A study of nonenzymatic-glycosylated-low-density-lipoprotein binding using the calf adrenocortical low-density lipoprotein receptor.

Peter Catomeris
University of Windsor

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A STUDY OF
NONENZYMATIC-GLYCOSYLATED-LOW-DENSITY-LIPOPROTEIN BINDING USING
THE CALF ADRENOCORTICAL LOW-DENSITY LIPOPROTEIN RECEPTOR

by

PETER CATOMERIS

A DISSERTATION
Submitted to the Faculty of Graduate Studies and Research through the
Department of Chemistry and Biochemistry in
Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy at the
University of Windsor

Windsor, Ontario, Canada
1992
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ABSTRACT

A STUDY OF

NONENZYMATIC GLYCOSYLATED-LOW-DENSITY LIPOPROTEIN BINDING USING

THE CALF ADRENOCORTICAL LOW-DENSITY LIPOPROTEIN RECEPTOR

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In vitro studies have shown that nonenzymatic glycosylation of low-density lipoprotein (LDL) reduces its ability to bind to the LDL receptor. It has been suggested that this may contribute to the increased risk of atherosclerosis associated with diabetes, since modified LDL is cleared from plasma by atherogenic pathways.

In order to test the ability of LDL from diabetics to bind to the LDL receptor, a competitive binding assay was developed in which the calf adrenocortical LDL receptor was used as the binder. The ability of LDL to bind to the receptor was determined indirectly by its ability to displace control $^{125}$I-LDL from the receptor.

It was shown that the calf adrenocortical LDL receptor is an acceptable substitute for the human LDL receptor for studying human LDL binding. It demonstrated the same binding specificity towards human lipoprotein fractions and had a similar dissociation constant ($8.8 \pm 1.0 \mu g ^{125}$I-LDL/mL) as the human LDL receptor. Furthermore, the calf adrenocortical receptor was able to distinguish between LDL preparations with different extents of nonenzymatic glycosylation.

The LDL receptor was purified to a semi-pure solubilized form in order to minimize the number of steps required in the purification and yet allow simple binding assays to be performed. The binding assay of the present study of nonenzymatic glycosylated LDL required shorter incubation periods and fewer steps than binding assays of previous studies which used intact
cultured cells.

$^{125}$I-LDL displacement of LDL isolated from subjects values did not correlate very well with any of the indicators of glycemic control (plasma glucose and fructosamine, and glycosylated hemoglobin). Correlation coefficients were -0.173, -0.133 and -0.067, respectively.

LDL from subjects (whether diabetic or not) whose plasma triglyceride concentrations were greater than 5.0 mmol/L had a reduced ability to bind to the LDL receptor when compared to LDL from subjects whose plasma triglyceride concentrations were less than 5.0 mmol/L.

The data suggests that hypertriglyceridemia rather than hyperglycemia plays a role in affecting LDL binding to the LDL receptor in diabetics.
ACKNOWLEDGEMENTS

Once again, I would like to express my sincerest gratitude to my supervisor, Dr. Roger J. Thibert. His role during my graduate studies was not simply as an intellectual leader, but more importantly as a friend who stood by my side during times of frustration and doubt.

I would also like to acknowledge the critical contributions made to this dissertation by Drs. N.F. Taylor, T.F. Draisey, K. Adeli, J.R. Monforte, L.R. Sabina, H.B. Fackrell and R.R. Calam.

The burden of typing this manuscript was once again placed upon the shoulders of Maeve Doyle-Catomeris. The professional quality of this document is a testament to her skill. (I also want to thank her for not filing for divorce in the midst of this horrendous chore.)

I would like to sincerely acknowledge the combined efforts of Dean D.T.N. Pillay, Dr. John Drake, Mr. Dave Hill, Mr. Jim Olsen, Mr. Archie Glasgow (and of course RJT) for getting me up and "spinning" after the untimely demise of the Beckman Type 65 rotor, Serial #3463.

The continued help and support of Dean Lois K. Smedick and the entire staff at the Faculty of Graduate Studies and Research is greatly appreciated.

The staff of the clinical laboratory at the Salvation Army Grace Hospital have always been gracious and welcoming. This research project could not have been completed without their assistance.

I would like to thank my friends Pardu Ponnapalli and Dave McKenzie, who by example taught me about leadership and commitment.

I would like to extend my best wishes to all the students, staff and faculty of the Department of Chemistry and Biochemistry. I thank you for your help, stimulating discussions and, most of all your cherished friendships.

To the University of Windsor, so long and thanks for all the fish!
DEDICATION

To All My Family,

for their continued love and encouragement.

To Maeve and Conor,

for the great joy they have brought me.
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<td>PAGE</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SR</td>
<td>specific radioactivity</td>
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<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
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</tr>
<tr>
<td>TNBS</td>
<td>2,4,6-trinitrobenzenesulfonic acid</td>
<td></td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) methylamine</td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>very-low-density lipoprotein</td>
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“Practice safe ultracentrifugation ... keep your O-rings clean and lubricated!!"
CHAPTER 1

INTRODUCTION

1.1 Atherosclerosis

1.1.1 Definition

Arteriosclerosis [from the Greek: \textit{arteria} meaning artery, and \textit{skeiros}, meaning hard (1)] is a generic term, referring to the three types of vascular disease which cause the thickening and inelasticity of arteries (2). \textit{Mönckeberg's medial calcific sclerosis} is encountered in medium-sized muscular arteries, but is not of clinical significance since the lesions do not encroach on the vessel lumen (3). \textit{Arteriolosclerosis} involves small arteries and arterioles and occurs most prominently in the kidneys (3,4). \textit{Atherosclerosis}, the most dominant form of arteriosclerosis, affects primarily the large arteries (aorta and main branches), but also the small arteries, particularly the coronary and cerebral arteries (3,4). The disease is marked by atheromas, which are focal areas of lipid deposition, smooth muscle cell proliferation, enhanced collagen formation and sometimes calcification (3-5).

1.1.2 Clinical Significance

The link between coronary heart disease and atherosclerosis was first made in 1912 by James Herrick, a cardiologist (6). It is now known that the formation of atheromas in atherosclerosis results in narrowing of the vascular lumens, and a reduction in blood supply to the heart as well as other tissues and organs of the body (3,4). In middle age and in later life, the obstruction may be sufficient to result in serious or fatal ischemia of vital organs. As mentioned previously, atherosclerosis has a predilection to develop in the aorta and the arteries of the heart and brain. Coronary heart disease, the most important form of which is myocardial infarction, accounts for about 90\% of all forms of heart disease (3). A large percentage of cerebral strokes are the result of atheromatous involvement of the arteries supplying the brain.
(3). The cause of most aneurysms of the abdominal aorta has also been attributed to atherosclerosis (3). Organ injuries induced by atherosclerosis account for most deaths in Canada (see Figure 1.1) (7).

1.1.3 Pathogenesis

Although many aspects of the pathogenesis of atherosclerosis remain unclear, there appear to be four stages in the disease process: 1) development of the fatty streak; 2) formation of a fibrous plaque; 3) formation of a complex lesion; and 4) thrombosis (3-6,8). (See Figure 1.2.)

The first step in atherosclerosis is chronic mild injury to the arterial endothelium. This is caused mainly by a disturbance in the pattern of blood flow in certain parts of the arterial tree, such as bending points and areas near branching vessels (9). Chronic minimal endothelial injury can also be brought about by hypercholesterolemia, circulating vasoactive amines, immunocomplexes, infection, and chemical irritants in tobacco smoke (9).

The earliest recognized gross lesion in atherosclerosis develops at the sites of endothelial injury. This is a lipid deposit which appears as an opaque yellow dot or streak just beneath the endothelium of affected arteries, and hence has been called a "fatty streak". The fatty streak is characterized by an accumulation of cells loaded with cholesteryl esters. These cells are referred to as "foam cells" and are derived mainly from circulating monocytes and, to some extent, from medial smooth-muscle cells (10).

The lesion which appears in the second stage of atherosclerosis is made up of a core of lipid-rich debris surrounded by capsules of smooth muscle and connective tissue, and is known as a "fibrous plaque". While it is believed that the fibrous plaque is the lesion that leads to clinical disease, it is not clear whether it arises by a process dependent on the formation of the fatty streak (3,6).

Fibrous plaques, once formed, undergo a number of qualitative changes to produce more
FIGURE 1.1
Age-Standardized Mortality Rates by Cause of Death,
Canada 1987-1988

Legend
Age-standardized mortality rates (ASMR) by cause of death for Canada in the two-year period of 1987-1988 are shown. ASMR were highest for coronary heart disease in both males and females. Major disease categories were selected using the ninth revision of the International Classification of Disease. [Reproduced from Wilkins and Mark (7) with the permission of the authors.]
FIGURE 1.2

Stages of Atherosclerosis

Legend

Injury to the arterial endothelium causes accumulation of monocytes and low-density lipoprotein (LDL) at the site of damage. Monocytes penetrate the intima to become macrophages and ingest LDL. The monocytes become so loaded with cholesteryl esters as to develop into "foam cells". The fatty streak, the earliest gross lesion in atherosclerosis, is characterized by an accumulation of foam cells. Release of growth factors by macrophages and platelets leads to proliferation and migration of smooth muscle cells into the intima. These cells secrete collagen, forming a fibrous plaque. Growth of the plaque causes distortion of the arterial-wall architecture and the development of a more complicated lesion. Ulceration of the lesion leads to thrombus formation. When thrombi are small, they can become organized and contribute to the growth of the atherosclerotic plaque. When the thrombi are large and occlusive, they can cause myocardial infarction and sudden cardiac death. [Adapted from Schaefer (5).]
complex lesions which lead to arterial occlusion. Growth of the plaque causes distortion of the
arterial-wall architecture, with the subsequent rupture of arterioles that have grown into the
plaque, calcification within the arterial wall, and ulceration of the inner portion of the fibrous-
plaque capsule (5,6).

The final stage in atherosclerosis is the terminal occlusive episode. Examples of
suggested mechanisms are: 1) an atheromatous embolism due to rupture of a fibrous plaque; 2)
hemorrhage and rapid swelling of a plaque; and 3) platelet aggregation and thrombosis on
ulcerated surface of plaque (5,6,11). In most cases of myocardial infarction, fatal or non-fatal,
and in many cases of sudden cardiac death without infarction, thrombosis appears to be the
immediate cause of the occlusive episode (6,9).

1.1.4 Risk Factors

Atherosclerosis is a complex disease process governed by genetic, cellular, molecular and
environmental components. Nevertheless, the major risk factors for coronary atherosclerosis
identified by The Expert Panel of the National Cholesterol Education Program (12) are listed in
Table 1.1. Since it is beyond the scope of this introduction to discuss all of these factors, the
reader is referred to current reviews (3,4,6,12,13).

The development of atherosclerosis has been strongly linked to elevated blood cholesterol
levels in a large number of epidemiologic, clinical and biochemical studies (13-15). Furthermore,
it has been well established that elevated serum cholesterol due to high levels of low-density-
lipoprotein-cholesterol significantly increases the risk for atherosclerosis (3-6,12-15). On the
other hand, decreased high-density-lipoprotein-cholesterol has been related to an increased risk
in some, but not all, studies (5,13,15). Although many patients with coronary heart disease have
elevated triglyceride levels, it has yet to be shown that this is a cause of the disease (6,13,15).
TABLE 1.1
Major Risk Factors for Coronary Atherosclerosis*

- LDL** cholesterol of 160 mg/dL or greater
- Male Sex
- Family history of premature coronary heart disease
defined as definite myocardial infarction
or sudden death in a parent or sibling
prior to age 55
- Cigarette smoking (> 10 cigarettes/day)
- Hypertension
- HDL-cholesterol of < 35 mg/dL, confirmed by repeat measurement
- Diabetes Mellitus
- History of cerebrovascular or occlusive peripheral
vascular disease
- Severe obesity (30% or more above ideal body weight)

* As defined by the Expert Panel of the National Cholesterol Education Program (12)

** LDL: low-density lipoprotein; HDL: high-density lipoprotein
A great deal of research has been devoted to explaining the mechanism by which elevated cholesterol, particularly LDL-cholesterol, in plasma leads to the acceleration of atherosclerosis.

1.2 Low-Density Lipoprotein

1.2.1 Definition

The transport of lipids in plasma is facilitated by lipoproteins, spherical particles consisting of an inner core of neutral fat (triglycerides and cholesteryl esters) surrounded by a surface of polar molecules (proteins, phospholipids, and unesterified cholesterol). There are five major classes of lipoproteins in normal plasma, defined in terms of their hydrated densities or electrophoretic mobilities (see Table 1.2) (5,16-18). The classes also differ in the relative amounts of their constituents as well as the particular proteins (designated apolipoproteins) they possess.

LDL can be defined as the total population of lipoproteins in normal human plasma within the density range of 1.019-1.063 g/mL (17).

1.2.2 Composition and Physical Characteristics

Although normal LDL isolated in an analytical centrifuge appears as a single symmetrical peak, as shown by Schlieren curves, the size and composition of the particles vary continuously across the whole density range (17). In fact, it has been shown, by methods with greater resolving power than flotation ultracentrifugation, that LDL from some normal subjects contains two or more subpopulations, with different mean densities and molecular weights (19).

LDL migrates in the β-globulin region upon zonal electrophoresis on agarose gel, and is sometimes referred to as β-lipoprotein. The molecular weight and size of LDL vary from 2.0-2.5 x 10^6 kDa and 190-250 Å, respectively (17).

In general, lipids comprise 75-80% of the mass of the LDL particle. Of the total lipid, 60% is cholesterol, approximately 80% of which is in the form of cholesteryl esters in the core
<table>
<thead>
<tr>
<th>Class</th>
<th>Density Range (g/mL)</th>
<th>Electrophoretic Mobility</th>
<th>Composition (% weight)</th>
<th>Apolipoproteins</th>
</tr>
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<td></td>
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</tr>
<tr>
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<td>Origin</td>
<td>1-2</td>
<td>85-95</td>
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<td>0.98-1.006</td>
<td>Pre-β</td>
<td>6-10</td>
<td>50-65</td>
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<tr>
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<td>β to pre-β</td>
<td>15-20</td>
<td>20-30</td>
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<tr>
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<td>β</td>
<td>18-22</td>
<td>4-8</td>
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<tr>
<td>HDL</td>
<td>1.063-1.21</td>
<td>α</td>
<td>45-55</td>
<td>2-7</td>
</tr>
</tbody>
</table>

* Information taken from Schaefer (5), Brewer et al. (16), and Myant (17)

** VLDL: very-low-density lipoprotein; IDL: intermediate-density lipoprotein; LDL: low-density lipoprotein; HDL: high-density lipoprotein
of the particle. Linoleate accounts for 50% of the total cholesteryl-ester fatty acids while oleate and palmitate comprise 20% and 15% of the total, respectively (18). Of the total lipid, 20% is phospholipid, primarily phosphatidylcholine (65%) and sphingomyelin (25%) (18).

LDL contains essentially only apolipoprotein B100 (apoB100), although small amounts of apoCIII and apoE have been detected in human LDL (20). The molecular weight of apoB100 has been calculated to be 513 kDa, based on the deduced amino acid sequence of the DNA coding for it (21). However, the value determined by gel electrophoresis is approximately 550 kDa (17). Each particle of LDL contains one molecule of apoB100 monomer. Although the precise orientation of apoB100 in the outer shell of LDL has not been established, it is clear from its susceptibility to partial hydrolysis by proteolytic enzymes, as well as other evidence (17), that at least part of the apoB100 is exposed on the external surface of the LDL particle. Based on the fact that the hydrophobicity profile of apoB100 shows many regions in which hydrophobic sequences alternate with hydrophilic sequences, Knott et al. (22) suggested that apoB100 is woven in and out of the LDL particle at irregular intervals. The interaction of apoB100 with LDL lipid at multiple sites distinguishes it from other apolipoproteins, which only possess a single lipid-binding domain, and could explain why apoB100 does not exchange between lipoproteins (17).

1.2.3 Biological Function

It was first demonstrated by Bailey (23) that mammalian cells, grown in culture, do not produce their own cholesterol but preferentially use the exogenous cholesterol contained in the lipoproteins present in the culture medium. The majority, 60-70%, of the plasma cholesterol is carried in LDL (17,18). It was therefore, postulated by Brown and Goldstein (24), and later shown by others that LDL provided an important source of cholesterol for the cells of many tissues.

It should be pointed out at this time that, in man, HDL is the second major carrier of
plasma cholesterol. HDL containing apoE is recognized by LDL receptors and by hepatic apoE receptors (25). It is thought that uptake of HDL by these receptors on the liver provides the body with a mechanism for transporting surplus cholesterol from extra-hepatic cells to the liver.

1.2.4 Metabolism

1.2.4.1 Anabolism

Plasma LDL is produced from very-low-density lipoprotein (VLDL), one particle of VLDL generating one particle of LDL.

In hepatocytes, the apolipoproteins B100, C and E are synthesized on polyribosomes bound to the rough endoplasmic reticulum (ER) and enter the lumen of the ER (26). During synthesis, the growing peptide of apoB100 weaves in and out of the phospholipid monolayer of the luminal face of the ER membrane. After cleavage of the signal sequence and N-glycosylation, the protein migrate to the junctions of the rough and smooth ER. A VLDL particle is formed when the apolipoprotein-phospholipid complex encloses a droplet of triglycerides and cholesteryl esters, synthesized on the cytoplasmic surface of the smooth ER. Addition of O-linked oligosaccharides and processing of the N-linked oligosaccharides is then carried out on the newly-formed particles, the site of which has not been clearly established (17).

After its secretion by the liver, the nascent VLDL particles acquire additional apoE and apoC from high-density lipoprotein (HDL) (17). In addition, cholesteryl esters generated in HDL by the lecithin:cholesterol acyltransferase (LCAT) reaction, are then transferred to VLDL by a cholesteryl-ester transfer protein. In the plasma, more than 90% of the triglycerides are hydrolyzed and removed from VLDL particles by the action of lipoprotein lipase, located in the luminal surface of the endothelium of the blood capillaries. In the process, most of the apoC and some of the apoE is recycled to HDL. The resultant particles, known as VLDL remnants or intermediate-density lipoprotein (IDL), have a higher cholesteryl ester: triglyceride ratio and
density than VLDL.

A considerable amount of the IDL (50-90%) is converted into LDL, while the remainder is taken up by the liver and other tissues via the LDL-receptor pathway (17). The conversion of IDL to LDL involves the acquisition of additional cholesteryl esters from HDL, facilitated by a transfer protein, further triglyceride removal, as well as the loss of all apolipoproteins except apoB100. Hydrolysis of triglycerides in IDL is thought to occur primarily by the action of hepatic lipase, an enzyme attached to the luminal surface of the liver sinusoids, and to some extent by lipoprotein lipase.

The rate of production of LDL is dependent on two factors: 1) the rate of production of VLDL by the liver; 2) the rate of conversion of VLDL to LDL in the circulation. Long-chain fatty acids have been shown to stimulate VLDL secretion (27). In vitro studies have shown that insulin stimulates synthesis of triglyceride and other VLDL lipids, and inhibits the synthesis of apoB100 and the secretion of VLDL in hepatocytes (28,29). Some in vivo studies (30,31) also suggest that insulin decreases VLDL secretion. 3,5,3'-Triiodothyronine (T₃) stimulates the production of apoB100 in human hepatocytes in vitro (32). Glucagon inhibits VLDL triglyceride and cholesterol secretion, although the latter effect is less pronounced (33).

The factors which determine the rate of conversion of VLDL to LDL remain to be fully determined, but VLDL particle size appears to be one determinant. Large VLDL particles, such as those produced after carbohydrate feeding, are rich in triglyceride and are removed directly from the circulation by the liver, resulting in low LDL production (17). Small VLDL particles, such as those produced after cholesterol feeding, remain in the circulation, resulting in increased production of LDL (17).
1.2.4.2 Catabolism

**LDL-Receptor Pathway**

The studies of Brown and Goldstein (18,24,34) established the existence of an LDL receptor and the mechanism by which cells are able to transport non-polar cholesteryl esters from plasma lipoproteins to the interior of the cell. The pathway, shown in Figure 1.3, involves the following steps: 1) binding of LDL to the high-affinity LDL receptor on the cell surface; 2) internalization of the LDL/receptor complex by endocytosis; 3) delivery of LDL to lysosomes; 4) hydrolysis of cholesteryl esters to produce free cholesterol; 5) recycling of LDL receptor to the cell surface. The release of free cholesterol causes: 1) the inhibition of activity of the rate-limiting enzyme of cholesterol synthesis, β-hydroxy-β-methylglutaryl coenzyme A reductase (HMG-CoA reductase); 2) the stimulation of the enzyme leading to its reesterification, acyl coenzyme A:cholesterol acyltransferase (ACAT); 3) the down regulation of the number of LDL receptors expressed on the cell surface.

It has been established that the LDL receptor is also able to bind apoE, an apolipoprotein present in several lipoproteins other than LDL. Based on genetic analysis of apoE isoforms, as well as site-directed mutagenesis and antibody-competition studies (35-38), it has been concluded that the binding domain extends from residue 142 to 150 of apoE. As is shown in Figure 1.4, this region of the protein is rich in basic amino acids (lysine and arginine). Two regions of apoB100 (residues 3147-3157 and 3359-3367) contain high proportions of basic amino acids (39,40), with the latter sequence having a significant degree of homology with the receptor-binding domain of apoE (see Figure 1.4). Based on monoclonal antibody studies, it has been concluded that there is a single receptor-binding domain on apoB100 (17,41). Yang et al. (40), showed that a synthetic peptide, containing the sequence 3359-3367, binds with high affinity to the LDL receptor, supporting the suggestion that this sequence is part of the apoB100 receptor.
FIGURE 1.3

The LDL-Receptor Pathway

Legend

The sequential steps in the LDL-receptor pathway are: 1) LDL receptors, synthesized on the rough endoplasmic reticulum, are transported to Golgi cisternae (possibly in vesicles) for processing; 2) the mature receptor is transported in a small coated vesicle for insertion into the plasma membrane; 3) receptors become clustered in pits, coated with a variety of proteins, particularly clathrin; 4) LDL binds to the receptor through apoB100; 5) the coated pit (including the LDL/receptor complex) is internalized by endocytosis; 6) dissociation of the LDL from the receptor follows a rapid fall in pH in the endocytic vesicle containing the complex; 7) tubular extensions, carrying membrane-bound receptors, bud off the endocytic vesicle; 8) receptors and any undissociated LDL are transported to the plasma membrane; 9) the rest of the endocytic vesicle fuses with a lysosome; 10) LDL and any undissociated receptors undergo lysosomal degradation to produce amino acids and free cholesterol; and 11) the release of free cholesterol causes the stimulation of ACAT (the enzyme leading to its reesterification), the inhibition of HMG-CoA reductase (the rate-limiting enzyme of cholesterol synthesis), and the down regulation of the number of LDL receptors expressed on the cell surface. [Adapted from Myant (17) and Brown et al. (34).]
FIGURE 1.4

Binding Domains of ApoE and ApoB100

Legend

It is believed that the binding domain of apoE extends from residue 142 to 150, a region rich in basic amino acids (lysine and arginine). Two regions of apoB100 (residues 3147-3157 and 3359-3367) contain a high proportion of basic amino acids, with the latter having a significant degree of homology with the receptor-binding domain of apoE. Homologous basic amino acids of apoE and apoB100 are enclosed in boxes. [Adapted from Myant (17).]
FIGURE 1.4


ApoB100  Arg^{1}_{1}Leu-Thr^{1}_{1}Arg-Lys-Arg^{1}_{1}Gly-Leu^{1}_{1}Lys^{1}_{1}Leu-Ala-Thr-Ala-Leu---
           142  150

ApoE3    Arg^{1}_{1}Lys-Leu^{1}_{1}Arg-Lys-Arg^{1}_{1}Leu-Leu^{1}_{1}Arg^{1}_{1}Asp-Ala-Asp-Asp-Leu---
           1  16
binding domain. Milne et al. (41) have proposed a model for the binding domain of apoB100, in which the two basic-amino-acid sequences above are brought together by a disulphide bridge.

The mature LDL receptor is a transmembrane glycoprotein, consisting of a single polypeptide chain of 8–9 amino acids (24). Based on the deduced amino-acid sequence and the organization of its gene, Brown and Goldstein (34) suggested that the receptor is divided into five structural domains, each adapted to carrying out one component of the overall function of the receptor, including binding of ligands and participation in the endocytic cycle. The first domain consists of the N-terminal 292 amino acids, divided into seven imperfect repeats of about 40 amino acids each. Each repeat contains 6 cysteine residues, all of which are disulphide-bonded, suggesting that the domain exists in a tightly cross-linked, convoluted state. At the C-terminus of each repeat in the first domain is a cluster of negatively-charged amino acids, which forms a complement to the positively-charged receptor-recognition sites of apoE and apoB100.

In addition to the liver, a variety of tissues including the adrenal gland, corpus luteum, intestine and arterial smooth muscle, have been shown to possess functional LDL receptors (42).

