STUDIES ON THE COUPLING OF DNA TO LOW DENSITY LIPOPROTEINS (LDL) AND THE INTERACTION OF THESE COMPLEXES WITH EUKARYOTIC CELLS

by

ZAINUB KHAN

Submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry in the Faculty of Science at the University of Durban - Westville.

Date submitted : December 1987

Supervisor : Professor A. O. Hawtrey, Head, Department of Biochemistry.
Co-supervisor: Professor M. Ariatti, Department of Biochemistry.
University of Durban - Westville.

ABSTRACT OF THESIS

The application of Molecular Biochemistry for transfection studies in eukaryotic systems is well documented. Of the numerous methods employed for the introduction of foreign DNA into eukaryotic cells, the use of low density lipoproteins (LDL) as carriers of DNA into cells has not been reported.

LDL was isolated, characterized with respect to its protein and lipid components, and then variously modified in an attempt to enhance its affinity for DNA. It was found that both unmodified and modified LDL could interact with DNA, at physiological pH. The carbodiimide modified LDL (ECDI - LDL) showed the greatest affinity for DNA.

LDL and ECDI - LDL were used to study LDL receptor binding in skin fibroblasts. This was followed by a study of receptor binding activities of both unmodified LDL and ECDI - LDL complexed to DNA (pBR322). Although the extent of binding of ECDI - LDL and ECDI - LDL - DNA complexes to plasma membranes was greater, the internalization and degradation of both modified and unmodified LDL complexes were equivalent. This additional binding was attributed to non - receptor - specific affinity of the carbodiimide modified complexes for the plasma membrane.

The transfection of foreign DNA into eukaryotic cells in culture was monitored by assaying for the expression of the cloning vector, pSV2cat, complexed to LDL or ECDI - LDL and introduced into the

cells by LDL receptor - mediated endocytosis. Of the cell lines in which the expression of the pSV2cat recombinant DNA was monitored, the human lung fibroblasts showed the greatest activity of the expressed chloramphenicol acetyltransferase enzyme. Although transfection efficiency was lower than that of the calcium phosphate - DNA coprecipitation procedure, the LDL receptor - mediated transfection of eukaryotic cells was carried out under physiological conditions and may be applicable *in vivo*.

ACKNOWLEDGEMENTS

I wish to express my sincere thanks and appreciation to :

Professor A. O. Hawtrey, for his supervision, encouragement and enthusiasm throughout this project.

Professor M. Ariatti, for supervision and assistance in solving the many problems encountered in this project.

Professor E. Harley and Dr R. Rubenstein, of the Department of Chemical Pathology, University of Cape Town, for their gift of the pSV2cat plasmid and cells, and for their assistance with the calcium phosphate transfection technique.

Professor I. Jaylal and Mrs. R. Hanif, of the Department of Chemical Pathology, University if Natal, for their gift of skin fibroblasts.

Dr I. Windsor, Mr S. Singh and Mr W. Gopaul, for use of the gamma counter and other facilities in the Department of Virology, University of Natal, and for helpful advice during the study.

Mrs B. Huckett, for photography and helpful discussions with regard to this study.

Mr K. Postum, for his enduring patience, encouragement and support, and for typing and proof - reading the thesis.

C O N T E N T S

C H A P T E R O N E

1.1 Introduction

Research carried out by Baker and Carrell, as early as 1926, attributed certain adverse effects of aging to serum components which were possibly lipoproteins. The nature of lipoproteins and the solubility of serum lipids were poorly understood until methodologies for electrophoresis and ultracentrifugation were improved and utilized for separating molecules according to their size and density. Hitherto, it was believed that cholesterol was associated with serum globulins (Homburger and Bernfeld, 1958).

Machebauf (1929) was the first to isolate plasma proteins containing a high concentration of lipids, and thereby laid the foundation for research on the transport systems of lipids. Two distinct lipoproteins, namely the α- and β - lipoproteins, were isolated and characterized by Cohn and coworkers in 1946. The β - lipoprotein was found to contain about 75% of the serum lipids (Homburger and Bernfeld, 1958). A quantitative analysis of β - lipoproteins was made by isolating the low density component by preparative ultracentrifugation. The sedimentation coefficient (S) was then modified to S_f (Svedberg unit of floatation) in order to identify the lipoproteins separated by ultracentrifugation (Mann, 1958). By 1956 serum lipoproteins could also be separated successfully by electrophoresis and determined quantitatively.

However, the variation in lipoprotein levels in humans, from one individual to another, had hampered the use of lipoprotein profiles for diagnostic purposes, e.g., lipoprotein patterns in plasma were found to increase with age, diet, pregnancy as well as in disease conditions such as xanthomatosis, nephrosis, billiary cirrhosis and diabetic acidosis. In the case of idiopathic hypercholesterolemia, however, serum lipoprotein levels were greatly increased and could be detected by measuring serum cholesterol levels.

In the next three decades new methods for isolating lipoproteins were developed and existing methods were improved. The lipoproteins were also classified with a greater degree of accuracy. For ultra - centrifugation methods, density adjustments were made with NaCl, KBr, $NaNO_3$, glycine and heavy water. Electrophoresis was carried out on paper, starch and agar. Lipoproteins, especially β - lipoproteins, were isolated by precipitation methods such as high molecular weight dextrans, ethanol or zinc reagent (Oncley, 1958; Bragdon *et al.*, 1956; Edelstein, 1979; Burnstein and Scholnick, 1974; Lee and Alaupovic, 1974; Albers and Cheung, 1979). In addition to the existing methods, chromatographic and immunochemical methods were also developed for the isolation and characterization of serum lipoproteins (Fellini *et al.*, 1974; Seidel, 1979; Mahley *et al.*, 1979; Lee, 1976; Laggner, 1976; Carrol and Rudel, 1983; Kostner and Laggner, 1979).

1.2 The plasma lipoproteins

The circulatory systems of higher animals transport various components such as α - and β - globulins, albumin, glucose and other carbohydrates and ionic and hydrophobic components in solubilized form. The hydrophobic components are carried in plasma in the form of lipoproteins. These lipoproteins are required for both structural and functional aspects of the living cell's metabolism. The lipoproteins are lipid - associated proteins which transport various components throughout the body.

The lipoproteins are classified on the basis of their densities, floatation coefficients and electrophoretic mobilities (Cawley and Minard, 1969; Brown and Goldstein, 1974). They are conveniently separated by ultracentrifugation techniques using density gradients. Thus chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) are found to have the following densities : $<$ 0,940, 1,006 to 1,019, 1,019 to 1,063 and 1,063 to 1,21 g / ml respectively. The ultra - centrifugation floatation coefficient (S_f) values ascribed to the lipoproteins are as follows : S_f 0 - 12 for LDL, S_f 12 - 20 for VLDL and S_f 20 - 4 000 for chylomicrons, where S_f is the Svedberg unit of floatation (Bernfeld, 1958; Gofman, 1958; Cornwell and Kruger, 1961; Hatch and Lees, 1968; Cawley and Minard, 1969; Chiat *et al.*, 1974; Laggner, 1976). HDL have been isolated at S_f 0 - 9 (Lindgren *et al.*, 1972).

Since lipoproteins are charged molecules, they also behave like proteins, in electric fields, and can be classified according to electrophoretic mobilities. The HDL have been designated α - lipo - proteins as they migrate close to the α_1 - globulins, just behind albumin. The LDL have been designated β - lipoproteins as their electrophoretic mobility is that of β - globulin. VLDL move ahead of the LDL and are known as pre - β - lipoproteins, while chylomicrons do not migrate in the electric field (Bernfeld, 1958; Lees and Hatch, 1963; Noble, 1968; Cawley and Minard, 1969). The existence of α- and β - polypeptides in the lipoproteins has been confirmed immunologically. Although this classification of the lipoproteins historically preceded that by ultracentrifugation, the latter has become more popular because, from a practical point of view, it is easier to isolate and separate lipoproteins by the ultracentrifugation methods (Havel *et al.*, 1955; Bragdon *et al.*, 1956; Hinton *et al.*, 1973; Rudel *et al.*, 1974; Redgrave *et al.*, 1975; Foreman *et al.*1977; Chung *et al.*, 1980; Zechner *et al.*, 1984; Roberts *et al.*, 1985).

The general functions of plasma lipoproteins have been ascertained from studies *in vitro*. Chylomicrons transport dietary cholesterol, triglycerides and other lipids from the intestines to adipose tissue and liver. Chylomicrons are the largest of the lipoproteins and are rich in triglycerides. Thus they have very low density ($< 0{,}94$ g / ml) and a protein content of $< 2\%$. The triglycerides of chylomicrons are hydrolysed within a short time by serum

lipases in capillaries of adipose tissues and other peripheral tissues. The resultant remnant particles are taken up by the liver, where VLDL are synthesized. The VLDL particles transport endogenously synthesized triglycerides to adipose tissues. The removal of triglycerides from VLDL results in the formation of LDL particles, which are rich in cholesterol. Most of the cholesterol in LDL are esterified to cholesteryl linoleate. The LDL transport cholesterol to peripheral tissues and also regulate the synthesis of cholesterol in these cells by the enzyme 3 - hydroxy - 3 - methylglutaryl Co A reductase (Section 1.5). HDL are synthesized in the liver, and are rich in phospholipids and cholesterol. The HDL transport cholesterol from peripheral tissues to the liver.

