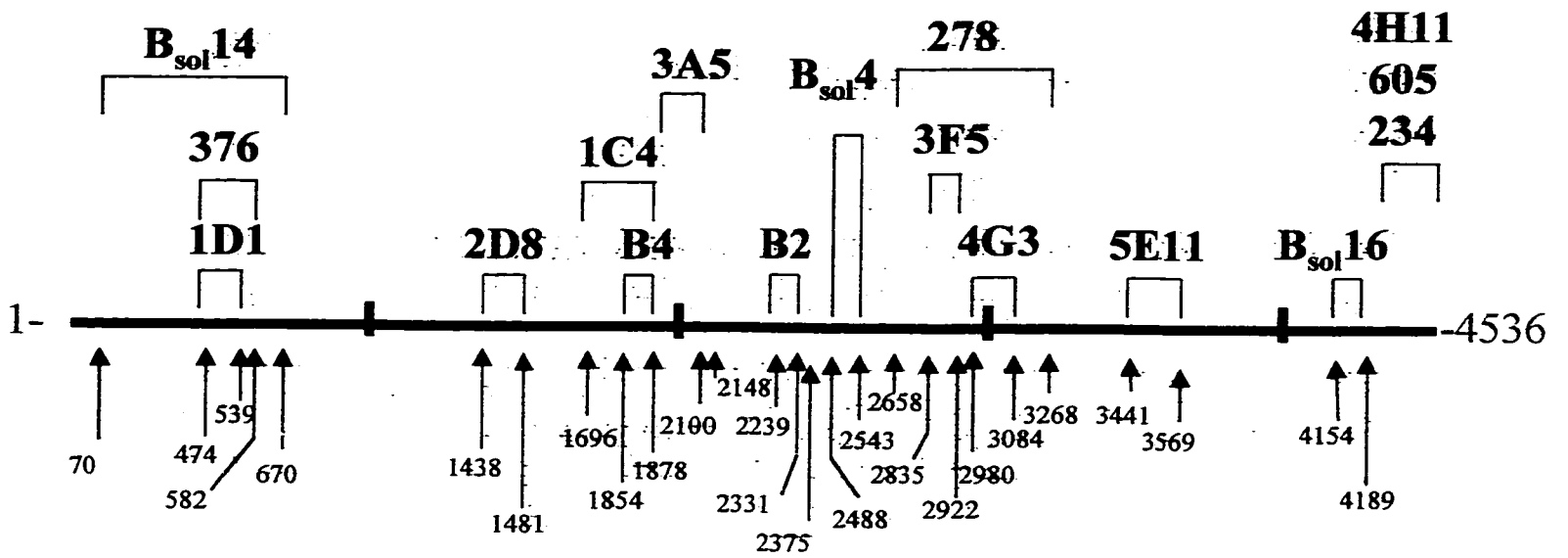
**Figure 2-1**



**Figure 2-2: Epitope map of human LDL.**

The position of the epitopes within apoB primary structure recognized by the panel of mAbs is shown (modified from Wang *et al*, 2000)

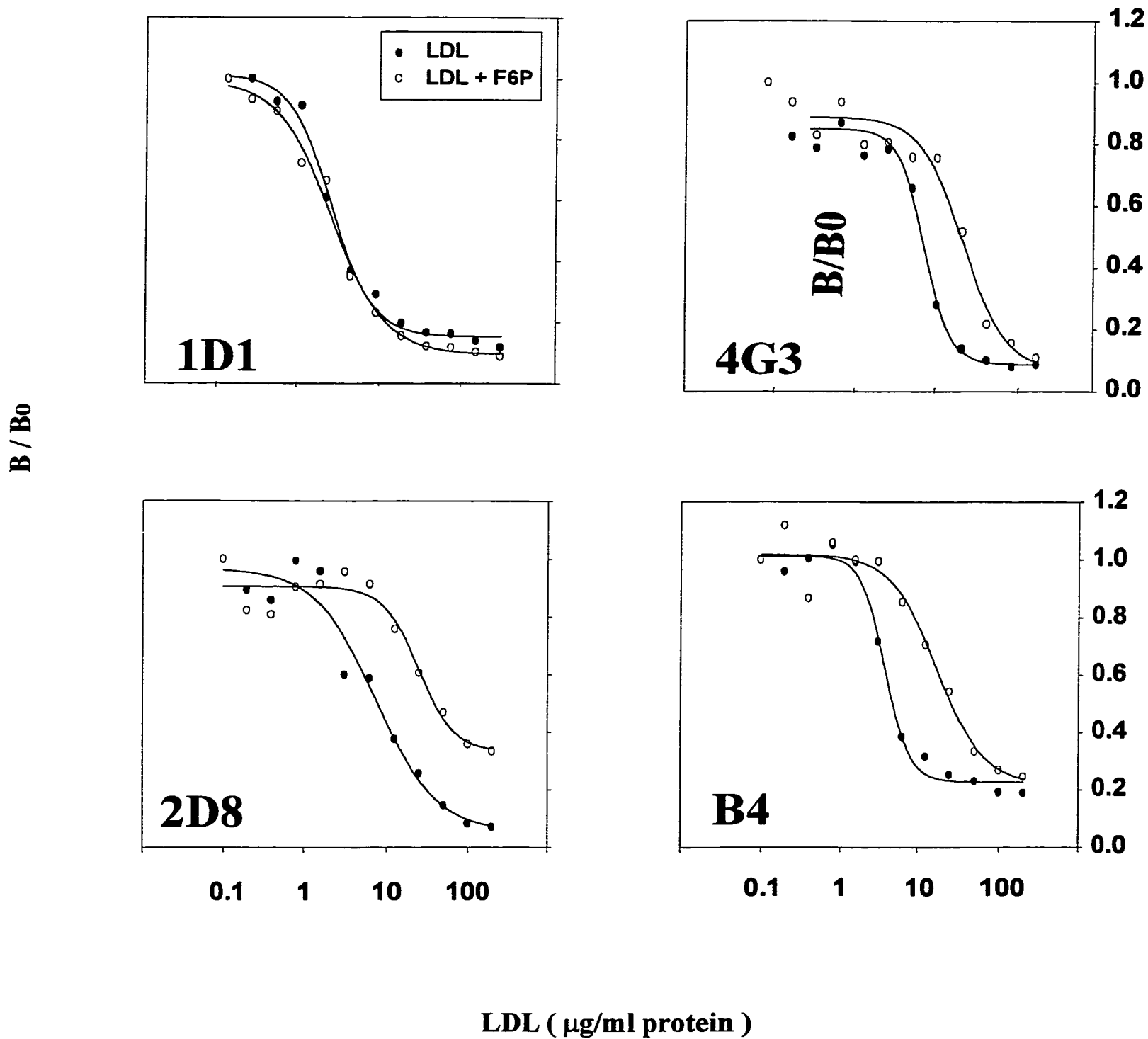
Figure 2-2



**Figure 2-3: The immunoreactivity of glycated LDL.**

The immunoreactivity of LDL that had been incubated for 4 days at 37° C without F6P (LDL), or with 0.145 mM F6P (LDL + F6P) was determined by a solid phase RIA using a panel of anti-apoB mAbs. Competition curves for mAbs 1D1, 2D8, 4G3, and B4 are presented. Results are presented as B/Bo, where B is radioactivity bound in the presence of competitor as a fraction of radioactivity bound in the absence of competitor.

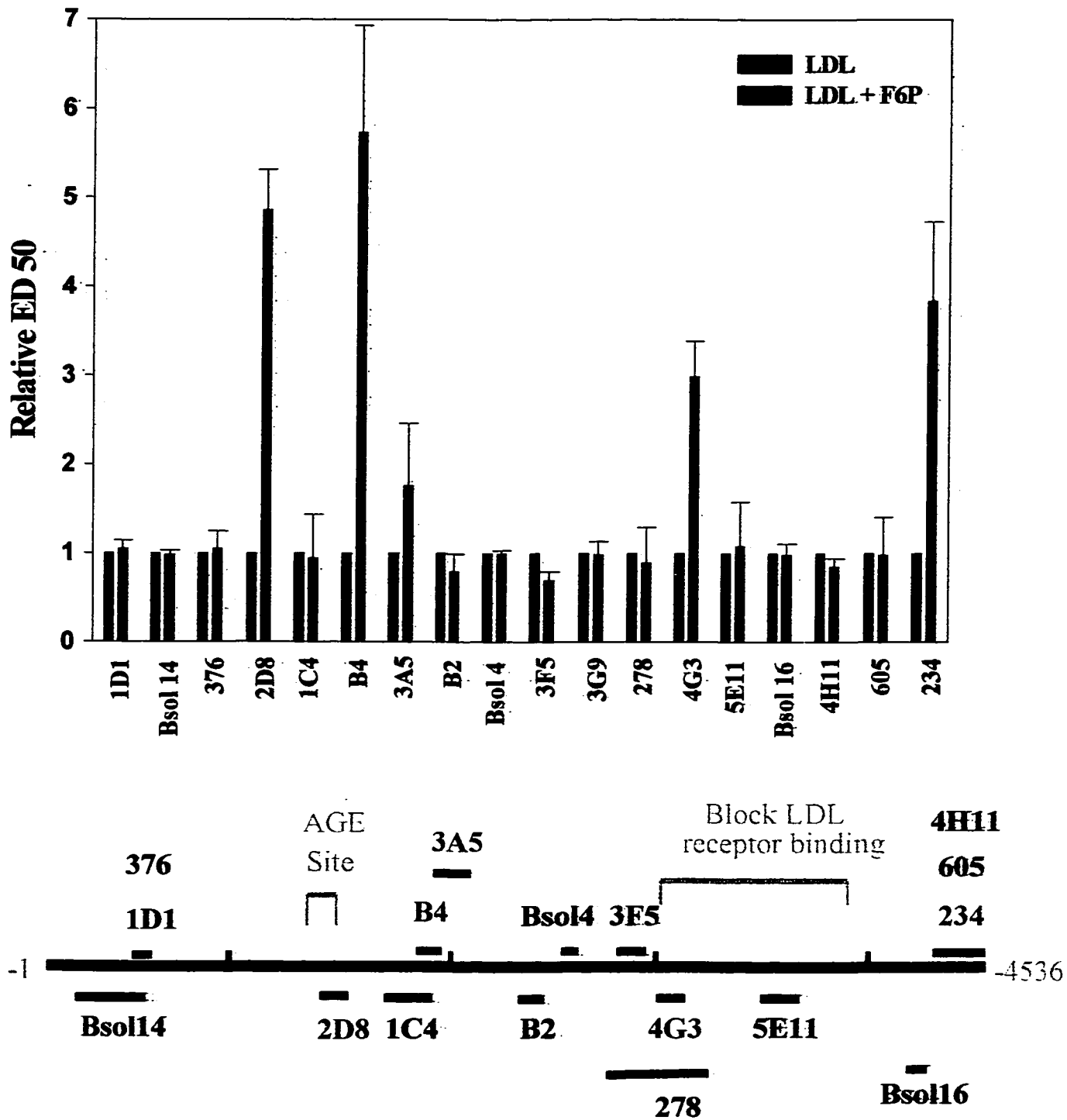
Figure 2-3



**Figure 2-4: The immunoreactivity of glycated LDL.**

The relative concentrations of LDL (LDL), or F6P-modified LDL (LDL + F6P) that were required to obtain 50% of maximum binding ( $ED_{50}$ ) in the RIA. LDL from 2 subjects were modified with F6P as described. The  $ED_{50}$  for non-modified LDL was normalized to a value of 1 for each mAb. The position of the epitopes within apoB primary structure recognized by the panel of mAbs is shown. The epitopes shown in red are those whose immunoreactivity is reduced in glycated LDL compared to control LDL.

Figure 2-4

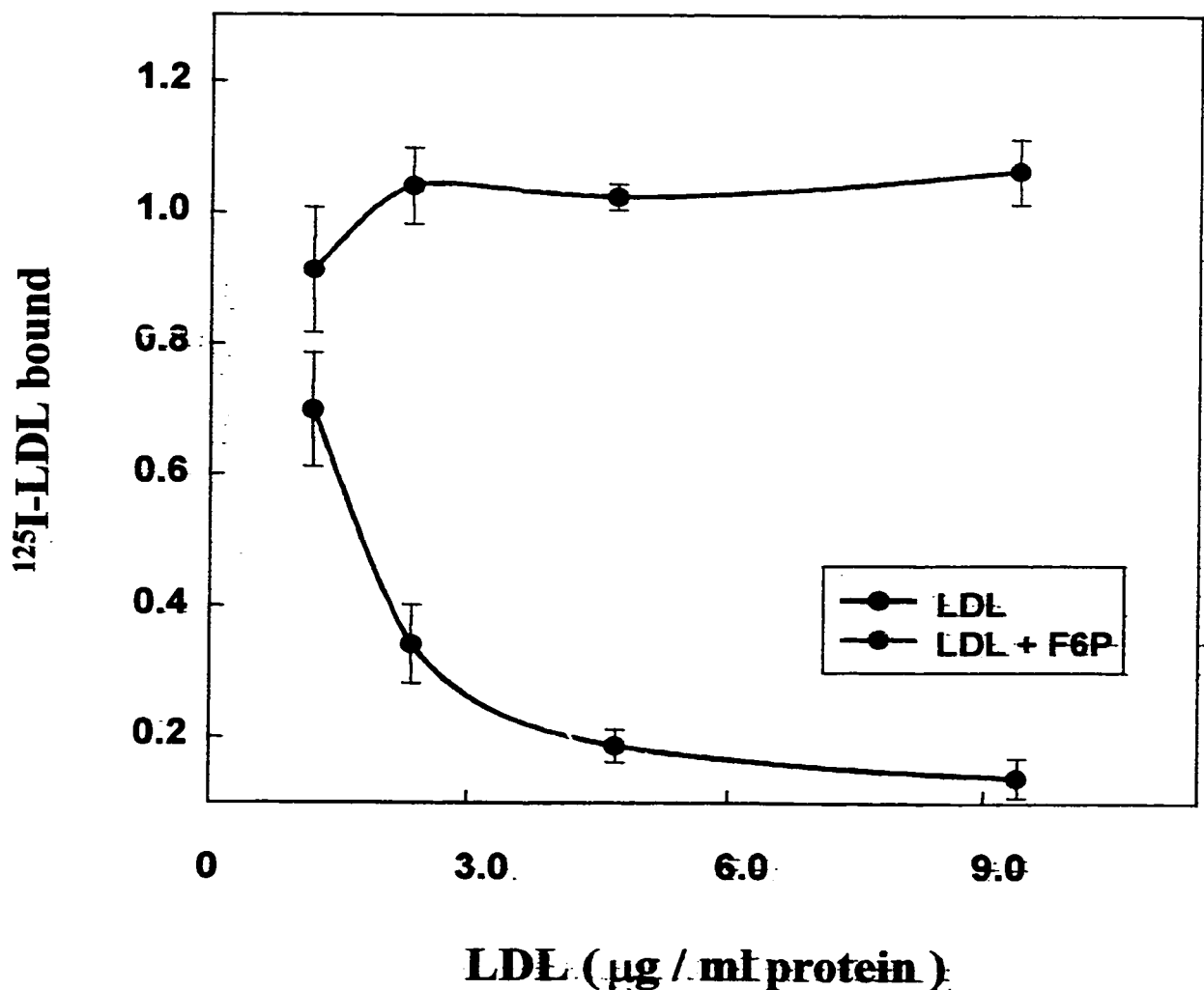


**Figure 2-5: The ability of glycated LDL to bind to the LDL receptor.**

LDL that had been incubated for 4 days at 37° C in the absence (LDL) or presence of 0.145 mM F6P (LDL + F6P) were tested for their ability to compete with <sup>125</sup>I-LDL for binding to the LDL receptor on cultured human fibroblasts. LDL from 2 subjects were modified as described and each experiment was done in triplicate. Results are presented as the ratio of radioactivity bound in the presence of competitor to that bound in the absence of competitor (B/Bo) as a function of the concentration of the competitor.