LDL receptor activity on cells is controlled by many factors. Brown and Goldstein (43) demonstrated that fibroblasts, incubated in the presence of LDL, express a progressive and concentration-dependent decrease in the number of receptors. Hepatocytes in culture are also subject to feedback regulation by LDL, but show only a 25-50% reduction in receptor activity by LDL concentrations that cause almost complete suppression of activity in fibroblasts (44). Kovanen et al. (45) showed that cholesterol feeding suppressed LDL-receptor activity in the liver of rabbits. Several hormones, including thyroxine (46), estrogens (47) and insulin (48) have been shown to stimulate LDL-receptor activity.

LDL-Receptor-Independent Pathway

Two lines of evidence support the presence of an LDL-receptor-independent pathway.
First, patients with homozygous familial hypercholesterolemia (FH), a disease in which there are no functional LDL receptors, are capable of catabolizing plasma LDL. Second, LDL which has been chemically modified so that LDL-receptor binding is completely abolished, can still be cleared from the plasma. In FH homozygotes, LDL-derived cholesterol accumulates selectively in cells of the reticuloendothelial system, especially in macrophages and other cells, known collectively as scavenger cells (17). The mechanisms by which this occurs are thought to be: 1) adsorptive endocytosis by cell-surface receptors with low-affinity for LDL; 2) pinocytosis; and 3) uptake of modified LDL by high-affinity receptors on cells, including the acetyl-LDL receptor. Degradation of LDL by the LDL-receptor-independent pathway is thought to be unregulated and nonsaturable, and therefore, the absolute rate of degradation of LDL by this route is directly proportional to plasma LDL concentration (17).

**Total Catabolic Pathways**

The contributions of the LDL-receptor-dependent and LDL-receptor-independent pathways *in vivo* have been estimated by several investigators. Total rate of catabolism of LDL by all routes in the whole body, *i.e.*, receptor-dependent + receptor-independent pathways, is determined from measurements of the rate of disappearance of radiolabelled native LDL from the circulation. The fractional clearance rate (FCR) of LDL by the receptor-independent pathway is determined similarly using LDL in which the receptor-binding domain of apoB100 has been blocked by chemical modification. The FCR of LDL by the receptor-dependent pathway is then calculated as the difference between the total FCR and the receptor-independent FCR. Values for the receptor-dependent FCR have varied from 60% using 1,2-cyclohexanediol-modified LDL (49) to 80% using glucose-modified LDL (50). As a comparison, Goldstein and Brown (18) determined that the receptor-dependent pathway is responsible for at least two thirds of total catabolism on the assumption that the receptor-independent FCR is equal to the total FCR of LDL.
in FH homozygotes, i.e., patients who have no receptor-dependent catabolism.

Studies by Pittman et al. (51) have shown that the liver has a dominant role in the catabolism of LDL, and is responsible for degrading 50-70% of the total LDL catabolized in the whole body. In addition, the LDL-receptor pathway accounts for up to 90% of total catabolism in the liver (52).

1.2.5 Role in Atherosclerosis

1.2.5.1 Elevation of Plasma LDL Levels

LDL functions to transport cholesterol from the liver to those cells of the body which cannot produce sufficient amounts to fulfill their needs. The LDL receptor may have evolved to provide the cell a means of taking up cholesterol from LDL for this purpose. The receptor binds LDL maximally at LDL levels of about 2.5 mg cholesterol/dL (53). Since the concentration of LDL in the interstitial fluid of man is only about 10% of that in plasma, a plasma concentration of 25 mg LDL-cholesterol/dL should be sufficient to maintain optimal function of the LDL receptor. Although the mean plasma LDL-cholesterol level is about fivefold higher than this "optimal" concentration in normal Western men, Brown and Goldstein (18,53) argue that a plasma level of LDL-cholesterol in the range of 25-60 mg/dL (total plasma cholesterol of 110-150 mg/dL) is an appropriate physiologic concentration. This is based on three arguments: 1) in other mammals that do not develop atherosclerosis, the plasma LDL-cholesterol level is less than 80 mg/dL; 2) the LDL level is 30 mg/dL in newborn humans, at a time before there are dietary and environmental stresses; 3) when humans are raised on a low fat diet, the plasma LDL tends to stay in the range of 50-80 mg/dL.

Diet and heredity are two major factors responsible for the elevation of plasma LDL levels. Although even moderate amounts of animal fat in the diet cause an increase in plasma cholesterol, the rise is not the same in every person, indicating the involvement of genetic factors
Entry of dietary cholesterol into the liver is mediated by the chylomicron remnant receptor, which is distinct from the LDL receptor (54). Since this receptor is not subject to feedback regulation (55), the liver can accumulate high levels of cholesterol when the diet contains excess fat. The liver, however, responds to this dietary cholesterol accumulation by decreasing the number of LDL receptors expressed (45). This suppression decreases the overall LDL-clearance capacity, invariably causing an increase in plasma LDL levels.

Dietschy and Spady (56) studied the metabolic consequences of increasing plasma-LDL concentration in rats under conditions where the number of LDL receptors are assumed to remain constant. They found that the rate of total uptake of LDL (sum of receptor-dependent and receptor-independent uptake) is related to the concentration of LDL in a curvilinear manner, i.e., at low LDL, the rate rises steeply but at concentrations greater than 200 mg/dL, the rate rises slowly and becomes directly proportional to the LDL concentration. On the other hand, the receptor-independent uptake of LDL is directly proportional to the LDL concentration at all plasma LDL levels. What this indicates is that at low LDL concentrations, receptor-dependent uptake dominates over receptor-independent uptake. Since the receptor-dependent pathway operates at high efficiency, FCR is high at low LDL concentrations. As the concentration of LDL increases above that required to saturate LDL receptors, receptor-independent uptake reaches a plateau and receptor-independent uptake makes an increasing relative contribution to the total uptake. Since this pathway operates at a low efficiency, the FCR at high LDL concentrations diminishes. This saturation phenomenon occurs independently of the number of LDL receptors.

1.2.5.2 Formation of Foam Cells

Newman and Zilveramit (57) showed that the cholesterol that accumulates in
atherosclerotic lesions originates primarily in plasma lipoproteins, including LDL. As mentioned in Section 1.1.3, these lesions are filled with scavenger cells that have accumulated so much cholesterol that they are converted into foam cells. Most of these scavenger cells are either resident macrophages of the arterial wall or from circulating monocytes that have taken residence in the wall at the site of endothelial damage.

That this process occurs by an LDL-receptor-independent pathway is supported by two lines of evidence. First, lesions rich with foam cells develop in patients with no functional LDL receptors (FH homozygotes). The second argument is that monocytes and monocyte-derived macrophages cannot be converted to foam cells by incubation even with high concentrations of native LDL (58).

It has been suggested that LDL must undergo some type of modification in order to be taken up by macrophages and generate foam cells. Brown and Goldstein (58) were the first to demonstrate that chemical acetylation converted LDL to a form recognized and taken up by macrophages. This uptake was attributed to a new receptor (designated the "scavenger" or "acetyl-LDL" receptor), which is distinct from the LDL receptor and does not recognize native LDL.

It was shown by subsequent in-culture studies that all three major types of cells in the arterial wall (endothelial cells, smooth muscle cells, macrophages) can induce changes in LDL which make the LDL recognizable by macrophages (59-61). The structural changes that occur to the LDL particle are initiated by the peroxidation of polyunsaturated fatty acids in the LDL lipids (62). The oxidative modification is absolutely dependent on low concentrations of copper or iron in the medium, and can be mimicked by the incubation of LDL in a serum-free medium in the presence of a sufficiently high concentration of copper or iron (63). Once the LDL contains fatty acid lipid peroxides, free-radical propagation can rapidly occur and lead to
extensive fragmentation of the fatty acid chains (10). Steinbrecher et al. (63) have demonstrated that the fragments of the oxidized fatty acids can attach covalently to apoB100, through ε-amino groups of lysine.

Macrophages ingest modified LDL and process the cholesteryl esters as follows: 1) in the lysosomes, the esters are hydrolyzed by an acid lipase; 2) the free cholesterol enters the cytoplasm where it is re-esterified, primarily with oleate as the fatty acid; 3) newly-formed cholesteryl esters are stored as droplets and undergo a continual cycle of hydrolysis and re-esterification (64). It should be pointed out that in the fatty streak, the principal cholesteryl ester is cholesteryl oleate (6) and it would appear, therefore, that this lesion arises from LDL processed by macrophages as described above.

On the other hand, in the fibrous plaque, the principal cholesteryl ester is cholesteryl linoleate (6), which is the primary ester present in LDL (18). In this lesion, it would appear that macrophage processing of LDL-cholesteryl esters has not taken place. In fact, it is known that these lesions have high levels of extracellular lipid accumulation due to intimal binding or trapping of LDL (65). Indeed, lesion progression is strongly associated with prominent extracellular lipid deposition rather than intracellular accumulation (65).

Brown and Goldstein (64) showed that HDL facilitates cholesterol excretion by macrophages. Under conditions where there is adequate plasma HDL, the free cholesterol produced by macrophages is not re-esterified but transported out of the macrophage and back to the liver, by a process termed "reverse cholesterol transport" (66). Under conditions where there is an excessive LDL load, the HDL is not sufficient to prevent the accumulation of cholesteryl esters by macrophages and their subsequent conversion into foam cells.
1.2.5.3 Development of the Fatty Streak

There is an increasing amount of evidence that oxidative modification of LDL greatly enhances its atherogenicity. The cytotoxicity of oxidized LDL, first observed by Hessler et al. (67) and Henriksen et al. (68) in culture, has been demonstrated in vivo by Kanazawa et al. (69). Studies by Quinn et al. have shown that oxidatively-modified LDL, but not native LDL, is a potent chemoattractant for circulating monocytes (70), as well as a potent inhibitor of the motility of the resident macrophage (71). In addition, it can induce cellular expression and secretion of colony stimulating factors, cytokines and growth factors (72).

Based on these types of observations, Steinberg et al. (10) proposed a hypothesis that the development of the fatty streak lesion could be accelerated by an increase in plasma LDL-cholesterol, and, therefore, an increase in the rate of oxidative modification.

1.3 Diabetes Mellitus

1.3.1 Blood-Glucose Regulation

Glucose is the major energy source for all cells of the body. The concentration of glucose in the blood is, therefore, maintained in a narrow range to ensure that all cells can maintain an adequate supply of energy at all times. This is accomplished by the coordinated effects of insulin, which tends to lower blood glucose, and several "counterregulatory" hormones (glucagon, cortisol, epinephrine, growth hormone) which tend to increase blood glucose (73,74).

1.3.2 Classification

High concentrations of glucose in blood, or hyperglycemia, due to diabetes mellitus (DM) is the most common disorder of carbohydrate metabolism. Diabetes is not a single disease but a heterogeneous group of disorders which have hyperglycemia or glucose intolerance in common. The National Diabetes Data Group (75) has proposed a classification for hyperglycemia disorders that has been widely adopted and is shown in Table 1.3.
# TABLE 1.3

**Classification of Diabetes Mellitus, and Other Types of Glucose Intolerance**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Diabetes Mellitus (DM)</td>
</tr>
<tr>
<td></td>
<td>I. Insulin-dependent type (IDDM)</td>
</tr>
<tr>
<td></td>
<td>II. Non-insulin-dependent type (NIDDM)</td>
</tr>
<tr>
<td></td>
<td>a. Obese NIDDM</td>
</tr>
<tr>
<td></td>
<td>b. Nonobese NIDDM</td>
</tr>
<tr>
<td></td>
<td>III. Glucose intolerance associated with certain conditions and syndromes</td>
</tr>
<tr>
<td>B</td>
<td>Impaired glucose tolerance (IGT)</td>
</tr>
<tr>
<td>C</td>
<td>Gestational diabetes (GDM)</td>
</tr>
<tr>
<td>D</td>
<td>Previous abnormality of glucose tolerance (Prev AGT)</td>
</tr>
<tr>
<td>E</td>
<td>Potential abnormality of glucose tolerance (Pot AGT)</td>
</tr>
</tbody>
</table>

* As defined by the National Diabetes Data Group (75).
The most common form of diabetes is non-insulin-dependent diabetes mellitus (NIDDM), which is also known as Type II, maturity onset, or non-ketotic diabetes (75). Nearly 90% of all diabetics are of Type II. Onset of NIDDM is usually in middle age (after age 40) and patients are usually obese (73,74,76). In spite of the fact that plasma insulin levels are often higher than normal, these patients are still hyperglycemic. In light of this, individuals with NIDDM are said to have a relative deficiency of insulin activity. This may be brought about by a number of factors: 1) production of mutant insulin with impaired biological activity; 2) decreased number of insulin receptor sites; 3) interference of insulin binding to receptor; 4) post-receptor defect (77). Most individuals with NIDDM are not dependent on insulin therapy, although some may require insulin injections or an oral anti-hyperglycemic agent if an improvement in obesity does not correct the hyperglycemic condition (73, 74, 76).

The second most common form of diabetes is insulin-dependent diabetes mellitus (IDDM), which is also known as Type I, juvenile onset, or ketosis-prone diabetes (75). Type I makes up only about 10% of all patients with DM. Onset of IDDM is usually before the age of 20, and patients are rarely obese (73,74,76). Individuals with IDDM produce very little insulin and are said to have an absolute deficiency of insulin. This may be brought on by several factors: 1) increased susceptibility to pancreatic beta cell damage; 2) infective (viral or chemical agents); 3) antipancreatic cell-mediated autoimmunity (77). Without insulin therapy, IDDM patients would quickly die.

1.3.3 Clinical Significance

It is estimated that there are as many as ten million persons with DM in the United States (73). Diabetics have a 1.5 to 3-fold increased risk of coronary heart disease compared to non-diabetics (15,78) and about 80% will die of some sort of cardiovascular disease (3,74). The pathology of atherosclerosis appears to be the same in the diabetic as in the nondiabetic (79), and
many of the risk factors in the diabetic are the same as those in the nondiabetic. For example, a common feature in diabetics is abnormal metabolism of lipoproteins, including hypertriglyceridemia and decreased HDL-cholesterol (76,80-82). There are some atherogenic factors unique to the diabetic, including altered LDL metabolism. Only the role of LDL as a risk factor for atherosclerosis in diabetics will be considered here.

1.3.4 Altered LDL Metabolism

1.3.4.1 Nonenzymatic Glycosylation

Nonenzymatic glycosylation (or glycation) is the covalent attachment of a sugar to a protein without the aid of enzymes (83-88). It has been known since 1912 that this reaction occurs in heated or stored foodstuffs, and is referred to by food chemists as the "Maillard" (after the first chemist to report it) or the "Browning" reaction (83). It was not known until 1966, when Holmquist and Schroeder (89) demonstrated the existence of a Schiff-base linkage on hemoglobin, that nonenzymatic glycosylation could take place under physiological conditions. Almost ten more years passed before the attached molecule was identified as glucose (90).

The first step in the reaction involves a condensation between the aldehyde group of a reducing sugar (e.g., glucose) and a free amino group (α- or ε-) of a protein, resulting in the formation of a Schiff base (or aldimine) and one molecule of water. The labile Schiff base may be hydrolyzed back to glucose and protein, or it may undergo an Amadori rearrangement to form a relatively stable ketoamine (1-amino-1-deoxy-2-ketone). The straight-chain ketoamine can then undergo cyclization to a hemiketal furanose or pyranose ring structure for added stability (84,85). The process described above is illustrated in Figure 1.5. More recently, it has been shown that these early glycosylation products (aldimine and ketoamine) can undergo a slow complex series of chemical rearrangements to form irreversible advanced glycosylation end products (88). In addition, some of these products can form covalent bonds with amino groups on other proteins,
FIGURE 1.5
Nonenzymatic Glycosylation of Proteins

Legend

Nonenzymatic glycosylation of proteins involves the reaction of glucose (through the
sidehyde group) and a free amino group (α- or ε-) of the protein, resulting in the formation of
a Schiff base (or aldimine) and a molecule of water. The labile Schiff base may be hydrolyzed
back to glucose and protein, or it may undergo an Amadori rearrangement to form a relatively
stable ketoamine (1-amino-1-deoxy-2-ketone). The straight chain ketoamine can then undergo
cyclization to a hemiketal furanose or pyranose ring structure for added stability. [Reproduced
from Armbruster (85), with permission of the author.]
Hypothetical stabilized furanose form

1 - amino - 1 - deoxyfructosamine
(isogluicosamine, the stable ketoamine)

Hypothetical stabilized pyranose form

Amadori rearrangement

1 - imino - 1 - deoxyglucose
(aldehyde; labile Schiff base)

Protein with free amine group, typically a lysine residue

HCOH
causing cross-linking of proteins (91).

Rahbar (92) was the first to report that nonenzymatic glycosylation of hemoglobin was appreciably increased in diabetics, to approximately double the values found in normal subjects. It has since been shown that a wide variety of proteins undergo increased nonenzymatic glycosylation in diabetic patients (83). The diagnostic value of glycosylated proteins, particularly hemoglobin, for assessment of glycemic control has been established (93). In addition, there is an increasing amount of evidence that protein glycosylation plays an important role in the development of the late complications of diabetes (83,88).

Schleicher et al. (94) were the first to demonstrate that purified human LDL incorporates glucose in vitro via ε-aminolysine groups in a manner dependent both on time of incubation and glucose concentration. It has been demonstrated that the LDL apoB100 of diabetics contains 2 to 33-fold increases in lysine-bound glucose compared to that from non-diabetics (94-97). Glycosylation of low-density lipoproteins in diabetics has also been significantly correlated with other parameters of glycemic control, including fasting blood glucose and glycosylated plasma protein levels (98,99).

Weisgraber et al. (100) showed that selective chemical modification of lysine residues of LDL apoB100 interfered with the recognition of LDL by LDL receptors on fibroblasts. Since it was known that nonenzymatic glycosylation of proteins involved Schiff base formation with free amino groups, such as the ε-amino groups of lysine, investigators studied the effect of in vitro glycosylation of LDL on its cell-interactive properties. It has been clearly established that in vitro glycosylation of LDL causes a reduction in the rate of binding, internalization and degradation by human fibroblasts, in proportion to the estimated degree of glycosylation (95,101-104). Whereas native LDL inhibits the activity of HMG-CoA reductase and stimulates ACAT (the enzymes regulating cholesterol synthesis and cholesterol esterification, respectively) in these
cells, glycosylated LDL (glcLDL) had no effects on these enzymes (95,101). Furthermore, in vitro glycosylation of LDL significantly impaired its binding with the LDL receptor of human endothelial cells (105).

The interaction of glcLDL with macrophages, the precursors of foam cells, has also been studied. Early studies indicated that glcLDL is not recognized by mouse peritoneal macrophages (94,101). More recent studies have shown that human monocyte-derived macrophages recognized and degraded glcLDL more extensively than native LDL (104,106). An increase in cholesteryl ester synthesis and accumulation in these macrophages were also observed. The above findings are in contrast, however, with those of Schleicher et al. (107) who did not find any difference between the metabolism of native and glcLDL by human macrophages.

Extensive glycosylation of LDL (approximately 40% of lysine residues) completely blocks receptor-mediated clearance from plasma in animals (95) and man (50). Modification of as few as 2-5% of lysines (on the order seen in diabetics) decreased LDL catabolism in guinea pigs by 5-25% (103). It should be pointed out, however, that there was no clear relationship between the fractional clearance rate and the extent of glycosylation, in the range of 2-5% glycosylation.

Witzum et al. (108) demonstrated that nonenzymatic glycosylation of LDL renders it immunogenic, leading to the uptake of the immunocomplex by macrophages via the Fc portion of the antibody (58).

There is no evidence in the literature that the LDL receptors themselves may be glycosylated. To the contrary, Lorenzi et al. (105) showed that human endothelial cells cultured in high glucose concentrations retained the ability to bind, internalize and degrade native LDL.

1.3.4.2 Glucose Autoxidation

Glucose can undergo enediol isomerization and thereby reduce molecular oxygen under physiological conditions, producing α-ketoaldehydes (109,110). These ketoaldehydes can
contribute substantially to the total monosaccharide attaching to protein during exposure to glucose (109).

In addition, glucose autoxidation produces \( \text{H}_2\text{O}_2 \) and free radical intermediates (109,110) and it was shown that added glucose stimulated lipid peroxidation in studies with isolated LDL \textit{in vitro} (111). These reactive oxidation products have been shown to cause protein fragmentation when proteins are incubated with glucose \textit{in vitro} (110). Since elevated levels of plasma peroxides have been found in diabetics (112,113), glucose autoxidation may play a role in LDL modification.

1.3.4.3 Changes in LDL Composition

Hypertriglyceridemia is a common biochemical manifestation in diabetes (75,114,115). It has been shown that the LDL separated from diabetic hypertriglyceridemic plasma is triglyceride enriched (116-118). Triglyceride-enriched LDL has an impaired ability to bind to LDL receptors and to downregulate LDL-receptor activity and \textit{de novo} sterol synthesis (119,120). An important determinant of the binding affinity of a lipoprotein particle for the LDL receptor is the extent to which the binding domain of apoB100 is masked or is oriented in a favourable conformation (17). Triglyceride enrichment may place certain conformational constraints on the apoB100, as it has been shown (121,122) that the immunoreactivity of certain antigenic determinants of apoB100 decreases directly with increased lipoprotein triglyceride.

1.3.4.4 Carbamylation

Carbamylation is the chemical reaction of urea with lysine residues of proteins. It has been shown that carbamylation of LDL causes decreased LDL-receptor binding by human fibroblasts (123,124) and hepatic membranes from guinea pigs (125), but increased interaction with scavenger receptors of human macrophages (123). Since uremia is a biochemical manifestation commonly seen in diabetes (74), increased modification of LDL by carbamylation
may occur.

1.3.4.5 *In vivo* Studies

Findings have been inconsistent regarding LDL-cholesterol concentrations in DM. Many studies have shown that levels are similar to those of controls, while others have shown increases (76, 80-82).

Kissebah *et al.* (126), studying patients with NIDDM, found that those with mild diabetes cleared their LDL at a faster rate than normal subjects, whereas those with moderately severe diabetes had a significant reduction in LDL FCR. Insulin-dependent diabetics have a similar LDL FCR to those of controls (127).

The decrease in FCR of LDL in NIDDM suggests an interference with the LDL-receptor pathway. Chait *et al.* (128) isolated fibroblasts from diabetic patients and found that there were no abnormalities in LDL binding by the LDL receptor. As discussed previously, *in vitro* studies have shown that there are many factors in the diabetic state which may modify LDL and result in decreased binding by the LDL receptor. Only three studies have reported on the LDL-receptor binding of LDL isolated from diabetics. Kraemer *et al.* (118), using cultured human fibroblasts, found no differences between either the apparent binding affinities or capacities of LDL isolated from normal Type 2 diabetic subjects. Lopes-Virella *et al.* (129) showed that LDL isolated from Type 1 diabetics bound to LDL receptors of cultured human fibroblasts to the same extent as LDL isolated from normals, but were internalized and degraded to a lesser extent. On the other hand, Hiramatsu *et al.* (115) reported that the binding of LDL from hypertriglyceridemic diabetic (both Type 1 and Type 2) patients was significantly reduced compared with that of normals. They attributed the decreased binding to the higher triglyceride to protein ratio in the LDL based on two observations: 1) LDL from normolipidemic diabetic patients did not show a decreased binding; 2) hypertriglyceridemic LDL from non-diabetics also showed a decreased binding. It
should be pointed out that in the studies of Kraemer et al. (118) and Lopes-Virella et al. (129) the diabetic patients were also hypertriglyceridemic (3 to 4-fold increase), but not to the same extent as that in the study of Hiramatsu (20-fold increase) (115). Interestingly, in the studies of Lopes-Virella (129), the LDL from diabetics showed normal internalization and degradation after the patients achieved metabolic control. Since the triglyceride/protein ratio in LDL decreased after metabolic control, they also suggested that a high triglyceride content may interfere with LDL metabolism.

Although LDL-receptor clearance appears to be reduced in diabetics, it has been suggested that LDL-receptor-independent clearance is enhanced. In support of this, autoantibodies against glycosylated LDL have been demonstrated in the plasma of some diabetics (130), suggesting an immune-mediated mechanism for LDL removal.

The reason that many diabetics do not show increases in their LDL concentrations may be due to the fact that LDL production is also decreased in diabetes (76). It has been shown that the VLDL particles produced in NIDDM are triglyceride rich. These particles are less efficiently converted to LDL (131,132) and a higher proportion is directly removed by the liver (76,126).

1.4 Research Proposal

Identification and assessment of the factor(s) responsible for the increased risk of atherosclerosis in diabetics would be invaluable to the proper treatment and monitoring of these patients, and would hopefully lead to an enhanced quality of life.

LDL is known to play a critical role in the development of atherosclerosis (12-15). Evidence suggests that impaired binding of LDL to its receptor may increase the risk for atherosclerosis, since LDL catabolism by LDL-receptor-independent (or atherogenic) pathways is enhanced (10).

In diabetes, there are several biochemical manifestations (hyperglycemia,
hypertriglyceridemia, uremia) which can lead to modification of LDL and, hence, decreased receptor binding (95,115,123).