Chromatographic, electrophoretic and other separation methods have shown that lipoproteins are heterogeneous, and contain more than one type of protein, e.g., HDL contain mainly apoprotein A (apo A) but also apoprotein C (apo C). LDL contain apoprotein B (apo B or apo B - 100) as the major protein component and apoprotein C (apo C) as the minor component. VLDL contain apo B and apo C. The apoproteins isolated to date, from the lipoproteins, are apoproteins A I, A II, B - 100, C I, C II, C III, apo D, apo E, apo F and apo G (Alaupovic *et al.*, 1972; Nyström and Sjöval, 1975; Morrisett *et al.*, 1975; Lee, 1976; Gidez *et al.*, 1979; Alaupovic, 1981; Cardin *et al.*, 1984; Galton *et al.*, 1985). Some apoproteins play a structural role in the formation and stabilization of

lipoproteins, while others, e.g., A I, B - 100, C II and E have functional roles in the catabolic pathways of lipoproteins in non - hepatic tissue. The major enzymes responsible for lipoprotein breakdown are lipoprotein lipases (and hepatic lipases) which hydrolyse neutral lipids in the lipoproteins. The enzymes are found most abundantly in adipose tissue and heart muscle. They catalyse the hydrolysis of triglycerides to provide fatty acids to the cells (Korn, 1955; Goldstein *et al.*, 1975; Speake *et al.*, 1985). Lipoprotein lipase is sometimes present in the blood, where it hydrolyses lipoprotein - and chylomicron - associated triglycerides. In hyperlipemic patients levels of this enzyme may be increased by administering heparin thereby reducing plasma triglyceride levels (Bragdon, 1958; Jansen and Hulsman, 1985). The serum enzyme lecithin : cholesterol acyltransferase (LCAT) is responsible for the esterification of plasma cholesterol. It is an enzyme of hepatic origin, which transfers a long fatty acyl group from the 2 - position of lecithin to the 3 - β - hydroxy - group of cholesterol. The cholesterol esters formed are then transferred to LDL and VLDL (Assman and Jabs, 1985; Owen *et al.*, 1985). The major receptor proteins found in peripheral cells are the LDL receptors. These bind apo B - and apo E - containing lipoproteins (Vogel *et al.*, 1985). Receptor binding studies of lipoproteins have been carried out in a number of cell lines, including monocytes, fibroblasts, adipocytes, adrenal cells and rat aortic smooth muscle cells (Galton *et al.*, 1985; Fong *et al.*, 1984; Hui *et al.*, 1984; Mazzone and Chiat, 1982; Pitas *et al.*,

1983; Chiat *et al.*, 1979; Bierman *et al.*, 1974; Kovanen *et al.*, 1979).

A considerable number of disorders have been associated with lipoprotein metabolism and function, and characterized by the excess, deficiency or absence of specific lipoproteins, e.g., the absence of the β - polypeptides (apo B) results in the disorder abetalipoproteinemia, while the absence of α - polypeptide causes Tangier disease (HDL is absent). The HDL deficiency is due to an autosomal recessive inheritance (Fredrickson *et al.*, 1967e).

Lipoprotein disorders may be detected from abnormal electrophoretic patterns, which have proved invaluable in diagnoses. Pre - β - lipids (i.e. VLDL) are increased in patients with coronary heart disease and diabetes. Alpha - and beta - lipoproteins increase in nephrotic syndrome; β - lipoproteins are elevated in patients with hypothyroidism (Papadopoulos, 1978).

Abetalipoproteinemia is characterized by the congenital absence of LDL (Fredrickson *et al.*, 1967c). This causes serious disorders in various organs, e.g., severe malabsorption in the intestine, neuromuscular disorders, mental retardation and retinitis pigmentosa. There is also a marked decrease in cholesterol levels in plasma. Since abetalipoproteinemia is coupled with a lack of chylomicrons and VLDL, and an accumulation of triglycerides in intestinal mucosa and liver, it is believed that the LDL is

essential for the transport of triglycerides, in addition to cholesterol, in plasma and lymph (Hatch and Lees, 1968; Shore and Shore, 1972). In abetalipoproteinemia the β - polypeptide synthesis is defective but serum HDL levels are normal (Cawley and Minard, 1969).

Also associated with genetic abnormalities of β - lipoprotein are the type II and type III hyperlipoproteinemias (Fredrickson *et al.*, 1967b, d; Wieland and Seidel, 1973; Brown and Goldstein, 1974; Papadopoulos, 1978). The type III hyperlipoproteinemia is characterized by subcutaneous lipid deposits and increased serum VLDL levels and reduced LDL levels. The type II hyperlipoprotein - emia (familial hypercholesterolemia) is associated with a decreased catabolism of LDL. The metabolic error is the result of LDL receptor deficiency (Brown and Goldstein, 1984).

1.3 Low density lipoprotein : structure

It was previously thought that LDL was a large globular molecule containing core lipids and peripherally arranged proteins, containing hydrophilic exteriors which accounted for its solubility and protein - like chemical behaviour (Mann, 1958). Subsequently it was proposed that the LDL component (apoprotein B) consisted of several subunits which formed globules and were embedded in the phospholipid coat of the LDL. Part of these globules were buried in the lipid and part were exposed to the aqueous medium. The basis

for this proposal was that upon delipidation the apoprotein B (apo B) was found to disaggregate into a number of subunits ranging from 8 000 to 25 000 daltons (Goldstein and Brown, 1977).

A more recent hypothesis for the structure of β - or low density lipoproteins is the location of a single large globular protein (apo B, molecular weight approximately 500 000 daltons) embedded in an array of lipid molecules. The protein presents a hydrophilic exterior while the hydrophobic parts are embedded in the lipids. The lipids, on the other hand, are arranged such that the non -polar or neutral lipids are located in the core of the particle, while the phospholipids and unesterified cholesterol are arranged on the periphery of the spherical macromolecule (Figure 1.1). The orientation of the peripheral components is such that their hydrophobic ends (fatty acyl chains and steroid groups) face towards the core, while their hydrophilic head groups face outwards (the aqueous medium)(Brown and Goldstein, 1976; Brown and Goldstein, 1977; Brown and Goldstein, 1984). It is the hydrophilic exterior of the LDL particle that confers solubility to the LDL particle in aqueous media such as blood, extracellular fluid and buffers. The phosphorylcholine groups of LDL are accessible to the surround - ing medium and display a high degree of motional freedom. The phospholipid polar head groups protrude slightly higher than the protein component of LDL (Laggner, 1976). The core lipids of LDL have weak lipid - lipid hydrophobic interactions and are surrounded by the partly hydrophilic protein, phospholipids and cholesterol.

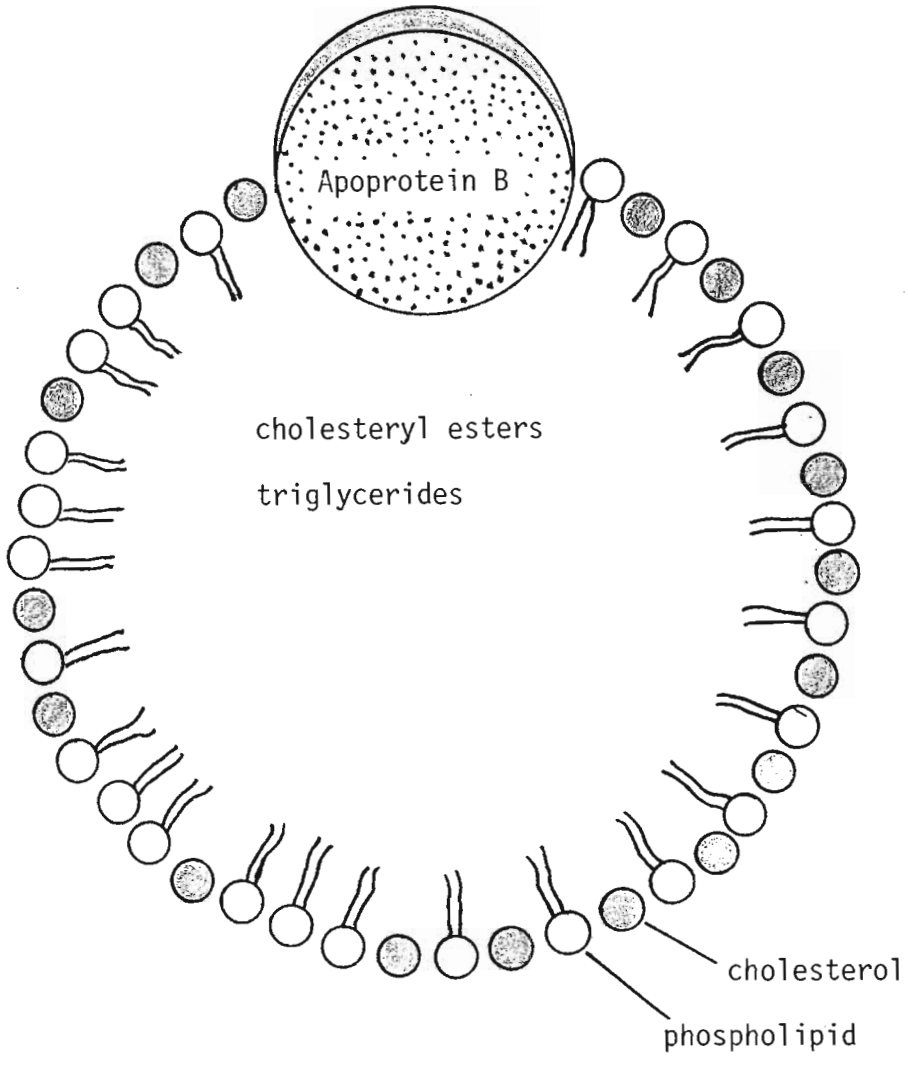


Figure 1.1 : Structure of a low density lipoprotein particle in cross - section.

The LDL particle allows an exchange of lipids (cholesteryl esters and triglycerides) from its core and surrounding medium (Zilversmit and Johnson, 1975; Zilversmit *et al.*, 1975; Roberts *et al.*, 1985). There is also an exchange of cholesterol between different lipo - protein classes. LDL reacts with a large number of substances such as cations (e.g., Ca^{++}), polyanions (dextran sulfate, heparin) and other macromolecules (e.g., mucopolysaccharides, proteoglycans) (Bernfeld *et al.*, 1957; Ghosh, 1976; Margolis and Capuzzi, 1976).