To assess the LDL receptor-binding activity of F6P-mediated AGE-modified LDL, we tested the abilities of LDL that had been incubated in the presence or absence of F6P to compete for binding to the LDL receptor on cultured human fibroblasts. Even at the highest concentration tested (9.375  $\mu\text{g}$  of apoB / ml), LDL that had been incubated with F6P could not compete with  $^{125}\text{I}$ -LDL for binding to the LDL receptor of cultured human fibroblasts (Figure 2-5).

**Figure 2-5**

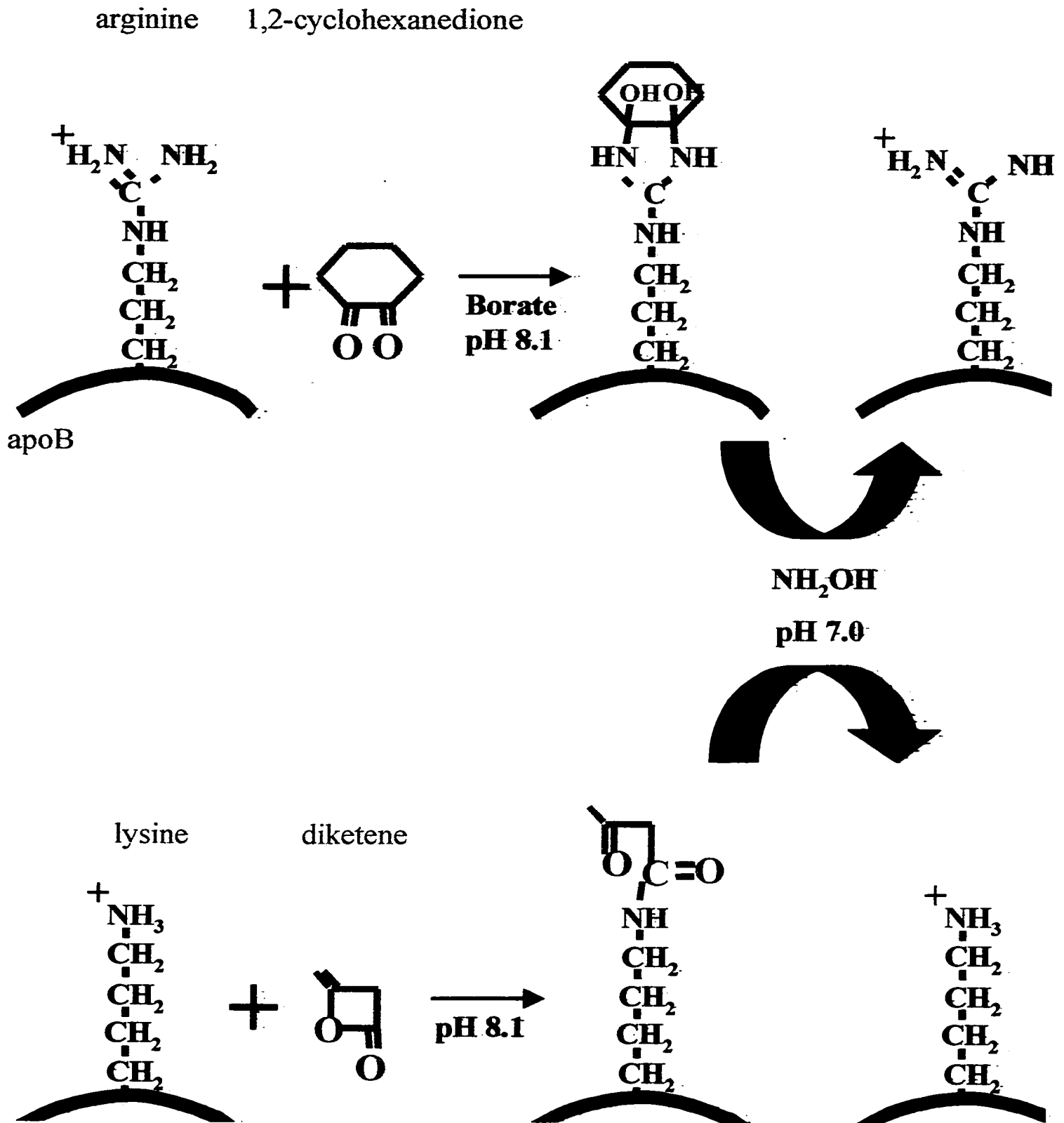


*Modification of arginine residues of apoB may not be solely responsible for the defective binding of glycosylated LDL to the LDL receptor*-In order to determine the possible role of arginine residues of apoB in defective binding of AGE-LDL to the LDL receptor, arginine residues were reversibly protected from modification by reducing sugar. One reagent which reacts specifically with arginine or the arginyl residues of proteins under very mild conditions is 1,2-cyclohexanedione (CHD). It has been demonstrated that CHD reacts only with the guanido groups of arginine and not with other amino acid residues (Patthy *et al*, 1975). The product is stable but CHD may be released quantitatively by incubation of the modified protein with hydroxylamine (HDA) (Figure 2-6).

Using the conditions described by Patthy and Smith (Patthy *et al*, 1975), we found that the treatment of human LDL with 0.1 M CHD, abolished their ability to compete with <sup>125</sup>I-LDL for binding to human fibroblasts (not shown). With this concentration of CHD, however, modification of arginyl residues of apoB was incomplete. It was reported that treatment of human LDL with 0.1 M CHD modifies approximately half of the arginyl residues in apoB (Mahley *et al*, 1977). To establish the conditions that maximize the modification of arginyl residues, different concentrations of CHD were tested for their abilities to modify apoB as monitored by a change in the electrophoretic mobility of the CHD-modified LDL (CHD-LDL) particles. Increasing the concentration of CHD from 0.3 to 1.0 M CHD resulted in an increased migration rate of the CHD-LDL towards the anode (Figure 2-7). Since concentrations greater than 1.0 M CHD were not any more effective (not shown), subsequent experiments were performed with 1.0 M CHD. It was also confirmed that CHD-modified LDL could be regenerated with hydroxylamine (Figure 2-7).

**Figure 2-6: Reversible modification of arginine and lysine residues of apoB.**  
Scheme for the formation of 1,2-cyclohexanedione-modified arginine and diketene-modified lysine residues in apoB, followed by their regeneration by hydroxylamine.

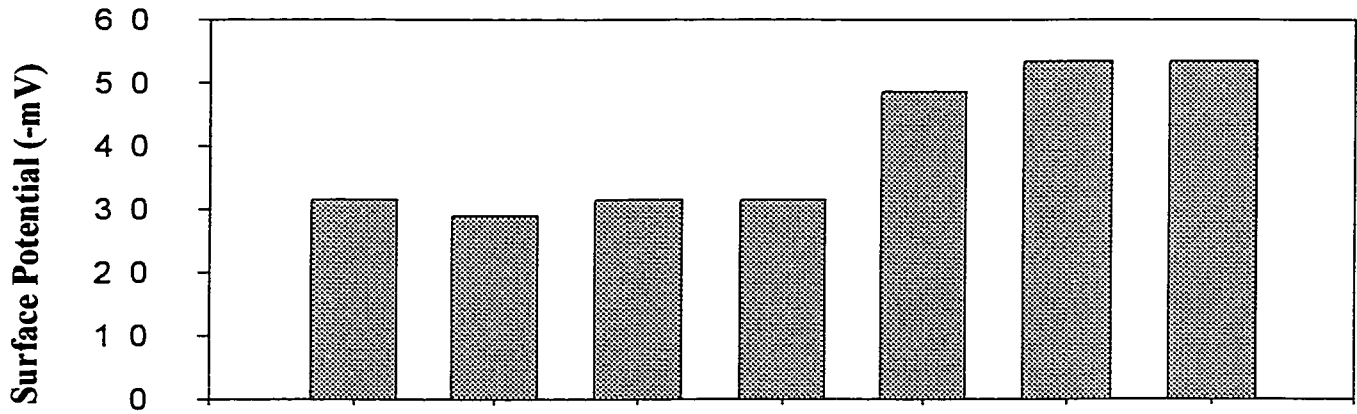
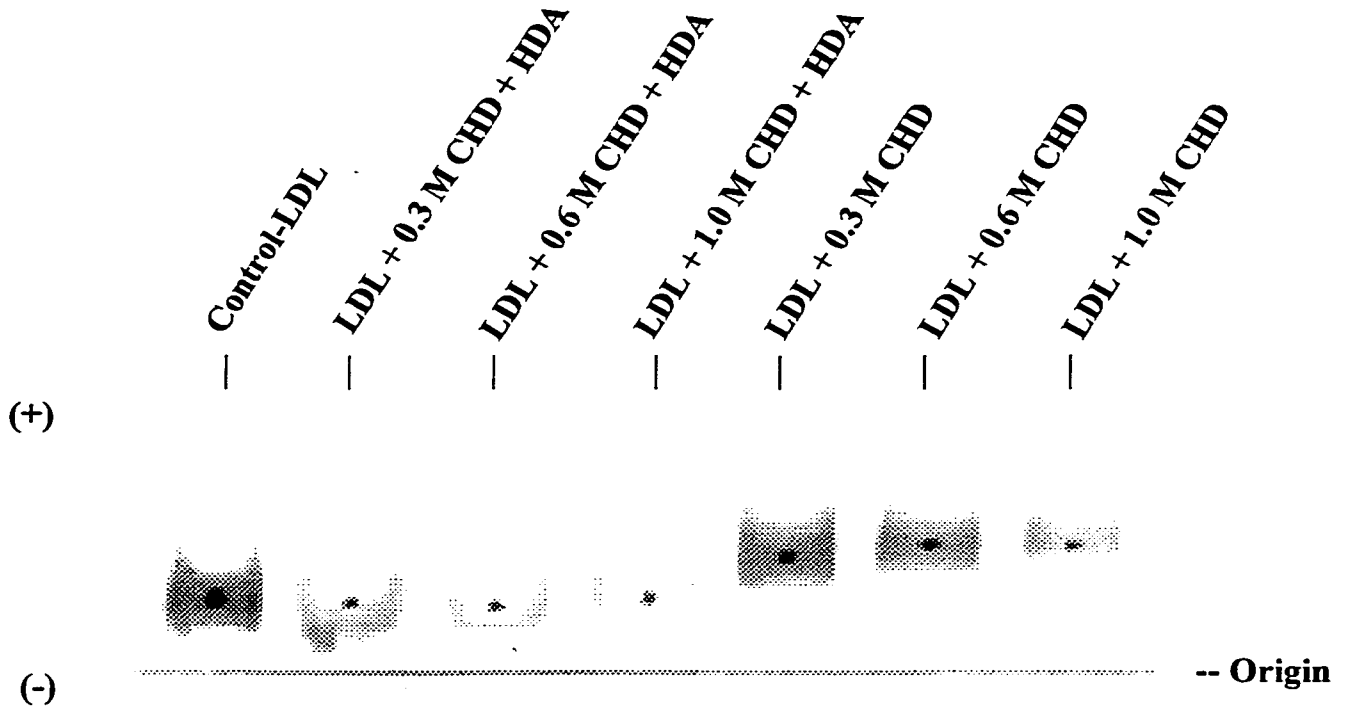
Figure 2-6



**Figure 2-7: Electrophoretic mobility of LDL as a function of 1,2-Cyclohexanedione modification, and regeneration.**

LDL that had been incubated with 0.3, 0.6, and 1.0 M 1,2-cyclohexanedione (LDL + CHD) with (LDL + CHD + HDA) or without regeneration by 4M hydroxylamine (LDL + CHD) or those incubated in absence of CHD and HDA (Control-LDL) were subjected to agarose (0.5 %) gel electrophoresis. The gel was stained for lipid according to the manufacturer's recommendations. Similar results were seen in other agarose gels.

**Figure 2-7**

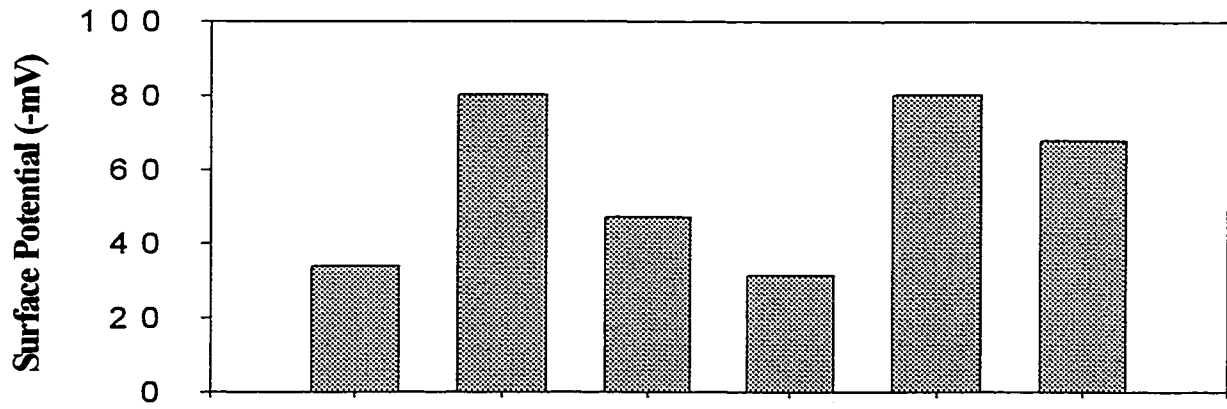
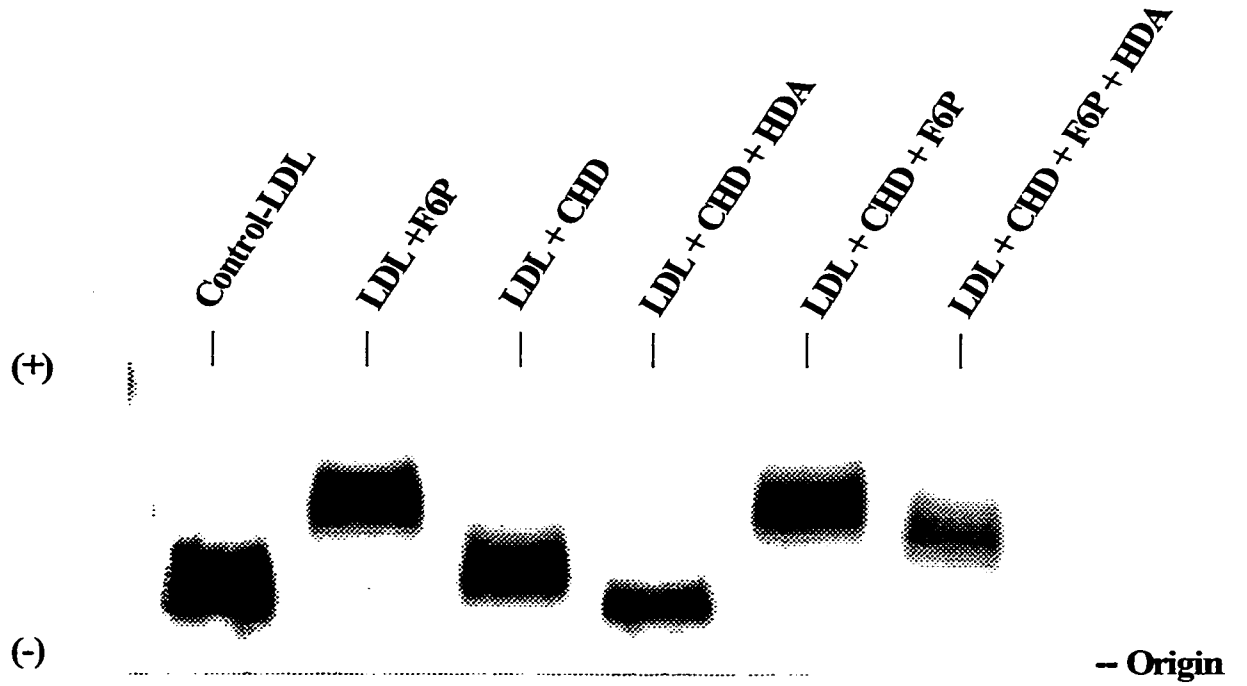


To determine if CHD could protect arginine residues from AGE modification, LDL were first treated with 1.0 M CHD followed by a 4 day incubation with 0.145 mM F6P. As seen in figure 2-8 LDL treated with CHD before AGE modification (LDL + CHD + F6P) showed a similar mobility to AGE-modified LDL (LDL + F6P). Treatment of the CHD-protected, AGE-modified LDL with hydroxylamine (LDL + CHD + F6P + HDA) resulted in a decrease in the negative charge due to the regeneration of the protected arginine residues. Even after HDA treatment, the LDL exhibited a greater electrophoretic mobility than control LDL, which indicates that CHD pretreatment does not interfere with AGE modification of lysine residues. Reaction of 1.0 M CHD with LDL completely eliminated the ability of the CHD-LDL to compete with <sup>125</sup>I-LDL for binding to human fibroblasts. This defective binding was almost completely regenerated by treatment with HDA (Figure 2-9). However, subsequent regeneration of CHD-modified AGE-LDL did not restore its binding ability (Figure 2-9). This demonstrates that protecting the arginine residues of apoB from glycation is not sufficient to protect its binding to the LDL receptor on fibroblasts.