The purpose of the present research study is to develop a competitive binding assay in order to measure the ability of modified LDL to compete with native radiolabelled LDL for the LDL receptor. Binding studies involving the LDL receptor on intact cultured cells can be lengthy and labour intensive (115,118,129). They involve: 1) careful maintenance and growth of cells in culture; 2) long incubation periods (12-24 h) for binding and internalization of LDL; 3) multiple washing of cell layers to remove unbound LDL; and 4) a second incubation of cells with a releasing agent (heparin or dextran) to dissociate LDL from its receptor. Rather than using cells in culture, a semi-purified LDL receptor from beef adrenocortical cells will be used in order to try to simplify the assay.

The first part of the study will involve the isolation of the LDL receptor and optimization of the binding assay.

In the second part, the ability of the bovine LDL receptor to recognize modifications of human LDL will be established. In turn, the ability of LDL isolated from diabetics to compete with control radiolabelled LDL for the LDL receptor will be measured. The relationship, if any, of this parameter with other parameters of glycemic control will be determined.
CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and Biochemicals

N-α-Acetyl-L-lysine, bovine serum albumin (Fraction V), Coomassie Brilliant Blue R-250, deoxycholic acid (sodium salt), Fein & Ciocalteu’s phenol reagent (2.0 N), gentamicin sulfate, glycerol, leupeptin (hemisulfate salt) {N-acetyl-leucine-leucine-argininal}, maleic acid (free acid), n-octylβ-D-glucopyranoside, phenylmethylsulfonyl fluoride, L-α-phosphatidyl choline (Type XIII-E from egg yolk; ethanol solution), Ponceau S [3-hydroxy-4-(2-sulfo-4-[4-sulfophenylazo]-phenylazo)-2,7-naphthalenedisulfonic acid], protein molecular weight markers, 5-sulfosalicylic acid [2-hydroxy-5-sulfobenzoic acid], thimerosal {Mercurochrome®}, and 2,4,6-trinitrobenzenesulfonic acid {picrylsulfonic acid} were purchased from Sigma Chemical Co. (St. Louis, MO).

Neuraminidase {acylneuraminyl hydrolase (EC 3.2.1.18) from Clostridium perfringens} was purchased from Sigma Chemical Co. (St. Louis, MO). One unit of enzyme will liberate 1.0 μmole of N-acetylneuraminic acid (NANA) per min at pH 5.0 at 37°C using NANA-lactose as the substrate.

Calcium chloride (dihydrate), copper(II) sulfate (pentahydrate), ethylenediamine tetraacetic acid (disodium salt), D-glucose, glycine (free base), potassium chloride, potassium phosphate dibasic (anhydrous), potassium phosphate monobasic (anhydrous), sodium azide, sodium hydroxide, sodium phosphate dibasic (anhydrous), sodium phosphate monobasic (monohydrate), sodium potassium tartrate, trichloroacetic acid, and urea were purchased from Fisher Scientific Canada Ltd. (Toronto, ON).

Acetic acid (glacial), acetone, chloroform, diethyl ether, hydrochloric acid, iodine
monochloride (98% purity), methanol, potassium bromide, sodium acetate, sodium borate, sodium carbonate, sodium chloride, and tris(hydroxymethyl)methylamine were purchased from BDH Chemicals (Toronto, ON).

Acrylamide, ammonium persulfate, Bromophenol Blue, 2-mercaptoethanol, N,N'-methylene-bis-acrylamide, and sodium dodecyl sulfate were purchased from Bio-Rad Laboratories (Richmond, CA).

Unless otherwise indicated, all chemicals were of ACS grade.

Na\textsuperscript{125}I (100 mCi/mL in 0.01 N NaOH) was purchased from ICN Biomedicals (Mississauga, ON). Specific radioactivity was 17 Ci/mg I.

Formula - 963 liquid scintillation cocktail was purchased from DuPont Canada, Inc. (Mississauga, ON).

Triton\textsuperscript® X-100 (especially purified for membrane research) was purchased from Boehringer Mannheim Canada Ltd. (Laval, PQ).

Double distilled deionized water was obtained by first passing distilled water through an ion-exchange cartridge and then a Barnstead Fi-stream\textsuperscript® 2 distillation apparatus (Sybron Corporation, Boston, MA). All references to "water" in this paper indicate water obtained in this manner.

2.1.2 Chromatography Resins

Sephadex G-10 (bead size: 40-120 \(\mu\)m) was purchased from Sigma Chemical Co. (St. Louis, MO).

DE-52 (microgranular) [diethylaminoethyl cellulose] was purchased from Whatman Ltd. (Maidstone, England).

2.1.3 Test Kits

Glyc-Affin\textsuperscript® GHb test kits were purchased from Isolab Inc. (Akron, OH).
Roche Fructosamine Test Plus test kits were purchased from Hoffmann-LaRoche Ltd. (Etobicoke, ON).

2.1.4 Supplies

Cellulose membrane dialysis tubing (average flat width: 10 mm; retains protein of MW > 12,000) was purchased from Sigma Chemical Co. (St. Louis, MO).

Cellulose acetate syringe filters (0.2 µm pore size; 4 mm and 25 mm diameter) were purchased from Nalge Company (Rochester, NY).

Cellulose acetate membrane filters (0.45 µm pore size; 25 mm diameter) were purchased from Sartorius Filters, Inc. (Hayward, CA).

Nitrocellulose membrane (0.45 µm pore size) was purchased from Bio-Rad Laboratories (Richmond, CA).

Blotting paper was purchased from Mandel Scientific Co., Ltd. (Ville St. Pierre, PQ).

Borosilicate glass culture tubes, polyethylene and borosilicate scintillation vials were purchased from Fisher Scientific Canada Ltd. (Toronto, ON).

Fuji RX X-ray film was purchased from Fuji Photo Film Co., Ltd. (Tokyo, Japan). Kodak X-ray Exposure Cassettes were purchased from Sigma Chemical Co. (St. Louis, MO). Cronex® Lightening Plus Enhancing Screens were purchased from DuPont Canada, Inc. (Mississauga, ON).

Superseal® sealable plastic containers were purchased from Accent (Montreal, PQ).

Proseal® plastic food wrap was purchased from Northern Plastics Ltd. (Markham, ON).

2.1.5 Apparatus

Ultraviolet/visible readings and recordings were obtained using a Response® UV-VIS spectrophotometer (Ciba-Corning Diagnostics Corp., Toronto, ON), and a 1.00-cm quartz (UV range) or 1.00-cm glass (visible range) cuvette (Hellma Canada Ltd., Concord, ON).
All liquid scintillation counting was performed on an LS 7500 Liquid Scintillation System (Beckman Instruments Inc., Fullerton, CA). Unless otherwise indicated, polyethylene scintillation vials and 10 mL of scintillation cocktail were used.

Centrifugations requiring less than 10,000 x g were carried out in a Superspeed RC2-B centrifuge (Sorvall Inc., Newton, CT). Polypropylene centrifuge bottles (250 mL) were used with the Sorvall GSA rotor. Polycarbonate centrifuge tubes (20 mm x 102 mm; 50 mL) were used with the Sorvall SS-34 rotor.

Centrifugation requiring greater than 10,000 x g were carried out in either an L8-55 or L5-65 ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). Thick-wall polyallomer Ultratubes® (16 mm x 76 mm; 13.5 mL), purchased from Nalge Co. (Rochester, NY), were used with the Beckman Type 65 rotor. Thick-wall polycarbonate Ultratubes® (25 mm x 89 mm; 38.5 mL), purchased from Nalge Co. (Rochester, NY) were used with the Beckman Type 30 rotor. Thin-wall thermoplastic Ultra-Clear® tubes (11 mm x 60 mm; 4.0 mL), purchased from Beckman Instruments Inc. (Palo Alto, CA) were used with the Beckman Type SW60Ti rotor.

All microcentrifugations were performed using a Model 235-C microcentrifuge (Fisher Scientific Canada Ltd., Toronto, ON) and 1.5-mL polypropylene conical microcentrifuge tubes (Elkay Products Inc., Shrewsbury, MA). The centrifuge operates at a fixed speed of 12,400 rpm (13,600 x g).

Micropipetting was carried out using Gilson Pipetman (P-20, P-100, P-1000) variable-volume pipettors (Mandel Scientific Co. Ltd., Ville St. Pierre, PQ) and a Socorex Calibra® digital 20-200 µL pipettor (Terochem Scientific, Toronto, ON). Blue and yellow propylene pipette tips (Canlab Scientific Products, Mississauga, ON) were used.

An Eppendorf Repeater® pipette with Eppendorf Combitips® (Brinkmann Instruments Inc., Westbury, NY) was used for repetitive micropipetting of less than 2-mL volumes. Repipet®
and Repipet® Jr. dispensers (Labindustries, Berkeley, CA) were used for repetitive pipetting of greater than 2-mL volumes.

All pH measurements were made with an Accumet® pH meter (Fisher Scientific Canada Ltd., Toronto, ON) and a combination [Ag/AgCl] reference/pH electrode (VWR Scientific Canada Ltd., London, ON). The electrode was standardized before each measurement with pH standard solutions (BDH Chemicals, Toronto, ON).

Measurements of mass were made with Mettler P1000 and H16 balances (Fisher Scientific Canada Ltd., Toronto, ON).

Measurements of refractive index were made with a Zeiss Abbe-refractometer (Fisher Scientific Canada Ltd., Toronto, ON), connected to a Type 01 temperature controlled water-bath (Heto Inc., Birkerod, Denmark).

Slab-gel electrophoresis was carried out with an in-house constructed system (based on that of Hoefer Scientific Instruments, San Francisco, CA) with 1.5-mm thick plastic spacers and Teflon slot formers. Mini slab-gel electrophoresis was carried out on an in-house constructed system (based on the Mini-PROTEAN II® Cell of Bio-Rad Laboratories, Richmond, CA), using 0.75 mm plastic spacers. Slab-gels were dried onto blotting paper using a Drygel Jr. (Hoefer Scientific Instruments, San Francisco, CA). Tube-gel electrophoresis was carried out on a Hoefer Scientific Instruments (San Francisco, CA) system, using glass tubes (6 mm inside diameter x 160 mm).

Agarose electrophoresis was carried out on a Corning electrophoresis system (Corning Medical Instruments, Toronto, ON).

Electrophoretic transfer of proteins was carried out on a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad Laboratories, Richmond, CA).

Dissection of adrenal glands was carried out with a #4 stainless steel surgical blade handle
(Feather Industries Ltd., Japan) and #22 surgical blade (W.R. Swann & Co. Ltd., Sheffield, England).


2.2 Methods

2.2.1 Protein Assays

2.2.1.1 Reagents

*Copper-Tartrate-Carbonate Solution.* Sodium carbonate (10.0 g) was dissolved in approximately 50 mL of water. Copper(II) sulfate pentahydrate (100 mg) and sodium potassium tartrate (200 mg) were dissolved in approximately 40 mL of water. The sodium carbonate solution was added slowly to the latter with stirring, and the mixture was then made up to 100 mL with water. The solution was stored in an amber glass bottle at room temperature and was stable for at least 3 months.

*10% Sodium Dodecyl Sulfate (SDS).* SDS (2.5 g) was dissolved in 25.0 mL of water.

*0.8 N Sodium Hydroxide.* Sodium hydroxide (1.6 g) was dissolved in 50.0 mL of water.

*Reagent A.* On the day of the assay, equal volumes of copper-tartrate-carbonate, 10% SDS, 0.8 N sodium hydroxide, and water were mixed. The formation of a white flocculent
precipitate did not affect the assay, as it redissolved upon dilution.

Reagent B. On the day of the assay, one volume of Folin & Ciocalteu phenol reagent (2.0 N) was mixed with five volumes of water.

0.15% (w/v) Sodium Deoxycholate. Sodium deoxycholate (37.5 mg) was dissolved in 25.0 mL of water. The solution was stored at 4°C.

72% (w/v) Trichloracetic Acid (TCA). TCA (18.0 g) was dissolved in 25.0 mL of water.

0.921 mg/mL Bovine Serum Albumin (BSA). BSA (approximately 25 mg) was dissolved in 25 mL of 0.15 M NaCl. Exact concentration was determined using an extinction coefficient of 0.668 mL x mg⁻¹ x cm⁻¹ at 279 nm (133). Aliquots were placed in microcentrifuge tubes, quick frozen in liquid nitrogen and stored at -70°C.

0.184 mg/mL BSA. The solution was prepared by diluting 1.0 mL of 0.921 mg/mL BSA up to 5.0 mL with 0.15 M NaCl. Aliquots were stored at -70°C.

0.184 mg/mL BSA in 40 mM n-Octyl β-D-Glucopyranoside (octylglucoside). The solution was prepared by dissolving 58 mg of octylglucoside in 1.0 mL of 0.921 mg/mL BSA and making up to 5.0 mL with 0.15 M NaCl. Aliquots were stored at -70°C.

2.2.1.2 Procedures

The procedures described below are based on the modifications of the Lowry protein assay (134) described by Peterson (135).

Macroassay

Samples containing between 5 and 100 μg of protein were made up to a total volume of 1.0 mL with water. A reagent blank and standards were similarly prepared using various volumes (0 to 100 μL) of the 0.921 mg/mL BSA. After adding 1.0 mL of Reagent A to the protein solution, tubes were vortexed, and allowed to stand for 10 min at room temperature. After adding 0.5 mL of Reagent B, the tubes were vortexed and allowed to stand for 30 min.
Absorbances were read at 750 nm. Protein concentrations of samples were calculated from the standard curve generated (see Figure 2.1.)

Microassay

This procedure was used for samples containing 1-20 μg of protein. The procedure was the same as that used for the macroassay, except that the quantities of reagents were 0.4 times that used in the macroassay, i.e., samples were made up to 0.4 mL with water and 0.4 mL of Reagent A and 0.2 mL of Reagent B were used. A reagent blank and standards were similarly prepared using variable amounts (0 to 100 μL) of 0.184 mg/mL BSA. Protein concentrations of samples were calculated from the standard curve generated (see Figure 2.2).

Microassay with Precipitation

Samples containing 1 to 20 μg of protein were made up to a total of 1.0 mL with water in a microcentrifuge tube. A reagent blank and standards were similarly prepared using variable volumes (0 to 100 μL) of 0.184 mg/mL BSA in 40 mM octylglucoside. After adding 0.1 mL of 0.15% sodium deoxycholate, the tubes were mixed and allowed to stand for 10 min at room temperature. After adding 0.1 mL of 72% TCA, tubes were vortexed and centrifuged for 5 min. The supernatants were drawn off using a syringe and 22-gauge needle (bevel removed with a file). The pellets obtained from this centrifugation were then subjected to the microassay exactly as described above. Protein concentration of samples were calculated from the standard curve generated (see Figure 2.2).

2.2.2 Electrophoresis

2.2.2.1 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Slab Gels

For 6% to 10% SDS-PAGE, slab gels were prepared according to a modification (136) of the Laemmli discontinuous Tris/glycine/0.1% SDS buffer system (137). After electrophoresis,
FIGURE 2.1

Protein Macroassay Standard Curve

Legend

The figure shows a typical standard curve generated by the protein macroassay.

A higher correlation coefficient is obtained from second-order regression analysis than from first-order regression.
FIGURE 2.2

Protein Microassay Standard Curves

Legend

The figure shows typical standard curves generated by the protein microassay (○—○) and microassay with precipitation (•——•).

For both microassays, higher correlation coefficients are obtained from second-order regression analysis than from first-order regression.
FIGURE 2.2

- o w/ o TCA precipitation

\[ y = 0.001 + 2.52 \times 10^{-2} x - 2.06 \times 10^{-4} x^2 \]
\[ r = 0.9997 \]

- - w/ TCA precipitation

\[ y = 0.002 + 2.40 \times 10^{-2} x - 1.54 \times 10^{-4} x^2 \]
\[ r = 0.9992 \]
gels were stained in 1% Coomassie Brilliant Blue R-250, containing 40% methanol and 10% acetic acid. Gels were destained with 40% methanol and 10% acetic acid solution. Protein markers (lysozyme: 14.3 kDa; carbonic anhydrase: 29.0 kDa; ovalbumin: 45.0 kDa; bovine serum albumin: 66.0 kDa; phosphorylase b: 97.4 kDa; β-galactosidase: 116 kDa, myosin: 200 kDa) were run on each gel. A standard curve (see Figure 2.3) was generated using these markers and used to determine MW of unknown proteins.

Tube Gels

For 3% SDS-PAGE, tube gels were prepared as described in Technical Bulletin No. MWS-877X (138) using a continuous phosphate buffer system. After electrophoresis, gels were immersed in fixative solution (40% methanol, 10% acetic acid) for 10 h, after which they were stained in 1% Coomassie Brilliant Blue R-250, containing 40% methanol and 10% acetic acid. Gels were destained with 40% methanol/10% acetic acid and stored in 5% methanol/7.5% acetic acid. Protein markers (cross-linked phosphorylase b: 97.4, 194.8, 292.2, 389.6, 487.0, and 584.4 kDa) were included with each run. A standard curve (see Figure 2.4) was generated using these markers, and used to determine the molecular weight of unknown proteins.

2.2.2.2 Agarose-Gel Electrophoresis

Agarose electrophoresis was carried out on Corning Universal Gel plates (1% w/v agarose, 5% w/v sucrose) using 0.065 M barbital buffer, pH 8.6 (containing 0.035% w/v EDTA). Gels were: 1) run at 90 V for 20 min; 2) dried in oven for 20 min; 3) stained with Fat Red 7B for 5 min; 4) destained in 50% methanol for 15-20 sec; 5) dried in oven for 20 min.

2.2.3 Collection of Serum

2.2.3.1 Reagents

1.75% Phenylmethylsulfonyl Fluoride (PMSF). PMSF (17.5 mg) was dissolved in 1.0 mL of dimethyl sulfoxide. The tube containing the solution was wrapped in aluminum foil and
FIGURE 2.3

Slab SDS-PAGE Molecular Weight Standard Curves

Legend

The figure shows typical standard curves generated by running protein molecular weight markers (MW of 14.3 to 200 kDa) on 7% (☉) and 10% (●) SDS-PAGE slab gels.
FIGURE 2.3

\[ y = 5.53 - 1.02x \]
\[ r = -0.997 \]

\[ y = 5.04 - 0.978x \]
\[ r = -0.997 \]
FIGURE 2.4

Tube SDS-PAGE Molecular Weight Standard Curve

Legend

The figure shows a typical standard curve generated by running protein molecular weight markers (MW of 96.4 to 584.4 kDa) on a 3% SDS-PAGE tube gel.
FIGURE 2.4

\[ y = 4.01 - 0.639x \]

\[ r = -0.998 \]
stored in a dessicator at 4°C.

*Preservative Cocktail.* Sodium azide (20 mg), ethylenediaminetetraacetic acid (EDTA) (40 mg), gentamicin sulfate (5 mg), and thimerosal (20 mg) were dissolved in 1.0 mL of water. The tube containing the solution was wrapped in aluminum foil and stored at 4°C.

### 2.2.3.2 Procedure

Blood samples were obtained from specimens sent to the clinical laboratory for routine analyses. Both serum (red-top Vacutainers®) and plasma (lavender-top Vacutainers) were used. After low-speed centrifugation (3000 x g) for 10 min, serum or plasma was separated from cells. To the serum were added 10 µL of 1.75% PMSF per mL of serum, and 10 µL of preservative cocktail per mL of serum. Samples were placed in polypropylene tubes, quick frozen in liquid nitrogen, and stored at -70°C.

### 2.2.4 Isolation of LDL

#### 2.2.4.1 Reagents

*Potassium Bromide.* Prior to each run, potassium bromide was dried overnight in an oven at 120°C. During the course of the run, the KBr was kept in a dessicator at room temperature.

*Density Standard (d = 1.019 g/mL).* Potassium bromide (2.964 g) and EDTA (10 mg) were dissolved in 100 mL of water. Density was confirmed by measuring the refractive index of the solution and by using Formula 2.2 (see p. 58).

*Density Standard (d = 1.063 g/mL).* Potassium bromide (9.455 g) and EDTA (10 mg) were dissolved in 100 mL of water. Density was confirmed by measuring the refractive index of the solution and by using Formula 2.2 (see p. 58).

*Buffer A (5 mM Tris, 0.15 M NaCl, 0.01% EDTA, pH 7.4 at 4°C).* Tris [hydroxymethyl]methylamine (Tris) (1.8 g), NaCl (26.3 g), and EDTA (0.3 g) were dissolved
in approximately 2.5 L of water at 4°C. The solution was adjusted to pH 7.4 with HCl and made up to 3.0 L with 4°C water.

2.2.4.2 Procedure

Low-density lipoprotein was isolated from 1 to 6 mL of serum using sequential flotation ultracentrifugation. The procedure (see Figure 2.5) is based on modifications (139,140) of the original Havel method (141). The serum (initial density = 1.006 g/mL) was adjusted to a final density of 1.019 g/mL by adding solid potassium bromide according to the Radding-Steinberg (142) formula:

\[ X = [V \times (d' - d)]/[1 - (v \times d')] \quad [2.1] \]

where \( X \) = amount of solid potassium bromide required (grams), \( V \) = initial volume, \( d \) = initial density, \( d' \) = final (desired) density, \( v \) = apparent volume of displacement of potassium bromide at final density. (See APPENDIX A for calculation of \( v \).) If necessary, the serum was overlaid with standard KBr solution (\( d = 1.019 \) g/mL). The serum was centrifuged at 4°C in a Beckman Type 65 rotor at 55,000 rpm (264,000 x g) for 21 h. (See APPENDIX B for rotor/spin-time correction.) A standard KBr solution (\( d = 1.019 \) g/mL) was run in parallel with serum for density determination.

After centrifugation, the top layer ("VLDL/IDL" or "\( d < 1.019 \) g/mL fraction") was harvested with a syringe and 22-gauge needle. The bottom fraction ("\( d > 1.019 \) g/mL fraction") was harvested with a Pasteur pipette and its mass was determined, using a tared tube. The portion of the \( d = 1.019 \) g/mL standard, corresponding to the bottom fraction, was also harvested with a Pasteur pipette and thoroughly mixed. To the bottom fraction and the \( d = 1.019 \) g/mL standard was added 10 \( \mu \)L of 1.75% PMSF per mL of solution. The refractive index of the \( d = 1.019 \) g/mL standard was then measured at 20°C, and its density calculated using the
FIGURE 2.5

Isolation of LDL from Human Serum

Legend

After the addition of preservative cocktail (protease inhibitor, antibiotics, antioxidant), serum was adjusted to a density of 1.019 g/mL with solid KBr and subjected to ultracentrifugation in a Beckman Type 65 rotor at 55,000 rpm (264,000 x g) for 21 h*. The top layer contained VLDL and IDL. The bottom layer (containing LDL, HDL, albumin and other proteins) was adjusted to a density of 1.063 g/mL with solid KBr and subjected to ultracentrifugation in a Beckman Type 65 rotor at 55,000 rpm (264,000 x g) for 29 h. The bottom layer contained HDL and other proteins. The top layer (containing LDL and some HDL contamination) was readjusted to a density of 1.063 g/mL with solid KBr and "washed" by ultracentrifugation in a Beckman Type 65 rotor at 55,000 rpm (264,000 x g) for 19 h. The top layer contained pure LDL and the bottom of the tube contained HDL.

* When LDL was isolated in a Beckman Type SW60Ti rotor, ultracentrifugations were carried out at 48,000 rpm (310,000 x g) and the duration of the first, second and third centrifugations were 18, 24 and 17 h, respectively.
Serum
(d = 1.006 g/mL)

Add PMSF and preservative cocktail
Adjust to d = 1.019 g/mL with KBr
Spin for 21 h (264,000 x g)

Bottom Layer
d > 1.019 g/mL
(contains LDL, HDL, albumin and other proteins)

Top Layer
d < 1.019 g/mL
(contains VLDL, IDL)

Adjust to d = 1.063 g/mL
Spin for 29 h (264,000 x g)

Bottom Layer
d > 1.063 g/mL
(contains HDL, albumin and other proteins)

Top Layer
1.019 < d < 1.063 g/mL
(contains LDL and some HDL contamination)

Adjust to d = 1.063 g/mL
Spin for 19 h (264,000 x g)

Bottom Layer
d > 1.063 g/mL
(contains LDL)

Top Layer
1.019 < d < 1.063 g/mL
(contains pure LDL)
formula:*

\[ \text{Density} = \frac{(\text{Refractive Index} - 1.1699)}{0.16347} \]  \[\text{[2.2]}\]

This value was assumed to be the density of the d > 1.019 g/mL fraction, and the volume of the d > 1.019 g/mL fraction was calculated using the formula:

\[ \text{Volume} = \text{Mass}/\text{Density} \]  \[\text{[2.3]}\]

The d > 1.019 g/mL fraction was then adjusted to a final density of 1.063 g/mL with solid potassium bromide according to Formula 2.1 above. If necessary, this was overlaid with standard KBr solution (d = 1.063 g/mL). The solution was then centrifuged at 4°C in a Beckman Type 65 rotor at 55,000 rpm (264,000 x g) for 29 h. A standard KBr solution (d = 1.063 g/mL) was run in parallel for density determination.