LDL is isolated from human plasma by ultracentrifugation, within the density range 1,019 - 1,063 g / ml. The molecular weight of LDL has been determined to be between 2×10^6 and $3,5 \times 10^6$ daltons (Shore and Shore, 1972; Goldstein and Brown, 1977). Its diameter varies between 193 Å and 250 Å. The size and molecular weight variations have been attributed to variation of lipids in the core and not the protein component. The average level of LDL in normal human plasma is 400 mg / 100 ml and 340 mg / 100 ml for males and females respectively.

The LDL lipids comprise about 75% of the LDL particle, while the proteins comprise 22% of LDL. About 5 - 9% of the protein component consists of carbohydrates (galactose, mannose, glucosamine and sialic acid residues). Cholesterol constitutes 40 - 60% of the total lipid of LDL, 80% of which is esterified and located in the apolar core (Skipski, 1972). The major fatty acid component of the cholesteryl esters is linoleate. This constitutes 50% of the

total fatty acids. Other fatty acids found in the LDL are oleate (20%), palmitate (15%) and small amounts of stearate, palmitoleate and arachidonate (Brown and Goldstein, 1976a; Goldstein and Brown, 1977). The phospholipids comprise 30% of total LDL lipids. The major phospholipids are phosphatidylcholine (65%) and sphingomyelin (25%). LDL also contains about five different glycosphingolipids. The triglycerides constitute 7 - 11% of LDL lipids (Shore and Shore, 1972; Goldstein and Brown, 1977: Skipski, 1972).

A LDL variant, designated Lp (a) has also been isolated from the plasma of normolipemic donors and found to band at densities 1,019 - 1,21 g / ml and comprise three species. The Lp (a) lipoproteins contain additional disulfide - linked, carbohydrate - rich components which vary from the apo B component of LDL (Fless *et al.*, 1984). The protein of Lp (a) is designated apoprotein (a). The origin and metabolism of Lp (a) has yet to be determined.

In addition to the preparative ultracentrifugation methods used for the isolation of LDL (and other lipoproteins; Section 1.2), selective methods such as immunoprecipitation and heparin - Mn^{++} precipitation methods have also been developed for isolation and purification of low density lipoproteins (Burnstein *et al.*, 1970; Burnstein and Scholnick, 1974). Affinity chromatography on Sepharose gels containing coupled cholesterol and cholic acid have also been used in the isolation and purification of LDL (Carroll and Rudel, 1983).

1.4 Apoprotein B

The apoprotein B of human plasma LDL has been designated apo B - 100 and has a molecular weight of between 5×10^5 and 5.5×10^5 daltons (Brown and Goldstein, 1976; Cardin *et al.*, 1984). It is partly embedded in the hydrophilic coat of LDL lipids and is recognized by receptors located on the plasma membrane of cells. Minor quantities of apo B - 74 (M_r 410 000) and apo B - 26 (M_r 145 000) have been detected in LDL preparations and are believed to be due to the proteolytic degradation of apo B - 100 (Cardin *et al.*, 1984) during exsanguination. LDL apo B - 100 contains a highly specific cleavage site which is recognized by both plasma and tissue kallikreins(Cardin *et al.*, 1984).

The extraction of lipids from LDL renders the apoprotein B insoluble in aqueous medium. LDL contain a total of 22% protein. A portion of this is hydrophobic in nature and embedded in the lipid core of LDL. The hydrophobic portion is insoluble in aqueous medium and in the absence of lipids. The solubility of apo B is increased by chemical modifications such as succinylation and maleylation.

Due to the difficulty in solubilizing the apo B without the use of denaturing solvents, the amino acid sequence of the apoprotein has not been determined. However, chemical studies have thrown light on various aspects of the apoprotein structure. Infra red and circular dichroism spectra have shown that apo B contains a large amount of anti - parallel, β - chain structure (Shore and

Shore, 1972). Apo B also contains single amino - terminal glutamic acid and carboxy - terminal serine residues. Thus Brown and Goldstein (1984) have recently proposed a structure for the LDL particle which contains a single apoprotein. Apo B contains 5 - 9% carbohydrates, however, their exact role and significance is not known.

The extraction of lipids from LDL results in the formation of intermolecular disulfide bonds in apo B. It is this disulfide bridge formation that results in aggregates of the apoprotein. The cross - linking reaction occurs by a sulfhydryl - disulfide exchange between free sulfhydryl groups present in the original apo B of LDL (Cardin *et al.*, 1982). Disulfide bond formation occurs in the absence of lipids and renders the apo B insoluble.

Attempts to isolate apo B by delipidation of LDL affords unstable and aggregated forms of the apoprotein. Solubilization is achieved by various denaturation techniques. Gustafson (1965) extracted LDL lipids with heptane and used soluble starch to stabilize the apoprotein. Sodium dodecylsulfate, sodium deoxycholate, Nonidet P 40, cetyltrimethylammonium bromide, guanidine hydrochloride and other solvents have been used to solubilize the delipidated apoproteins (Gotto *et al.*, 1969; Helenius and Simons, 1971; Cardin *et al.*, 1982; Shore and Shore, 1972; Krieger *et al.*, 1978a, b; Kane, 1973; Lee and Alaupovic, 1974a). The removal of LDL lipids with ether and ethanol at high salt concentration and subsequent dialysis at lowered

salt concentrations has also been carried out in an attempt to isolate soluble, non - denatured apo B (Lee and Alaupovic, 1974b)

LDL is generally isolated in the density range 1,019 - 1,063 g / ml, and is regarded as being homogeneous. Lee and Alaupovic (1974b) have separated LDL into six subfractions, all of which contained apo B as the major protein component and apo A and apo C as the minor components. Of these LDL I, II and III have apo A and apo C in an associated form while LDL IV, V and VI contain apo A and apo C in the free form.

Stoichiometric binding studies using Fab fragments of 8 monoclonal antibodies were carried out to determine the number of epitopes present per molecule of LDL. Wickland and co - workers (1985) found that one antibody bound per LDL for each of the 8 different molecular loci in apo B. Thus immunologic data also point to the presence of a single large protein monomer of apo B per LDL particle.

Young and co - workers (1986) prepared a monoclonal antibody (MB 47) directed against an epitope in the LDL receptor - binding domain of human apo B. The Fab fragments of the purified antibody were prepared by papain digestion, and could react with LDL from human and various animal species (lion, bear, shark, seal, whale and tuna). This cross - reactivity shows a possible common ancestral origin of apoprotein B.

1.5 LDL metabolism and function

Although LDL is the major cholesterol - carrying lipoprotein in human plasma, it is not secreted as such in the blood. The protein component of LDL, apoprotein B, is synthesized in the intestine and liver. It is secreted by the liver as part of the triglyceride - rich lipoprotein, VLDL, which also contains other proteins. VLDL is transported to adipose tissue where a large amount of triglycerides and proteins , except apo B, are removed from the particles. In addition, the cholesterol content is increased to yield the LDL particle (Margolis and Capuzzi, 1972; Coleman, 1973; Goldstein and Brown, 1977).

Turnover studies with [^{125}I] LDL show that about 700 mg LDL appears in human plasma per day. This comprises about 40% of the plasma pool. Unused LDL is believed to be degraded in the liver as well as in other tissue. A number of non - hepatic cells in culture have been used to study the LDL metabolic pathway. Cell types utilized are skin fibroblasts, arterial smooth muscle cells, lymphoid cells and endothelial cells. These have helped in further elucidating LDL metabolism, function and associated abnormalities, and in understanding the significance and importance of low density lipoproteins.

Mammalian cells in culture preferentially utilize exogenous cholesterol, contained in LDL, obtained from serum added to the culture medium. The fate of LDL in normal skin fibroblasts, as

outlined by Goldstein and Brown (1977) begins with the binding of LDL to high affinity receptors on the cell surface. The receptor - bound LDL are then internalized by a process called receptor - mediated endocytosis (discussed in Section 1.7). The LDL particles are then delivered to the lysosomes, where the LDL components are broken down and made available to the cells (Figure 1.2).

LDL - derived cholesterol is essential for plasma membrane and steroid hormone synthesis. The accumulation of free intracellular cholesterol regulates the activities of two microsomal enzymes, namely, 3 - hydroxy - 3 - methylglutaryl Co A reductase (HMG Co A reductase) and acyl Co A : cholesteryl acyl transferase (ACAT). The presence of free cholesterol in the cell suppresses the HMG Co A reductase (thereby supressing endogenous cholesterol biosyn - thesis) and activates ACAT (which esterifies cholesterol). Cellular esterified cholesterol contains mainly oleate and palmitate. The cholesteryl esters of plasma LDL contain mainly the linoleoyl fatty acyl group. Thus the overall function of LDL is to transport cholesterol from the plasma to the cell (extra - hepatic) where part of it is utilized for synthesis of cell membrane and the remainder is re - esterified and stored in a liquid crystalline state.

More than 90% of the cholesterol produced in the body is synthesized in the liver and intestine. Although most cells require cholesterol, its synthesis is suppressed in non - hepatic cells, in favour of

exogenous cholesterol (Brown and Goldstein, 1976a), which is obtained from plasma LDL taken up by LDL receptor - mediated endocytosis (Section 1.7). However, the cholesterol synthesis in the liver is controlled by dietary cholesterol (which is taken to the liver in chylomicrons remnants) and not by LDL - associated cholesterol.