**Figure 2-8: Electrophoretic mobility of LDL as a function of glycation, and/or 1,2-cyclohexanedione modification, and regeneration.**

LDL that had been incubated in the absence of F6P and CHD (control-LDL), or with 1.0 M 1,2-cyclohexanedione (LDL + CHD), or 0.145 mM F6P (LDL + F6P), or with 1.0 M 1,2-cyclohexanedione and 4M hydroxylamine (LDL + CHD + HDA), or 1.0 M 1,2-cyclohexanedione prior to 0.145 mM F6P and then with 4M hydroxylamine (LDL + CHD + F6P + HDA) were subjected to agarose (0.5 %) gel electrophoresis. The gel was stained for lipid according to the manufacturer's recommendations. Similar results were seen in other agarose gels.

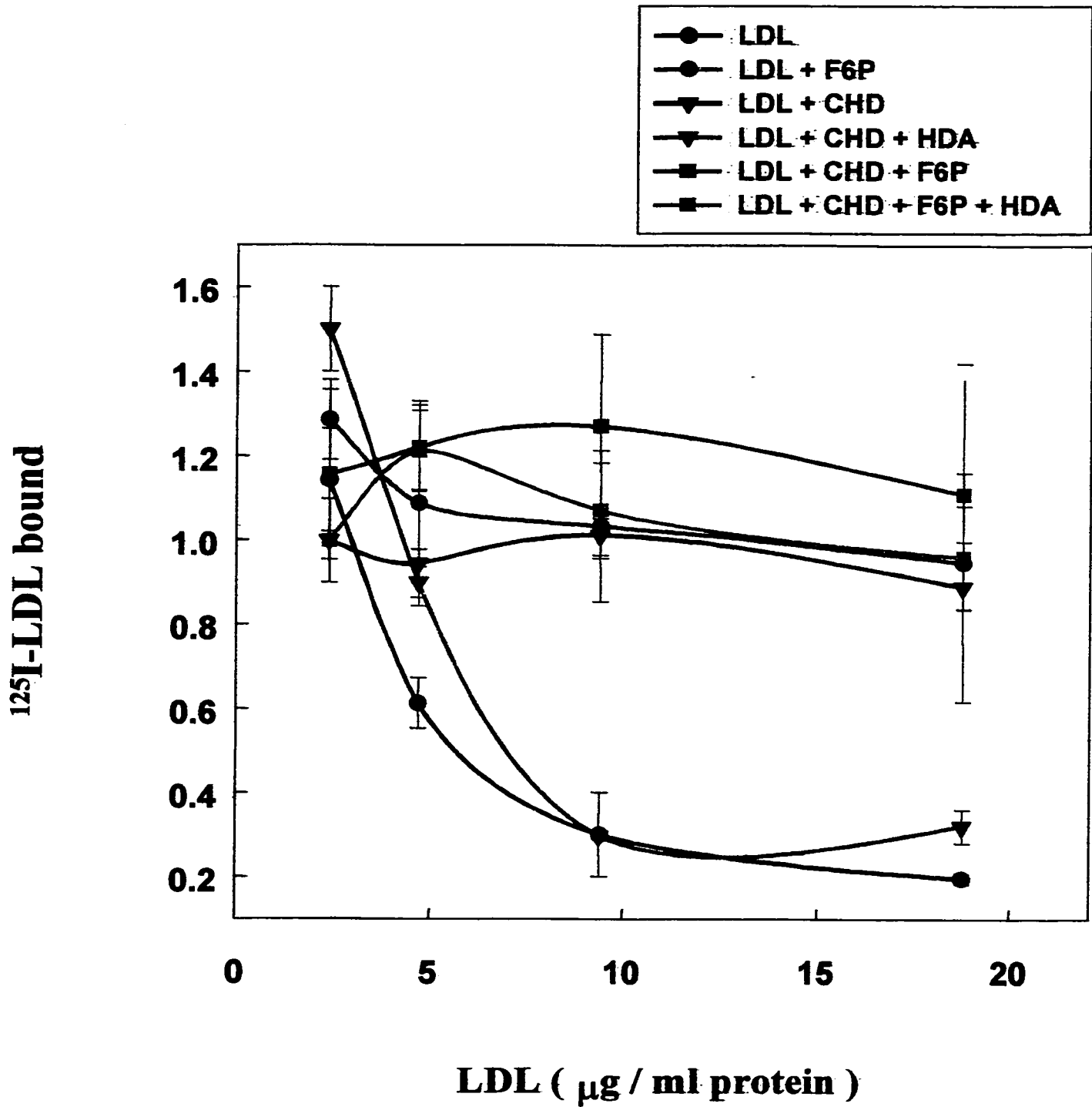
Figure 2-8



**Figure 2-9: Arginine-protected AGE-LDL is unable to bind to the LDL receptor.**

Control and modified LDL (those described in figure 2-8) were tested for their ability to compete with  $^{125}\text{I}$ -LDL for binding to the LDL receptor on cultured human fibroblasts. Results are presented as the ratio of radioactivity bound in the presence of competitor to that bound in the absence of competitor ( $B/B_0$ ) as a function of the concentration of the competitor. LDL from 2 subjects were modified as described before and each experiment was done in triplicates.

Figure 2-9



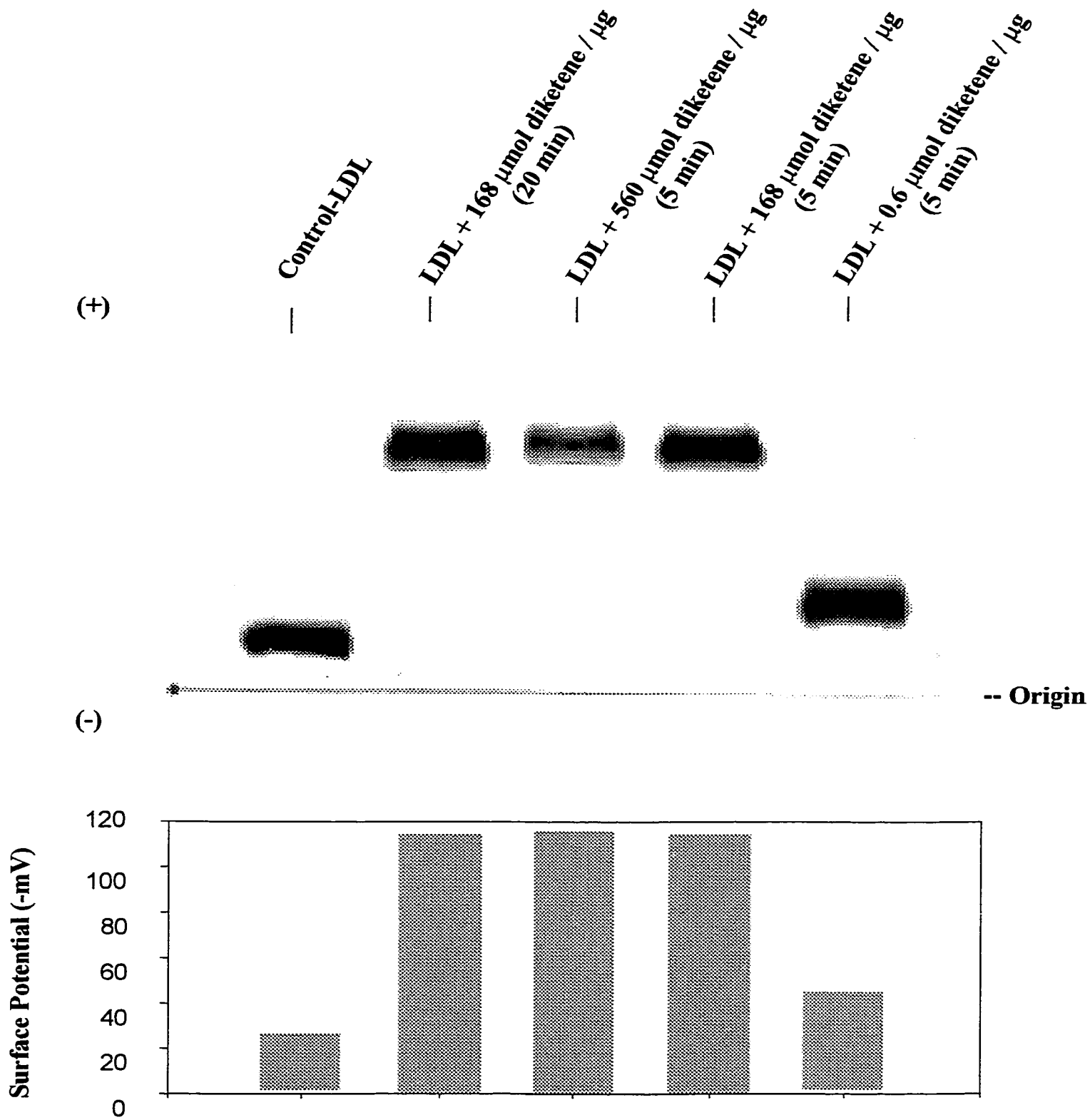
*Modification of lysine, and not arginine, residues of apoB may be largely responsible for the defective binding of glycosylated LDL to the LDL receptor-Diketene (DKT) has been described as a specific, reversible reagent for the modification of lysine residues of various proteins (Figure 2-6) (Weisgraber et al, 1978). The reaction, an acetoacetylation of amino groups, can be relatively specific for lysine depending on the conditions, but may also modify tyrosine and serine residues in some cases. However, the acetoacetylation of tyrosine and serine is rapidly and easily reversed by dialysis of the modified proteins in carbonate/bicarbonate buffer. By contrast, the modified lysine is stable not only in the carbonate/bicarbonate buffer but also under the conditions of the tissue culture experiments. The modification of lysine residues can be reversed by incubation with hydroxylamine.*

Reaction of 0.6  $\mu\text{mol}$  of DKT / mg of LDL protein was reported to modify approximately 25% of lysine residues in apoB (Weisgraber et al, 1978). We determined that the most extensive modification of lysine residues of apoB is possible with 168  $\mu\text{mol}$  of DKT / mg of LDL protein. Longer incubations or higher concentration of this reagent did not show further modification on gel electrophoresis (Figure 2-10). As shown in figure 2-10, the migration towards the anode reflects the neutralization of the positive charge on the modified lysyl residues. The migration observed with the DKT reagent is much greater than that shown with CHD, which reflects the abundance of lysine residues (357 residues) compared to arginines (144 residues) in apoB.

**Figure 2-10: Electrophoretic mobility of LDL as a function of Diketene modification.**

LDL that had been incubated with 0.6, 168, 560  $\mu\text{mol}$  of Diketene /  $\mu\text{g}$  apoB for 5 to 20 min (LDL + DKT), or without DKT (Control-LDL) were subjected to agarose (0.5%) gel electrophoresis. The gel was stained for lipid according to the manufacturer's recommendations. Similar results were seen in other agarose gels.

Figure 2-10



To determine if DKT can protect lysine residues of apoB from AGE modification, LDL were first treated with 168  $\mu\text{mol}$  of DKT /  $\mu\text{g}$  of LDL protein, followed by a 4 day incubation with 0.145 mM F6P. As seen in figure 2-11, LDL pretreated with DKT before AGE modification (LDL + DKT + F6P) showed a greater electrophoretic mobility than LDL treated with DKT alone (LDL + DKT). As this difference in migration approximates that between LDL treated with DKT (LDL + DKT) and LDL treated with both DKT and CHD (LDL + DKT + CHD) (Figure 2-12), chemical modification of lysine residues does not appear to prevent arginine residues from reacting with F6P. HDA treatment of DKT-treated LDL (LDL + DKT + HDA) resulted in a particle that exhibited an electrophoretic mobility that was similar to control LDL (Figure 2-11). In contrast, exposure of DKT-treated, F6P-modified LDL to HDA (LDL + DKT + F6P + HDA) generated a particle with an intermediate mobility of the DKT + F6P + HAD treated LDL. As it is unlikely that the increased net negative charge, giving rise to the intermediate mobility, is solely due to AGE modification of arginine residues, it is probable that DKT-treatment only partially protected lysine residues from AGE modification or that reversal of DKT protection by HDA in DKT-treated, F6P-modified LDL was only partial.