After centrifugation, the top layer ("unwashed 1.019 < d < 1.063 g/mL fraction") was harvested with a syringe and needle and its mass determined, using a tared tube. The corresponding top layer of the standard KBr solution was also harvested and thoroughly mixed. The refractive index of the standard KBr solution was measured at 20°C, and its density calculated using Formula 2.2 above. Volume of the 1.019 < d < 1.063 g/mL fraction was calculated using Formula 2.3. This fraction was then adjusted to a density of 1.063 g/mL with solid potassium bromide according to Formula 2.1 above. If necessary, this was overlaid with standard KBr solution (d = 1.063 g/mL). The solution was then centrifuged at 4°C in a Beckman Type 65 rotor at 55,000 rpm (264,000 x g) for 19 h.

After centrifugation, the top layer ("LDL" or "washed 1.019 < d < 1.063 g/mL fraction") was harvested carefully (to minimize dilution) with a syringe and 22-gauge needle.

* The formula was calculated by first-order linear regression, using data found in the CRC Handbook of Chemistry and Physics (143).
The LDL was then dialyzed* at 4°C against a total of 3 L of Buffer A for 36-48 h (6 changes of 0.5 L each). Vigorous stirring was avoided during dialysis to prevent precipitation of LDL (140). After dialysis, the LDL was centrifuged in a microcentrifuge for 30 min at 4°C, and the supernatant was sterilized by passage through a cellulose acetate syringe filter (pore size: 0.2 μm; diameter: 4 mm).

Protein content of LDL (typically 1-4 mg/mL) was determined using the macroassay (see Section 2.2.1.2, p. 43). Unless otherwise indicated, references to amounts of LDL in this paper indicate the amount of LDL protein.

LDL was stored in a microcentrifuge tube under nitrogen at 4°C, and used within 2 weeks.

2.2.5 Preparation of ¹²⁵I Quench Standards

2.2.5.1 Reagents

0.01 N NaOH. Sodium hydroxide (40 mg) was dissolved in 100 mL of water.

Stock Na¹²⁵I. Na¹²⁵I (100 mCi/mL in 0.01 N NaOH) was diluted with 0.01 N NaOH to obtain a 20 to 30-mCi/mL solution.

2.2.5.2 Procedure

Stock Na¹²⁵I was serially diluted with 0.01 N NaOH to obtain a solution of about 20 μCi/mL. To each of ten borosilicate glass scintillation vials containing 10 mL of scintillation cocktail were added 4 μL of 20 μCi/mL Na¹²⁵I. Radioactivity of vials was measured by liquid scintillation counting. Those vials containing radioactivity within ± 1% of the mean were used for preparation of the quench standards. Different volumes (0, 55, 130, 195, 250, 330, 440,

* In order to remove glycerol, sulphurous compounds, and heavy metal ions, dialysis tubing was washed with acetic acid and EDTA as described in Biochemical Research Techniques (136). Dialysis tubing was stored at 4°C in a wide-mouth polycarbonate bottle, containing distilled water with sodium azide to prevent bacterial growth.
600, and 800 μL) of water were added to each vial and mixed thoroughly.

The Beckman LS 7500 Liquid Scintillation System is equipped with an external standardization technique for determining the amount of quenching in a sample in which an H-number is generated for each sample counted (144). I\textsubscript{25}I quench standards were counted during each experiment. The counting efficiency (CE) of each standard was calculated from the theoretical dpm (CE = cpm/dpm) and plotted against its measured H-number (see Figure 2.6). The CE of every sample was then calculated from the standard curve and was, in turn, used to calculate the dpm (dpm = cpm/CE) in the sample.

Linearity of the liquid scintillation counter was checked by counting serial dilutions of Na\textsuperscript{125}I in 0.01 N NaOH. Radioactivity was linear up to 5 x 10\textsuperscript{6} dpm (see Figure 2.7).

2.2.6 Radioiodination of LDL

2.2.6.1 Reagents

Stock Na\textsuperscript{125}I. Na\textsuperscript{125}I (1μCi/mL in 0.01 N NaOH) was diluted with 0.01 N NaOH to obtain a 20 to 30 μCi/mL solution.

Buffer B (1.0 M Glycine, pH 10.0 at 4°C). Glycine (1.9 g) was dissolved in approximately 20 mL of water (4°C). The solution was adjusted to pH 10.0 with NaOH, and made up to 25.0 mL with water (4°C). The solution was kept at 4°C.

2 M NaCl. Sodium chloride (5.8 g) was dissolved in 50.0 mL of water. The solution was kept at 4°C.

42.1 mM Iodine Monochloride. Iodine monochloride (69.8 mg) was dissolved, with vortexing, in 10.0 mL of 1.0 M HCl in a stoppered, conical, glass tube. The solution was extracted several times with carbon tetrachloride until purple colour no longer appeared in the organic phase. The solution was kept over CCl\textsubscript{4} at 4°C and was stable indefinitely (145).
FIGURE 2.6

$^{125}I$ Quench Standard Curve

Legend

The figure shows a typical standard curve generated by counting the $^{125}I$ quench standards using a Beckman LS 7500 Liquid Scintillation System.
$y = 0.970 - 3.46 \times 10^{-3} x$

$r = 0.984$
FIGURE 2.7

Linearity of Liquid Scintillation Counter

Legend

Na$^{125}$I was serially diluted with 0.01 N NaOH and radioactivity was measured using a Beckman LS 7500 Liquid Scintillation System. Radioactivity was linear up to $5 \times 10^6$ dpm.
342 μM iodine Monochloride. On the day of radioiodination, 42.1 mM iodine monochloride was serially diluted (19-fold and 5-fold) with 2 M NaCl to obtain an 842 μM solution.

Buffer C (50 mM Tris, 0.15 M NaCl, 0.01% EDTA, pH 7.4 at 25°C). Tris (6.1 g), NaCl (8.8 g) and EDTA (100 mg) were dissolved in approximately 900 mL of water. The solution was adjusted to pH 7.4 with HCl and made up to 1.0 L with water. The solution was kept at room temperature.

Sephadex G-10. A fresh Sephadex G-10 column was prepared for each radioiodination. Sephadex G-10 (5.0 g) was added slowly to approximately 80 mL of water and stirred gently with a stirring rod. The mixture was placed in a 70°C water bath for 1 h (146). After cooling to room temperature, the water was aspirated off. Approximately 80 mL of Buffer C were added to Sephadex and the mixture was gently stirred. After allowing the gel to settle, the fines and supernatant were removed by aspiration. Fresh Buffer C was added and the above process (i.e., removal of fines) was repeated two or three times. After the last wash, enough Buffer C was left with the Sephadex to produce a 75% slurry, which was poured into a chromatography column (1.0 cm x 20 cm). Buffer C was allowed to flow through the column at 0.5 mL/min to pack the resin (final bed size: 1.0 cm x 15 cm).

2.2.6.2 Procedure

The procedure described below is based on modifications (140,147) of the iodine monochloride method of MacFarlane (148,149). Radioiodination was carried out on ice in a fumehood behind lead bricks. To 50 μL of Buffer B in a microcentrifuge tube, was added 0.44 mCi of Na125I. This solution was transferred to 250 μg of LDL (in Buffer A) in a microcentrifuge tube. Into this mixture, 14.8 μL of 842 μM iodine monochloride were added with mixing. The mixture was incubated on ice for 5 min, after which it was applied to the
Sephadex G-10 column. The column was eluted with Buffer C at a flow rate of 0.15 mL/min. Eluate was collected as 3-min (0.45-mL) fractions for Fractions 1-20 and 7.5-min (1.13-mL) fractions thereafter. Radioactivity of each fraction (10-μL aliquots) was measured by liquid scintillation counting.

Fractions corresponding to the early peak of radioactivity were pooled and designated \(^{125}\text{I}\)-LDL. \(^{125}\text{I}\)-LDL was sterilized by filtration through a cellulose acetate syringe filter (pore size: 0.2 μm).

An aliquot (1.0 mL) of the \(^{125}\text{I}\)-LDL was added to 40 mg of BSA in a microcentrifuge tube and mixed until the BSA had dissolved. The \(^{125}\text{I}\)-LDL was stored under nitrogen at 4°C for 7 to 10 days.

2.2.7 Characterization of \(^{125}\text{I}\)-LDL

2.2.7.1 Reagents

0.15% Sodium Deoxycholate. Sodium deoxycholate (37.5 mg) was dissolved in 25.0 mL of water. The solution was stored at 4°C.

72% Trichloracetic acid (TCA). TCA (18.0 g) was dissolved in 25.0 mL of water. The solution was kept at room temperature.

2:1 (v/v) Chloroform/Methanol. On the day of the experiment, 2.0 mL of chloroform were thoroughly mixed with 1.0 mL of methanol.

3:48:47 (v/v/v) Chloroform/Methanol/Water. On the day of the experiment, 12 μL of chloroform, 192 μL of methanol, and 188 μL of water were thoroughly mixed.

2.2.7.2 Procedure

TCA Precipitation

A 10-μL aliquot (in duplicate) of \(^{125}\text{I}\)-LDL (no BSA added) was added to 990 μL of water in a microcentrifuge tube. Sodium deoxycholate (100 μL of 0.15%) was added with mixing to
this solution. After 10 min, 180 µL of 72% TCA were added. After vortexing, the tube was centrifuged for 5 min. The supernatant was removed with a syringe and 22-gauge needle (bevel removed with a file). The microcentrifuge tube containing the pellet was placed in a scintillation vial, scintillation cocktail was added, and the tube was counted. A 10-µL aliquot (in duplicate) of ¹²⁵I-LDL was also counted to determine the total radioactivity.

*Lipid Extraction*

The procedure is based on that of Folch *et al.* (150). Stock ¹²⁵I-LDL (no BSA added) was diluted 5-fold with 0.15 M NaCl. A 50-µL aliquot of dilute ¹²⁵I-LDL (in duplicate) was added with vortexing to 950 µL of 2:1 (v/v) chloroform/methanol in a microcentrifuge tube. Water (200 µL) was added with vortexing to this solution. The tube was centrifuged for 5 min, after which the top (aqueous) layer was removed with a syringe and 22-gauge needle (bevel removed). The inside of the microcentrifuge tube was washed by allowing 150 µL of 3:48:47 chloroform/methanol/water (v/v/v) to flow down the inside of the tube while slowly rotating the tube. The top layer was then removed with syringe and needle. After the bottom layer was evaporated under nitrogen gas, the tube was placed in a scintillation vial. Scintillation cocktail was added and the tube was counted. A 50-µL aliquot (in duplicate) of the dilute ¹²⁵I-LDL was also counted to determine the total radioactivity.

*Specific Radioactivity*

Protein concentration of the ¹²⁵I-LDL was determined by subjecting 10 µL (in triplicate) of ¹²⁵I-LDL (no BSA added) to the protein microassay (see Section 2.2.1.2, p. 43). Specific radioactivity (SR) was then calculated using the formula:

\[
SR \ (dpm/ng) = \frac{Radioactivity \ precipitated \ (dpm/µL)}{Protein \ concentration \ (ng/µL)}
\]  

[2.4]

The SR was used in all calculations where it was necessary to convert radioactivity (dpm) values into protein values.
 Autoradiography

$^{125}$I-LDL was subjected to 10% SDS-PAGE at 100 V for 7 h and stained with Coomassie Blue R-250 (see Section 2.2.2.1, p. 44). After destaining and drying, the gel was exposed at -80°C to X-ray film with enhancing screens for 1–4 h.

2.2.8 Isolation of LDL Receptor

2.2.8.1 Reagents

0.15 M NaCl. NaCl (8.8 g) was dissolved in 1 L of water. The solution was kept at 4°C.

0.5 M Tris-Maleate. Tris (15.2 g) and maleic acid (14.5 g) were dissolved in 250 mL of water. The solution was kept at 4°C.

100 mM Calcium Chloride. Calcium chloride dihydrate (7.35 g) was dissolved in 500 mL of water. The solution was kept at 4°C.

200 mM Phenylmethylsulfonyl Fluoride (PMSF). PMSF (0.871 g) was dissolved in 25 mL of anhydrous ethanol. The solution was stored dessicated at -20°C in an amber bottle.

Triton X-100. Triton X-100, especially purified for membrane research, was used. It was stored under nitrogen at 4°C in an amber bottle with a tight-fitting septum lid.

5% (v/v) Triton X-100. Just prior to use, 2.5 mL of Triton X-100 were made up to 50.0 mL with water (4°C).

4 M NaCl. NaCl (5.8 g) was dissolved in 25.0 mL of water. The solution was stored at 4°C.

2 M Maleic Acid. Maleic acid (5.8 g) was dissolved in 25 mL of water. The solution was stored at 4°C.

Buffer D (20 mM Tris, 0.15 M NaCl, 1 mM CaCl$_2$, 1 mM PMSF, pH 8.0 at 4°C). Just prior to use, 0.727 g of Tris, 2.630 g of NaCl, 2.0 mL of 100 mM CaCl$_2$, and 1.5 mL of
200 mM PMSF were dissolved in approximately 250 mL of water (4°C). The solution was adjusted to pH 8.0 with HCl, and made up to 300 mL with water (4°C).

Buffer E (250 mM Tris-maleate, 2 mM CaCl₂, 1 mM PMSF, pH 6.0 at 4°C). Just prior to use, 30 mL of 0.5 M Tris-maleate, 1.2 mL of 100 mM CaCl₂, 0.3 mL of 200 mM PMSF, and approximately 20 mL of water (4°C) were thoroughly mixed. The solution was adjusted to pH 6.0 with NaOH, and made up to 60 mL with water (4°C).

Buffer F (10 mM Tris-maleate, 2 mM CaCl₂, 1% (v/v) Triton X-100, 1 mM PMSF, pH 6.0 at 4°C). Just prior to use, 10 mL 0.5 M Tris-maleate, 10 mL of 100 mM CaCl₂, 5 mL of Triton X-100, 2.5 mL of 200 mM PMSF, and approximately 400 mL of water (4°C) were thoroughly mixed. The solution was adjusted to pH 6.0 with NaOH, and made up to 500 mL with water (4°C).

Buffer G (50 mM Tris-maleate, 2 mM CaCl₂, 1% (v/v) Triton X-100, 1 mM PMSF, pH 6.0 at 4°C). Just prior to use, 80 mL of 0.5 M Tris-maleate, 16 mL of 100 mM CaCl₂, 8 mL of Triton X-100, 4 mL of 200 mM PMSF, and approximately 600 mL of water (4°C) were thoroughly mixed. The solution was adjusted to pH 6.0 with NaOH, and made up to 800 mL with water (4°C).

Buffer H (50 mM Tris-maleate, 2 mM CaCl₂, 40 mM octylglucoside, 1 mM PMSF, pH 6.0 at 4°C). Just prior to use, 8 mL of 0.5 M Tris-maleate, 1.6 mL of CaCl₂, 0.935 g of octylglucoside, 0.4 mL of 200 mM PMSF, and approximately 60 mL of water (4°C) were thoroughly mixed until the octylglucoside had dissolved. The solution was adjusted to pH 6.0 with NaOH, and made up to 80 mL with water (4°C).

Gradient Buffer - 0 mM NaCl. Just prior to use, 5 mL of 0.5 M Tris-maleate, 1.0 mL of 100 mM CaCl₂, 0.585 g of octylglucoside, 0.25 mL of 200 mM PMSF, and approximately 40 mL of water (4°C) were thoroughly mixed until the octylglucoside had dissolved. The
solution was adjusted to pH 6.0 with NaOH, and made up to 50 mL with water (4°C).

*Gradient Buffer* - 250 mM NaCl. Just prior to use, 5 mL of 0.5 M Tris-maleate, 1.0 mL of 100 mM CaCl₂, 0.585 g of octylglucoside, 0.25 mL of 200 mM PMSF, 0.731 g of NaCl, and approximately 40 mL of water (4°C) were thoroughly mixed. After pH adjustment to 6.0, the solution was made up to 50 mL with water (4°C). Only upon adjustment of the pH with NaOH did the octylglucoside completely dissolve.

*Diethylaminoethyl (DEAE) - cellulose.* A fresh DEAE-cellulose column was prepared for each LDL-receptor isolation. DEAE-cellulose was preswollen as described by others (136,151). DEAE-cellulose (15 g) was suspended in 150 mL of 0.5 N HCl for 1 h. The suspension was filtered on Whatman #1 filter paper using a Büchner funnel and washed with water until filtrate had a pH of about 4. The DEAE-cellulose was resuspended in 150 mL of 0.5 N NaOH for 30 min after which the NaOH was removed by aspiration. The DEAE-cellulose was resuspended in 150 mL of fresh 0.5 N NaOH. After 30 min, the suspension was filtered and washed as above, until the filtrate had a pH near neutral. The DEAE-cellulose was gently resuspended in 150 mL of Buffer G and allowed to settle until a bed was formed (about 15 min). The cloudy supernatant, containing fines, was removed by aspiration. The washing of the DEAE-cellulose with Buffer G was repeated 3 or 4 times. After the last wash, enough Buffer G was left with the DEAE-cellulose to produce a 75% slurry, which was poured into a chromatography column (2.5 cm x 20 cm). Buffer G was allowed to flow through the column at 60 mL/h to pack the resin (final bed size: 2.5 cm x 8.0 cm).

### 2.2.8.2 Procedure

The procedure for isolating LDL receptors from calf adrenal glands is based on a procedure described by Schneider *et al.* (152). The procedure is summarized in Figure 2.8.
FIGURE 2.8

Isolation of LDL Receptor from Calf Adrenal Glands

Legend

The figure summarizes the steps involved in isolating the LDL receptor from calf adrenal glands, based on the procedure of Schneider et al. (152). For a more detailed description, see Section 2.2.8.2, p. 70.
Bovine Adrenal Glands
Dissect
  Cortex
  Medulla (discard)
  Scrape
  Capsule (discard)
  Homogenize (Polytron homogenizer)
  Centrifuge (800 x g)
  Supernatant
    Filter
    Centrifuge (100,000 x g)
    Membrane Pellet
      Suspend in buffer
      Solubilize in 1% Triton X-100
      Centrifuge (100,000 x g)
      Supernatant
        Pellet (discard)
        Supernatant (discard)
        Dilute 4-fold
        Centrifuge (5,000 x g)
        Supernatant
          Pellet (discard)
          Load supernatant onto DEAE-cellulose
          Wash column
          Elute column with 0-250 mM NaCl gradient, containing octyl-glucoside.

Fractions
Preparation of Calf Adrenal Cortices

Immediately after calves (15 weeks to 1 year old; 200-250 kg) were slaughtered, 36 adrenal glands were removed with a sharp knife and placed in ice-cold 0.15 M NaCl. After the removal of extraneous fat, the adrenal gland was sliced open with a #22 surgical blade using a glass plate on ice. The medulla was separated from the cortex either by gentle prying (with the aid of the surgical blade) or by sharp dissection, when the presence of nodules prevented the former. The cortex was scraped from the outer capsule with a blade and transferred into liquid nitrogen. After all cortices had been prepared in this way, they were stored at -70°C for up to 3 months (152).

Preparation of Calf Adrenocortical Membrane Pellets

The following steps in the preparation of the membrane pellets were carried out at 4°C.

When required, half of the cortices (approximately 36 g) were thawed in 5 mL of Buffer D (containing 0.1 mM leupeptin) per gram of frozen tissue. The tissue was homogenized with two 30-sec pulses (settings #5 and 8, respectively) of a Polytron homogenizer, and centrifuged in a Sorvall GSA rotor at 2500 rpm (800 x g) for 10 min. The supernatant was filtered through cheesecloth, and the filtered suspension was centrifuged in a Beckman Type 30 rotor at 27,000 rpm (85,600 x g) for 70 min. Supernatants were removed by aspiration. The surface of each pellet and the inside wall of each centrifuge tube were rinsed with 15 mL of Buffer D to remove the lipid film present. The membrane pellets were frozen in liquid nitrogen and stored at -70°C for up to 1 month (152).

Preparation of "DEAE Fraction" Containing LDL Receptor

The following steps in the preparation of the "DEAE fraction" (152) were carried out at 4°C.

When required, membrane pellets prepared from 18 adrenal glands, as described above,
were suspended in 60 mL of Buffer E by sequential aspiration through a 16-gauge and a 22-gauge needle. The suspension was sonicated twice for 25 sec with a probe at a setting of 60%. To the suspension were added 4.9 mL of 4 M NaCl, 31 mL of water and 24 mL of 5% (v/v) Triton X-100. After stirring for 10 min, the suspension was centrifuged in a Beckman Type 30 rotor at 27,000 rpm (85,600 x g) for 70 min. The supernatants were pooled into a graduated cylinder and the total volume measured (approximately 125 mL). The supernatant was then diluted into 3 volumes (about 375 mL) of Buffer F. After stirring for 10 min, the mixture was centrifuged in a Sorval SS-34 rotor at 6,500 rpm (5,000 x g) for 10 min to remove undissolved matter. Supernatants were pooled, and the solution of solubilized membranes was adjusted to pH 6.0 with 2 M maleic acid.

The solution of solubilized membranes was then applied to a DEAE-cellulose column at a rate of 40 mL/h. The column was then washed with 80 mL of Buffer G, followed by 80 mL of Buffer H, both at a flow rate of 40 mL/h. The column was eluted with a 100-mL linear gradient of 0 to 250 mM NaCl, using the Gradient Buffer at a flow rate of 40 mL/h. Fractions (4 mL) were collected at 6-min intervals. Aliquots (20 μL) of each fraction (ir duplicate) were added to 980 μL of water in a microcentrifuge tube, and these were used for protein determination by the microassay with precipitation (see Section 2.2.1.2, p. 44). The fractions were transferred to polypropylene tubes, frozen in liquid nitrogen, and stored at -70°C. LDL-receptor activity was measured using the standard binding assay (see Section 2.2.10.2, p. 77) using 200-μL aliquots of only those fractions containing protein. Fractions containing LDL-receptor activity were pooled and this material was designated as the DEAE fraction. Aliquots (400-800 μL) were placed in microcentrifuge tubes, frozen in liquid nitrogen, and stored at -70°C for up to 2 months.
2.2.9 Reconstitution of LDL Receptor

2.2.9.1 Reagents

Buffer I (50 mM Tris, 2 mM CaCl₂, 50 mM NaCl, pH 8.0 at 25°C). Tris (0.606 g), 100 mM CaCl₂ (2 mL), NaCl (0.292 g) were dissolved in approximately 90 mL of water (4°C). The solution was adjusted to pH 8.0 with HCl and made up to 100 mL with water (4°C). The solution was stored at 4°C.

1.5 mg/mL Phosphatidylcholine Liposomes. Using a 100-μL Hamilton syringe, 150 μL of 100 mg/mL phosphatidylcholine (in ethanol) were withdrawn from a septum-covered bottle, and placed in 25-mL round-bottom flask. After the ethanol was evaporated under a stream of nitrogen gas, the dry phospholipid was redissolved in about 20 mL of diethyl ether. The ether was evaporated under a stream of nitrogen, leaving a film of the phospholipid on the inner walls of the flask. Buffer I (10 mL) was added to the flask, and a suspension of multi-lamellar liposomes (153) was formed by hand shaking at room temperature for 5 min. The suspension was stored at 4°C for up to 1 month (151).

1.5 M NaCl. NaCl (2.2 g) was dissolved in 25 mL of water. The solution was stored at 4°C.

Acetone. Acetone was precooled to -20°C prior to use.

2.2.9.2 Procedure

In microcentrifuge tubes, equal volumes (100-300 μL) of 1.5 mg/mL phosphatidylcholine liposomes, 1.5 M NaCl and DEAE fraction (containing LDL receptors) were thoroughly mixed. After 5 min, 0.62 volumes of acetone (-20°C) were added with vortexing to the mixtures with vortexing. Tubes were centrifuged at 4°C for 20 min. Supernatants were drawn off with a syringe and 22-gauge needled (bevel removed) and discarded. Pellets were designated as "phospholipid/acetone precipitates" (152).
Using a 20-μL aliquot of the DEAE fraction (in duplicate), protein concentration was determined by the microassay with precipitation (see Section 2.2.1.2, p. 44). Phospholipid/acetone precipitates were obtained as above using 100-μL aliquots (in duplicate) of the DEAE fraction. The precipitates were resuspended in 1.0 mL of water and the amount of protein was also determined using the microassay with precipitation. Percent incorporation of protein into liposomes was then calculated using the equation:

\[
\% \text{ Incorporation} = \frac{\text{Protein precipitated (μg/μL DEAE fraction used)}}{\text{DEAE fraction protein (μg/μL)}} \times 100 \quad [2.5]
\]

2.2.10 LDL-Binding Assay

2.2.10.1 Reagents

40 mg/mL BSA. BSA (0.4 g) was dissolved in 10 mL of 0.15 M NaCl. The solution was stored at 4°C for up to two weeks.

100 μg/mL \(^{125}\text{I}-\text{LDL}. Just prior to use, stock \(^{125}\text{I}-\text{LDL} was diluted with 40 mg/mL BSA to obtain a final concentration of 100 μg LDL protein/mL.

Buffer J (20 mM Tris, 1 mM CaCl\(_2\), 50 mM NaCl, pH 8.0 at 25°C). Tris (0.121 g), 100 mM CaCl\(_2\) (0.5 mL) and NaCl (0.146 g) were dissolved in approximately 40 mL of water. The solution was adjusted to pH 8.0 with HCl and made up to 50 mL with water. The solution was stored at 4°C for up to two weeks.