In vitro studies have shown that when adequate amounts of cholesterol are accumulated by the cell, the LDL binding activity is reduced. This constitutes a feedback mechanism for the regulation of cholesterol levels in the cell. The accumulation of cholesterol in the cell suppresses *de novo* synthesis of the LDL receptors, thus reducing, and finally blocking, further transport of cholesterol (LDL) into the cell. This protects the cell against the abnormal accumulation of cholesterol. Cholesterol synthesis, in the absence of exogenous cholesterol, is regulated by HMG Co A reductase. When cholesterol is available from an exogenous source, the enzyme activity is suppressed. Esterification of cholesterol, catalysed by ACAT, is stimulated, thus preventing excess accumula - tion of free cholesterol in the cell (Brown and Goldstein, 1976 a; Dempsey, 1974; Goldstein and Brown, 1977; Brown and Goldstein, 1984).

1.6 Abnormalities associated with low density lipoproteins

Familial hyperlipoproteinemias are disorders associated with lipoprotein metabolism. Clinically significant hyperlipoproteinemia

can be classified into 3 major types : hypercholesterolemia, hypertriglyceridemia and the combined hyperlipemia (hypercholesterolemia and hypertriglyceridemia). Of these hypercholesterolemia is due to increased concentration of LDL in serum. The classical disorder of familial hypercholesterolemia is due to a genetic defect in the LDL receptor - mediated uptake of LDL by the cells. Manifestations of the heterozygous form are xanthomas of the tendons (hand and Achilles), xanthelasma and corneal arcus. The homozygous form is characterized by the same manifestations as for the heterozygous form, but also by premature ischaemic heart disease.

It has long been suggested that the accumulation of cholesteryl esters, from LDL, on the walls of arteries lead to the formation of atherosclerotic plaques. This predisposition to atherosclerosis is also due to increased plasma levels of LDL. Atherosclerotic plaques may inhibit blood flow and eventually lead to thrombosis, which may finally block arteries and lead to heart attack or stroke (Hanig, 1958; Goldstein and Brown, 1977; Brown and Goldstein, 1984). Thus, the removal of LDL, from serum, by the LDL receptor, maintains normal levels of LDL and reduces the risk of atherosclerosis.

Plasma LDL levels also increase in women with breast carcinoma (Petermann *et al.*, 1958) and bilateral oophorectomy. Estrogens maintain normal levels of LDL, and this is disrupted when ovaries are removed. The turnover of LDL is increased in patients suffering from rheumatoid arthritis, cancer, leukemia and hyper -

thyroidism (Margolis and Capuzzi, 1972). Elevated plasma LDL in hypothyroidic patients can be corrected by administration of thyroid hormone.

Serum LDL levels in hypercholesterolemic individuals may be reduced by both dietary and pharmaceutical approaches. Thus it is known that the presence of unsaturated fats in the diet leads to decreased LDL levels. It has been shown that administration of thyroid hormone also normalizes serum LDL levels albeit temporarily (Gofman, 1958). More recently, familial hypercholesterolemias have been treated with cholestyramine, a resin which sequesters bile acids in the gut, thereby stimulating bile acid synthesis from cholesterol. Inhibitors of the enzyme HMG Co A reductase have also proved to be useful therapeutic agents.

1.7 LDL receptors and endocytosis

LDL receptors were discovered in 1973 during LDL binding studies carried out on human skin fibroblasts in culture (Brown *et al.*, 1973). The finding that fibroblasts utilized cholesterol only from LDL gave the first indication that cells utilized specific receptor - mediated mechanisms for internalizing the cholesterol - containing LDL (Brown *et al.*, 1974; Goldstein and Brown, 1974).

In order to study the cell surface receptors which carried LDL into the cells, LDL was labelled with iodine. Goldstein and co - workers (1979a) found that [^{125}I] - labelled LDL bound to the

receptors of normal skin fibroblasts at 4°C but were not internalized. Internalization and degradation were facilitated at 37°C.

Fibroblasts produce between 20 000 and 50 000 receptors per cell and require the presence of divalent cations (Ca^{++} or Mn^{++}) to bind LDL. The receptors are sensitive to proteases (Pronase, trypsin and chymotrypsin) but resistant to glycosidases.

The LDL receptor recognizes and binds the apoprotein B component of LDL and VLDL. It also binds apoprotein E of HDL_c, which shows some sequence homology to apo B. Apoproteins A I and A II of HDL do not bind to the receptors.

The LDL receptor is a glycoprotein which is embedded in the plasma membrane. It has a recognition site for apo B - 100, and due to its high affinity for the LDL particle, the receptor can bind to LDL at a concentration of less than 10^{-9} M (Brown and Goldstein, 1984).

The LDL receptors are located in specialized indented regions of the cell membrane called coated pits, the interior surfaces of which are coated with the protein called clathrin. The coated pits invaginate and 'pinch off' from the membrane to form coated vesicles. LDL bound to the receptors is thus carried into the cell inside the clathrin - coated vesicles. The entry of LDL particles into the cell by this method is known as receptor - mediated endocytosis (Figure 1.2).

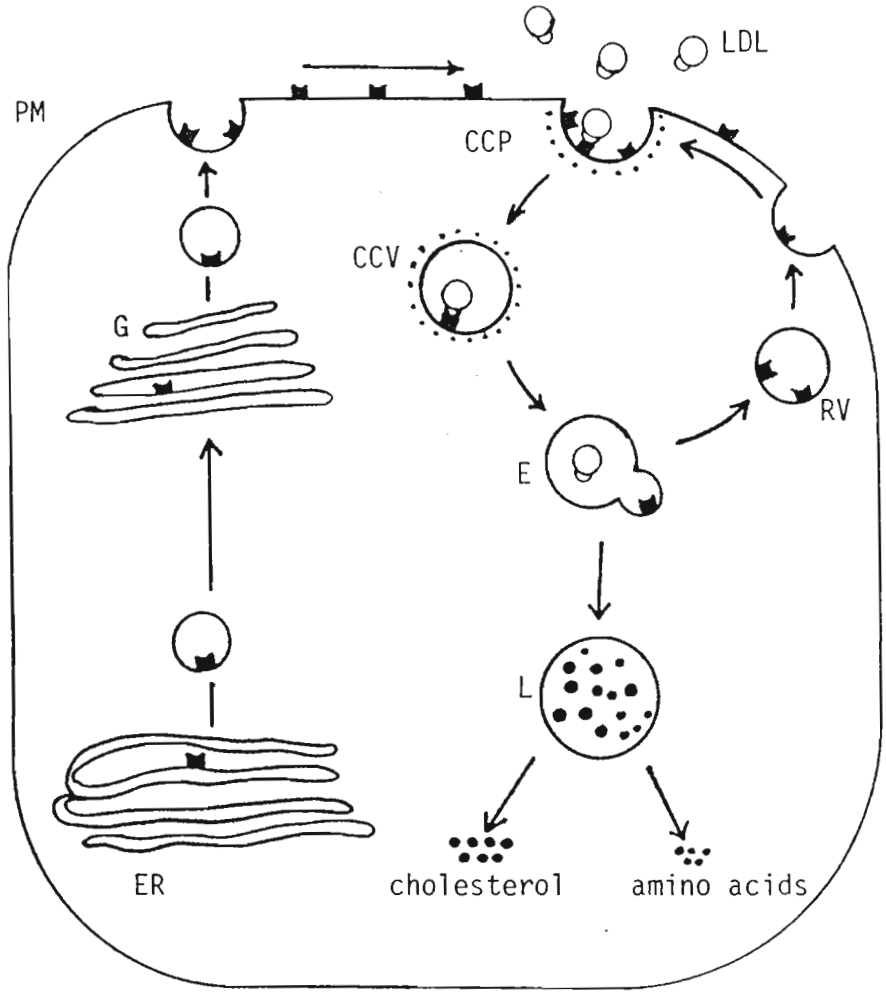


Figure 1.2 : Synthesis of LDL receptors and the internalization of LDL by receptor-mediated endocytosis. The LDL receptor is synthesized on polyribosomes bound to endoplasmic reticulum (ER), glycosylated in the Golgi apparatus (G) and inserted into the plasma membrane (PM) at random sites. The LDL receptors gather at clathrin coated pits (CCP) and internalize LDL into clathrin coated vesicles (CCV) by receptor - mediated endocytosis (Section 1.7). The LDL is delivered via the endosomes (E) to lysosomes (L) where they are degraded. The LDL receptors are returned to the cell surface in recycling vesicles (RV).

The LDL receptor is synthesized on membrane - bound polyribosomes and glycosylated in the Golgi apparatus. The receptor is initially inserted into the plasma membrane at random sites. Experimental data suggest that the LDL receptor has two recognition sites : one for binding LDL (LDL binding site) and the other for binding to coated pits (internalization site). The LDL binding site is located on the extracellular surface whilst the internalization site is positioned on the cytoplasmic side. Receptors with normal internalization sites gather at coated pits (Figure 1.2).

Receptor - bound ligands are first internalized into coated vesicles which contain a polyhedral protein of molecular weight 180 000 daltons, originally designated clathrin (Pearse, 1975; Brown and Goldstein, 1979). The coat can be dissociated into hexameric protein complexes that consist of three heavy chains (112 000 - 125 000 daltons) and three light chains (30 000 - 36 000 daltons). Electron microscopy studies have revealed that each hexameric molecule is organized into a three legged structure termed clathrin triskelion (Ungewickell and Branton, 1981). Purified triskelions reassemble into cage - like structures in which the arrangement is similar to the original coat. The clathrin heavy chains exhibit substantial α - helical conformation and are stabilized by the trypsin - labile monomeric light chains (Ungewickell, 1984). Clathrin cages also bind to 100 000 dalton and 50 000 dalton polypeptides which connect clathrin to the membrane of coated vesicles.