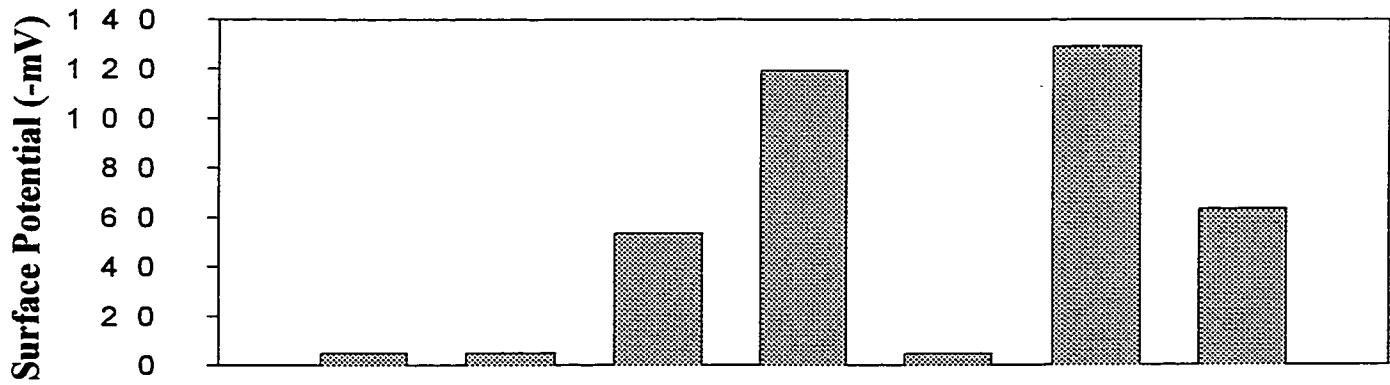
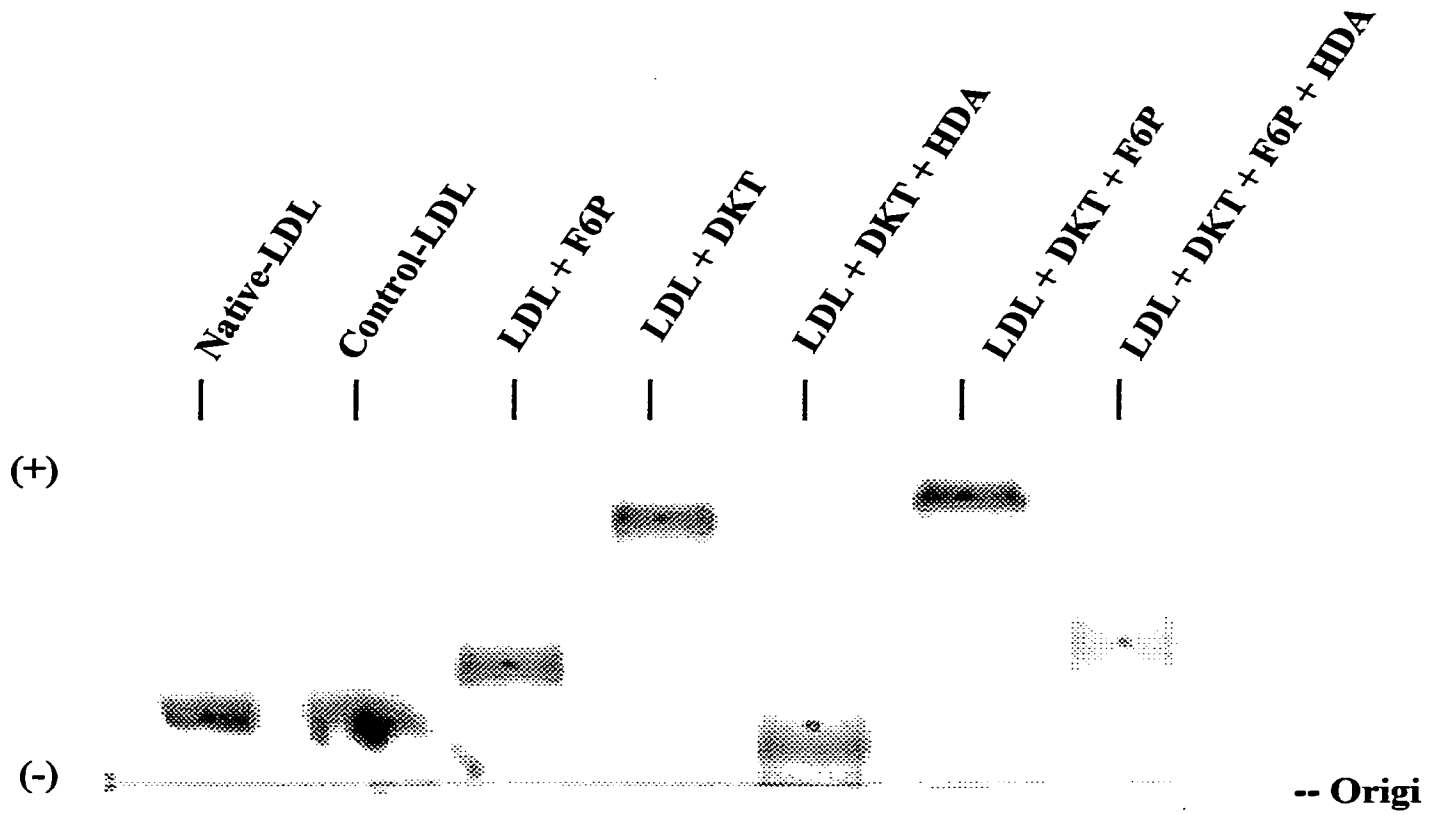
Oxidized LDL is known to migrate faster than native LDL on agarose gel electrophoresis. Anti-oxidants, EDTA, and BHT are present in all incubations. The possibility that we may, nevertheless, be generating oxidized LDL during incubation is unlikely as the electrophoretic mobility of native LDL and control LDL (incubated in absence of DKT and F6P) are similar (Figure 2-11).

The mAb, 4G9, is known for its specificity for AGE adducts (Makita, 1992). As shown in figure 2-13, 4G9 recognized LDL that had been incubated in presence of F6P (LDL + F6P). In contrast, lysine-protected AGE-LDL (LDL + DKT + F6P + HDA) shows no detectable immunoreactivity with 4G9. As arginine residues were likely modified in lysine-protected AGE-LDL, this mAb may be specific for AGE-modified lysine residues. Alternatively, the lack of 4G9 reactivity of lysine-protected AGE-LDL may result from the lack of sensitivity of the antibody. While higher amounts of AGE (20 mg / ml AGE-BSA) are easily detected, we are unable to detect immunoreactivity with a 100 fold diluted sample (0.2 mg / ml AGE-BSA). Thus, mAb may not have a sensitivity sufficient for the detection of arginine-bound AGEs in the lysine-protected AGE-LDL sample.

**Figure 2-11: Electrophoretic mobility of LDL as a function of glycation, and/or diketene modification, and regeneration.**

Isolated fresh LDL (Native-LDL), LDL that had been incubated in absence of diketene and F6P (Control-LDL), or those incubated with 168  $\mu$  mol diketene /  $\mu$ g protein (LDL + DKT), or 0.145 mM F6P (LDL + F6P), or 168  $\mu$  mol diketene /  $\mu$ g protein and 4M hydroxylamine (LDL + CHD + HDA), or 168  $\mu$  mol diketene /  $\mu$ g protein in prior to 0.145 mM F6P and 4M hydroxylamine were subjected to agarose (0.5 %) gel electrophoresis. The gel was stained for lipid according to the manufacturer's recommendations. Similar results were seen in other agarose gels.

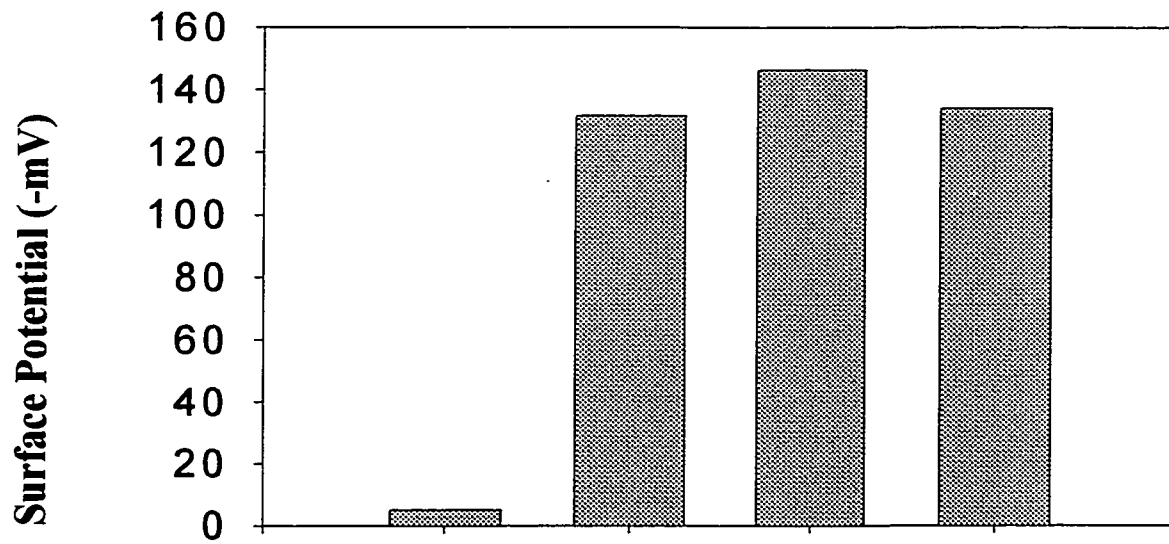
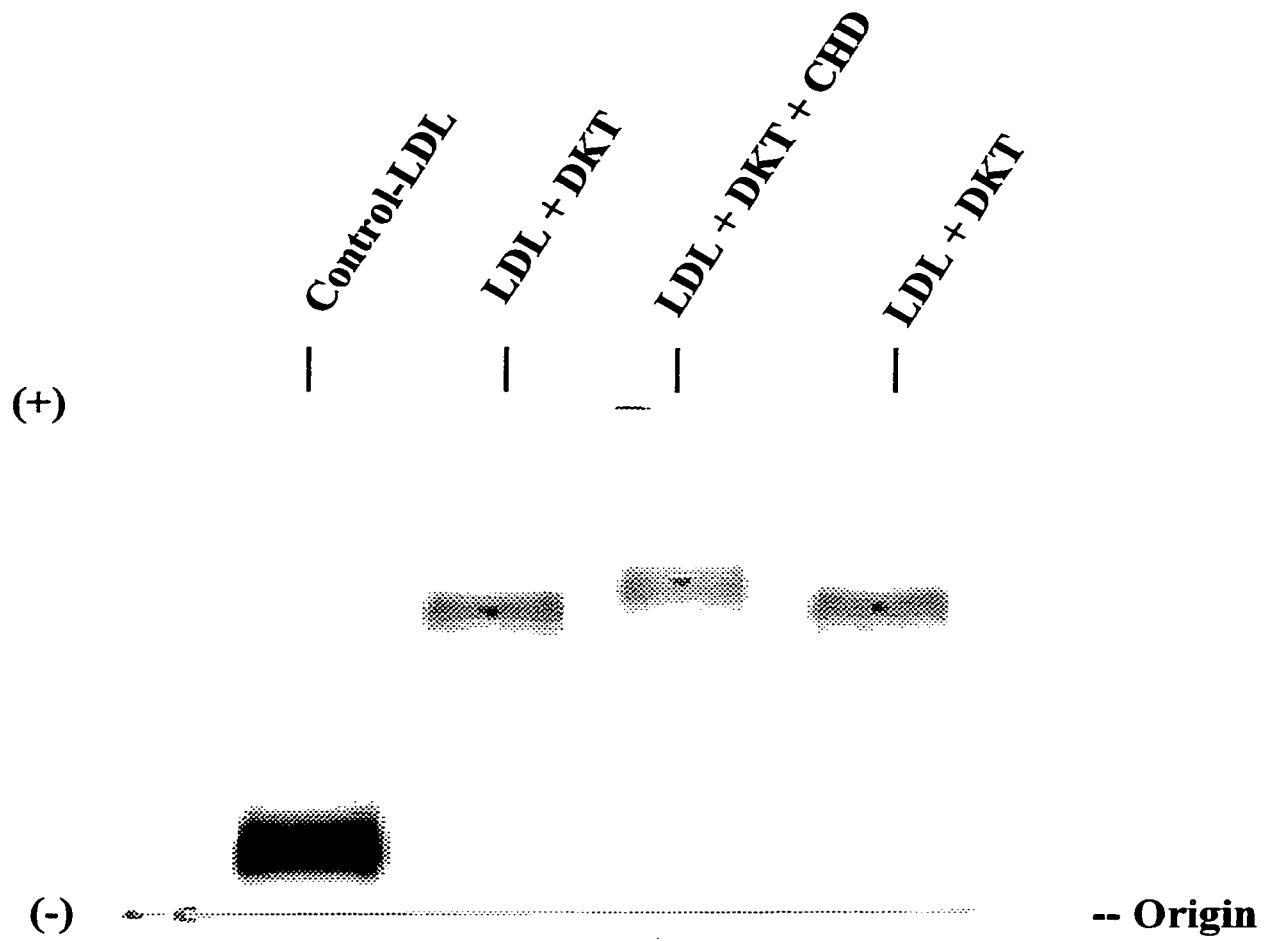
**Figure 2-11**



**Figure 2-12: Electrophoretic mobility of LDL as a function of Arginine and Lysine residues modification.**

LDL that had been incubated with 168  $\mu$  mol diketene /  $\mu$ g protein (LDL + DKT) were further incubated with 1.0 mM 1,2-cyclohexanedione (LDL + DKT + CHD) and LDL incubated in absence of DKT and CHD (Control-LDL) were subjected to agarose (0.5%) gel electrophoresis. The gel was stained for lipid according to the manufacturer's recommendations. Similar results were seen in other agarose gels.

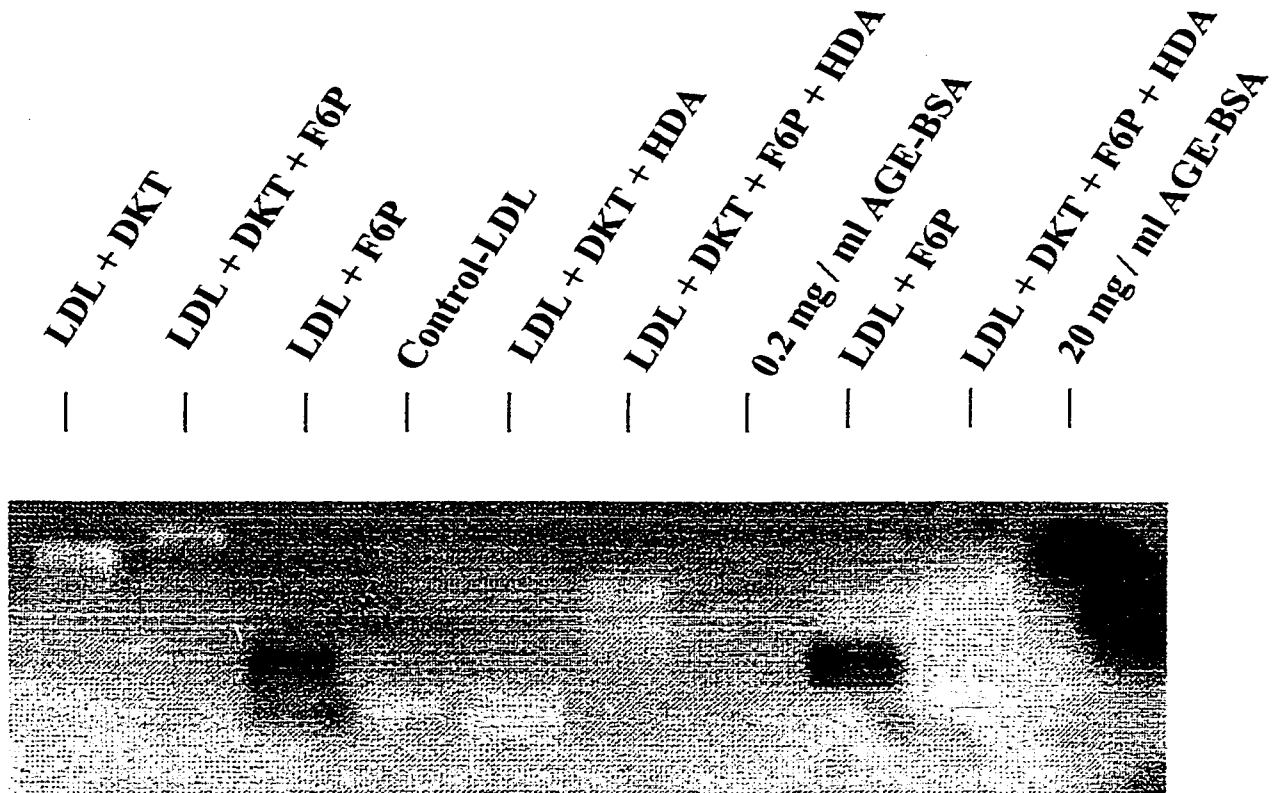
Figure 2-12



**Figure 2-13: Immunoreactivity of AGE-LDL and lysine-protected AGE-LDL**

Equal quantities of LDL and modified LDL (those described in figure 2-11) were subjected to agarose (0.5%) gel electrophoresis and transferred to nitrocellulose membranes. Immunoreactivity of the transferred proteins was tested with mAb 4G9 that is specific for AGE adducts. Similar results were seen in two separate experiments.

**Figure 2-13**



The expression of apoB epitopes on lysine-protected AGE-LDL was tested using the panel of 18 anti-apoB mAbs. Competition curves for two of the mAbs (4G3 and 1D1) are presented in figure 2-14. As we had anticipated, LDL incubated in presence of DKT lost immunoreactivity with the majority of anti-apoB mAbs. This loss of immunoreactivity was almost fully recovered when the chemical modification was reversed by incubating lysine-protected LDL with HDA. As described above, glycated LDL (LDL + F6P) are as reactive as LDL that had been incubated in absence of F6P (LDL) with certain mAb such as, 1D1 (Figure 2-14). However, they are much less reactive than non-glycated LDL with other mAbs, such as 4G3. Incubation of LDL with DKT does not fully protect the 4G3 epitope from subsequent modification by F6P-mediated glycation. This was not entirely unexpected as it had been previously shown that modifying the arginine residues of apoB, by incubating LDL with cyclohexanedione, could almost completely eliminate 4G3 immunoreactivity (Milne, 1983). Therefore, both lysines and arginines contribute to the 4G3 epitope and both could be susceptible to AGE modification. Similar results to that with the 1D1 epitope were observed with AGE-insensitive mAbs (i.e. Bs04, 1C4, 5E11), whereas other AGE-sensitive mAbs (B4, 3A5, 588, 234) showed similar results to that observed with the 4G3 epitope (not shown).