Buffer K (150 mM Tris, 1.5 mM CaCl\(_2\), 40 mg/mL BSA, pH 8.0 at 25°C). Tris (0.182 g), 100 mM CaCl\(_2\) (150 μL), and BSA (0.400 g) were dissolved in approximately 9 mL of water. The solution was adjusted to pH 8.0 with HCl and made up to 10 mL with water. The solution was stored at 4°C for up to two weeks.

Buffer K' (150 mM Tris, 1.5 mM CaCl\(_2\), 40 mg/mL BSA, 25 mM EDTA, pH 8.0 at 25°C). This solution was prepared exactly as Buffer K above, except that 0.093 g of EDTA
(disodium salt) was also added. Solution was stored at 4°C for up to two weeks.

Buffer L (20 mM Tris, 1 mM CaCl₂, 50 mM NaCl, 1 mg/mL BSA, pH 8.0 at 4°C). Just prior to use, 1.2 g of Tris, 5 mL of 100 mM CaCl₂, 1.5 g of NaCl, and 0.5 g of BSA were dissolved in approximately 450 mL of water (4°C). The solution was adjusted to pH 8.0 with HCl and made up to 500 mL with water (4°C).

2.2.10.2 Procedure

Standard Binding Assay

The LDL-binding assay described below is based on a method described by Schneider et al. (152,154,155).

Buffers J, K, and K' were allowed to come to room temperature. Enough cellulose acetate membrane filters (pore size: 0.45 μm; diameter: 25 mm) to perform the experiment were placed in approximately 25 mL of Buffer L and left at room temperature for at least 90 min before use.

LDL receptors (DEAE fraction) were reconstituted into phosphatidylcholine liposomes (as described in Section 2.2.9.2, p. 75) and phospholipid/acetone precipitates were obtained. The amount of phospholipid/acetone precipitates prepared in this way depended on the number of assay mixtures required for the experiment (approximately 10 μg protein required per assay mixture). Phospholipid/acetone precipitates were resuspended in Buffer J by passage through a 22-gauge needle to obtain a final protein concentration of 150 to 300 μg/mL.

Unless otherwise indicated, blank and sample assay mixtures were prepared as described in the following protocol:
<table>
<thead>
<tr>
<th>Solution</th>
<th>Non-Specific Binding (BLANK)</th>
<th>Total Binding (SAMPLE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer K (no EDTA)</td>
<td>--</td>
<td>40 μL</td>
</tr>
<tr>
<td>Buffer K' (with EDTA)</td>
<td>40 μL</td>
<td>--</td>
</tr>
<tr>
<td>100 μg/mL ¹²⁵I-LDL</td>
<td>20 μL</td>
<td>20 μL</td>
</tr>
<tr>
<td>Resuspended phospholipid/acetone precipitates</td>
<td>40 μL</td>
<td>40 μL</td>
</tr>
</tbody>
</table>

Binding was initiated by the addition of the resuspended phospholipid/acetone precipitates at 3.5-min intervals. After a 1 h incubation at room temperature, bound ¹²⁵I-LDL was separated from free as follows: 1) a presoaked cellulose acetate membrane filter was placed on a stainless steel filter holder, which was connected to a vacuum line, and 3 mL of ice-cold Buffer L were added without suction to the filter holder; 2) upon the addition of 80 μL of the reaction mixture to the above, suction was applied immediately; 3) with the aid of a Repipet® dispenser, the filter was then washed four times under vacuum with ice-cold Buffer L (3 mL per wash); 4) suction was allowed to continue for 1 min after the last wash, and the filter was then transferred to a scintillation vial. After all samples had been processed in this way, scintillation cocktail was added to each filter. At least 30 min were allowed to pass before the radioactivity on the filters was measured by liquid scintillation counting.

The amount of ¹²⁵I-LDL non-specifically retained by the filter is represented by those incubations containing EDTA (152). Therefore, high-affinity binding of ¹²⁵I-LDL to receptors was calculated by subtracting the value for non-specific binding (blank) from that for total binding (sample).

**Saturation Binding Assay**

The saturation binding studies were performed as described above for the **standard**
binding assay except that variable volumes (0-20 μL) of stoichi 125I-LDL (250-400 μg/mL) were added to the assay mixtures. Each assay mixture was made up to 100 μL with the appropriate volume of 40 mg/mL BSA.

**Competitive Binding Assay**

In the competitive binding studies, unlabelled lipoproteins were diluted with 0.15 M NaCl to a final protein concentration of 200-250 μg/mL. The competitive binding assay was performed as described above in the *standard binding assay* except that 10 μL of 125I-LDL (100 μg/mL) were added to each assay mixture and variable volumes (between 0 and 10 μL) of the dilute unlabelled lipoprotein were added. Each assay mixture was made up to 100 μL with the appropriate volume of 0.15 M NaCl.

**2.2.11 Neuraminidase Treatment**

**2.2.11.1 Reagents**

*Buffer M (1.0 M acetate, pH 6.0 at 25°C).* Sodium acetate (0.82 g) was dissolved in approximately 9.5 mL of water. The solution was adjusted to pH 6.0 with HCl and made up to 10 mL with water.

*Buffer N (50 mM phosphate, pH 6.5 at 25°C).* Potassium phosphate monobasic (57 mg) and potassium phosphate dibasic (15 mg) were dissolved in approximately 9.5 mL of water. The solution was adjusted to pH 6.5 and made up to 10 mL with water.

**Neuraminidase.** Just prior to use, 0.86 units of neuraminidase were dissolved in 24 μL of Buffer M and 100 μL of Buffer N.

**2.2.11.2 Procedure**

The procedure used is based on that of Schneider et al. (156).

Equal volumes of DEAE fraction containing LDL-receptor activity (see Section 2.2.8.2, p. 70) and neuraminidase were mixed and incubated at 37°C for 2 h. Unless otherwise indicated,
the mixture was then incubated at 4°C for an additional 1 h.

2.2.12 Western Blot

2.2.12.1 Reagents

*Electrophoretic Sample Buffer.* Tris (0.020 g), SDS (0.650 g), sucrose (6.5 g) and Bromophenol Blue (0.030 g) were dissolved in approximately 45 mL of water. The solution was adjusted to pH 6.8 with HCl and made up to 50 mL with water.

*Buffer P (25 mM Tris, 192 mM Glycine, 20% (v/v) Methanol, pH 8.3 at 25°C).* Tris (3.0 g), glycine (14.4 g), and methanol (200 mL) were dissolved in approximately 750 mL of water. The pH was checked but not adjusted. The solution was then made up to 1.0 L with water and stored at 4°C.

*Buffer Q (10 mM Tris, 150 mM NaCl, 0.5 mM CaCl₂, pH 8.0 at 25°C).* Tris (1.21 g), NaCl (8.77 g) and 100 mM CaCl₂ (5.0 mL) were dissolved in approximately 950 mL of water. The solution was adjusted to pH 8.0 with HCl and made up to 1.0 L with water.

*Buffer Q’ (10 mM Tris, 150 mM NaCl, 0.5 mM CaCl₂, 10 mM EDTA, pH 8.0 at 25°C).* The solution was prepared exactly as Buffer Q above, except that 3.72 g of EDTA (disodium salt) were also added.

*Blocking Buffer.* On the day of use, 1.0 g of BSA was dissolved in 20 mL of Buffer Q. The solution was kept at 37°C.

*Stock Ponceau S Solution.* Ponceau S (2 g), TCA (30 g), and 5-sulfosalicylic acid (30 g) were dissolved in water and made up to 100 mL with water.

*Working Ponceau S Solution.* Just prior to use, one volume of Stock Ponceau S Solution was diluted with nine volumes of water.

2.2.12.2 Procedure

The procedure used is based on that described by Kroon et al. (157).
Aliquots (5-15 µg) of the DEAE fraction containing LDL-receptor activity (see Section 2.2.8.2, p. 70) were made up in duplicate to 45 µL with Electrophoretic Sample Buffer. In contrast to the protein molecular weight standards (29-200 kDa), the samples did not receive a reducing agent and were not heated prior to electrophoresis. Samples were subjected to 7% SDS-PAGE (mini slab gel) at 40 V for 4 h at room temperature (see Section 2.2.2.1, p. 44).

Proteins were electrophoretically transferred from the slab gel onto nitrocellulose membranes (158), using Buffer P as the transfer buffer. Electrophoresis was carried out at 4°C, at 30 V for 16 h and subsequently at 50 V for an additional 1 h.

The region of the nitrocellulose membrane containing the molecular weight standards was stained with the Working Ponceau S Solution.

The region of the nitrocellulose membrane containing the samples was incubated in the Blocking Buffer at 37°C for 1 h, and was subsequently cut into two strips. The strips were incubated at room temperature for 90 min with ^125^I-LDL in the absence (Buffer Q) or presence (Buffer Q') of EDTA. Incubations were performed in sealable plastic containers using 10 mL of buffer containing 3 µg/mL ^125^I-LDL (300-600 dpm/µg). The nitrocellulose strips were then washed for 10 min in 200 mL of the appropriate Buffer Q (with or without EDTA), followed by two 20 min washes in 200 mL of the same buffer. After the last wash, the membrane was blotted dry, wrapped in plastic food wrap and exposed at -80°C to X-ray film with enhancing screens for 20-40 h.

2.2.13 Nonenzymatic Glycosylation of LDL

2.2.13.1 Reagents

2.0 M Glucose. D-Glucose (3.5 g) was dissolved in 10 mL of water. The solution was stored at 4°C.
Phosphate-Buffered Saline (10 mM Phosphate, 0.15 M Na\(^+\), 5 mM K\(^+\), 0.15 M Cl\(^-\), 0.3 mM EDTA, pH 7.5 at 4\(^\circ\)C). Sodium chloride (24 g), potassium chloride (0.6 g), anhydrous sodium phosphate dibasic (4.5 g), anhydrous potassium phosphate monobasic (0.3 g), and EDTA (0.3 g) were dissolved in approximately 2.5 L of water (4\(^\circ\)C). The solution was adjusted to pH 7.5 and made up to 3 L with water (4\(^\circ\)C).

Buffer R (0.1 M Sodium Borate, pH 9.3 at 25\(^\circ\)C). Sodium borate decahydrate (9.5 g) was dissolved in approximately 200 mL of water. The solution was adjusted to pH 9.3 with HCl and made up to 250 mL with water.

0.03 M Trinitrobenzenesulfonic Acid (TNBS). TNBS (0.088 g) was dissolved in 10 mL of water.

N-\alpha-Acetyl-L-Lysine (Acetyllysine) Standards. Acetyllysine (29.5 mg) was dissolved in 25 mL of Buffer R. The solution (1.0 mL) was made up to 25 mL with Buffer R to obtain an acetyllysine concentration of 2.5 \times 10^{-4} M. To five different 25-mL volumetric flasks were added 1.0, 2.0, 3.0, 4.0, and 5.0 mL of 2.5 \times 10^{-4} M acetyllysine, respectively. Each was made up to volume with Buffer R to obtain final acetyllysine concentrations of 10, 20, 30, 40 and 50 \mu M.

2.2.13.2 Procedure

Serum samples containing normal triglyceride levels (0.90-1.90 mmol/L), normal cholesterol levels (2.00-5.20 mmol/L) and normal glucose levels (3.5-6.0 mmol/L) were pooled. LDL was isolated from this pooled serum as previously described (see Section 2.2.4.2, p. 55) and dialyzed at 4\(^\circ\)C against a total of 3 L of Phosphate-Buffered Saline for 36-48 h (6 changes of 0.5 L each). After dialysis, the LDL was centrifuged in a microcentrifuge for 30 min at 4\(^\circ\)C, and the supernatant was sterilized by passage through a cellulose acetate syringe filter (pore size: 0.2 \mu m; diameter: 4 mm). Water and 2.0 M glucose were similarly sterilized by filtration.

Incubation mixtures were prepared as described in the following protocol:
<table>
<thead>
<tr>
<th>Solution</th>
<th>Assay Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>LDL (3-4 mg/mL)</td>
<td>100 μL</td>
</tr>
<tr>
<td>Glucose (2.0 M)</td>
<td>-</td>
</tr>
<tr>
<td>Water</td>
<td>11 μL</td>
</tr>
</tbody>
</table>

Assay mixtures 1, 2 and 3 contained final glucose concentrations of 0, 100 and 200 mM, respectively.

Nitrogen gas was allowed to pass into each tube for one minute, after which the tubes were closed and incubated at 37°C for 7 days. The tubes were placed in a microcentrifuge and centrifuged for a couple of seconds to recover all the liquid. The tubes were then stored at 4°C for no longer than 1 day.

Aliquots of each assay mixture were subjected to the competitive binding assay (see Section 2.2.10.2, p. 79). The extent of non-enzymatic glycosylation of LDL was measured indirectly by the TNBS assay, *i.e.*, as a decrease in the number of reactive amino groups relative to native LDL. The procedure is based on a modification (159) of the original method of Habeeb (160). Briefly, duplicate aliquots (10 μL) of native or glycosylated LDL were each added to 990 μL of Buffer R. To this solution were added 25 μL of 0.03 M TNBS with thorough mixing. After incubation at room temperature for 30 min, the absorbance was read at 420 nm. Since LDL absorbs at this wavelength, it was necessary to prepare a sample blank, in which 25 μL of water were added instead of TNBS. The number of reactive amino groups was then determined with the use of a standard curve (see Figure 2.9), generated by assaying 1.0 mL of acetyllysine standards (0, 10, 20, 30, 40 and 50 μM) as above. Extent of glycosylation was then calculated by the formula:

\[
\% \text{ Glycosylation} = \left(1 - \frac{\text{Glycosylated LDL amino groups}}{\text{Native LDL amino groups}}\right) \times 100
\]

[2.6]
FIGURE 2.9

TNBS Assay Standard Curve

Legend

The figure shows a typical standard curve generated by the TNBS assay for the determination of free amino groups.

A higher correlation coefficient is obtained from second-order regression analysis than from first-order regression. Error bars represent $\pm$ standard deviation (SD) of the mean, arising from duplicate analyses.
$y = 0.002 + 5.89 \times 10^{-3} x + 7.27 \times 10^{-5} x^2$

$r = 0.9989$
2.2.14 Patient Study

Samples used for the patient study were primarily selected from those sent to the clinical laboratory from the Windsor Diabetic Clinic for analysis of glycosylated hemoglobin. The study also included three hypertriglyceridemic samples of unknown cause.

Control serum for the study was a pool of sera which contained normal serum concentrations of glucose, triglycerides and cholesterol.

Concentrations of glucose, urea, triglycerides, total cholesterol, HDL-cholesterol, LDL-cholesterol, glycosylated hemoglobin and fructosamine were determined as described in Section 2.2.15 below.

LDL was isolated from serum or plasma as described in Section 2.2.4.2, p. 55. The isolated LDL was then subjected to the competitive binding assay (see Section 2.2.10.2, p. 79).

2.2.15 Clinical Chemistry Assays

Glucose, urea, triglycerides, total cholesterol, HDL-cholesterol, and LDL-cholesterol concentrations in serum were determined using a Kodak Ektachem 700 Analyzer. Test methodologies are summarized in Table 2.1.

Glycosylated hemoglobin was determined by the Glyc-Affin® GHb test, according to the manufacturer’s instructions (162). The test uses spectrophotometric measurement at 415 nm after separation of glycosylated hemoglobin from other hemoglobins by boronate affinity chromatography.

Fructosamine concentration in serum was determined using the Roche Fructosamine Test Plus, according to the manufacturer’s instructions (163). The test uses spectrophotometric measurement at 530 nm based on the ability of ketoamines (glycosylated proteins) to reduce nitroblue tetrazolium in alkaline medium.
TABLE 2.1

Test Methodologies for the Kodak Ektachem 700

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>$\beta-D$-Glucose + $O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} D$-Glutamic acid + $H_2O$</td>
</tr>
<tr>
<td></td>
<td>$2H_2O_2 + 4$-Aminooantipyrine + 1,7-Dihydroxynaphthalene \xrightarrow{\text{Peroxidase}} \text{Red dye (540nm)}</td>
</tr>
<tr>
<td>Urea</td>
<td>Urea + $H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>NH$_3$ + Ammonia indicator $\xrightarrow{\text{Dye (670 nm)}}$</td>
</tr>
<tr>
<td></td>
<td>Lipoproteins $\xrightarrow{\text{Surfactant}}$ Triglycerides + Proteins</td>
</tr>
<tr>
<td></td>
<td>Triglycerides + $O_2 \xrightarrow{\text{Lipase}}$ Glycerol + Fatty acids</td>
</tr>
<tr>
<td></td>
<td>Glycerol + ATP $\xrightarrow{\text{Glycerol kinase}} L-\alpha$-Glycerophosphate + ADP</td>
</tr>
<tr>
<td></td>
<td>$L$-\alpha-Glycerophosphate + $O_2 \xrightarrow{\text{L-\alpha-Glycerophosphate oxidase}}$ Dihydroxyacetone phosphate + $H_2O$</td>
</tr>
<tr>
<td></td>
<td>$H_2O_2 + \text{Triarylimidazole leuco dye} \xrightarrow{\text{Peroxidase}}$ Dye (540 nm) + $H_2O$</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>Lipoproteins $\xrightarrow{\text{Surfactant}}$ Lipids + Proteins</td>
</tr>
<tr>
<td></td>
<td>Cholesteryl esters + $H_2O \xrightarrow{\text{Cholesteryl ester hydrolase}}$ Cholesterol + Fatty acids</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>Cholesterol + $O_2 \xrightarrow{\text{Cholesterol oxidase}}$ Cholest-4-ene-3-one + $H_2O_2$</td>
</tr>
<tr>
<td></td>
<td>$H_2O_2 + \text{Triarylimidazole leuco dye} \xrightarrow{\text{Peroxidase}}$ Dye (540 nm) + $H_2O$</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>VLDDL and LDL are first precipitated using dextran sulfate/MgCl$_2$.</td>
</tr>
<tr>
<td></td>
<td>HDL is then analyzed for cholesterol as described above for Total cholesterol.</td>
</tr>
<tr>
<td></td>
<td>Calculated using the Friedewald equation (161):</td>
</tr>
<tr>
<td></td>
<td>$\text{LDL-cholesterol} = \text{Total cholesterol} - \text{HDL-cholesterol} - \text{Triglycerides/2.22}$,</td>
</tr>
<tr>
<td></td>
<td>where all analytes are expressed as mmol/L.</td>
</tr>
</tbody>
</table>
CHAPTER 3
RESULTS AND DISCUSSION

3.1 Isolation of LDL

3.1.1 Collection of Blood and Storage

LDL has been isolated equally well from plasma and serum (139,164,165). There are some artifacts originating after blood collection which can affect LDL. In our studies, a "cocktail" recommended by Schumaker and Puppione (139) was added to the serum to minimize or eliminate these effects. The cocktail included EDTA, gentamicin, thimerosal, sodium azide, and PMSF.

Lipid peroxidation leads to gross denaturation of LDL, resulting in changes in the physical properties of the particles, and alterations in apoB100 (164). EDTA prevents lipid peroxidation by chelating heavy metals, such as Cu$^{2+}$, which promote autoxidation of unsaturated fatty acids and cholesterol. The antibiotics were added to prevent the growth of bacteria. Bacterial endopeptidases, neuraminidases, and phospholipase C can attack LDL, producing physicochemical changes (164).

ApoB100 can undergo limited digestion by plasma proteases, to generate two smaller fragments apoB74 (about 407 kDa) and apoB26 (about 143 kDa). It should be pointed out that LDL particles in which apoB100 has been cleaved in this manner bind normally to the LDL receptor (166). Nevertheless, PMSF was added to serum in the present study to minimize the action of proteolytic enzymes.

Recommendations for storage have been based on immunoreactivity (164,165) and the same guidelines were applied in the present study. Serum was stored for no longer than seven days at 4°C. For longer-term storage, serum was stored at -70°C.
3.1.2 Sequential Flotation Ultracentrifugation

Since lipoproteins have lower hydrated densities relative to other plasma proteins, sequential flotation ultracentrifugation has been the most common method used for their isolation (139).

As mentioned previously (see Section 1.2.1, p. 9), LDL is defined as the total population of lipoproteins within the density range 1.019-1.063 g/mL. The critical step in the sequential-ultracentrifugal isolation of LDL (or any other lipoprotein for that matter) is proper density adjustment of the sample. Improper adjustment can lead to contamination of the LDL preparation or a failure of the LDL to "float" during centrifugation.

According to the Radding-Steinberg formula (see Section 2.2.4.2, p. 55), the volume and initial density of the sample must be known for density adjustment (142). In early studies, density was calculated by placing the sample into a volumetric flask and measuring the mass of the sample, i.e., density = mass/volume. This method was unreliable, since small errors in filling the volumetric flask gave unacceptable errors in the calculated density. A more reliable method was to run a standard KBr solution in parallel with the sample and determine density by measuring the refractive index of the standard. After measurement of the mass, the volume of the sample was calculated, i.e., volume = mass/density.

3.1.3 Characterization

3.1.3.1 Slab-Gel Electrophoresis

Fractions obtained during the sequential-ultracentrifugal isolation of LDL were subjected to 10% SDS-PAGE (see Figure 3.1) as described previously (see Section 2.2.2.1, p. 44).

The d < 1.019 g/mL (see lane 2) contains several proteins, including a 35 kDa protein, corresponding to apoE (characteristic of VLDL and IDL).

Although the 1.019 < d 1.063 g/mL fraction (see lane 4) contains essentially one high
FIGURE 3.1

10% SDS-PAGE of Lipoprotein Fractions From Normolipemic Human Serum

Legend

Aliquots (20 μg) of fractions obtained during the sequential flotation ultracentrifugation of normolipemic human serum (see Section 2.2.4.2, p. 55) were subjected to 10% SDS polyacrylamide slab-gel electrophoresis (34 V for 17 h) and stained with Coomassie Brilliant Blue R-250 (see Section 2.2.2.1, p. 44).

Lane 1, 8: Protein molecular weight (MW) standards (lysozyme: 14.3 kDa; carbonic anhydrase: 29.0 kDa; ovalbumin: 45.0 kDa; bovine serum albumin: 66.0 kDa; phosphorylase b: 97.4 kDa; β-galactosidase: 116 kDa); lane 2: top layer of first centrifugation (d < 1.019 g/mL); lane 3: bottom layer of first centrifugation (d > 1.019 g/mL); lane 4: top layer of second centrifugation (1.019 < d < 1.063 g/mL); lane 5: bottom layer of second centrifugation (d > 1.063 g/mL); lane 6: top layer of third ("wash") centrifugation (1.019 < d < 1.063 g/mL); lane 7: bottom layer of third ("wash") centrifugation (d > 1.063 g/mL).
MW band (characteristics of apoB100), it is evident that this has undergone some degree of proteolysis to produce two smaller fragments. The smaller fragment (130-140 kDa) corresponds to apoB26 suggesting that the larger fragment is apoB74. Since apoB100 which has undergone this type of proteolysis stills binds normally to the LDL receptor (166), it was not of concern in the present study.

The 1.019 < d < 1.063 g/mL fraction was free of apoE contamination. This is important since apoE also binds to the LDL receptor and is an interferent in the LDL-binding assay.

It should be pointed out that the 1.019 < d < 1.063 g/mL fraction contained two bands with MW of 28 and 18 kDa. These MW's correspond to those of apoAII and apoAI, respectively, and are characteristic of HDL. When the 1.019 < d < 1.063 g/mL fraction was subjected to a "wash" centrifugation; these fractions appeared in the bottom fraction (see lane 7). This finding indicated that the third centrifugation was necessary to remove all traces of HDL contamination and obtain pure LDL (see lane 6).

3.1.3.2 Tube-Gel Electrophoresis

Lipoprotein (a) [Lp(a)] is a plasma lipoprotein which contains a single molecule of apoB100 disulphide-linked to a single molecule of apolipoprotein(a) [apo(a)] (167,168). Apo(a) is a glycoprotein with a heterogeneous MW range of 400 to 800 kDa, depending on the individual from which it was isolated (168). Controversy remains on whether or not Lp(a) can bind to the LDL receptor (167-169). Since Lp(a) has a hydrated density of 1.06-1.08 g/mL, the presence of this particle in the LDL preparations was checked by 3% SDS-PAGE (see Section 2.2.2.1, p. 49). As shown in Figure 3.2, the 1.019 < d < 1.063 g/mL fraction contained a single protein, with a calculated MW of 520 ± 7 kDa. This corresponds to apoB100, with a literature value of 550 kDa (17), and indicated the absence of Lp(a) in the LDL preparation.
FIGURE 3.2

3% SDS-PAGE of LDL Isolated from Normolipemic Human Serum

Legend

An aliquot (40 μg) of LDL isolated by sequential flotation ultracentrifugation of normolipemic human serum (see Section 2.2.4.2, p. 55) was subjected to 3% SDS polyacrylamide tube gel electrophoresis (50 mA for 6 h) and stained with Coomassie Brilliant Blue R-250 (see Section 2.2.2.1, p. 49).