The liver has by far the largest concentration of LDL receptors and degrades a large amount of cholesterol. LDL receptors have been purified from bovine adrenal cortex membranes, cultured human fibroblasts and cultured human epithelioid carcinoma A - 431 cells (Schneider *et al.*, 1979; Schneider *et al.*, 1982). The receptors have been solubilized in a non - ionic detergent, octyl - β - D - glucoside, followed by affinity chromatography on LDL - coupled Sepharose 4B. The receptor was eluted with suramin. The LDL receptor was found to be a single glycoprotein (molecular weight 164 000 daltons) which displayed the binding properties of intact LDL receptors.

The coated pits on the plasma membranes are areas which contain LDL receptor protein and other migrant proteins. The reason for gathering of LDL receptors in coated pits is possibly to isolate them from other membrane proteins, which are permanent constituents of the plasma membrane and must not be carried, in the endosomes, to the lysosomes. Other receptors found in coated pits include those for transferrin (Omary and Trowbridge, 1981), insulin (Kasuga *et al.*, 1982), epidermal growth factor (Ushiro and Cohen, 1980) and asialoglycoprotein (Drikamer, 1981; Drikamer and Mamon, 1982; Steer *et al.*, 1981). Unlike the transferrin receptor which is a 160 kilodalton dimer, the mature LDL receptor is a single glyco - protein chain of 164 kilodaltons, bearing O - linked as well as N - linked carbohydrate chains which have been added post - translationally (Schneider *et al.*, 1982).

Receptor - bound LDL particles are internalized as a complex, with their receptors, in small coated vesicles. The small vesicles lose their clathrin and fuse together to form large vesicles called endosomes or receptosomes. In the receptosomes, the LDL particles are released from their receptors which return to the cell surface. The LDL particles are carried to the lysosomes where the lipid and protein components are degraded. The internalization of LDL by receptor - mediated endocytosis is illustrated in Figure 1.2. The LDL receptors are migrant glycoproteins which complete a single cycle of internalizing LDL every 12 minutes (Brown *et al.*, 1983).

It has been found that cells can internalize receptor - bound macromolecules or ligands at a steady state without the depletion of cell surface receptors. Even when cycloheximide was used to block the synthesis of new receptors, the cells continued to internalize macromolecules by receptor - mediated endocytosis. (Goldstein and Brown, 1974). This could only have been possible if the receptors were recycled. The occurrence of the recycling phenomenon is supported by the use of chloroquine, which raises the pH of intracellular compartments, including lysosomes. Under these conditions, the receptors, once internalized, cannot return to the cell surface. LDL receptors trapped intracellularly can be detected by suitable tagging (with ligand or antibodies) and electron microscopy.

Endocytosis has been defined as the process by which internalization of extracellular molecules occurs by invagination of the plasma membrane, giving rise to a vesicle. Depending on the type of cell in which endocytosis occurs (and the type of internalization), the vesicle formed may be a phagosome (phagocytosis), pinosome (pino - cytosis) or coated vesicle (receptor - mediated endocytosis) (Besterman and Low, 1983).

Endocytosis has been classified into two types : phagocytosis (or eating) and pinocytosis (drinking). The former defines internalization by the cell of particles larger than a few μm in diameter. Pinocytosis has been further subdivided into fluid - phase pinocytosis (smaller substances enter cells in the fluid) or adsorptive pinocytosis (bound to vesicle membranes). Adsorptive pinocytosis includes the endocytosis of ligands by specific cell membrane receptors (receptor - mediated endocytosis). Molecules taken up by receptor - mediated endocytosis include polypeptide hormones (epidermal growth factor. insulin, thyroid hormone), plasma transport proteins (LDL, transferrin, asialoglycoproteins) and α_2- macroglobulins (Cuatrecasas, 1974; Silverstein *et al.*, 1977; Besterman and Low, 1983; Brown *et al.*, 1983; Dean *et al.*, 1984).

Electron microscopic studies indicate that receptor - ligand complexes are located in endosomes for up to 20 minutes before the ligands are delivered to the lysosomes. The endosomes are

heterogeneous (round, cup - shaped or tubular) when viewed under the electron microscope. Biochemical analysis has shown that the endosomes do not exhibit acid phosphatase and arylsulfatase activity (lysosomal marker enzymes). It has been found that at 20°C fusion of endosomes is inhibited (Dunn *et al.*, 1980). This also illustrates that the endosomal and lysosomal compartments are functionally different.

The pH within the endosomal compartment has been found to be acidic. It has been demonstrated that isolated endosomes become acidified *in vitro* in the presence of ATP. An ATP - driven proton pump, resembling that of lysosomes (Schneider *et al.*, 1981) may also exist in the endosomes. At pHs below 6 receptor - ligand dissociation has been shown to occur with LDL, lysosomal enzymes, epidermal growth factor, asialoglycoproteins and α_2 - macroglobulin. The dissociated receptor is then returned to the cell surface to bind another ligand (i.e., the receptor is re - used or recycled).

Some bacterial toxins such as diphtheria and tetanus toxin have been called 'opportunistic ligands' as they bind to cell surface receptors and enter the endosome, where the acidic pH induces a conformational change in the toxins, thus allowing them to cross the endosomal membrane and enter the cytoplasm (Donovan *et al.*, 1981).

The receptor - ligand dissociation phenomenon accounts for the

rapid return (3 minutes) of the LDL to the cell surface. It also explains how the LDL receptor can make up to 150 rounds into the cell and back without undergoing proteolysis. The proteolytic enzymes are contained in the lysosomes. Only 10% of the internalized LDL is regurgitated intact. Thus 90% of the endosomes must pass on their contents to the lysosomes.

Genetic defects in LDL receptor function produce familial hyper - cholesterolemia. Because LDL is inefficiently internalized, or not internalized by defective receptors, an accumulation of LDL occurs in the plasma, eventually leading to atherosclerosis (Section 1.6).

Receptor deficiency is inherited and can lead to homozygous or heterozygous familial hypercholesterolemia (FH). Patients diagnosed as FH homozygotes show abnormality in both alleles of the gene for the LDL receptor. Three classes of mutant alleles have been identified by Brown and Goldstein : R^{b°, which specifies a receptor that has no binding ability, R^{b-}, which specifies a receptor that has reduced binding ability and R^{b+/i°, which specifies a receptor capable of binding LDL normally but incapable of internalizing the LDL (Brown and Goldstein, 1979). In the heterozygous form of FH, patients have one normal allele and one (of the three above) mutant allele at the receptor locus. Since binding and internalization are mediated by a single protein, the two alleles, whether normal or mutant, must reside in a single locus. The heterozygous form of FH is more common (1 in 500) than

the homozygous form (1 in 10^6). Both forms of FH are characterized by elevated plasma LDL levels.

[^{125}I] labelled monoclonal antibodies (IgG - C7), raised in mice, against LDL receptors of bovine adrenal cortex, have been shown to bind to receptors of abnormal human fibroblasts in molar amounts equivalent to that of human [^{125}I] LDL. However, fibroblasts from FH homozygotes showed a tendency to bind negligible amounts of [^{125}I] LDL and [^{125}I] IgG - C7, thus illustrating that the receptors were defective (Beisiegel *et al.*, 1982).

Mazzone and Chiat (1982) have described two types of receptor - mediated systems in monocyte - derived macrophages. One type recognizes native LDL and internalizes the LDL by the receptor - mediated pathway described (Goldstein and Brown, 1977). The second is a unique pathway which contains receptors which recognize chemically modified LDL (acetyl - or malondialdehyde - treated LDL) and degrade them at a more rapid rate than native LDL. Thus a study of the literature shows that the LDL receptor serves two major functions : it provides cholesterol for plasma membrane synthesis and maintains normal LDL levels in plasma by continuously binding and internalizing plasma LDL.

1.8 Interaction of LDL with mammalian cells

The number of receptors on the surface of the cell is under feedback control (Brown and Goldstein, 1975). This control is exerted on the synthesis of LDL receptors. The LDL internalized by receptor - mediated endocytosis provide cholesterol for plasma membrane synthesis. As a feedback control the cholesterol not utilized for plasma membrane synthesis is esterified and cholesterol synthesis is suppressed by ACAT and HMG Co A reductase respectively (Section 1.5). This system is dependent on exogenous cholesterol brought to the cell by LDL. The entry of cholesterol (from LDL) is controlled by the synthesis of LDL via a feedback loop. The LDL receptors are essential in the control of cholesterol synthesis by the cell, however, the receptors can be by - passed in culture by free cholesterol or oxygenated analogues (15 - hydroxycholesterol, 7 - ketocholesterol) added to the culture medium (Brown and Goldstein, 1974b).

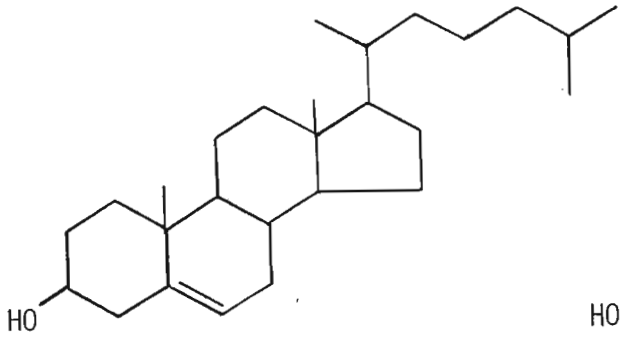
Basu and co - workers (1976) have reported that LDL, cationized by reaction with N,N - dimethyl - 1,3 - propanediamine, increased LDL degradation in homozygous FH fibroblasts *in vitro* 100 - fold. This degradation was inhibited by chloroquine, suggesting that the degradation occurred in the lysosomes. The cationized LDL was shown to regulate cholesterol metabolism in the cells. The authors have suggested that an alternate method of internalization occurs, which is charge - related. However, LDL which is not modified will also bind to negatively charged material, such as

glass beads (Dana *et al.*, 1977) and sulfated glycosaminoglycans and polyphosphates (Burnstein and Scholnick, 1974; Burnstein *et al.*, 1970).