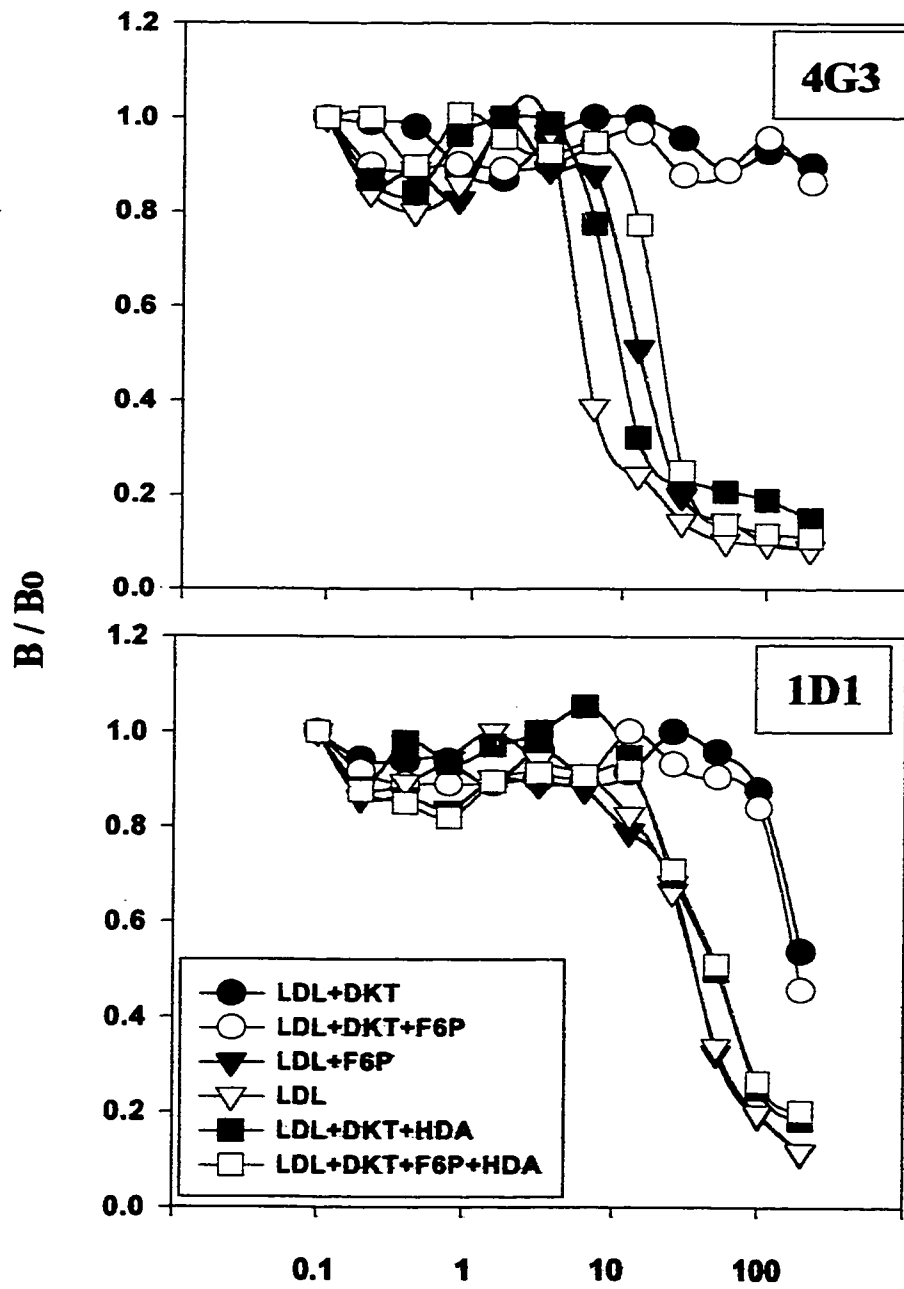
Reaction of 168  $\mu$ mol of DKT / mg of LDL protein with LDL completely eliminated the ability of the DKT-LDL to compete with  $^{125}$ I-LDL for binding to the LDL receptor on human fibroblasts. LDL that had been sequentially treated with DKT and HAD showed about 75% of the LDL receptor-binding activity of untreated LDL. Also the subsequent regeneration of DKT-modified AGE-LDL restored its binding ability to that of LDL treated with DKT & HDA (Figure 2-15). This suggests that protecting, even

**Figure 2-14: The immunoreactivity of modified and control LDL.**

The immunoreactivity of modified (those described in figure 2-11) and control (LDL) was determined by a solid phase RIA using a panel of anti-apoB mAbs. Competition curves for mAbs 4G3 and 1D1 are presented. Results are presented as  $B/B_0$ , where B is radioactivity bound in the presence of competitor as a fraction of radioactivity bound in the absence of competitor. Similar result was seen in another RIA.

2-15). This suggests that protecting, even partially, the lysine residues of apoB from glycation protects it's ability to bind to the LDL receptor on fibroblasts.

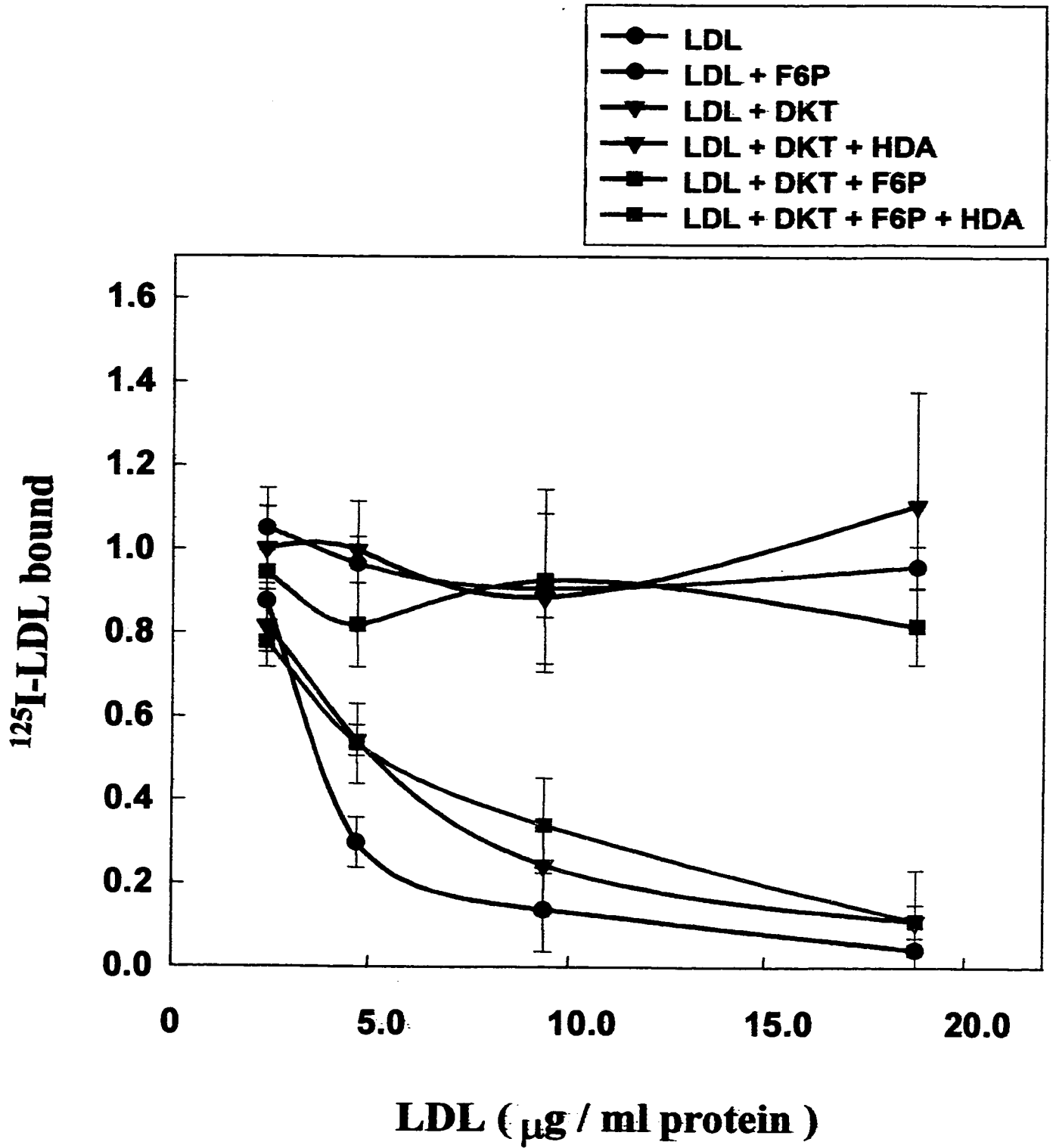
Figure 2-14



**Figure 2-15: Lysine-protected AGE-LDL is able to bind to the LDL receptor.**

Control (LDL) and modified LDL (those described in figure 2-11) were tested for their ability to compete with  $^{125}\text{I}$ -LDL for binding to the LDL receptor on cultured human fibroblasts. Results are presented as the ratio of radioactivity bound in the presence of competitor to that bound in the absence of competitor (B/Bo) as a function of the concentration of the competitor. LDL from 2 subjects were modified as described before and each experiment was done in triplicate.

Figure 2-15



partially, the lysine residues of apoB from glycation protects its ability to bind to the LDL receptor on fibroblasts.

*AGE-peptide modified LDL*- Recently it was reported that AGE-LDL can also be prepared *in vitro* by incubating native LDL with AGE-peptides (Bucala *et al*, 1994). AGE-peptides have been shown to crosslink protein amino groups and to accumulate in plasma as a consequence of renal insufficiency (Makita *et al*. 1994). Bucala and co-workers (Bucala *et al*, 1994), observed that LDL which had been modified by AGE-peptides *in vitro* exhibited markedly impaired clearance kinetics when injected into transgenic mice expressing the human LDL receptor. We hypothesized that LDL modified *in vitro* by AGE-peptides may have different immunochemical characteristics than those modified by reducing sugar *in vitro*. We prepared AGE-peptides by proteolysis of AGE modified albumin as has been described (Bucala *et al*, 1994). However, we were unable to produce any AGE-peptide-modified LDL. The LDL incubated with AGE-peptides did not show any change in electrophoretic mobility, or reduced binding to LDL receptor on the cultured fibroblasts, or reduced immunoreactivity to any of the 18 anti-apoB mAbs (not shown). Although AGE-peptides are known to be very reactive molecules *in vivo*, they did not show any reactivity in our *in vitro* model. We later learned that other laboratories have experienced similar difficulties in producing AGE-peptide modified LDL (R. Bucala, personal communication).

*Immunochemical and functional characterization of LDL isolated from normal subjects, diabetic patients, and patients with renal insufficiency*-In order to compare LDL that had been modified *in vitro* by incubation with F6P (LDL + F6P), with LDL modified

*in vivo* under patho-physiological conditions, we characterized LDL isolated from normal subjects and from patients with diabetes and renal insufficiency. Table 2-1 summarizes some of the physical and biochemical features of the nine subjects that were studied. Hemoglobin Amadori product HbA<sub>1c</sub> reflects ambient glycemia over the preceding 4-5 weeks. HbA<sub>1c</sub> ranges from 3.65 to 4.35% of total hemoglobin in normoglycemic individuals. The HbA<sub>1c</sub> values of the diabetic subjects were approximately three times normal values (Table 2-1).

As described earlier, size and composition of LDL may modulate apoB conformation and function. Furthermore, it is known that the LDL particle diameter and chemical composition can be abnormal in subjects with diabetes or renal insufficiency. We have, therefore, investigated the size and lipid composition of the LDL from all subjects. When the LDL were subjected to non-denaturing gradient gel electrophoresis, the LDL from both diabetic and renal insufficient patients appeared to be somewhat smaller than LDL from normal subjects (Figure 2-16). However, no differences in lipid composition were observed between LDL from normal subjects and LDL from the patients (Figure 2-17).

**Table 2-1**

**Table 2-1: Physical and biochemical profile of the normal, diabetic, and renal insufficient subjects**

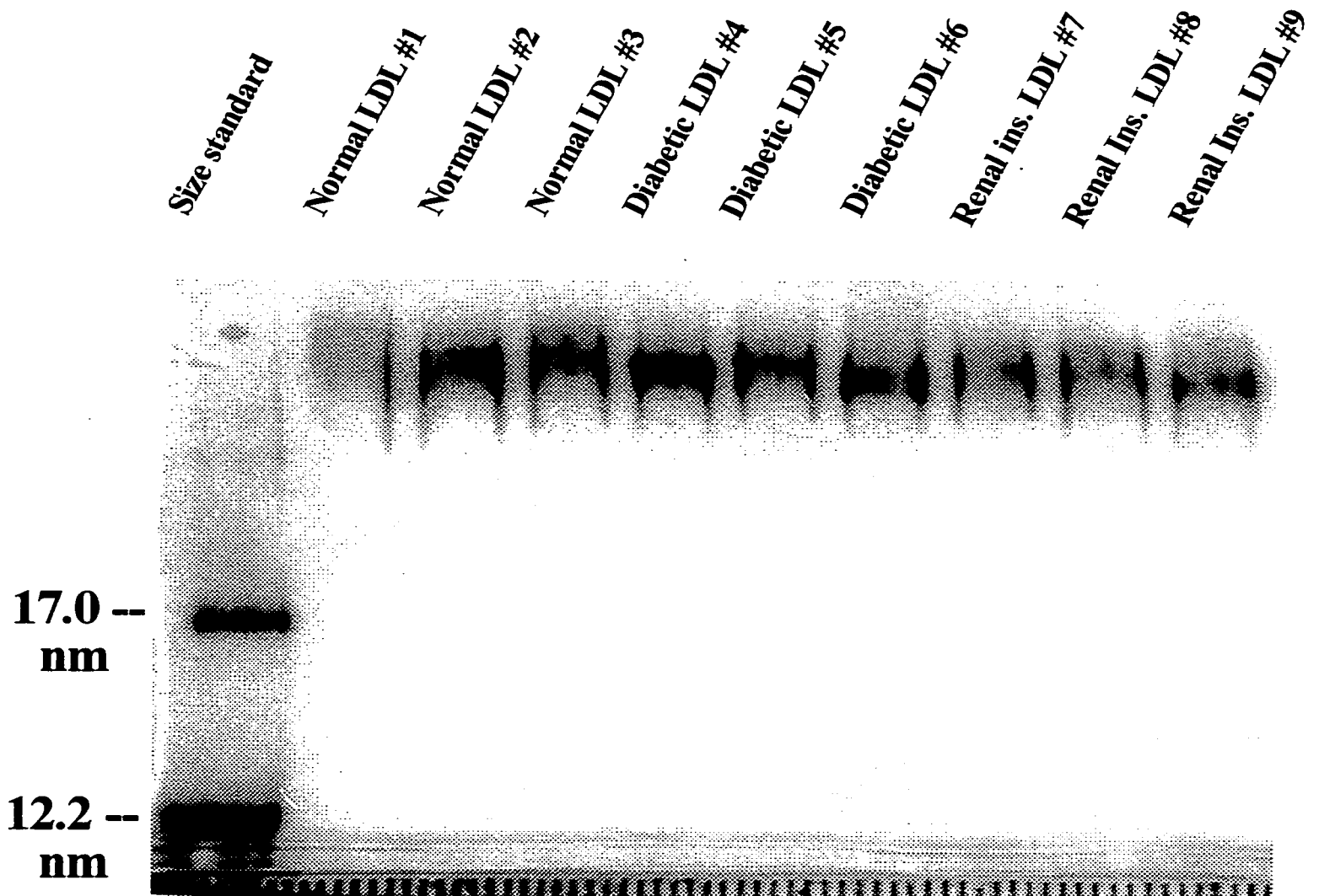
<b>sample no</b>	<b>subject</b>	<b>sex</b>	<b>age(year)</b>	<b>HB A1c(%)</b>
1	control	F	-	*
2	control	F	-	*
3	control	M	-	*
4	diabetic (I)	M	37	12.45
5	diabetic(II)	F	69	11.50
6	diabetic(II)	M	65	10.80
7	Renal Ins.	F	51	-
8	Renal Ins.	F	41	-
9	Renal Ins.	M	68	-

\* HBA1c = 3.65-4.35

**Figure 2-16: Non denaturing polyacrylamide gradient gel electrophoresis to determine the size of LDL samples.**

1.5  $\mu\text{g}$  of LDL samples from three normal, diabetic, and renal insufficient subjects were subjected to non-denaturing 3-8% polyacrylamide gel electrophoresis under non-denaturing conditions according to manufacturer's specification. The gel was stained for protein. Similar results were seen in other gel electrophoresis.