Tube 1: Protein MW standards (phosphorylase b monomer: 97.4 kDa; phosphorylase b dimer: 194.8 kDa; phosphorylase b trimer: 292.2 kDa; phosphorylase b tetramer: 389.6 kDa; phosphorylase b pentamer: 487.0 kDa; phosphorylase b hexamer: 584.4 kDa); tube 2: LDL.
FIGURE 3.2

MW (kDa)

- 584.4
- 487.0
- 389.6
- 292.2
- 194.8
- 97.4
3.1.3.3 Agarose-Gel Electrophoresis

Further evidence that the $1.019 < d < 1.063$ g/mL fraction isolated is LDL was given by agarose-gel electrophoresis (see Section 2.2.2.2, p. 49). As shown in Figure 3.3, the $1.019 < d < 1.063$ g/mL fraction (lanes 3 and 7) had $\beta$-mobility, characteristic of LDL. The $d < 1.019$ g/mL fraction (lanes 2 and 6) had pre-$\beta$-mobility (characteristic of VLDL) and the $d > 1.063$ g/mL fraction (see lanes 4 and 8) had $\alpha$-mobility (characteristic of HDL). It was clear that the LDL was not contaminated with these other lipoproteins.

3.2 Radioiodination of LDL

3.2.1 Reaction Conditions

Shepherd et al. (147) compared four methods for incorporating $^{125}$I into LDL and found the iodine monochloride method to be the best for several reasons: 1) it was simple; 2) it produced no disruption of the lipoprotein complex; 3) it produced a labelled product with less than 5% of the label associated with lipid.

The percent incorporation of iodine into proteins using the iodine monochloride method is determined by the amount of iodine monochloride used in the reaction, since only iodine in the $I^+$ form can take part in the iodination reaction. Some of the $I^+$ is made radioactive by the inclusion of $^{125}$I in the reaction mixture, which undergoes rapid isotope exchange to become $^{125}$I$^+$ (145). The specific radioactivity of the final product can be enhanced by increasing the amount of $^{125}$I. However, it should be noted that with increasing $^{125}$I, the fraction of $^{125}$I in the $I^+$ form will decrease according to the formula below:

$$ Fraction \left( ^{125}I^+ \right) = 1 - \left[ \frac{A \times SR}{ng \ T} \right] $$  \[3.1\]

where $A = mCi$ $^{125}$I used and $SR = \text{specific radioactivity of } ^{125}I \ (ng/mCi)$ (145).

The I:aPoB100 ratio in the final LDL product is given by the formula:

$$ \langle I:aPoB100 \rangle_{PRODUCT} = \frac{\% \text{ incorporation}}{100} \times \langle I^+\text{aPoB100} \rangle_{REACTION} $$  \[3.2\]
FIGURE 3.3

Agarose-Gel Electrophoresis of Lipoprotein Fractions from
Normolipemic Human Serum

Legend

Aliquots of lipoprotein fractions obtained during the sequential flotation ultracentrifugation of normolipemic human serum (see Section 2.2.4.2, p. 55) were subjected to agarose-gel electrophoresis (90 V for 20 min) and stained with Fat Red 7B (see Section 2.2.2.2, p. 49).

Lanes 1,5: 1 μL of serum; lane 2,6: 1 μL and 0.5 μL, respectively, of VLDL/IDL (d < 1.019 g/mL fraction); lanes 3,7: 1 μL and 0.5 μL, respectively, of LDL (washed d < 1.019 < d < 1.063 g/mL fraction); lanes 4,8: 1 μL and 0.5 μL, respectively, of HDL (washed d > 1.063 g/mL fraction).
The percent incorporation can be increased by increasing the amount of ICI used, but as the \((I:\text{apoB100})_{\text{REACTION}}\) increases, so does \((I:\text{apoB100})_{\text{PRODUCT}}\) and an undesirably high incorporation can take place. Goldstein and Brown (140) suggest that \(^{125}\text{I}\)-LDL used for binding studies contain < 1 mole of I per 100 kDa of apoB100, or 5.5 moles I per 1 mole apoB100.

Previous workers have suggested \((I^+:\text{apoB100})_{\text{REACTION}}\) ratios of 38:1 (140) and 22:1 (147)* to obtain satisfactory incorporation into apoB100 and to minimize incorporation into the lipid moiety of LDL. For the standard radiiodination procedure in this study (see Section 2.2.6.2, p. 65), the \(I^+:\text{apoB100}\) ratio in the reaction mixture was 25:0.45 or 28:1. It was desired that the \(^{125}\text{I}\) produce have a ratio of less than 5.5 I:1 apoB100 as stated above. To calculate the percent incorporation required to satisfy this requirement, Formula 3.2 can be rearranged to give:

\[
\% I \text{ incorporation} = \frac{(I:\text{apoB100})_{\text{PRODUCT}}}{(I^+:\text{apoB100})_{\text{REACTION}}} \times 100
\]  

[3.3]

Substituting the values 5.5 and 28 into the formula gives an incorporation of 20%. Therefore, as long as the incorporation was less than 20%, the requirement of Brown and Goldstein (140) was fulfilled.

Goldstein and Brown (140) also suggest that \(^{125}\text{I}\)-LDL used for binding studies contain 200-600 cpm/ng protein. In an initial radiiodination attempt, 50 \(\mu\)Ci of \(^{125}\text{I}\) were used, resulting in a low specific radioactivity of the \(^{125}\text{I}\)-LDL (see Table 3.1). The amount of \(^{125}\text{I}\) used was increased to 0.44 mCi, resulting in increased specific radioactivities.

Interestingly, the incorporation on one particular attempt (see Table 3.1, attempt 8) fell to < 1%. Even after fresh reagents (glycine buffer, iodine monochloride) were prepared, the incorporation remained less than 1%. Bolton (170) suggests that in this type of situation, the

* The authors suggest a 2.5:1 ratio, assuming a MW of 64 kDa for apoB100. Correcting for a MW of 550 kDa, the 1:apoB100 becomes 22:1.


**TABLE 3.1**

Radioiodination of LDL

<table>
<thead>
<tr>
<th>Attempt</th>
<th>Stock $^{125}$I (mCi/mL)</th>
<th>I:LDL (pmole:mmole)</th>
<th>$^{125}$I:LDL (μCi:mmole)</th>
<th>%I Incorporation *</th>
<th>Specific Radioactivity (dpm/ng LDL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>28</td>
<td>110</td>
<td>15</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>28</td>
<td>470</td>
<td>10</td>
<td>175</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>28</td>
<td>980</td>
<td>9.4</td>
<td>143</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>28</td>
<td>980</td>
<td>9.8</td>
<td>160</td>
</tr>
<tr>
<td>5</td>
<td>21/100</td>
<td>28</td>
<td>980</td>
<td>5.0</td>
<td>203</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>28</td>
<td>980</td>
<td>18</td>
<td>683</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>28</td>
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<td>23</td>
<td>28</td>
<td>980</td>
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<td>1</td>
</tr>
<tr>
<td>9</td>
<td>23</td>
<td>28</td>
<td>980</td>
<td>0.56</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>38</td>
<td>980</td>
<td>2.0</td>
<td>29</td>
</tr>
<tr>
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<td>25</td>
<td>38</td>
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<td>125</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>38</td>
<td>980</td>
<td>25</td>
<td>680</td>
</tr>
<tr>
<td>13</td>
<td>100</td>
<td>28</td>
<td>980</td>
<td>17</td>
<td>375</td>
</tr>
</tbody>
</table>

* The fraction of $^{125}$I in the I⁺ form is given by:

\[
\text{Fraction} = 1 - \left[ \frac{A \times SR}{\text{ng I}} \right]
\]

where \(A\) = mCi $^{125}$I used and \(SR\) = specific radioactivity of $^{125}$I (ng/mCi) (145). For radioiodinations in this study, the fraction is 0.98 or greater, indicating that the $^{125}$I is essentially all in the I⁺ state. Therefore, % I incorporation was equivalent to the % $^{125}$I incorporation.
radioiodide solution may be substandard and that a new batch should be tried. Even when fresh radioiodide was purchased, incorporation remained extremely low. Upon review of previous attempts at radioiodination, it was discovered that the most successful iodinations (attempts #6, 7) were those in which the radioiodide was purchased as a 100 mCi/mL stock solution. By comparison, those attempts in which the radioiodide was purchased as a 20-30 mCi/mL stock solution gave lower incorporations and specific radioactivities (see Table 3.1). It was thought that perhaps there was an inhibitor present in the radioiodide solution at the time of dilution by the manufacturer that could interfere with the radioiodination. It was reasoned that if there were inhibitors present, and if less $^{125}\text{I}$ were added to the reaction mixture, then less inhibitor would also be added and the incorporation would increase. Indeed, that is what happened (compare attempt #11 with #10, in which the only difference is the amount of $^{125}\text{I}$ added). For this reason, all subsequent radioiodinations were carried out using $^{125}\text{I}$ purchased as 100 mCi/mL solution and the results were satisfactory with good incorporations and high specific radioactivities (see attempt #13).

3.2.2 Separation of Free and Bound $^{125}\text{I}$

Many different methods, including dialysis and gel filtration, have been used to remove free radiotracer from bound. In this study, dialysis was first used for this purpose. The procedure required approximately 6 h to remove 95% of the free $^{125}\text{I}$ and produced several litres of radioactive waste.

In order to reduce the time required and the amount of waste produced, gel filtration was employed (see Section 2.2.6.2, p. 65). With a flow rate of 0.4 mL/min, 90-95% of the free $^{125}\text{I}$ was removed. Although a slower flow rate of 0.15 mL/min increased the time needed for separation, this was chosen for subsequent radioiodinations since almost 100% of the free $^{125}\text{I}$ was removed. This is illustrated in Figure 3.4, in which close to 100% of the early peak
FIGURE 3.4

Sephadex G-10 Chromatography of LDL-Radioiodination Mixture

Legend

LDL was radioiodinated using the iodine monochloride method (see Section 2.2.6.2, p. 65). The mixture was applied to a Sephadex G-10 gel-filtration column (1 cm x 15 cm bed) and eluted with Buffer C (50 mM Tris, 0.15 M NaCl, 0.01% EDTA pH 7.4 at 25°C). The flow rate was 0.15 mL/min and 3-min fractions were collected for fractions 1-20, and 7.5-min fractions thereafter. A 10-μL aliquot (in duplicate) of each fraction was used for measurement of radioactivity by liquid scintillation counting and two peaks of radioactivity were observed. A 10-μL aliquot of each fraction from the early peak was subjected to TCA precipitation (see Section 2.2.7.2, p. 66). Fractions of the early peak with the highest radioactivity were pooled and designated 125I-LDL.
FIGURE 3.4

Radioactivity Precipitated (% of total) [●—●]

[●—●] Radioactivity (cpm/fraction x 10^-7)
(radioiodinated LDL) was precipitated by 10% TCA.

### 3.2.3 Characterization of $^{125}$I-LDL

#### 3.2.3.1 Autoradiography

Figure 3.5 shows an autoradiograph of $^{125}$I-LDL subjected to 10:5 SDS-PAGE. The majority of the radioactivity was present in one high MW band (see lanes 3-6) indicating that the apoB100 remained essentially intact after radiiodination. Fragmentation of apoB100 is more evident with increasing concentration of protein (see lanes 7 and 8).

#### 3.2.3.2 Lipid Extraction

Less than 10% of the radioactivity was extractable into 2:1 chloroform/methanol, indicating that the majority of the label was in the protein rather than the lipid moiety of LDL.

#### 3.2.3.3 Stability

One problem that arose from using gel filtration was the dilution of the $^{125}$I-LDL. Since LDL is most stable in concentrated protein solutions (165), it was decided to add bovine serum albumin (BSA)s (40 mg/mL) after the removal of free $^{125}$I. The addition of BSA has also been suggested by Bolton (170) to help minimize denaturation of labelled proteins by self-radiolysis. $^{125}$I-LDL was stored under liquid nitrogen in order to minimize lipid peroxidation. $^{125}$I-LDL stored in this manner was stable in terms of binding to the LDL receptor for 7 to 10 days (see Figure 3.6).

### 3.3 Optimization of LDL-Binding Assay

In preliminary experiments of the present study, precision of the LDL-binding assay was poor. The steps of the assay which included pipetting, liquid scintillation counting, and preincubation of membrane filters, therefore, were studied in order to improve precision.

#### 3.3.1 Pipetting

In the binding assay of Schneider et al. (152) it was necessary to pipette four different
FIGURE 3.5
Autoradiograph of $^{125}$I-LDL

**Legend**

LDL was radioiodinated using the iodine monochloride method (see Section 2.2.6.2, p. 65) and subjected to 10% SDS-PAGE (see Section 2.2.2.1, p. 44). After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 and dried onto blotting paper. The gel was then exposed to X-ray film in the presence of enhancing screens for 3 h at -80°C (see Section 2.2.7.2, p. 68).

*Lane 1*: protein MW standards (lysozyme: 14.3 kDa; carbonic anhydrase: 29.0 kDa; ovalbumin: 45.0 kDa; bovine serum albumin: 66.0 kDa; phosphorylase b: 97.4 kDa; β-galactosidase: 116 kDa); *lane 2*: LDL (20 μg), before radioiodination; *lanes 3 to 8*: $^{125}$I-LDL (359 dpm/μg), containing 0.15, 0.30, 0.60, 1.2, 2.4, 4.8 μg of protein, respectively.
FIGURE 3.6

Stability of $^{125}$I-LDL

Legend

LDL was radioiodinated using the iodine monochloride method and stored in 40 mg/mL BSA under liquid nitrogen (see Section 2.2.6.2, p. 65). Specific radioactivity of $^{125}$I-LDL was 683 dpm/μg on day "zero". At various times, thereafter, the $^{125}$I-LDL was subjected to the *standard binding assay* (see Section 2.2.10.2, p. 77). Binding assays were carried out using 9 μg of DEAE-fraction protein (2.7 μg of phospholipid/acetone-precipitate protein).

Error bars represent ± SD of the mean, arising from duplicate analyses.
solutions. In order to minimize errors due to pipetting, two solutions were combined and the number of pipetting steps was reduced to three per tube.

3.3.2 Liquid Scintillation Cocktail Incubation

A critical source of error was the length of time that the cellulose acetate membrane filters were left in the scintillation cocktail before the measurement of radioactivity. Figure 3.7 shows that up to 30 min, the radioactivity detected increased the longer the membrane filter was left in the cocktail. Thereafter, the radioactivity measured remained constant. For this reason, all radioactivity measurements of membrane filters were performed at least 30 min after the addition of scintillation cocktail.

3.3.3 Non-Specific Binding

$^{125}$I-LDL is non-specifically retained by cellulose acetate membrane filters. Since binding of LDL to the receptor is Ca$^{2+}$-dependent, the presence of EDTA in the reaction mixture inhibits binding. For each binding assay a sample (no EDTA) and blank (with EDTA) were prepared. The sample represented the Ca$^{2+}$-dependent + Ca$^{2+}$-independent binding of $^{125}$I-LDL, while the blank represented the Ca$^{2+}$-independent binding. Therefore, the high-affinity or Ca$^{2+}$-dependent binding of the $^{125}$I-LDL to the receptor was obtained by subtracting the blank from the sample.

In order to minimize non-specific binding of $^{125}$I-LDL to membrane filters, the filters were preincubated in a buffer containing 1 mg/mL BSA. Figure 3.8 shows the effect of the length of preincubation on non-specific binding. Minimum non-specific binding was obtained when the filters were preincubated for at least 90 min. When $^{125}$I-LDL containing no BSA was applied, less than 0.2% of the applied radioactivity was retained by the filters. This is in agreement with the finding of Schneider et al. (154) who observed < 0.2% non-specific binding with cellulose acetate filters. However, when $^{125}$I-LDL containing BSA was applied, substantially higher non-specific binding was obtained. This is consistent with the finding that reaction
FIGURE 3.7

Effect of Length of Incubation of Membrane Filters in Scintillation Cocktail on Radioactivity Measured

Legend

An aliquot (20 μL) of $^{125}$I-LDL (100 μg/mL; 575 dpm/μg) was applied to a cellulose acetate membrane filter (pore size: 0.45 μm; diameter: 25 mm) on a stainless steel filter holder. The filter was washed four times under vacuum with 3 mL of ice-cold Buffer L (20 mM Tris, 1 mM CaCl$_2$, 50 mM NaCl, 1 mg/mL BSA, pH 8.0 at 4°C) per wash. The vacuum was left on for 1 min after the last wash. The filter was placed in a scintillation vial and scintillation cocktail added (time "zero"). Radioactivity trapped on the filter was then measured by liquid scintillation counting at various times thereafter.

Error bars represent the counting error which was obtained by multiplying the percent counting error (see APPENDIX C for calculation) by the count rate.
FIGURE 3.8

Effect of Length of Preincubation of Membrane Filters with

BSA on Non-Specific Binding of $^{125}$I-LDL

Legend

Cellulose acetate membrane filters (pore size: 0.45 μm) were placed in ice-cold Buffer L (20 mM Tris, 1 mM CaCl$_2$, 50 mM NaCl, 1 mg/mL BSA, pH 8.0 at 4°C) and left at room temperature. At various times, a filter was removed and placed on a stainless steel holder. An aliquot (20 μL) of $^{125}$I-LDL (100 μg/mL; 575 dpm/ng) was applied to the filter. The $^{125}$I-LDL was either in 0.15 M NaCl (○——○) or 40 mg/mL BSA, 0.15 M NaCl (●——●). The filter was washed four times under vacuum with 3 mL of ice-cold Buffer L per wash. The vacuum was left on for 1 min after the last wash. Radioactivity trapped on filters was measured by liquid scintillation counting. The above procedure was carried out in duplicate.

Error bars represent ± SD of the mean, arising from duplicate analyses.
FIGURE 3.8

- $^{125}$I-LDL in 40 mg/mL BSA, 0.15 M NaCl
- $^{125}$I-LDL in 0.15 M NaCl
mixtures containing BSA led to higher non-specific binding of $^{125}$I-LDL to the membrane filters.

The buffer used to wash the membrane filters after application of the $^{125}$I-LDL/receptor incubation mixture contained BSA. Within one day of the preparation of the buffer, a precipitate would form in the buffer, leading to reduced flow rates in the washing procedure and substantially higher non-specific binding of $^{125}$I-LDL to the membrane filters. For this reason, the buffer was prepared fresh for every binding assay performed.

3.4 Isolation of LDL Receptor

3.4.1 Choice of Tissue

Kovanen et al. (42) showed that of the 18 bovine tissues they studied, the highest specific activity of LDL receptors was in the adrenal cortex, and that binding activity in this tissue was 6-fold higher than that in the adrenal medulla. The adrenal cortex has, therefore, been the choice of tissue for the preparation of chemical amounts of LDL receptors (152).

Although adult glands were suggested by previous workers (154-156), the unavailability of sufficient quantities of the adult glands from local slaughterhouses necessitated the use of calf adrenals.

Substantially less adrenal cortex (approximately 2 g/gland) was obtained from the calves used in this study compared with approximately 4 g/gland obtained from adult bovine adrenal glands (155).

3.4.2 Isolation

It has been shown that the LDL receptor in crude solubilized extracts retains the properties (i.e., affinity, specificity) characteristic of the receptor in whole cells and its purified form (156). For the present study, it was decided to purify the LDL receptor to a semi-purified solubilized form in order to minimize the number of steps in the purification and yet allow simple binding studies to be performed.
Schneider et al. (154) showed that the LDL receptor could not be extracted from adrenocortical membranes by procedures such as freeze-thawing or sonication, or by washing with high concentrations of salts, urea or guanidine hydrochloride. Although several detergents were effective in solubilizing the receptor, only octylglucoside was satisfactory in producing a receptor in a form which allowed assay of its binding activity.

Schneider et al. (155) showed that ion-exchange chromatography on DEAE was the most useful technique as a first step in the purification of the LDL receptor. This technique was used in the preparation of the solubilized LDL receptor in the present study. Solubilized adrenocortical membranes were subjected to DEAE-cellulose chromatography at pH 6 to adsorb the LDL receptor. Upon elution of the DEAE with a NaCl gradient of 0-250 mM, the LDL receptor appeared in a single peak between 125 and 150 mM NaCl (see Figure 3.9). In the studies of Schneider et al. (155, 156), the LDL receptor from adult bovine adrenocortical cells also eluted at a NaCl concentration of 125-150 mM.

3.4.3 Characterization of LDL Receptor

3.4.3.1 Reconstitution of LDL Receptor into Liposomes

*Liposome/Receptor Incubation*

The receptor activity recovered by reconstitution into liposomes was influenced by the length of time that the liposomes and DEAE fraction were incubated before acetone precipitation (see Figure 3.10). Maximum activity was obtained after 5 min of incubation.

*Phosphatidylcholine Concentration*

The receptor activity recovered by reconstitution into liposomes was influenced by the concentration of phosphatidylcholine used in the reconstitution (see Figure 3.11). Maximum activity was obtained in the presence of greater than 0.3 mg/mL phosphatidylcholine. In the absence of phosphatidylcholine, less than 4% of receptor activity was recovered in the
FIGURE 3.9
DEAE-Cellulose Chromatography of
Solubilized Calf Adrenocortical Membranes

Legend

Descending chromatography was carried out at 4°C. DEAE-cellulose was equilibrated
with Buffer G (50 mM Tris-maleate, 2 mM CaCl₂, 1% v/v Triton X-100, 1 mM PMSF, pH 6.0
at 4°C) at 60 mL/h to obtain a final bed of 2.5 cm x 8.5 cm. The LDL receptor was solubilized
from 26 calf adrenal glands with the use of Triton X-100 (see Section 2.2.8.2, p. 70). The
solubilized-receptor solution (approximately 480 mL) was applied to the column at 46 mL/h, after
which the column was washed with 80 mL of Buffer G and then 80 mL of Buffer H (50 mM
Tris-maleate, 2 mM CaCl₂, 40 mM octylglucoside, 1 mM PMSF, pH 6.0 at 4°C) at 40 mL/h.
The column was eluted with a 100-mL linear gradient of 0-250 mM NaCl in Buffer H at 40 mL/h
and six-minute (4.0-mL) fractions were collected. Aliquots of each fraction (<20 μL) were
removed for duplicate assay of protein content using the microassay with precipitation (see
Section 2.2.1.2, p. 44) and a 200-μL aliquot was removed for assay of ¹²⁵I-LDL binding activity
using the standard binding assay (see Section 2.2.10.2, p. 77). The tubes containing high-affinity
¹²⁵I-LDL binding activity (fractions 12-18) were pooled and designated as DEAE fraction.
FIGURE 3.9

$\text{NaCl (mM)}$ [--- --- ---]

$^{125}\text{I-LDL Binding (\mu g/fraction)}$ [• --- •]
FIGURE 3.10

Effect of Length of Incubation of DEAE Fraction with Liposomes on Receptor Activity Recovered

Legend

Equal volumes (150 μL) of phosphatidylcholine liposomes (2.0 mg/mL), NaCl (1.5 M), and DEAE fraction (190 μg protein/mL) were mixed in microcentrifuge tubes. At various times after the mixing of the above, 270 μL of freezing-cold acetone were added with vortexing. Mixtures were centrifuged in a microcentrifuge for 20 min, after which supernatants were removed with a syringe and needle. Each precipitate was resuspended in 100 μL of Buffer J (20 mM Tris, 1 mM CaCl₂, 50 mM NaCl, pH 8.0 at 25°C) and high-affinity ¹²⁵I-LDL binding was determined using the standard binding assay (see Section 2.2.10.2, p. 77). The above procedure was carried out in duplicate.

The value for maximum activity represents the activity recovered when the liposomes and DEAE fraction were incubated for 10 min before acetone precipitation. Error bars represent ± SD of the mean, arising from duplicate analyses.
FIGURE 3.10

125I-LDL Bound (% of maximum)

Length of Incubation (min)
FIGURE 3.11
Effect of Phosphatidylcholine Concentration on Receptor Activity Recovered

Legend

In microcentrifuge tubes, equal volumes (150 µL) of NaCl (1.5 M) and DEAE fraction (190 µg protein/mL) were added. Variable volumes (0-150 µL) of phosphatidylcholine liposomes (2.0 mg/mL) in Buffer I (50 mM Tris, 2 mM CaCl₂, 50 mM NaCl, pH 8.0 at 25°C) were added. Appropriate volumes of Buffer I were added to bring volumes up to a total of 450 µL. After 5 min, 270 µL of freezing-cold acetone were added with vortexing to each tube. Tubes were centrifuged for 20 min, after which the supernatants were removed with a syringe and needle. Each precipitate was resuspended in 100 µL of Buffer J (20 mM Tris, 1 mM CaCl₂, 50 mM NaCl, pH 8.0 at 25°C), and high-affinity ¹²⁵I-LDL binding was determined using the standard binding assay (see Section 2.2.10.2, p. 77). The above procedure was carried out in duplicate.

Phosphatidylcholine concentrations are those in the mixture before the addition of acetone. The value for maximum activity represents the activity recovered in the presence of 0.33 mg/mL phosphatidylcholine. Error bars represent ± SD of the mean, arising from duplicate analyses.
phospholipid/acetone precipitate.

In the study of Schneider et al. (155), maximum activity was recovered in the presence of greater than 0.2 mg/mL phosphatidylcholine, and about 10% of activity was recovered in the absence of phosphatidylcholine.