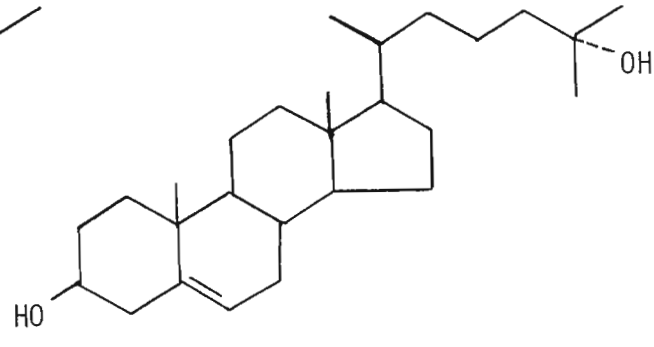
Of interest is the finding that ascorbate increases the receptor - mediated internalization and degradation of LDL in cultured bovine smooth muscle cells (Aulinskas *et al.*, 1983). This is inhibited by cycloheximide, suggesting that ascorbate stimulates *de novo* synthesis of LDL receptors. Although maximum receptor activity occurs about 12 - 24 hours after treatment, and is transient, ascorbic acid therapy is being considered in patients with familial hypercholesterolemia or those predisposed to atherosclerosis.

LDL particles have been modified and reconstituted in order to probe *in vitro* metabolism of LDL. Thus, Krieger and co - workers (1978) have removed cholesteryl esters from the LDL core and replaced them with labelled cholesteryl linoleate. The reconstituted LDL exhibited the same biological activity as native LDL, in cultured human fibroblasts. It was also able to regulate the activities of HMG Co A reductase and ACAT.

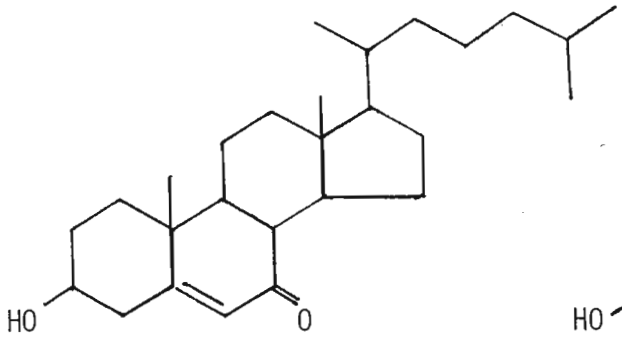
Cholesterol analogues, 7 - ketocholesterol and 25 - hydroxycholesterol (Figure 1.3a) have been utilized to study receptor - mediated endocytosis *in vitro*. The oxygenated sterols have been found to enter the cell, replace the cholesterol of plasma membranes and suppress the activity of the LDL receptor (Brown and Goldstein, 1974b).



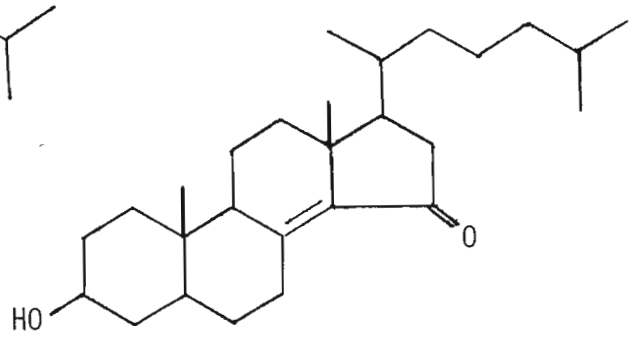
cholesterol



25 - hydroxycholesterol

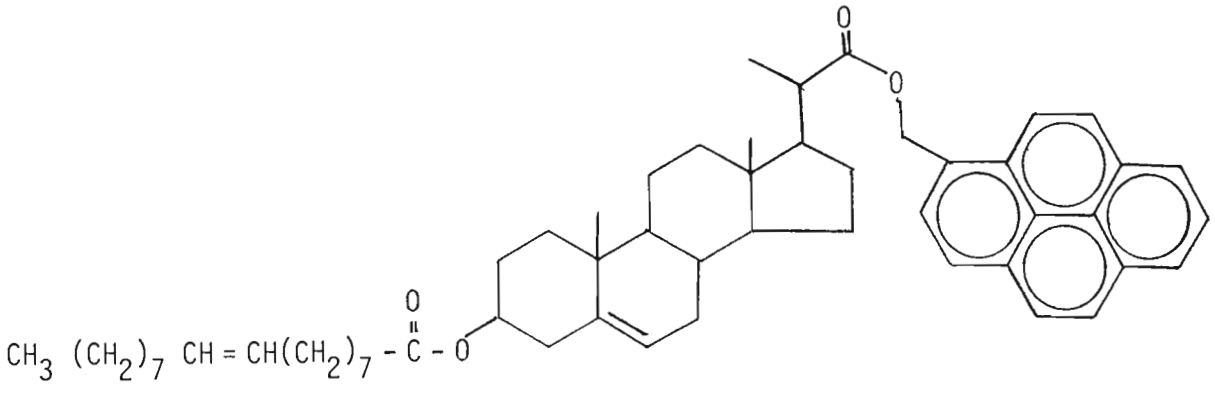


7 - ketocholesterol

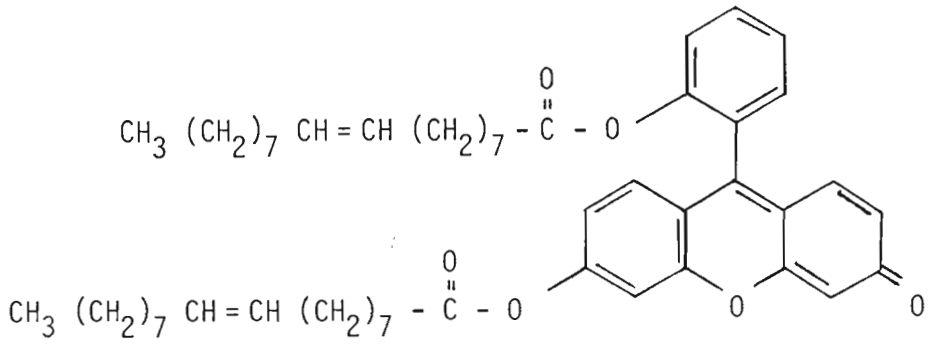


5α - cholest - 8 (14) en - 3β - ol - 15 one

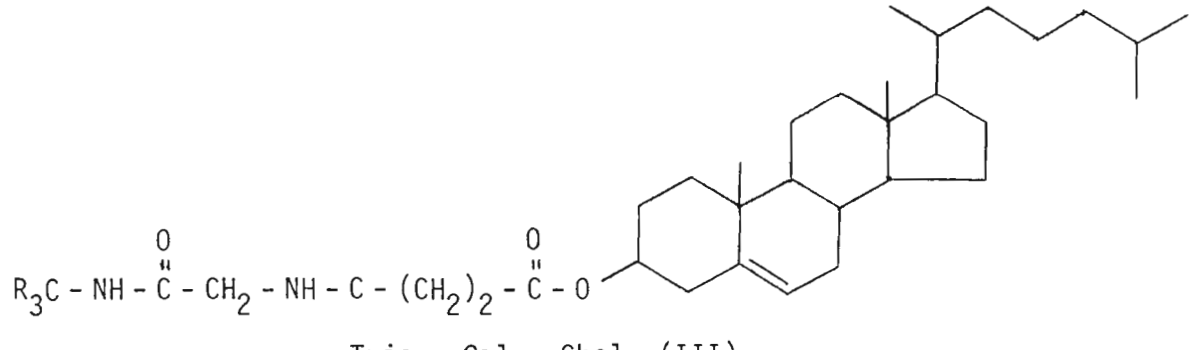
Figure 1.3a : Structures of cholesterol and its analogues used *in vitro* to study LDL receptor activity and LDL receptor - mediated regulation of HMG Co A reductase and ACAT enzyme activities.



PMCA oleate; I



dioleoyl fluorescein; II



Tris - Gal - Chol (III)

Figure 1.3b : Cholesterol derivatives (I, III) and fluorescein probe (II) incorporated in LDL and used in the study of LDL receptor function discussed in Section 1.8. R = (Gal - OCH_3); Gal = galactose.

Another cholesterol analogue, 5α- cholest - 8 (14) - *en* - 3β ol - 15 one, also reduced plasma cholesterol levels, in cholesterol fed Rhesus monkeys. This was coupled with the decrease in LDL and VLDL levels (Schroepfer *et al.*,1984).

Fluorescent probes, 3 - pyrene methyl - 23,24 - *dinor* - 5 cholen - 22 - oate - 3 - β - yl oleate (PMCA oleate) and dioleoyl fluorescein (Figure 3b) were incorporated into LDL and added to cultured human fibroblasts to visualize receptor - mediated endocytosis (Krieger *et al.*, 1979). These reconstituted LDL probes were used to show that cells from homozygous FH patients lacked LDL receptors.

A derivatized cholesterol, N - (tris - (β - D - galactopyranosylmethyl) methyl) - N^{α} - 4 - (5 - cholesten - 3β - yloxy) succinyl glycinamide (abbreviated Tris - Gal - Chol; Figure 3b) was introduced into LDL and liposomes. These were injected into rats and found to be incorporated into the liver parenchymal cells 10 minutes after injection (van Berkel *et al.*, 1985). The cholesterol derivative was rapidly catabolized by the hepatic cells. The authors proposed this as a method for targeting drugs, hormones and other substances into specific cells. However, since the liver is a 'detoxifying' organ, substances intended for targeting may be broken down before reaching the target organs.