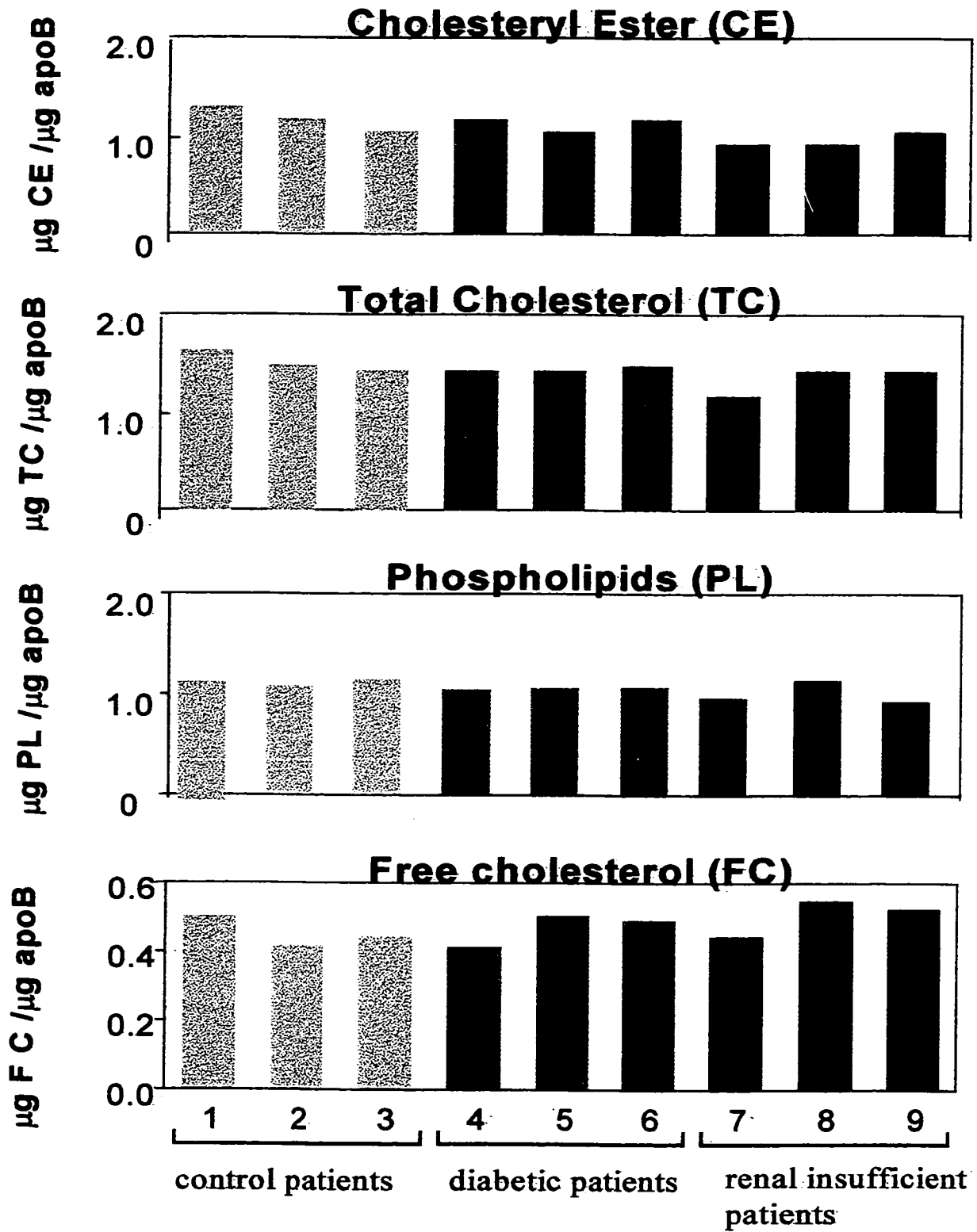
Figure 2-16



**Figure 2-17: Lipid composition of LDL isolated from three control, diabetic, and renal insufficient subjects**

4  $\mu\text{g}$  of LDL samples from three normal, diabetic, and renal insufficient subjects were used for lipid analysis according to manufacturer's specification. Similar results were seen in a second lipid analysis.

Figure 2-17



It has been shown that AGE modification of LDL occurs *in vivo* in diabetic and renal insufficient patients; however, the extent of modification is considerably less than that seen in LDL that has been modified *in vitro* (Steinbrecher and Witztum, 1984). Diabetic patients have been reported to have higher levels of glycated LDL than their euglycemic counterparts (2 to 8% versus 1 to 2% respectively) (Lyons, 1986). When the LDL were subjected to agarose gel electrophoresis, it was observed that the LDL from diabetic and renal insufficient subjects did not show a major increase in electrophoretic mobility compared to LDL from normal subjects (Figure 2-18). We also tested immunoreactivity of the LDL samples with mAb 4G9 that is specific for AGE adducts. Using this mAb, neither by western blot technique (not shown), nor by the competitive ELISA did we detect any evidence for presence of AGE modification. Figure 2-19 demonstrates ELISA competition curves for the anti-AGE mAb (4G9) antiserum. None of the LDL samples isolated from the diabetic and renal insufficient subjects competed with the immobilized AGE-BSA for the binding of the anti-AGE antibody. In contrast, *in vitro*-AGE-modified LDL (LDL + F6P), but not control LDL, competed efficiently.

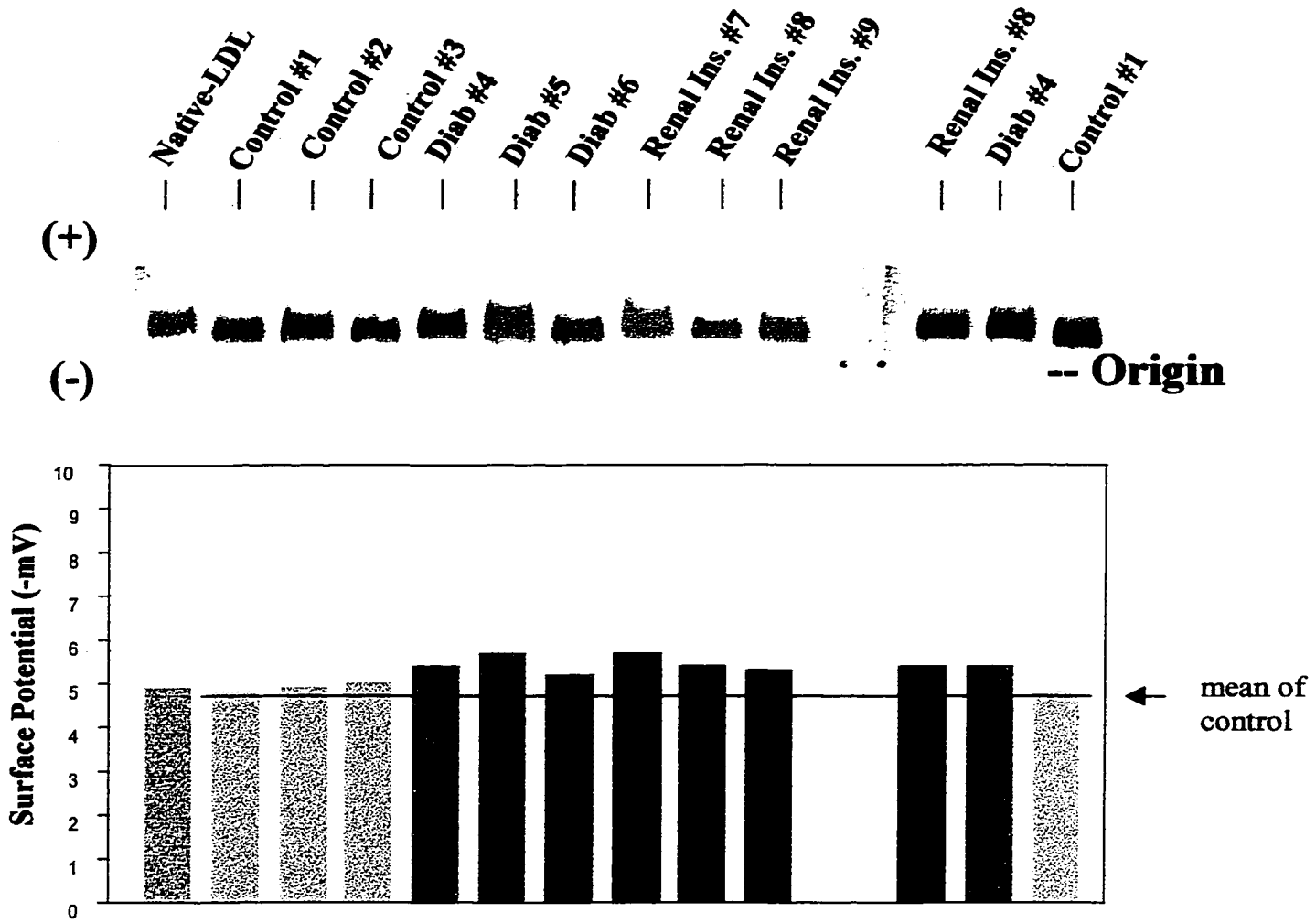
Patients with diabetes and renal insufficiency frequently suffer from a complex dyslipidemia which is thought to result from defective lipoprotein binding, uptake, and catabolism (Bucala *et al*, 1994). In other studies, however, Schleicher *et al* failed to demonstrate significant changes in the binding of moderately glycated LDL by fibroblasts, endothelial cells, hepatocytes, and macrophages. We have conducted similar fibroblast cell surface binding studies to compare the binding ability of the diabetic or renal insufficient patients' LDL to the LDL receptor, to that of LDL from control subjects. We were, unable to detect any significant difference in the receptor-binding

ability of patients' LDL compared to that of the healthy control subjects' LDL (Figure 2-20). To eliminate the possibility that apoE mediated binding of some remnant LDL may have masked any difference in binding of patient versus control LDL, we incubated all LDL samples with anti apoE mAb (1D7) prior to the fibroblast cell surface binding assay. 1D7 is shown to completely block apoE mediated binding of lipoproteins to the LDL receptor (Weisgraber *et al*, 1983). Once again the diabetic and the renal insufficient patients' LDL did not show any significant decrease in binding to the LDL receptors on fibroblast cells (not shown)

**Figure 2-18: Electrophoretic mobility of LDL isolated from three control, diabetic, and renal insufficient subjects**

LDL that had been isolated from the control, diabetic, and renal insufficient subjects, and LDL isolated from non-frozen plasma (Native-LDL) were subjected to agarose (0.5%) gel electrophoresis. Samples were applied to a thin film agarose gel and were allowed to migrate for 30 min at 100V. The gel was stained for lipid according to the manufacturer's recommendations.

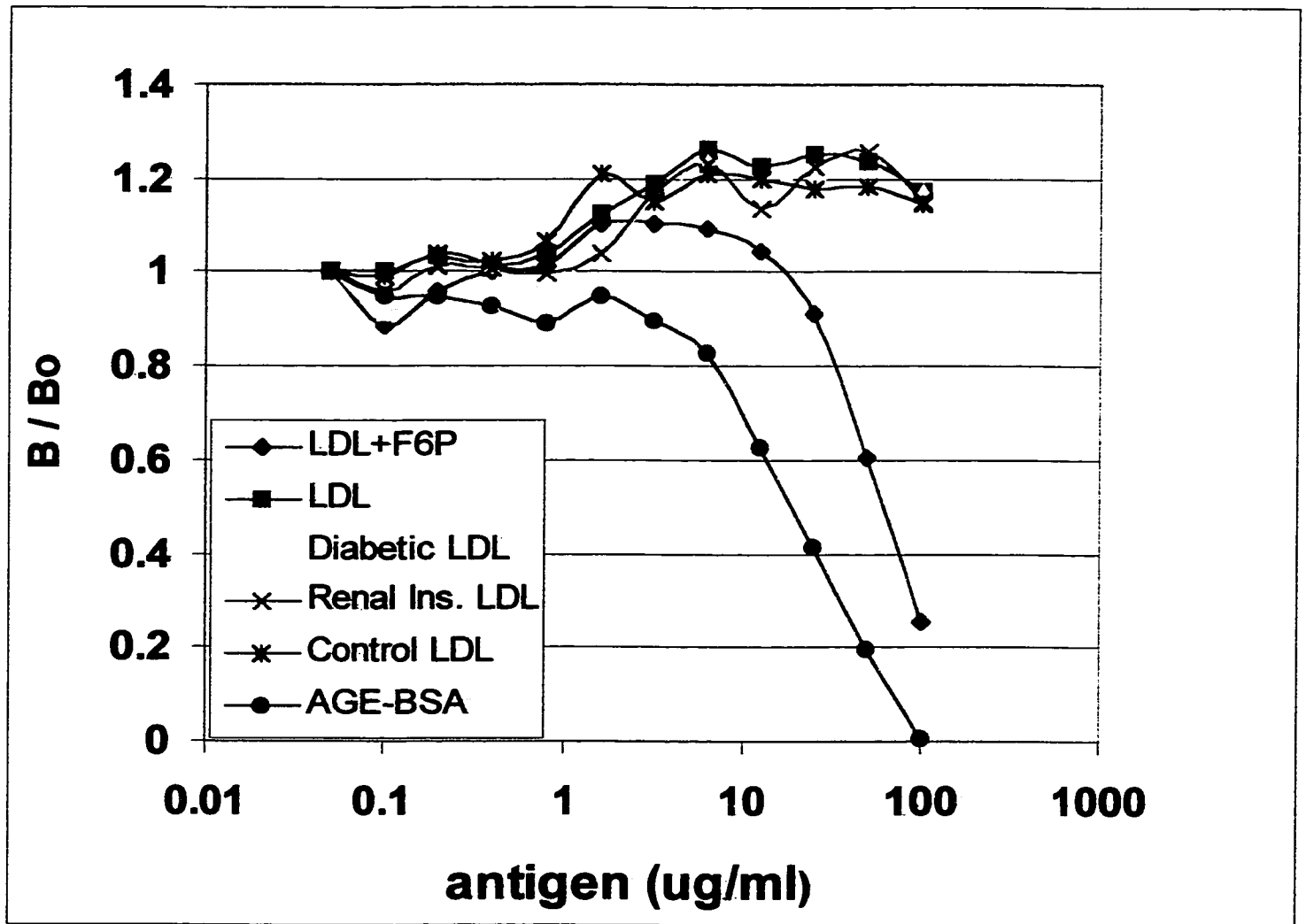
Figure 2-18



**Figure 2-19: AGE measurements using competitive ELISA**

The immunoreactivity of LDL that had been incubated for 4 days at 37° C without F6P (LDL), with 0.145 mM F6P (LDL + F6P), LDL isolated from diabetic (Diabetic LDL), renal insufficient (Renal Ins. LDL), and healthy control subjects (Control LDL) was determined by a competitive ELISA using a anti AGE mAb (4G9). Glycated BSA (AGE-BSA) was used as the standard competition curve. Results are presented as B/Bo, calculated as experimental OD - background OD (no antibody) / total OD (no competitor) - background OD. Similar results were seen in other ELISA.