*Acetone Concentration*

The receptor activity recovered by reconstitution into liposomes was influenced by the concentration of acetone used to precipitate the liposome/receptor complex (see Figure 3.12). Maximum activity was obtained in the presence of 37-39% v/v acetone. In the absence of acetone, less than 4% of receptor activity was recovered in the phospholipid precipitate.

In the study of Schneider et al. (155), maximum activity was recovered over a broader range of acetone (about 30-40% v/v), and about 10% of activity was recovered in the absence of acetone.

*Percent Reconstitution*

In the presence of optimal amounts of phosphatidylcholine (0.5 mg/mL) and acetone (38% v/v) about 34% ± 3.7% of the protein in the DEAE fraction was reconstituted into the liposomes.

In the study of Schneider et al. (155), about 25% of the protein was incorporated. It should be pointed out that protein determination in that study was based on a modified Lowry assay in which BSA was reconstituted into liposomes and processed similarly to the samples to generate a standard curve. The authors indicated that the standard curves obtained were identical with those obtained with BSA by the standard Lowry assay. However, when it was attempted to reconstitute BSA into liposomes in the present study, absorbances of the BSA standards were less than 5% of those obtained without reconstitution, indicating a poor incorporation of BSA into the liposomes.
FIGURE 3.12

Effect of Acetone Concentration on
Receptor Activity Recovered

Legend

In microcentrifuge tubes, equal volumes (150 μL) of NaCl (1.5 M), phosphatidylcholine liposomes (1.5 mg/mL) and DEAE fraction (190 μg protein/mL) were mixed. After 5 min, variable volumes (0-440 μL) of freezing-cold acetone were added with vortexing. Tubes were centrifuged for 20 min, after which the supernatants were removed with a syringe and needle. Each precipitate was resuspended in 100 μL of Buffer J (20 mM Tris, 1 mM CaCl₂, 50 mM NaCl, pH 8.0 at 25°C), and high-affinity ¹²⁵I-LDL binding was determined using the standard binding assay (see Section 2.2.10.2, p. 77). The above procedure was carried out in duplicate.

Acetone concentrations are expressed as a % (v/v) of the total mixture. Data was obtained from two separate experiments, indicated by the open or closed circles. The value for maximum activity represents the activity recovered in the presence of 37.5% (v/v) acetone. Error bars represent ± SD of the mean, arising from duplicate analyses.
Liposome Non-Specific Binding Site

The observation discussed in Percent Reconstitution above, suggested that a difference existed between the liposomes used in this study and those used in the study of Schneider et al. (155). It may be that some of the differences observed in terms of optimal phosphatidylcholine and acetone concentrations were related to a difference in the liposomes rather than the LDL receptor itself.

It has been shown that LDL can interact with liposome binding sites on cell surfaces (171). In order to assure that this binding did not interfere with the LDL-binding assay, phospholipid/acetone precipitates were prepared as described in Section 2.2.9.2, p. 75 in the absence of protein. When phospholipid/acetone precipitates prepared in this way were subjected to the LDL-binding assay (see Section 2.2.10.2, p. 77), the radioactivity of the sample and blank were identical. Furthermore, phospholipid/acetone precipitates, prepared from DEAE fractions that did not contain LDL receptors but did contain other proteins (see fractions 10 and 24 in Figure 3.9, p. 116) showed no difference between the sample and blank in the binding assay. This suggested that if $^{125}\text{I}$-LDL was binding to the liposomes, then the binding was Ca$^{2+}$- independent and accounted for in the blank. This finding is in agreement with that of Brissette et al. (172), who found that the non-specific binding site on liver membranes was Ca$^{2+}$-independent.

3.4.3.2 Specific Activity

The specific activity of the DEAE fraction was found to be $5.2 \pm 2.1 \, \mu g \, ^{125}\text{I}$-LDL bound/mg protein. Precipitation of the receptor with phosphatidylcholine and acetone gave a 3-fold purification since only $34 \pm 3.7\%$ of the protein was recovered in the phospholipid/acetone precipitate. Specific activity of this fraction was found to be $15 \pm 5.7 \, \mu g \, ^{125}\text{I}$-LDL bound/mg protein.
In the study of Schneider et al. (155), specific activities of the DEAE fraction and phospholipid/acetone precipitate were 4.3 and 17.2 μg $^{125}$I-LDL bound/mg protein, respectively. In that study, only 25% of the protein was recovered in the phospholipid/acetone precipitate and, therefore, the specific activity of this fraction was greater than that observed in the present study.

It should be pointed out that although the same concentration of $^{125}$I-LDL (18-20 μg/mL) was used in both studies, this concentration was equal to the dissociation constant in the study of Schneider et al. (155) whereas it was about two times the apparent dissociation constant in the present study.

3.4.3.3 Western Blot

Figure 3.13A shows the DEAE fraction prepared from calf adrenocortical cells, as visualized by ligand blotting on nitrocellulose. Two bands (major and minor) capable of binding $^{125}$I-LDL were visualized (see lanes 2 and 3). Binding of $^{125}$I-LDL to both bands was inhibited by 10 mM EDTA (see lane 6), characteristic of LDL-receptor binding.

The major band had a calculated MW of about 135 ± 2 kDa, which is considerably less than the value of 160 kDa found for the purified receptor (156). It should be pointed out that the MW of the purified receptor was determined in the presence of a reducing agent (2-mercaptoethanol) whereas the present ligand blotting experiments were performed in the absence of a reducing agent. Under non-reducing conditions, other workers (157,173,174) have found that the MW of the LDL receptor is 130 kDa. This is consistent with the finding in the present study, suggesting that the major band corresponds to the intact mature receptor.

In the ligand ($^{125}$I-LDL) blotting study of Kroon et al. (157), on which the present blotting experiments were based, only one LDL-binding band was present in the DEAE fraction. The existence of the minor band was, therefore, investigated in the present study. The minor band had a calculated MW of 125 ± 2 kDa. Since it has been shown that the removal of sialic
FIGURE 3.13

Western Blot of DEAE Fraction

Legend

Aliquots of the DEAE fraction (30 μL containing 10.8 μg of protein) were mixed with 6 μL of 1.0 M sodium acetate (pH 6.0 at 25°C) and 24 μL of 50 mM potassium phosphate (pH 6.5 at 25°C) in the absence or presence of neuraminidase (0.22 units) and incubated under various conditions (see protocol below). After incubation 30 μL of each assay mixture were made up to 45 μL with Electrophoretic Sample Buffer (3.3 mM Tris, 1.3% SDS, 13% sucrose, 0.03% Bromophenol Blue, pH 6.8). Protein MW standards (carbonic anhydrase: 29.0 kDa; ovalbumin: 45.0 kDa; bovine serum albumin: 66.0 kDa; phosphorylase b: 97.4 kDa; β-galactosidase: 116 kDa; myosin: 200 kDa) were made up in a reducing buffer (53 mM Tris, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.1% Bromophenol Blue, pH 6.8) and placed in a boiling water bath for 2 min. Samples and MW standards were subjected to 7% SDS-PAGE (mini-gel) at 40 V for 4 h (see Section 2.2.2.1, p. 44). Proteins were electrophoretically transferred from the slab gel onto nitrocellulose membrane (see Section 2.2.12.2, p. 80). The region of the nitrocellulose membrane containing the MW standards (lane 1) was stained with Ponceau S. The region of the nitrocellulose membrane containing the samples was cut into two strips and incubated with 125I-LDL (3 μg/mL, 398 dpm/ng) in the absence or presence of 10 mM EDTA (see Section 2.2.12.2, p. 80). The membrane was then exposed to X-ray film with enhancing screens for 40 h at -80°C.

(A): autoradiograph of nitrocellulose membrane. (B): polyacrylamide gel after electrophoretic transfer of proteins. Lane 1: protein MW standards; lanes 2-7: DEAE fraction treated as described in the protocol below.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Neuraminidase Treatment</th>
<th>Condition</th>
<th>125I-LDL Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>not added</td>
<td>4°C for 10 min</td>
<td>no EDTA</td>
</tr>
<tr>
<td>3</td>
<td>not added</td>
<td>37°C for 2 h; 4°C for 14 h</td>
<td>no EDTA</td>
</tr>
<tr>
<td>4</td>
<td>added</td>
<td>37°C for 2 h; 4°C for 14 h</td>
<td>no EDTA</td>
</tr>
<tr>
<td>5</td>
<td>added</td>
<td>37°C for 2 h</td>
<td>no EDTA</td>
</tr>
<tr>
<td>6</td>
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<tr>
<td>7</td>
<td>added</td>
<td>37°C for 2 h; 4°C for 14 h</td>
<td>EDTA added</td>
</tr>
</tbody>
</table>
acid residues from bovine adrenal receptors by treatment with neuraminidase results in a decrease in MW of 7-11 kDa (156), it was thought that the minor band may be an immature form of the LDL receptor. This idea was tested by treatment the DEAE fraction with neuraminidase. After treatment with neuraminidase, two bands capable of binding $^{125}$I-LDL were still present (see lanes 4 and 5), and binding of $^{125}$I-LDL to both bands was inhibited by 10 mM EDTA (see lane 7). Both of these proteins had a MW of about 5-6 kDa lower than those without neuraminidase treatment, indicating that both proteins were susceptible to neuraminidase attack. This finding clearly ruled out the possibility that the lower MW band was a de-sialated immature form of the LDL receptor.

It was thought that the minor band may correspond to another form (possibly a fetal form) of the receptor since the bovine adrenal glands were obtained from calves. In the study of Beisiegel et al. (175), preparations of LDL receptor from human fetal adrenal gland also produced two bands (major and minor) which demonstrated immunoreactivity towards a monoclonal anti-(LDL receptor) antibody. The two bands had identical MW's to the two bands visualized in preparations from bovine adrenal cortex. This suggests that the same forms of the receptor exist in both the fetal and adult gland. The authors do not comment on the presence of the second band.

In the study of Daniel et al. (173), a second lower-MW minor band was also observed by ligand (LDL) blotting, but its existence was also not commented on by the authors. The study demonstrated that the minor band was not visible in the presence of less than 2 $\mu$g/mL LDL in the incubation mixture. In the present study, 3 $\mu$g/mL of $^{125}$I-LDL were used, but in the study of Kroon et al. (157), only about 0.3 $\mu$g/mL $^{125}$I-LDL was used, which may explain why the second band was observed in the present study, but not in the latter.

The identity of the minor band has not been established. The possibility that it may be
a "fetal" form of the LDL receptor (c.f. the existence of adult and fetal hemoglobin) has not been ruled out at this time. It seems more likely that the minor band corresponds to a proteolytic fragment of the mature LDL receptor. Figure 3.14 shows a model of the LDL receptor and a possible site of fragmentation. This would explain three properties of the protein demonstrated in this study: 1) it has LDL-binding activity; 2) it is susceptible to the action of neuraminidase; and 3) it has a MW of about 7 kDa less than the mature LDL receptor.

Schneider et al. (156) reported that "the ability of the purified receptor to bind $^{125}$I-LDL was not affected by neuraminidase treatment", but the authors did not provide any experimental information or data to further clarify this. An interesting finding which arose from the Western-blot experiments in the present study is that the intensity of the $^{125}$I-LDL-binding bands increased after neuraminidase treatment (compare lanes 4 and 5 with lanes 2 and 3 in Figure 3.13). Since electrophoretic transfer of proteins from the SDS-PAGE gel to the nitrocellulose membrane was essentially complete (see Figure 3.13B), the result does not appear to be an artifact of the procedure. Rather, the result would suggest that the binding activity of the LDL receptor is increased after neuraminidase treatment.

Although the exact role of the domain containing O-linked sugars (see Figure 3.14) is not known, Goldstein et al. (176) suggested that in vivo the oligosaccharide chains act as struts to keep the ligand-binding domain at a distance from the cell membrane. However, it may be that in vitro, under conditions when the receptor is solubilized, removal of sialic acid residues by neuraminidase alters the LDL-receptor conformation to one which favours LDL binding.

3.4.3.4 Binding Properties

*Dissociation Constant*

Figure 3.15 shows the effect of $^{125}$I-LDL concentration on non-specific and high-affinity binding to the phospholipid/acetone precipitate of the DEAE fraction. The high-affinity binding
FIGURE 3.14

Model of the LDL Receptor and Possible Proteolytic Site

Legend

The DEAE fraction obtained from calf adrenocortical cells in the present study contained two bands (major and minor) capable of binding $^{125}$I-LDL as visualized by ligand blotting (see Figure 3.13). The major band corresponds to the intact mature LDL receptor. The minor band is possibly a proteolytic fragment of the intact mature receptor.

The arrow on the model of the LDL receptor indicates a possible site of proteolytic cleavage which would account for three properties of the minor band demonstrated in the present study: 1) it has LDL-binding activity; 2) it is susceptible to the action of neuraminidase; and 3) it has a MW of about 7 kDa less than the mature LDL receptor. [Adapted from Brown and Goldstein (53)].
FIGURE 3.14

<table>
<thead>
<tr>
<th>DOMAIN</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>2</td>
<td>EGF precursor homology (~400 amino acids)</td>
</tr>
<tr>
<td>3</td>
<td>O-linked sugars (58 amino acids)</td>
</tr>
<tr>
<td>4</td>
<td>Membrane-spanning (22 amino acids)</td>
</tr>
<tr>
<td>5</td>
<td>Cytoplasmic (50 amino acids)</td>
</tr>
</tbody>
</table>
FIGURE 3.15

Effect of $^{125}$I-LDL Concentration on $^{125}$I-LDL Binding to DEAE Fraction

Legend

Total (■) and non-specific (○) $^{125}$I-LDL binding were determined by the saturation binding assay (see Section 2.2.10.2, p. 78) in the absence or presence of 10 mM EDTA, respectively. High-affinity binding (■) was calculated by subtracting non-specific binding (○) from total binding (●). Each assay mixture contained 6.4 µg of phospholipid/acetone-precipitate protein and the indicated concentration of $^{125}$I-LDL (specific radioactivity: 327 dpm/ng).

Error bars represent ± SD of the mean, arising from duplicate analyses.
was saturable, characteristic of receptor/ligand interaction (177). This saturable binding was abolished by the presence of 10 mM EDTA in the reaction, as shown by the non-specific binding curve. Non-specific binding was directly proportional to the $^{125}$I-LDL concentration over the entire range tested.

The high-affinity saturable data was subjected to Scatchard-plot analysis (see Figure 3.16) in order to determine the dissociation constant (177). The LDL receptor from calf adrenocortical cells had an apparent dissociation constant of $8.8 \pm 1.0 \mu g$ $^{125}$I-LDL/mL (or $16.0 \pm 1.7 \times 10^{-9}$ M, assuming a MW of 550 kDa for apoB100). It has been shown (152,155,156) that the LDL receptor from adult bovine adrenocortical cells has an apparent dissociation constant range from 8 to 20 $\mu g$/mL (15 to $36 \times 10^{-9}$ M), although most values quoted are in the higher part of this range.

The value for the dissociation constant determined in the present study may be in the low end of the quoted range in the literature for two reasons: 1) calf adrenocortical cells were used in the present study, whereas adult bovine adrenocortical cells were used in these other studies; and 2) different protein assays were used to measure the concentration of LDL protein.

It has been shown (178) that LDL receptors on puppy liver membranes exhibit a slightly lower dissociation constant ($11 \mu g$/mL) than those on adult canine membranes ($15 \mu g$/mL).

Previous studies (152,155,157) have measured LDL protein by the original Lowry assay (134). It has been shown (135) that lipids are a positive interferent in the Lowry assay, but in the presence of SDS they are solubilized and become noninterfering. Measurement of LDL in the present study was measured by a modified Lowry assay (see Section 2.2.1.2, p. 43) which includes SDS in the reagent. It was shown in the present study that the original Lowry assay had a positive bias of about 10% over the modified Lowry assay in the measurement of LDL protein.
FIGURE 3.16
Scatchard Plot of $^{125}$I-LDL Saturation-Binding Data

Legend

The $^{125}$I-LDL high-affinity binding data from Figure 3.15 was subjected to Scatchard-plot analysis (177).

The dissociation constant ($K_d$) is equal to $-(1$/slope$)$. 
$y = 0.162 - 0.135x$
$r = -0.98$

$K_d = 7.4 \mu g/mL$
In a more comprehensive study (179), this bias has been shown to be about 16%. Since values of LDL protein are overestimated by the standard Lowry assay, the value for the dissociation constant of the LDL receptor quoted by Schneider et al. (152,155,156) may be too high.

**Specificity**

It has been demonstrated (180) that the human LDL receptor has an apparent dissociation constant of about 2 to 4 μg LDL/mL (3.6 to 7.3 x 10^{-9} M) at 4°C and 10 to 15 μg/mL (18-27 x 10^{-9}M) at 37°C, using human LDL as the ligand. It is evident from the dissociation constant determined in the present study that the calf adrenocortical LDL receptor has a similar affinity for human LDL. Furthermore, it has been shown (181) that the binding domain of the LDL receptor is functionally conserved in the human and beef, since the beef LDL receptor can distinguish between normal and familial binding-defective human apoB100 as effectively as the human LDL receptor.

The binding of ^{125}I-LDL to the phospholipid/acetone of the DEAE fraction was inhibited competitively by unlabelled human LDL (see Figure 3.17). Human HDL (devoid of apoE) did not compete for binding, but human VLDL/IDL (containing apoE) was a more effective competitor than LDL. This specificity for human lipoproteins is identical with that of the human LDL receptor (17), reinforcing the idea that the binding domain is functionally conserved in the two species.

3.5 Nonenzymatic Glycosylation of LDL

3.5.1 Reaction Conditions

The extent of nonenzymatic glycosylation in diabetics has been reported as being 2 to 5% (95,96,103). The extent of nonenzymatic glycosylation is influenced both by the degree and duration of the hyperglycemia, as well as the residence time of the LDL in the plasma (96). Fasting serum glucose concentrations in diabetics may be as high as 25 mM. Although after a
FIGURE 3.17

Competition of Unlabelled Lipoproteins with $^{125}$I-LDL for the LDL Receptor

Legend

Unlabelled VLDL/IDL, LDL and HDL were obtained from normolipemic human serum (see Section 2.2.4.2, p. 55) and diluted with 0.15 M NaCl to obtain protein concentrations of 250 $\mu$g/mL. $^{125}$I-LDL high-affinity binding was determined by the competitive binding assay (see Section 2.2.10.2, p. 79) in the presence of variable amounts of the unlabelled lipoproteins. Specific radioactivity of $^{125}$I-LDL was 143 dpm/ng and the final $^{125}$I-LDL concentration was 10 $\mu$g/mL. Each assay mixture contained 8.6 $\mu$g of phospholipid/acetone-precipitate protein.

The value for maximum activity represents the amount of $^{125}$I-LDL bound in the absence of any unlabelled lipoprotein. Error bars represent ± SD of the mean, arising from duplicate analyses.
glucose load, where serum glucose concentrations may double, they return to fasting levels within 3 to 4 h in mild diabetes and 6 to 8 h in severe diabetes (182). The circulating half-life of LDL is approximately 3 days in normals (183), but may be slightly longer in moderately severe diabetes (127).

Significantly higher concentrations of glucose (up to 200 mM) and longer incubation periods (7 to 9 days) are necessary in vitro (94,95,101,103) to achieve the same extent of glycosylation seen in vivo.

When nonenzymatic glycosylation of LDL was carried out in vitro in the present study, the maximum extent of modification attained was about 5-6%, even when LDL was incubated in the presence of as high as 200 mM glucose for 7 days. This finding is consistent with that of Steinbrecher and Witzum (103), although they found that in some experiments substantially higher derivatization (up to 17%) was achieved with a lower concentration (80 mM) of glucose. The authors comment that the reason for the great variability in the extent of glycosylation under apparently identical conditions was not known.

The discrepancy between in vivo and in vitro nonenzymatic glycosylation of LDL and the variability of in vitro glycosylation may be explained by the role of metal ions in monosaccharide attachment to protein. Hunt et al. (110) found that incorporation of monosaccharide into protein increased in the presence of added Cu\(^{2+}\). Presumably, this occurred by the production of autoxidation-derived ketoaldehydes since this effect was inhibited by the addition of a metal chelator (109,110).

In vitro glycosylation of LDL in the present study as well as others (100-104) are carried out in the presence of EDTA, a metal chelator. It may be that slight variations from experiment to experiment in the amount of EDTA present cause variations in the extent of glycosylation. Similarly, it may be that in vivo glucose is more susceptible to peroxidation and, therefore,
incorporation into protein. There is evidence (112,113) that lipid peroxidation is elevated in diabetics, and this state would favour the glycosylation of proteins.

3.5.2 Binding to LDL Receptor

It has been shown (101,103) using intact normal human fibroblasts that LDL nonenzymatically glycosylated in vitro had a reduced ability to compete for the binding and degradation of native $^{125}$I-LDL.

In the present study, LDL nonenzymatically glycosylated in vitro also had a reduced ability to compete for the binding of $^{125}$I-LDL to the calf adrenocortical LDL receptor, when compared with control LDL (see Figure 3.18). With increasing glycosylation, LDL had a decreased ability to compete for the binding of $^{125}$I-LDL. The study showed that the calf adrenocortical LDL receptor was capable of recognizing subtle changes in human LDL on the order of what is seen in diabetics, i.e., modification of 2-5% of lysine residues.

3.6 Patient Study

3.6.1 Subject Descriptions

In order to test the effect of hyperglycemia in vivo on the ability of LDL to bind to the LDL receptor, three types of subjects were chosen. The control sample was 5 pooled serum from ten normoglycemic (4.7 ± 0.4 mmol/L; 4.2 - 5.5 range), normocholesterolemic (4.29 ± 1.30 mmol/L; 4.44 - 5.03 range), and normotriglyceridemic (0.56 ± 0.04 mmol/L; 0.53 - 0.61) subjects.

The second group consisted of diabetic subjects in good control (glycosylated hemoglobin less than 10%) with a mean plasma glucose (± SD) of 6.2 (± 1.1) mmol/L. The third group consisted of diabetic subjects in poor control (glycosylated hemoglobin greater than 10%) with a mean plasma glucose (± SD) of 10.9 (± 1.7) mmol/L.

Since it has been shown by others (115) that hypertriglyceridemia can affect LDL binding
FIGURE 3.18

Competition of Nonenzymatic Glycosylated LDL with

$^{125}$I-LDL for the LDL Receptor

Legend

LDL isolated from normolipemic, normoglycemic human serum (see Section 2.2.4.2, p. 55) was incubated in the presence of 0 (▲), 100 (●), or 200 (■) mM glucose at 37°C for 7 days (see Section 2.2.13.2, p. 82). These LDL preparations were diluted with 0.15 M NaCl to obtain protein concentrations of 200 µg/mL. $^{125}$I-LDL high-affinity binding was determined by the competitive binding assay (see Section 2.2.10.2, p. 79) in the presence of variable amounts of the unlabelled LDL. Specific radioactivity of $^{125}$I-LDL was 122 dpm/ng and final $^{125}$I-LDL concentration was 10 µg/mL. Each assay mixture contained 8.2 µg of phospholipid/acetone-precipitate protein.

The value for maximum activity represents the amount of $^{125}$I-LDL bound in the absence of any unlabelled lipoprotein. Error bars represent ± SD of the mean, arising from duplicate analyses. The percent glycosylation of LDL as determined by the TNBS assay (see Section 2.2.13.2, p. 82) is 3.0 ± 1.2% and 5.3 ± 1.0% for the preparations incubated with 100 mM and 200 mM glucose, respectively.
to the LDL receptor, a fourth group was also included in the patient study. This consisted of three hypertriglyceridemic subjects of unknown cause, with a mean plasma triglyceride (± SD) of 5.14 (± 0.27) mmol/L.

A more complete clinical chemistry profile of the individual subjects tested is given in Table 3.2.

3.6.2 Binding of Subject LDL to LDL Receptor

The ability of LDL to bind to the LDL receptor was measured indirectly by its ability to displace control 125I-LDL from the receptor. A lower displacement value indicates a reduced ability to compete with the control radiolabelled LDL for the receptor.

Displacement values for the subjects tested as well as means for each group are given in Table 3.3.

3.6.2.1 Correlation with the Degree of Glycemic Control

The LDL isolated from most of the diabetic subjects had an equal or better ability to compete with the control 125I-LDL than LDL from non-diabetic subjects. Analysis of the data by a t-test (184) showed that the means of the two groups were not statistically different (p = 0.01).

For the entire population studied, there was a negative correlation* between 125I-LDL displacement values and all three indicators of glycemic control (plasma glucose and fructosamine, and glycosylated hemoglobin), suggesting that LDL binding decreases as the plasma glucose concentration increases. However, correlation coefficients were low (-0.173, -0.133, -0.067 for glucose, fructosamine and glycosylated hemoglobin, respectively), indicating a high degree of randomness in the results.