It has been reported that a maximum of 250 000 molecules of [^{125}I] LDL can be bound per cell in normal skin fibroblasts with high affinity and specificity (Brown and Goldstein, 1974). Fibroblasts from

patients with abetalipoproteinemia, on the other hand, do not bind [^{125}I] LDL. This indicates a complete absence of LDL receptors. In the normal cells HMG Co A reductase activity is regulated by the LDL in the culture medium. HMG Co A reductase is the rate - controlling enzyme in cholesterol biosynthesis. The enzyme activity is not regulated or suppressed in FH homozygous cells *in vitro*, by exogenous LDL. Consequently, these cells are resistant to LDL - mediated suppression of HMG Co A reductase activity and continue to carry out cholesterol synthesis.

Scavenger receptors of human monocytes and macrophages recognize human LDL containing derivatized lysine residues and take up the acetylated, succinylated or malonylated LDL particles if the modified components comprise more than 16%. The recognition of the modified LDL was shown to be due to the presence of positive charges on the lysine residues (Davis and Malone - McNeal, 1985).

1.9 Lysosomes

In 1955 de Duve discovered a distinct type of subcellular organelle which he called the lysosome. He conducted latency studies and found that the lysosomes carried out the digestion of intracellular and extracellular macromolecules. Since then more research has been carried out to elucidate the functions of the lysosomes. It has been found that vesicles containing various endocytosed macromolecules fuse with primary lysosomes to form secondary lysosomes. The hydrolytic enzymes are contained in the primary

lysosomes after being synthesized in the endoplasmic reticulum (Reijngoud and Tager, 1977). The lysosome functions as an intra - cellular digestive system, and in it the hydrolytic enzymes have a working optimum pH which is acidic (pH 6).

All lysosomal enzymes are synthesized as inactive precursors which contain oligosaccharides and are activated by cleavage of the precursors at specific sites (Tager *et al.*, 1984). It has been found that a transmembrane pH gradient is generated by a proton - translocating ATPase of intracellular organelles such as lysosomes, endosomes, storage granules and Golgi apparatus. This proton gradient maintains a more acidic pH in the interior of the organelles (Harikumar and Reeves, 1984). The acidic pH is required for the optimal functioning of the lysosomal enzymes, which degrade macromolecules. The efflux of degradation products has also been shown to be dependent on ATP (Schneider *et al.*, 1984). A specific Mg^{++} - dependent ATPase is an integral component of lysosomal membranes (Schneider, 1981).

In the lysosomes, components internalized by receptor - mediation are selectively broken down by processes which complement cellular biosynthesis. About 50 lysosomal enzymes have been detected in the lysosomes (Neufeld *et al.*, 1975; Dobrota and Hinton, 1984; Barrett, 1984; Tager *et al.*, 1984). Lysosomal metabolism, involving these enzymes, has been divided into the proteolytic,

glycanolytic, nuclease and lipolytic pathways.

DNA and RNA entering the lysosome by the process of autophagy or endocytosis are broken down via the nuclease pathway (Barrett,1984; Dean, 1984; Duncan *et al.*, 1984). Lysosomal ribonuclease and deoxyribonuclease have an acid pH optimum. The lysosomes also contain acid phosphatase, phosphodiesterase and phosphatase activities to complete the hydrolysis of DNA and RNA (Dobrota and Hinton, 1984).

The lysosomes also contain various lipolytic enzymes, e.g., tri - acylglycerol lipase (which cleaves fatty acids from triglycerides and cholesteryl esters), phospholipase A_1 and A_2, phosphatidate phosphatidase (degrade phospholipids), sphingomyelin phosphodiesterase and acylsphingosine deacylase (hydrolyse sphingolipids).

Lysosomal enzyme deficiencies with respect to the lipolytic and nuclease pathways manifest serious inherited diseases such as Gaucher, Tay Sachs (sphingolipidosis) and Wolman's disease (cholesteryl ester and triglyceride accumulation). The lysosomal proteolytic pathway has, to date, shown no hereditary deficiencies. Proteolytic activity of the lysosome makes it the largest source of amino acids for new protein synthesis. The proteolytic enzymes also have a reaction maximum at acid pH. Lysosomal preparations are characterized by acid phosphatase and arylsulfatase activities. These are the lysosomal marker enzymes.

1.10 Cloning vehicles

Recombinant DNA techniques have been useful for the structural analysis of the chromosome. Specific segments of the chromosome, bearing genes of interest, can be isolated, inserted into cloning vehicles and propagated in suitable hosts.

The most commonly used cloning vehicles or vectors are plasmids and bacteriophages (Glass, 1982). The plasmids are double - stranded circular DNA molecules which can replicate in the host (usually specific strains of *E. coli*). Plasmid vectors have been derived from naturally occurring plasmids such as pSC101 and ColE1. Depending on the size of foreign DNA inserts, these plasmids can vary in size from 3 000 to 30 000 base pairs. The plasmids, carrying specific genes of interest, have been used to transform selected strains of *E. coli* (C600, HB101, h303, RR1) by adsorption of the plasmid to the host in the presence of calcium ions at reduced temperature, followed by temperature elevation to 42°C (Sinsheimer, 1977; Wilson and Goulding, 1986).

The plasmids may be 'stringent' or 'relaxed' with respect to DNA replication. The former type produces 6 - 8 plasmid molecules per chromosome in exponentially growing cells and requires new protein synthesis. Plasmid pSC101 and its derivatives fall into this category. The ColE1 and its derivatives are relaxed plasmids which produce 20 - 30 plasmid molecules per chromosome and do not require new protein synthesis for their replication. Neither type

of plasmid requires an active DNA polymerase I for replication.

Plasmids used as vectors for recombinant DNA carry genes for antibiotic resistance, e.g., pBR322 plasmid carries genes for resistance against the antibiotics ampicillin and tetracycline, denoted by amp^R and tet^R respectively (Sutcliffe, 1978; Watson *et al.*, 1983; Maniatis *et al.*, 1982). A distinct advantage is the presence of restriction sites (Section 1.11) in the antibiotic resistance genes. The insertion of a foreign gene into such a restriction site destroys the antibiotic resistance gene. However, the loss of resistance can be detected easily, as a check for the successful insertion of the foreign gene. When the recombinant plasmid is then introduced into a host bacterium, it confers resistance to the host by virtue of the second resistance gene (Bolivar *et al.*, 1977). A scheme for the insertion of a foreign gene into the *Pst*1 restricted site of the amp^R gene of pBR322 plasmid is presented in Figure 1.4.

In the presence of an inhibitor of protein synthesis, e.g., chloramphenicol, which blocks host DNA replication as well, the plasmid can continue replication until the plasmid DNA constitutes 40 - 50% of the cellular DNA. Thus plasmid DNA molecules can be increased from 20 copies per cell to 1 000 copies per cell (Potter and Dressler, 1979).

The viral cloning vector most commonly used for gene transfer is bacteriophage λ (Philipsen *et al.*, 1978; Maniatis *et al.*, 1982).

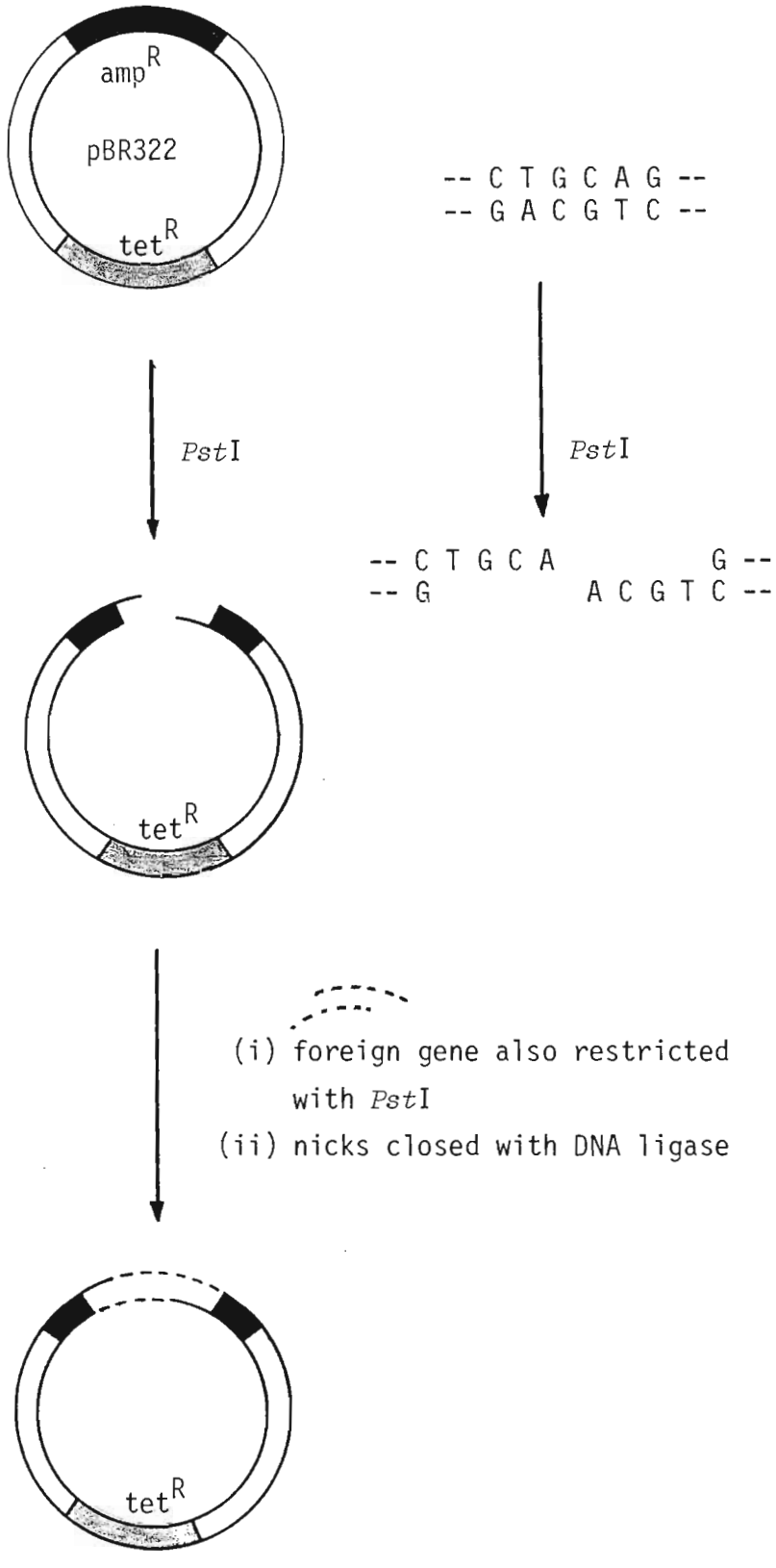


Figure 1.4 : Insertion of foreign DNA into amp^R gene of pBR322 at the *Pst*I restriction site. The recombinant is introduced into a bacterial host. Selection of transformed bacteria is by tetracycline resistance.