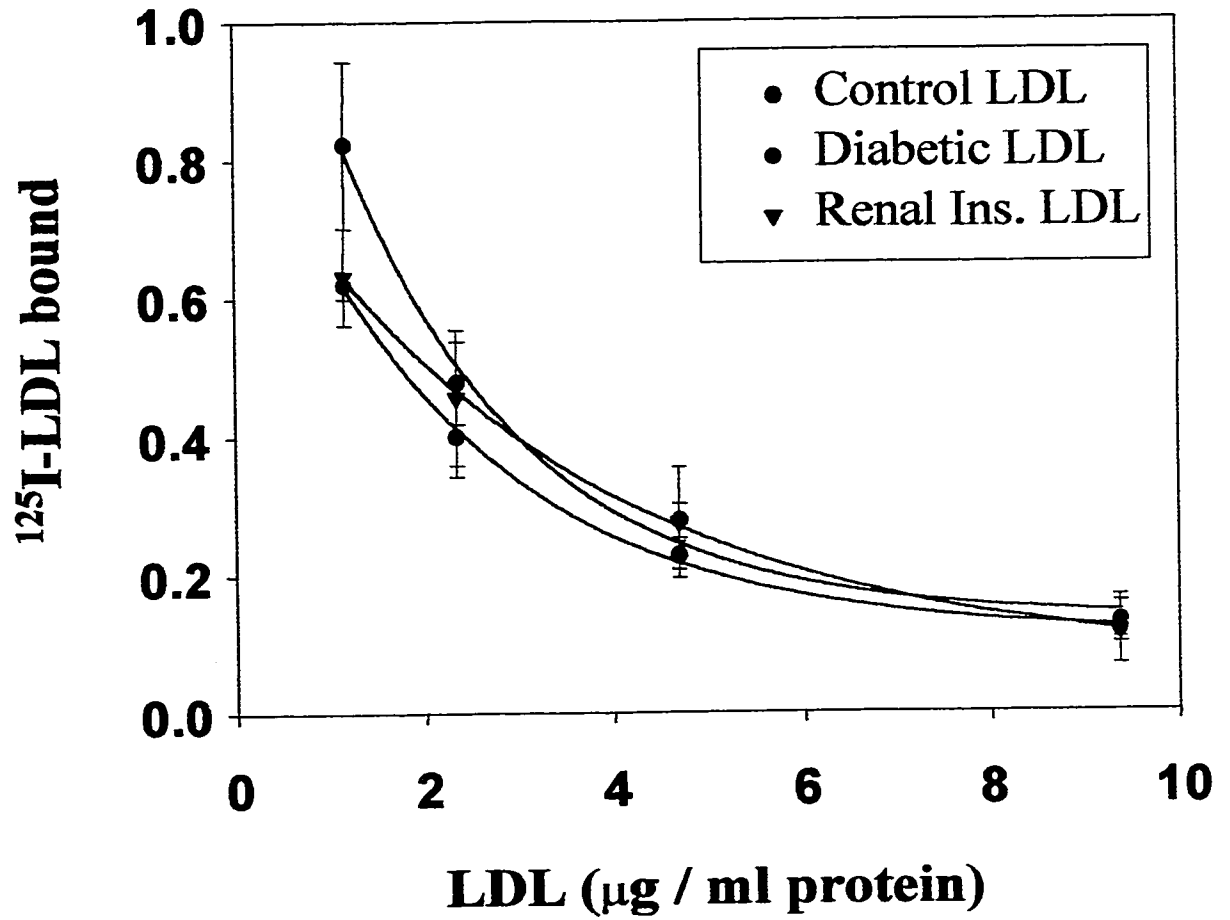
Figure 2-19



**Figure 2-20: binding ability to the LDL receptor.**

Control, diabetic, and renal insufficient LDL were tested for their ability to compete with  $^{125}\text{I}$ -LDL for binding to the LDL receptor on cultured human fibroblasts. LDL from 3 subjects were used and the experiment was done in triplicate. Results are presented as the ratio of radioactivity bound in the presence of competitor to that bound in the absence of competitor (B/Bo) as a function of the concentration of the competitor.

Figure 2-20

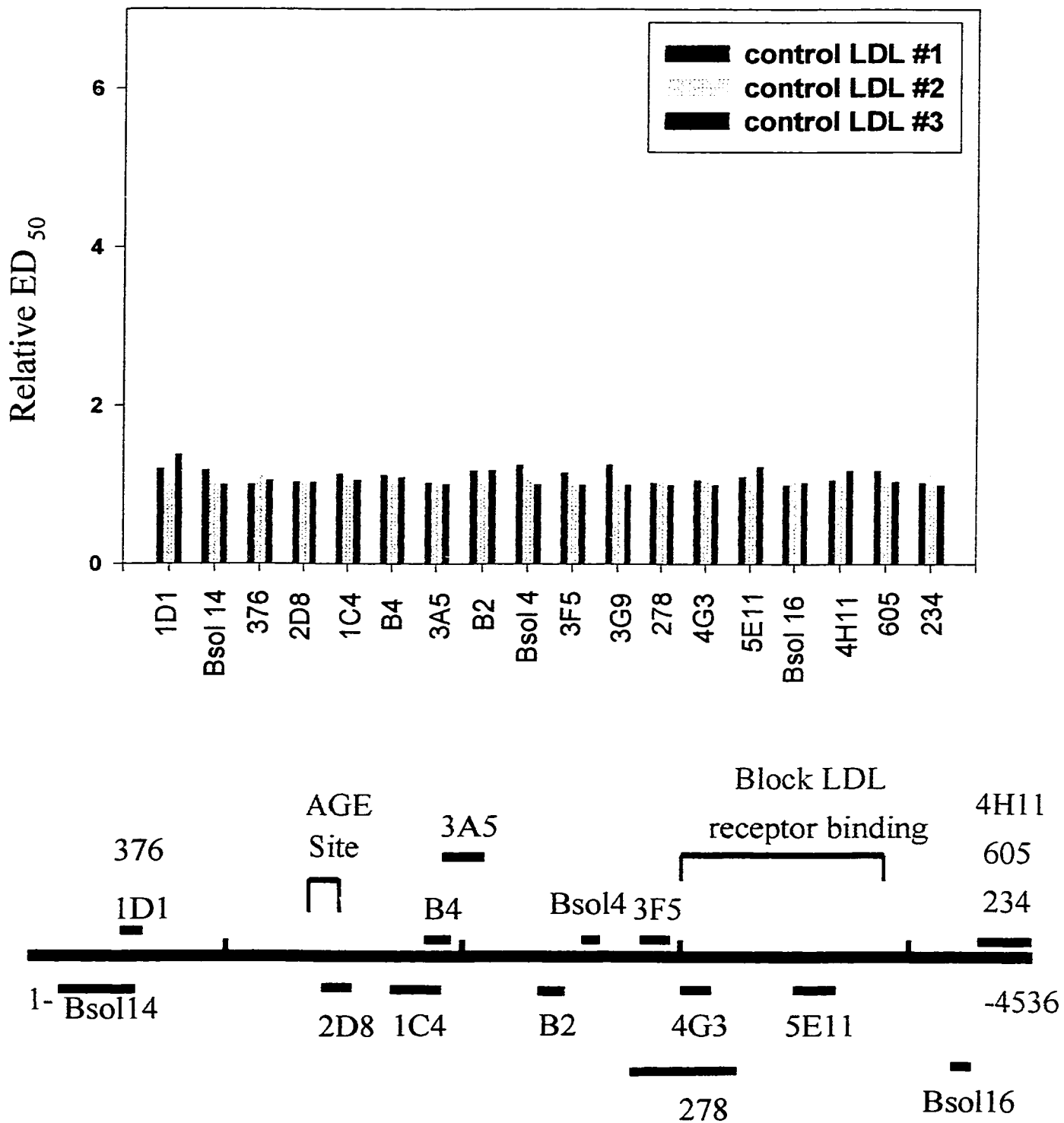


LDL from the nine subjects were tested in a competitive apoB radioimmunoassay. As it would be impossible to compare the reactivity of LDL samples isolated from each of the nine subjects with a panel of eighteen mAbs in a single solid phase RIA, we first compared the immunoreactivity of LDL samples from each of the three control subjects with the panel of mAbs. There was no difference in the expression of apoB epitopes among the control subjects (Figure 2-21). Therefore, we prepared a pooled control LDL sample that contained equal amounts of LDL from each of the three control subjects. Then in two separate experiments the immunoreactivity of apoB epitopes of LDL samples from the diabetic or renal insufficient subjects were compared to that of the pooled control LDL sample (Figure 2-22). LDL from the three diabetic subjects showed a loss of reactivity with mAbs 3F5 and 3A5. In addition, LDL from two of the three diabetic subjects, demonstrated a total loss of reactivity with mAb 376. LDL from all three renal insufficient subjects showed reduced immunoreactivity with mAbs 1C4 and B4. Both mAbs 3A5 and B4, which showed reduced reactivity with LDL from diabetic and renal insufficient subjects, respectively, have also been demonstrated (Figure 2-3) to have reduced immunoreactivity with *in vitro* AGE-modified LDL.

**Figure 2-21: The immunoreactivity of control subjects' LDL.**

The relative concentrations of control LDL that were required to obtain 50% of maximum binding ( $ED_{50}$ ) in the RIA. The  $ED_{50}$  for subject 2 was normalized to a value of 1 for each antibody. The position of the epitopes within apoB primary structure recognized by the panel of mAbs are shown.

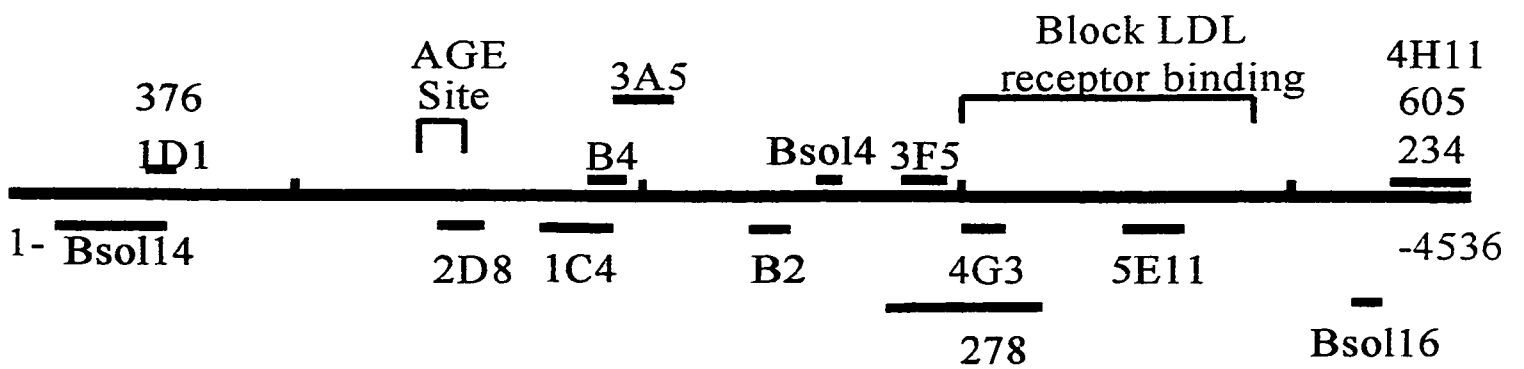
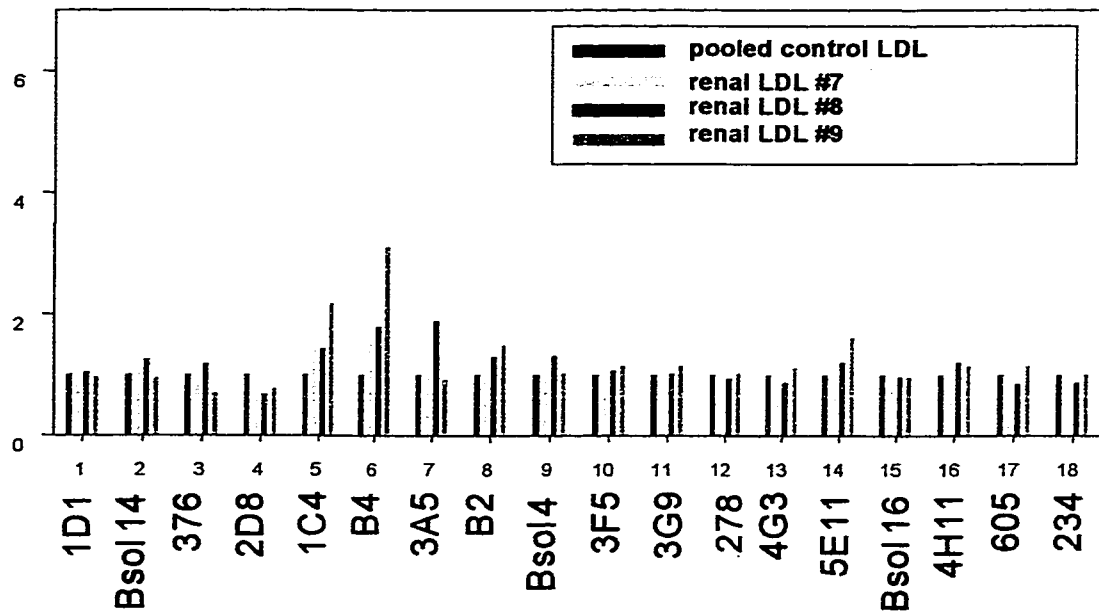
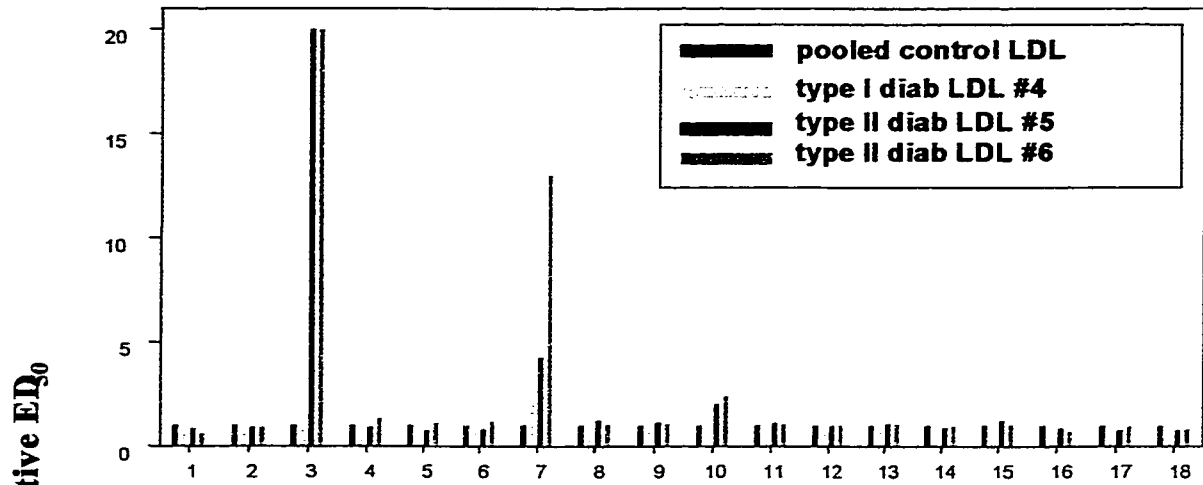
Figure 2-21



**Figure 2-22: The immunoreactivity of diabetic and renal insufficient subjects' LDL.**

The relative concentrations of pooled control LDL, diabetic LDL (diab LDL), and renal insufficient LDL (renal Ins. LDL) that were required to obtain 50% of maximum binding ( $ED_{50}$ ) in the RIA. The pooled control LDL sample contains equal amounts of LDL from each of the three control subjects. The  $ED_{50}$  for pooled control LDL was normalized to a value of 1 for each mAb. The positions of the epitopes within apoB primary structure recognized by the panel of mAbs are shown. The mAbs with red colour indicate the reduced immunoreactivity for glycated LDL compared to control LDL.

Figure 2-22



## Chapter IV

### Discussion

One of the mechanisms that has been proposed to be responsible for the association between diabetes and increased risk for the development of cardiovascular disease is that proteins, including apolipoproteins, can undergo non-enzymatic glycation. One of the consequences of AGE modification of apoB is a decreased ability to mediate LDL binding to the LDL receptor. This has been clearly demonstrated for LDL that had been subjected to *in vitro* AGE modification by incubation with reducing sugars and there is some evidence that LDL isolated from patients with diabetes and renal insufficiency are also defective in their binding to the LDL receptor. The principal goal of the present project was to demonstrate the structural basis by which AGE modification interferes with LDL function. A second goal was to compare LDL that had been AGE modified *in vitro* with LDL that had been modified *in vivo* under the patho-physiological conditions of diabetes and renal insufficiency.