* Correlation was determined using the computer programme StatView® SE+ (Abacus Concepts, Inc., Berkeley, CA), on a Macintosh® Plus personal computer system (Apple Computer, Inc., Cupertino, CA).
### TABLE 3.2

Glycosylated Hemoglobin, Plasma Glucose, Fructosamine, Lipid and Urea Concentrations in Control, Diabetic and Hypertriglyceridemic Subjects

<table>
<thead>
<tr>
<th>Subjects*</th>
<th>Glucose** (mmol/L)</th>
<th>Glycosylated Hemoglobin (% of Total Hb)</th>
<th>Fructosamine (µmol/L)</th>
<th>Cholesterol</th>
<th>Triglycerides (mmol/L)</th>
<th>Urea (mmol/L)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>4.7 ± 0.4</td>
<td>ND***</td>
<td>180</td>
<td>Total (mmol/L)</td>
<td>HDL (mmol/L)</td>
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<td></td>
<td>4.29 ± 1.30</td>
<td>1.28</td>
<td>2.76</td>
</tr>
<tr>
<td>normolipemic control</td>
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<td>(4.44 - 5.03)</td>
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<td>Diabetics in good control***</td>
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<td>Hypertriglyceridemc of</td>
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</table>

* Samples were obtained as described in Section 2.2.14, p. 86. Numbers 1 to 12 refer to individual patients.

** Glucose, cholesterol, triglycerides, and urea concentrations in plasma were measured using a Kodak Ektachem 700 (see Section 2.2.15, p. 86). Glycosylated hemoglobin and fructosamine were measured using appropriate test kits (see Section 2.2.15, p. 86).

*** ND: not determined

**** Subjects considered under good control were those with glycosylated hemoglobin values less than 10%. Subject 5 was considered an exception to this due to the extremely high plasma fructosamine concentration.
TABLE 3.3

Displacement of Control $^{125}$I-LDL from Calf Adrenocortical LDL Receptor by LDL Isolated from Control, Diabetic and Hypertriglyceridemic Subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>$^{125}$I-LDL Displaced* (% of Maximum)</th>
<th>Mean ± SD** for Each Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled normoglycemic, normolipemic control</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 ± 0.9</td>
<td>100 ± 0.9</td>
</tr>
<tr>
<td>Diabetics in good control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>101 ± 3.2</td>
<td>103 ± 1.9</td>
</tr>
<tr>
<td>2</td>
<td>106 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>102 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>104 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>Diabetics in poor control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>102 ± 0.9</td>
<td>104 ± 5.0</td>
</tr>
<tr>
<td>6</td>
<td>113 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>104 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>97.8 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>103 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>Hypertriglyceridemics of unknown cause</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>113 ± 1.2</td>
<td>100 ± 9.6</td>
</tr>
<tr>
<td>11</td>
<td>97.5 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>89.8 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

* High-affinity binding of $^{125}$I-LDL was determined by the competitive binding assay (see Section 2.2.10.2, p. 79), in the presence of 10 μg/mL unlabelled LDL from control subjects. The specific radioactivity of $^{125}$I-LDL was 390 dpm/ng and the final concentration was 10 μg/mL. Each assay mixture contained 16 μg of phospholipid/acetone-precipitate protein. Maximum $^{125}$I-LDL displaced represents the difference between the amount of $^{125}$I-LDL bound in the absence of any unlabelled lipoprotein and that bound in the presence of unlabelled control LDL.

** SD values are those arising from duplicate analyses.
Glucose, fructosamine and glycosylated hemoglobin are short-, medium- and long-term indicators of glycemic control, with time frames of 1 day, 2-3 weeks and 4 months, respectively. One of the problems of trying to correlate LDL binding with these parameters may be that LDL has a circulating half-life of approximately 3 days (183), a time frame in which none of these indicators can accurately predict the extent of glycemic control. Interestingly, \(^{125}\text{I}-\text{LDL}\) displacement values did show the best correlation with the short-term indicator (plasma glucose concentration) and the worst correlation with the long-term indicator (glycosylated hemoglobin).

3.6.2.2 Correlation with Plasma Triglyceride Concentration

In the study of Hiramatsu et al. (115), it was shown that LDL from hypertriglyceridemic subjects (both diabetic and non-diabetic) had a decreased ability to downregulate LDL receptor activity and sterol synthesis in cultured human skin fibroblasts. They suggest that this effect "is likely to be due to delivery of fewer LDL particles to the cells as a result of the lower binding of triglyceride-enriched LDL to the LDL receptor."

In the present study, \(^{125}\text{I}-\text{LDL}\) displacement values demonstrated a negative correlation with plasma triglyceride concentration, suggesting that LDL binding decreases as the triglyceride concentration increases. However, the correlation coefficient was low (-0.113), indicating a high degree of randomness in the results.

It should be pointed out that in the study of Hiramatsu et al. (115), the mean plasma triglyceride concentration of the hypertriglyceridemic subjects was 22 mmol/L, compared to 4.2 mmol/L in the present study. Furthermore, the effect of LDL-receptor-activity suppression by LDL from hypertriglyceridemic subjects was not observed in subjects whose plasma triglyceride concentrations were less than 5.6 mmol/L.

In the present study, the mean of the \(^{125}\text{I}-\text{LDL}\) displacement values from subjects with plasma triglyceride values greater than 5.0 mmol/L was lower than that from subjects with
plasma triglyceride values less than 5.0 mmol/L and the difference approached statistical significance ($p < 0.10$). This suggests that when plasma triglyceride concentrations are greater than 5.0 mmol/L modification of LDL by triglyceride-enrichment may be sufficient to affect its binding to the LDL receptor.

### 3.6.2.3 Correlation with Plasma Urea Concentration

It has been shown (123) that carbamylation (chemical reaction of urea with lysine residues of proteins) of LDL causes decreased LDL-receptor binding *in vitro*. Since uremia can manifest in diabetics, plasma urea concentrations of the subjects were also measured. For the entire population studied, there was a negative correlation between $^{125}$I-LDL displacement values and plasma urea concentrations, suggesting that LDL binding decreases with increasing plasma urea concentration. However, the correlation coefficient was low (-0.094), indicating a high degree of randomness in the results.

### 3.6.2.4 Multifactorial Correlation

Since glucose (101-104), triglycerides (115), and urea (123) have all been implicated in the modification of LDL binding to the receptor, the data in the present study was subjected to multiple regression analysis.* It was thought that the variables might predict $^{125}$I-LDL displacement values more when considered collectively rather than independently. In fact, the correlation coefficient (-0.201) was higher when the three variables were considered collectively, but it still indicates the high degree of randomness in the results.

* Analysis was carried out using the StatView® SE+ computer programme.
CHAPTER 4

SUMMARY AND CONCLUSIONS

4.1 Isolation and Characterization of LDL

LDL was isolated from human serum or plasma as the 1.019 < d < 1.063 g/mL fraction using sequential flotation ultracentrifugation. The procedure involves the sequential density adjustment and ultracentrifugation of samples to cause the sequential flotation of VLDL/IDL, LDL, and HDL (139,140). In the present study, it was found that the most reliable method for proper density adjustment of samples was to run a standard KBr solution in parallel with the sample and determine density by measuring the refractive index of the standard.

A "wash" centrifugation of the 1.019 < d < 1.063 g/mL fraction was necessary to remove all traces of HDL contamination. Agarose-gel electrophoresis also confirmed that this fraction was free of VLDL/IDL, while SDS-PAGE showed that it was not contaminated with apoE, an apolipoprotein which is an interferent in the LDL-binding assay (17).

The 1.019 < d < 1.063 g/mL fraction obtained in this manner contained only one apolipoprotein with a calculated MW of 520 ± 7 kDa. This corresponds to apoB100 with a MW of 550 kDa (17). This fraction also had β-mobility on agarose-gel electrophoresis, characteristic of LDL (17).

The most significant limitation (but not restricted) to this study was the isolation of LDL from serum. Although widely accepted in the literature as the procedure for isolating LDL, sequential flotation ultracentrifugation is a long and technically difficult procedure. A procedure such as affinity chromatography may prove to be a much simpler and faster means of purifying LDL from serum.

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4.2 Radioiodination of LDL

Radioiodinations of LDL were carried out using the iodine monochloride method (148,149) and proved to be extremely simple to carry out, i.e., it had few reagents and was fast.

The procedure was most successful in terms of percent I-incorporation, when Na$^{125}$I was purchased as a 100 mCi/mL stock solution rather than a 20-30 mCi/mL stock solution. It is believed that inhibitors of radioiodination were present in the dilute solution of Na$^{125}$I.

Gel-filtration (using Sephadex G-10) was a more satisfactory means of separating free and unbound $^{125}$I after radioiodination than dialysis for two reasons: 1) it only required about 1 h, compared with more than 6 h for dialysis; and 2) it produced only about 25 mL of radioactive waste, compared to about 6 L for dialysis.

Using an I$^+_{10}\text{apoB100}$ ratio of 28 moles:1 mole and an $^{125}$I:apoB100 ratio of 980 μCi: 1 n mole in the reaction mixture gave percent I-incorporations of less than 20% and produced $^{125}$I-LDL which satisfied the requirements of Goldstein and Brown (140): 1) it contained < 5.5 moles I per 1 mole of apoB100; and 2) it had specific radioactivities of 200-600 cpm/ng of protein.

$^{125}$I-LDL prepared in this manner had less than 10% of the radioactivity extractable into 2:1 chloroform/methanol, indicating that the majority of the label was in the protein rather than the lipid moiety.

The apoB100 remained essentially intact after radioiodination as shown by SDS-PAGE and autoradiography, but was stable in terms of LDL-receptor binding for only 7 to 10 days. This relatively short shelf-life of $^{125}$I-LDL was another limitation to the binding assays as the reagent had to be prepared often. A means of increasing the stability of this reagent would be extremely useful.
4.3 Optimization of LDL-Binding Assay

For radioactivity measurements of $^{125}$I-LDL trapped on cellulose acetate membrane filters by liquid scintillation counting, it was necessary to incubate the filters in the scintillation cocktail for at least 30 min for detection of maximum radioactivity.

Cellulose acetate membrane filters were preincubated in a buffer containing 1 mg/mL BSA for at least 90 min to minimize the non-specific binding of $^{125}$I-LDL to the filters. When aliquots of $^{125}$I-LDL not containing BSA were applied to preincubated filters, less than 0.2% of the applied radioactivity was retained by the filters. When aliquots of $^{125}$I-LDL containing BSA were applied to preincubated filters, substantially higher non-specific binding was observed. This effect was probably due to the retention of complexes of LDL/albumin resulting from protein/protein interactions.

4.4 Isolation and Characterization of LDL Receptor

The unavailability of sufficient quantities of adult bovine adrenal glands from local abattoirs necessitated the use of calf adrenals for the isolation of the LDL receptor. The purification is a modification of the procedure of Schneider et al. (152) and consisted of essentially four steps: 1) dissection of adrenal gland to obtain adrenal cortex; 2) homogenization of adrenal cortex to obtain adrenocortical membranes; 3) solubilization of membranes with Triton X-100; and 4) adsorption on to and elution off a DEAE-cellulose ion-exchange column.

Preparations of the LDL receptor from calf adrenocortical cells had some similarities to those from adult bovine adrenocortical cells, but also had some differences (see Table 4.1). Most notably, the apparent dissociation constant of the receptor ($8.8 \pm 1.0 \mu g^{125}$I-LDL/mL) was in the low end of the quoted range ($8-20 \mu g^{125}$I-LDL/mL) in the literature (152,155,156).

Two bands (major and minor) capable of binding $^{125}$I-LDL were visualized when the DEAE fraction was subjected to ligand blotting on nitrocellulose. The major band corresponded
TABLE 4.1
Comparison of Preparations of LDL Receptor from Calf and Adult Bovine Adrenocortical Cells

<table>
<thead>
<tr>
<th>PROPERTY</th>
<th>CALF (Present Study)</th>
<th>ADULT (Literature)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass of cortex</td>
<td>2 g/gland</td>
<td>4 g/gland</td>
</tr>
<tr>
<td>[NaCl] to elute receptor off DEAE-cellulose</td>
<td>125-150 mM</td>
<td>125-150 mM</td>
</tr>
<tr>
<td>Optimum concentration to reconstitute receptor into liposomes:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylycholine</td>
<td>&gt; 0.3 mg/mL</td>
<td>&gt; 0.2 mg/mL</td>
</tr>
<tr>
<td>Acetone</td>
<td>37-39 % (v/v)</td>
<td>30-40% v/v</td>
</tr>
<tr>
<td>Receptor activity recovered in absence of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylycholine</td>
<td>&lt; 4 %</td>
<td>10%</td>
</tr>
<tr>
<td>Acetone</td>
<td>&lt; 4 %</td>
<td>10%</td>
</tr>
<tr>
<td>Percent of DEAE-fraction protein reconstituted into liposomes</td>
<td>34 ± 3.7%</td>
<td>25%</td>
</tr>
<tr>
<td>Specific binding activity:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE fraction</td>
<td>5.2 ± 2.1 μg ¹²⁵I-LDL/mg</td>
<td>4.3 μg ¹²⁵I-LDL/mg</td>
</tr>
<tr>
<td>Phospholipid/acetone precipitate</td>
<td>15 ± 5.7 μg ¹²⁵I-LDL/mg</td>
<td>17.2 μg ¹²⁵I-LDL/mg</td>
</tr>
<tr>
<td>Molecular weight of receptor:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before neuraminidase treatment</td>
<td>135 ± 2 kDa</td>
<td>130 kDa</td>
</tr>
<tr>
<td>After neuraminidase treatment</td>
<td>129 ± 2 kDa</td>
<td>120 kDa</td>
</tr>
<tr>
<td>Apparent dissociation constant</td>
<td>8.8 ± 1.0 μg/mL</td>
<td>8-20 μg/mL</td>
</tr>
</tbody>
</table>
to the intact mature LDL receptor. The minor band demonstrated the following properties: 1) it had LDL-binding activity; 2) it was susceptible to the action of neuraminidase; and 3) it had a MW of about 7 kDa less than the mature LDL receptor, as determined on SDS-PAGE under non-reducing conditions. The identity of the minor band was not established but may correspond to a proteolytic fragment of the mature LDL receptor. Further studies are necessary to confirm this hypothesis.

The binding activity of solubilized LDL receptor appeared to increase after neuraminidase treatment, as shown by ligand blotting.

The calf adrenocortical LDL receptor is a suitable substitute for the human LDL receptor for studying human LDL binding. The apparent dissociation constant of the LDL receptor from calf adrenocortical cells was very similar to that reported for the human LDL receptor (4-10 µg LDL/mL), using human LDL as the ligand (180). Furthermore, the calf adrenocortical LDL receptor demonstrated binding specificity towards human lipoprotein fractions which was identical with that of the human LDL receptor. This may suggest that the binding domain of the LDL receptor is functionally conserved in the human and beef.

Previous studies using intact culture cells examined the ability of nonenzymatic glycosylated LDL (101,103) and LDL from diabetic subjects (115,118,119) to bind to the LDL receptor. The studies involved: 1) careful maintenance and growth of cells; 2) long incubation periods (12-24 h) for binding and internalization of LDL; 3) multiple washings of cell layers to remove unbound LDL; and 4) a second incubation of the cells with a releasing agent to dissociate LDL from its receptor. The use of semi-purified solubilized calf adrenocortical LDL receptors in the present study has facilitated the study of nonenzymatically glycosylated LDL by a binding assay which is much simpler than these previous studies. It only involves: 1) a 1-h incubation of LDL with the receptor; and 2) a simple filtration procedure to remove unbound LDL. The
use of a "slot-blot" apparatus may allow the processing of binding mixtures even more quickly and make the procedure more amenable to automation.

4.5 Nonenzymatic Glycosylation of LDL

When nonenzymatic glycosylation of LDL was carried out in vitro, the maximum extent of modification attained was about 5-6%, even when LDL was incubated in the presence of as high as 200 mM glucose for 7 days.

Nonenzymatic glycosylated LDL prepared in this way had a reduced ability to compete for the binding of $^{125}$I-LDL to the calf adrenocortical LDL receptor when compared with control LDL. With increasing extent of glycosylation, LDL had a decreased ability to compete for the binding of $^{125}$I-LDL.

The calf adrenocortical LDL receptor was capable of recognizing changes (nonenzymatic glycosylation) to human LDL on the order of what is seen in diabetics, i.e., modification of 2-5% of lysine residues (95,96,103).

4.6 Patient Study

The ability of LDL from subjects to bind to the calf adrenocortical LDL receptor was measured indirectly by its ability to displace control $^{125}$I-LDL from the receptor. A lower displacement value indicates a reduced ability to compete with the control radiolabelled LDL.

Analysis of the data by a t-test indicated that there was no difference between LDL binding from diabetics and non-diabetics. Similarly, there was no difference between diabetics in poor control and those in good control.

Although LDL incubated with increasing amounts of glucose in vitro had a decreasing ability to bind to the LDL receptor, LDL binding did not correlate very well with any of three indicators of in vivo glycemic control (plasma glucose and fructosamine, and glycosylated hemoglobin). The reason for this may be two-fold. First, for the in vitro studies, the LDL was
incubated in a constant known concentration of glucose over the entire incubation period, whereas in the subjects the concentration of glucose is not fixed. Plasma glucose, fructosamine and glycosylated hemoglobin can give an indication of the average degree of hyperglycemia over a time period of 1 day, 2-3 weeks, and 4 months, respectively. However, none of them can accurately predict the glucose concentration in a specific time period of 3-5 days, the circulating half-life of LDL. The measurement of plasma glucose concentrations at various times over the period starting from 3-5 days prior to and ending at the time of collection of the plasma, would give a more accurate estimate of the average plasma glucose concentration over the lifespan of the LDL. LDL binding may show a higher correlation with this "average" value.

There is a second, and perhaps more critical, possibility for the discrepancy between the in vitro and in vivo results. The in vitro study involved changing one variable (glucose concentration) while keeping all others constant. In vivo, there are a number of other varying factors, including plasma triglyceride and urea concentration, which can simultaneously influence the extent of LDL binding. The correlation between LDL binding and plasma glucose, triglyceride and urea concentrations did increase when these three variables were considered collectively rather than independently, but not high enough to predict $^{125}$I-LDL displacement values with any degree of accuracy. It may be that there are other factors affecting LDL binding to the receptor which have not been identified. A more comprehensive clinical profile of the subjects (e.g., smoker or non-smoker, male or female) may help to identify these factors and should be considered in future clinical studies.

In addition, there may be varying factors in vivo which influence the extent of nonenzymatic glycosylation itself since the extent of in vivo nonenzymatic glycosylation of LDL does not demonstrate a high degree of correlation with fasting blood glucose (98,99). For example, triglyceride-enrichment of LDL may mask certain lysine residues preventing
nonenzymatic glycosylation at high plasma triglyceride concentrations. In uncontrolled diabetes, hypertriglyceridemia often accompanies hyperglycemia. Thus, nonenzymatic glycosylation may be low even though glucose concentrations are elevated.

The results from the patient study suggest that LDL from subjects whose plasma triglyceride concentrations are greater than 5.0 mmol/L has a decreased ability to bind to the LDL receptor, presumably because of triglyceride-enrichment of the LDL as suggested by others (115). The effect of hypertriglyceridemia on LDL binding may be a partial explanation for the increased risk of coronary heart disease associated with elevated serum triglycerides which was recently reported (185). An LDL-binding study of a larger population, including grossly-hypertriglyceridemic subjects, is necessary to confirm this finding.
APPENDIX A

CALCULATION OF VOLUME OF DISPLACEMENT OF POTASSIUM BROMIDE

The volume of displacement (ν) is defined as the volume (mL) of water displaced per gram of solute and is calculated by the formula:

\[ \nu = \frac{(C_o - C_w)}{(0.99823 \times C_s)} \]  \hspace{1cm} [A.1]

where \((C_o - C_w)\) is the water (g/L) displaced by anhydrous solute; 0.99823 is the density (g/mL) of water at 20°C; \(C_s\) is the anhydrous solute concentration (g/L) (143). The values \((C_o - C_w)\) and \(C_s\) are dependent upon the density of the solution, and can be calculated using the formulae below:

\[ C_o - C_w = -4.2191 \times 10^2 + (4.2217 \times 10^2 \times \text{Density}) \]  \hspace{1cm} [A.2]

\[ C_s = -1.4200 \times 10^3 + (1.4220 \times 10^3 \times \text{Density}) \]  \hspace{1cm} [A.3]

Sample Calculation

When \(d = 1.019\) g/mL,

\[ C_o - C_w = 8.28\ \text{g/L} \]

\[ C_s = 29.0\ \text{g/L} \]

\[ \therefore \nu = 0.286\ \text{mL/g.} \]

When \(d = 1.063\) g/mL,

\[ C_o - C_w = 26.9\ \text{g/L} \]

\[ C_s = 91.6\ \text{g/L} \]

\[ \therefore \nu = 0.294\ \text{mL/g.} \]

* The formulae were calculated by first-order linear regression, using data given in the CRC Handbook of Chemistry and Physics (143).
APPENDIX B

ROTOR/SPIN-TIME CORRECTION

Since the rotors quoted in the literature were not available, it was necessary to make corrections in the spin time. This was done using the following equation:

\[ t_1 = \frac{t_2 \times RCF_2}{RCF_1} \]  \hspace{1cm} [B.1]

where \( t_1 \) = run time needed for this study; \( t_2 \) = run time specified in the literature; \( RCF_1 \) = RCF for rotor used in this study; \( RCF_2 \) = RCF specified in the literature.

RCF values were calculated from the formula:

\[ RCF = 1.12 \times r_{max} \times \left( \frac{Speed}{1000} \right)^2 \]  \hspace{1cm} [B.2]

where \( r_{max} \) = maximum radius of rotor (mm); \( Speed \) = speed of rotation (rpm). Values of \( r_{max} \) were obtained from a Beckman manual (186).

Sample Calculation

Spin 1 for the isolation of LDL from serum by sequential centrifugation calls for a 16 h spin at 59,000 rpm in a Beckman Type 60 Ti rotor (140). For the present study, a Beckman Type 65 rotor (derated to a maximum of 55,000 rpm) was used. For the 60 Ti rotor,

\[ RCF = 1.12 \times 89.9 \times \left( \frac{59,000}{1,000} \right)^2 = 351,000 \times g \]

For the 65 rotor:

\[ RCF = 1.12 \times 77.8 \times \left( \frac{55,000}{1,000} \right)^2 = 264,000 \times g \]

Therefore,

\[ t_1 = \frac{16 \times 351,000 \times g}{264,000 \times g} = 21 \, h \]
APPENDIX C

RADIOACTIVITY COUNTING ERROR

The counting error of radioactivity measurements is calculated from the formula (187):

\[ \text{Counting error} \, (\%) = \frac{\sqrt{\text{Total counts accumulated}}}{\text{Total counts accumulated}} \times 100 \]  \hspace{1cm} \text{(C.1)}

Radioactivity measurements on the Beckman LS 7500 Liquid Scintillation System were performed for 1 min or until 10,000 counts were accumulated, whichever happened first.

For count rates of less than 10,000 cpm, the counting error was

\[ \text{Counting error} \, (\%) = \frac{\sqrt{\text{Count rate (cpm) x 1 min}}}{\text{Count rate (cpm) x 1 min}} \times 100 \]  \hspace{1cm} \text{(C.2)}

For count rates of 10,000 cpm or greater, the error was always

\[ \frac{\sqrt{10,000}}{10,000} \times 100 \text{ or } 1\% \].
REFERENCES


42. Kovanen PT, Basu SK, Goldstein JL, Brown MS. Low density lipoprotein receptors in bovine adrenal cortex. II. Low density lipoprotein binding to membranes prepared from fresh tissue. *Endocrinology* 1979; 104: 610-6.


48. Wade DP, Knight BL, Soutar AK. Hormonal regulation of low-density lipoprotein (LDL) receptor activity in human hepatoma Hep G2 cells. Insulin increases LDL receptor activity and diminishes its suppression by exogenous LDL. *Eur J Biochem*


82. Steiner G. Effects of various lipid-lowering treatments in diabetics. *J Cardiovas Pharm* 1990; 16 (Suppl. 9); S35-9.


136. Barton R. *Biochemical research techniques*. Vancouver: Biochemistry Department, University of British Columbia.


175. Beisiegel U, Schneider WJ, Brown MS, Goldstein JL. Immunoblot analysis of low density lipoprotein receptors in fibroblasts from subjects with familial


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3. Catomeris P, Thibert RJ. An improved kinetic fluorometric enzymatic coupled assay
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Presentations:

July 23, 1992  "Measurement of Glycosylated Low-Density Lipoprotein Using the Calf Adrenocortical LDL Receptor". A poster to be presented at the 44th National Meeting of the American Association for Clinical Chemistry, Chicago, USA.

October 24, 1991  "LDL-Binding Studies Using the LDL Receptor". A seminar presented to the Michigan Section of the American Association for Clinical Chemistry, Detroit, USA.


October 25, 1990  "Non-enzymatically Glycosylated Low-density Lipoprotein and Atherosclerosis". A seminar presented to the Michigan Section of the American Association for Clinical Chemistry, Windsor, Canada.

July 27, 1989  "Degree of Lipid Peroxidation in Post Mortem Blood of Fire Victims". A poster presented at the 41st National Meeting of the American Association for Clinical Chemistry, Atlanta, USA.

October 27, 1988  "Study of the Degree of Lipid Peroxidation in Postmortem Blood of Fire Victims". A seminar presented to the Michigan Section of the American Association for Clinical Chemistry, Detroit, USA.

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