In the present study we report a significant simplification in studying the molecular basis of LDL glycation. Since glycation by glucose is usually a slow (weeks and months) process, we have investigated glycation with fructose 6-phosphate (F6P), a more reactive analogue of glucose, and an intermediate metabolite of the sorbital pathway. *In vitro* F6P-mediated non-enzymatic glycation may also be a more appropriate model for *in vivo* modifications of biomolecules which occur in diabetes. Although an elevated level of glucose has been thought to play a primary role via Maillard reaction in

enhanced glycation in diabetic tissue, the non-enzymatic glycation is known to result from the action of various metabolites other than glucose (Brownlee, 1986). Among the intermediates, F6P levels been shown to be increased in diabetic states from a few  $\mu\text{mol/l}$  to 100  $\mu\text{mol/l}$  (Petersen, 1990; Dills, 1993). Most *in vitro* studies of AGE formation so far have been in presence of high, non-physiological concentrations of glucose (200mM). Here, with the use of F6P, an extremely potent glycating agent, not only have we reduced the incubation time required to prepare AGE-modified LDL (AGE-LDL), but we have also reduced the required amount of reducing sugar to the concentrations (0.1mM) observed in uncontrolled diabetics.

It would appear that the initial stages of the Maillard reaction of proteins with fructose occur more rapidly than with glucose (Dills, 1993). The basis for the faster Maillard reactions is not entirely clear although one potential factor is that, fructose exists to a greater extent in the open-chain form than does glucose (Bunn, 1981).

It has been proposed that AGE modification at a single site in apoB, almost 1800 residues from the putative apoB LDL receptor-binding domain, induces a change in the conformation of apoB that prevents its binding to the LDL receptor (Bucala *et al*, 1995). This hypothesis was challenged by a study that used a panel of 29 anti-apoB monoclonal antibodies (mAbs) to demonstrate that *in vitro* glycation of LDL results in modification at multiple sites in apoB, including two in the vicinity of the apoB LDL receptor-binding domain (Wang, 1998). In the present study we demonstrate that LDL modified by F6P have similar properties to those modified with glucose. Furthermore, the apoB epitopes that are modified by F6P are identical to those previously reported to be modified in LDL that had been exposed to glucose (Wang *et al*, 1998).

Watkins and co-workers studied the non-enzymatic glycation of several proteins in various buffers in order to assess the effects of buffering ions on the kinetics and specificity of glycation of protein (Watkins *et al*, 1986). Phosphate was found to catalyze the glycation of protein in a concentration-dependent manner. The unexpected observation in this work was that different concentrations of phosphate also altered the specificity of modification of proteins by reducing sugar. Therefore, the discrepancies in the potential sites of apoB modification by *in vitro* LDL glycation described earlier may, in part, be attributed to differences in the phosphate buffer system that was used.

Chemical modification of functional groups in the apoB molecule has shown that basic lysine and arginine residues are involved in binding to the LDL receptor (Mahley *et al*, 1977; Weisgraber *et al*, 1978). Mahley and co-workers reported that modification of 40 to 50% of the arginyl residues of apoB of human LDL abolishes their ability to bind to the cell surface receptors of fibroblasts in culture (Mahley *et al*, 1977). Later, it was reported that modification of as few as 15%, of the total lysine residues of LDL prevents LDL binding, internalization, and degradation by fibroblasts (Weisgraber *et al*, 1978). It appears that the interaction of LDL with the receptor requires the presence of both arginine and lysine within the LDL receptor-binding site.

AGE modification is known to primarily target lysine and arginine residues of proteins. In fact, pentosidine, a major AGE adduct, has been shown to form on lysines and/or arginine residues *in vivo* (Bucala *et al*, 1995). The chemical structure of this molecule is shown in figure 1-9. LDL modified by AGEs are shown to exhibit markedly delayed clearance kinetics *in vivo* (Bucala *et al*, 1994), and uptake by fibroblasts *in vitro* (Bucala *et al*, 1995; Wang *et al*, 1998). In our chemical modification strategy we have

established the conditions that maximizes the modification of lysine or arginine residues. However, it is impossible to modify all 357 lysine and 144 arginine residues, as not all of these residues are exposed on the surface of LDL. For example, a  $^{13}\text{C}$  NMR characterization of lysine residues in apoB reveals that only 65% of these residues are solvent-accessible (Lund-Katz, 1998).

Our results parallel those of Mahley (Mahley *et al*, 1977), in that modification of LDL with cyclohexanedione (CHD) completely eliminated its ability to compete with  $^{125}\text{I}$ -LDL for binding to human fibroblasts. We also demonstrated that this defective binding is almost completely regenerated by quantitative release of CHD from apoB. However, when CHD was used to protect arginine residues from AGE modification, subsequent treatment of arginine-protected AGE-LDL with HDA did not restore its ability to bind to the LDL receptor. This suggests that protecting the arginine residues of apoB from AGE-modification is not sufficient to protect its ability to bind to the LDL receptor on cultured fibroblasts.

Using a similar strategy to protect lysine residues from AGE modification, we explored the possibility that it was the modification of lysine residues that accounted for the defective binding of AGE-LDL to the LDL receptor. Hydroxylamine (HDA) treatment of diketene (DKT)-treated LDL could almost completely restore both the electrophoretic mobility and LDL receptor-binding activity to that of control LDL. However, based on the electrophoretic mobility tests, DKT modification of LDL could only partially protect apoB lysine residues from AGE modification. As shown in figure 2-11, the lysine-protected AGE-LDL (LDL+DKT+F6P+HDA) demonstrates a greater electrophoretic mobility than expected. The loss of net positive charge resulting in this

electrophoretic mobility, is partly due to the glycation of arginines, and may partly be due to glycation of unprotected lysines. Nevertheless, we showed that even partially protecting the lysine residues of apoB from glycation protects its ability to bind to the LDL receptor on fibroblasts. Apparently a critical sub-population of lysine residues of apoB on LDL can be protected from glycation by DKT treatment. Therefore, our present data suggest that modification of lysine, and not arginine, residues of apoB may be largely responsible for the defective binding of AGE-LDL to the LDL receptor.

Several studies have shown that apoB residues 3,359-3,367 participate directly in binding to the LDL receptor (Knott, 1985; Boren, 1998). Wang *et al.* have presented evidence that lysine residues in this region are not modified in LDL that had been incubated for 15 days with 200 mM glucose (Wang *et al.*, 1998). It was not formally demonstrated in this study that arginine residues in this region escaped AGE modification. As a consequence, it could not be excluded that modification of arginines between apoB residues 3,359-3,367 are responsible for the defective binding activity of AGE-LDL. Our demonstration that protection of lysine residues from AGE modification is sufficient to maintain the LDL receptor-binding activity of apoB suggests that glycation-sensitive lysine residues outside of the sequence 3,359-3,367, are responsible for the defective binding of the LDL to the LDL receptor.

Although circulating AGEs form in part by the *in situ* reaction of reducing sugar with serum proteins, a large portion of these products enter the plasma compartment as AGE-modified peptides (AGE-peptides) via catabolism of AGE-modified tissue proteins (Bucala *et al.*, 1994). Thus, extremely high concentrations of circulating AGE-peptides occur even under non-hyperglycemic conditions if plasma filtration is impaired by renal

failure. This prompted us to consider the possibility that LDL may be modified differently under hyperglycemic conditions (i.e. diabetes) than in presence of high concentrations of reactive AGE-peptides (i.e. renal insufficiency).

We proposed to address this question by two approaches. First, we attempted to compare LDL that had been AGE modified by incubation with F6P to LDL that had been modified by exposure to AGE-peptides. However, in spite of having followed a published protocol (Bucala *et al*, 1994), we were unable to demonstrate AGE modification of LDL that had been incubated with AGE-peptides. Others have encountered similar difficulties (R. Bucala, personal communication). It is possible that AGE-peptides generated *in vivo* are more reactive than those generated *in vitro* or that other factors *in vivo* may promote AGE-peptide mediated modification of LDL.

As a second approach we have compared LDL that had been modified *in vitro*, with LDL that had been isolated from normal, diabetic, and renal insufficient subjects (Table 2-1). Diabetic and renal insufficient patients have been shown to exhibit higher levels of AGE-LDL than normal healthy subjects (Lyons, 1986). However, in the present study, by a number of criteria, we were unable to demonstrate AGE modification of LDL from the diabetic and renal insufficient subjects. While we show for LDL modified *in vitro* by F6P that there is a direct correlation between the level of apoB glycation and electrophoretic mobility of the LDL sample, the increases in the mobility of the LDL from diabetic and renal insufficient patients compared to LDL from controls were, at most, modest. Furthermore, the LDL from the diabetic and renal insufficient patients did not react with mAb 4G9, an antibody which has been shown to detect AGE adducts that had been formed either *in vitro* and *in vivo*. Based on our data (Figure 2-13) we know that

4G9 may be not sensitive enough to detect low concentrations of *in vitro* modified-AGE-BSA, and this may account for the lack of 4G9 immunoreactivity. A study by Makita *et al.* indicates that immunologically similar AGE structures form during the preparation of AGEs *in vitro*, regardless of whether the AGEs are prepared from proteins or amino acids (Makita *et al.* 1991). However, this has not been demonstrated to be true *in vivo*. In other words, due to the great heterogeneity of the AGE products, the mAb used in our study may not have been specific for AGE adducts present in LDL isolated from our cohort of diabetic and renal insufficient patients. This points to the need for further structural studies of the advanced glycation pathway as it occurs *in vivo*, under native conditions.

It is reported that circulating AGE-modified LDL (AGE-LDL) present in high levels in plasma of patients with diabetes or renal insufficiency exhibit impaired clearance kinetics when injected into transgenic mice expressing the human LDL receptor (Bucala *et al.*, 1994). In another study conducted by Kramer-Guth it was shown that the uptake of LDL prepared from the plasma of patients with non-insulin-dependent diabetes mellitus (NIDDM) in cultured human hepatoma cells is largely reduced (Kramer-Guth, 1997). LDL from patients with NIDDM was abnormal with regard to lipid composition and the conformation of the apolipoprotein B receptor-binding domain. We were, however, unable to detect any difference in the receptor-binding ability of patients' LDL compared to that of healthy control subjects' LDL. It is possible that the AGE modification of the LDL was insufficient to produce changes in LDL receptor-binding activity. LDL used for this study were prepared from frozen plasma. In order to see if freezing the plasma may have altered the LDL receptor-binding activity of LDL, we compared the receptor binding ability of LDL isolated from plasma donated by a healthy

subject that had been frozen at -80 °C for 24 hours or kept at 4 °C for 24 hours. The LDL isolated from a frozen plasma had a slightly better binding ability than that prepared from unfrozen batch (not shown). While the difference was small, we cannot exclude that alteration in the LDL receptor-binding due to freezing the plasma may have masked differences between the LDL of patients and controls.

LDL from the nine subjects were tested in a competitive apoB radioimmunometric assay. It has been found that glycation of hemoglobin *in vivo* and *in vitro* affects different sites (Shapiro, 1980). A clear representation of all the potential *in vivo* modification sites on apoB has not been shown. However, the present study demonstrates some of the possible differences and similarities in AGE-modification of LDL *in vitro* to that *in vivo*. Some of the mAbs which showed reduced reactivity with LDL from diabetic and renal insufficient subjects, have also been demonstrated earlier (Figure 2-3) to lose immunoreactivity with *in vitro* AGE-modified LDL. Nonetheless, other mAbs which showed reduced immunoreactivity with *in vitro* AGE-modified LDL did not show any changes in immunoreactivity with the LDL isolated from the subjects of this study. Although, incubation with F6P results in greater LDL modification than that seen *in vivo* some changes in epitope expression of apoB are apparent in patients that are not seen in *in vitro* modified LDL.

It is possible that the observed altered apoB immunoreactivity of LDL isolated from diabetic and renal insufficient subjects may reflect changes in LDL size or composition. This is unlikely to be the case. LDL from the controls and from the patients showed little difference in terms of either particle size (Figure 2-17) or particle composition (Figure 2-18). Furthermore, it has recently been demonstrated using the

same solid phase radioimmunoassay and the same panel of mAbs that the expression of most epitopes of apoB was remarkably similar in VLDL, IDL, and LDL sub-fractions (Wang *et al*, 2000). The only change in epitope expression occurs as large buoyant LDL are converted to smaller less buoyant particles, and is detected by mAbs 4H11 and 605 that are specific for epitopes in the carboxy terminus of apoB-100. Neither 4H11 nor 605 were amongst the epitopes whose expression differed between control subjects and patients in this study.

## Chapter V

### Conclusions and Future Work

In conclusion, the present data provide a molecular structural basis for beginning to assess how advanced glycation end-products can alter LDL metabolism. We have demonstrated that modification of lysine, and not arginine, residues of apoB may be largely responsible for the defective binding of AGE-LDL to the LDL receptor. Based on previous observations, it is probable that the critical lysine residues are situated outside of the apoB sequence 3,359-3,367 that is thought to be directly implicated in apoB-mediated binding to the LDL (Boren *et al*, 1998). In addition we have shown that there are both similarities and differences in apoB epitope expression in LDL that has been modified *in vitro* and LDL modified *in vivo* under patho-physiological conditions of diabetes or renal insufficiency. However, there is still a need for better characterization of AGE-LDL from a larger sample population of diabetic and renal insufficient patients or diabetic patients with end-stage renal disease. Furthermore, a study of an animal model such as, streptozotocin-induced diabetic human apoB transgenic mice would be very informative. The precise chemical nature of the apoB-AGE that are formed *in vivo* are needed to assist in the design of more specific pharmacological inhibitors of AGE-LDL.

It has previously shown that inclusion of the 2D8 mAb, which has been mapped to a region that overlaps the major AGE modification site on apoB, during the glycation of LDL could protect the 2D8 epitope from modification but could prevent neither the acquisition of AGE immunoreactivity by apoB nor the loss of receptor-binding activity of

the LDL (Wang X. and Milne R., Unpublished results). It will be interesting to repeat this experiment with other mAbs specific for glycation-sensitive activity, particularly those mAbs that recognize epitopes close to the LDL receptor-binding site.

Recently in our laboratory the expression of a functional soluble form of the LDL receptor in the Chinese hamster ovary (CHO) cells has been achieved. This soluble receptor binds to human LDL and not to acetylated human LDL (unpublished results). It will be possible to incubate human LDL with this soluble receptor prior to glycation, subsequently remove the receptor, and test the LDL for binding to the LDL receptor on cultured fibroblasts. This strategy will help evaluate the contribution of other AGE-sensitive sites outside the putative LDL receptor-binding site to the defective binding of the AGE-LDL to the LDL receptor.

In the present study we observed that incubation of LDL with DKT does not fully protect epitopes such as that recognized by mAb 4G3 from subsequent modification by F6P-mediated glycation. Both lysines and arginines contribute to these epitopes and both could be susceptible to AGE modification. It will be important to show that treating the LDL with both DKT and CHD, and therefore, protecting both the lysine and the arginine residues of apoB from glycation can protect these epitopes. As an accurate apoB epitope map has been established, these experiments will help to identify arginine and lysine residues that are susceptible to AGE modification within apoB.

In this study I have concentrated on the role of AGE-modification of apoB on LDL function. It is known, however, that AGE modification are present on both apoB and phospholipid components of LDL such as phosphatidylethanolamine (Bucala *et al*, 1994). Many studies have demonstrated that the lipid component of LDL can modulate

apoB interaction with the LDL receptor (Bucala *et al*, 1994). It will be important, therefore, to determine the possible contribution of lipid-AGE in defective binding of AGE-LDL to the LDL receptor.

## Chapter VI

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2, **Shermin Rahimkhani**, Xingyu Wang, Vinita Chauhan, Stephanie Walter, and Ross W. Milne. Immunochemical and functional characterization of advanced glycation end-products-modified low density lipoproteins (manuscript in preparation)

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