Since the phage λ DNA cannot be encapsulated unless at least 75% of its total genome is present, the λ DNA is excised and non - essential genome is replaced with the specified (foreign) gene under study. Large inserts in plasmids lead to large molecular weight plasmids which decrease the efficiency of transformation of host cells. Hence the use of bacteriophages (such as phage λ) as alternate vectors has become useful. Foreign DNA of up to 17 000 kilobases in length can be inserted into such a recombinant. The recombinant DNA molecules are then packaged into viral envelopes and allowed to infect bacterial cells on agar plates. The viral DNA, together with the insert, is injected into the bacterial cells, therefore the efficiency of transformation is very high and is detectable as viral plaques (Wilson and Goulding, 1986).

A cloning vehicle used for transforming eukaryotic cells is the derivative of the oncogenic virus SV 40 (Simian virus 40), which contains a 5 kilobase double stranded circular DNA. SV 40 mutants with intact origin of replication (ORI) region have had their deleted genome (late genes) replaced by foreign genes. However, such recombinant DNA can only be propagated with the aid of a helper SV 40 virus (Sinsheimer, 1977; De Pamphilis and Wasserman, 1980).

Very long DNA fragments have been inserted in viral vectors at the cos sites and given rise to recombinants known as cosmids. Since the only requirement for a DNA molecule to be packaged into a viral head is the presence of cohesive (cos) sites (12 bases in

length) spaced about 37 - 52 kilobases apart, long pieces of DNA located between cos sites and with plasmid origin of replication and unique restriction sites, for the insertion of foreign DNA, can be used as recombinants. The method of transformation by viral vectors is illustrated in Figure 1.5.

Plasmids have also been used as vehicles for transferring DNA into eukaryotic cells. These require a eukaryotic origin of replication gene (ORI) and a marker gene which, when expressed, is detecteble in eukaryotic cells. A useful cloning vehicle is the naturally occurring plasmid *2μ circle*, which is found in yeasts. Its origin of replication can be utilized, with further genetic manipulation, to produce a smaller plasmid containing a foreign insert.

A plant bacterium *Agrobacterium tumefaciens* contains a plasmid called Ti plasmid, which transfers part of its genetic material into the plant when the bacterium infects the plant. This plasmid has been used to introduce foreign genes into plants and forms the basis of genetic engineering in plants.

1.11 Methods of preparing recombinant DNA

With the advent of sequencing procedures as a means of studying DNA and RNA function, ideas for the production of recombinant genomes also arose. The first nucleic acid to be sequenced was yeast alanyl tRNA (Holley, 1966). As methodologies improved larger DNA

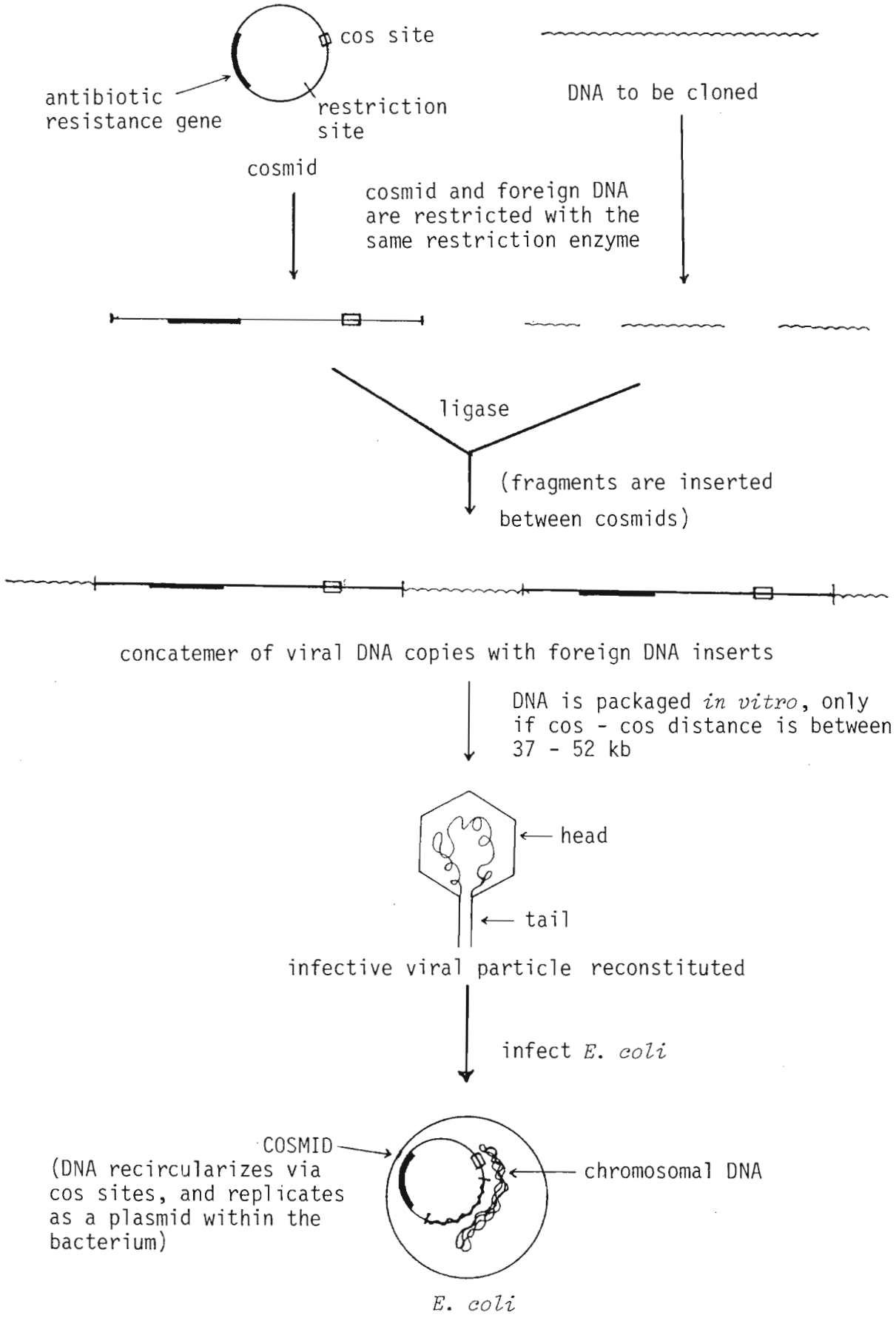


Figure 1.5 : Cloning in a cosmid vector.

and RNA molecules were sequenced (Sanger and Coulson, 1978; Fiers *et al.*, 1976; Sanger *et al.*, 1977; Maxam and Gilbert, 1977; Sutcliffe, 1978; Winter and Brownlee, 1978). Unlike the DNases and RNases which cleave DNA and RNA at random sites, other enzymes, which cleaved at sites of specific nucleotide sequences, were discovered. These were called restriction enzymes.

A restriction enzyme from the bacterium *Haemophilus influenzae* was found to cleave foreign DNA at the sequence :

$$\begin{array}{l} 5' - G\ T\ Py\ Pu\ A\ C - 3' \\ 3' - C\ A\ Pu\ Py\ T\ G - 5' \end{array}$$

(arrows indicate exact site of cleavage). The restriction enzyme was later called *Hin*dIII (Smith and Wilcox, 1970; Salser, 1974). Restriction enzymes that cut at other specific sequences have been isolated from different bacteria (Kessler *et al.*, 1985; Roberts, 1983; Kelley and Smith, 1970; Sharp *et al.*, 1973).

The discovery of restriction enzymes permitted reproducible restriction maps to be obtained for specific DNA fragments, using any chosen restriction enzyme. Restriction analysis was utilized in the determination of the sequence of small fragments of DNA such as pBR322 (which contains 4362 bases) and of Ø X174 DNA (Sutcliffe, 1979; Sanger *et al.*, 1977).

Restriction enzymes recognize specific sequences in DNA and cleave at these sites to produce restriction fragments which contain

either blunt ends or cohesive ends. For example, an enzyme like *Eco*R1 makes staggered cuts to produce short 4 - base single - stranded cohesive ends - T T A A $^{5'}$ (Figure 1.6) at the ends of each fragment (Dugiackzyk *et al.*, 1975). The cohesive or 'sticky' ends can reanneal with each other or with other DNA fragments that have been similarly restricted. After complementary base - pairing, the nicks on the DNA strand can be resealed by a ligase (Gefter, 1975) as shown in Figure 1.6. An example of a restriction enzyme producing blunt ends is *Hin*dIII (Kessler *et al.*, 1985) which cuts as follows :

$^{5'}$-- G T T A A C --$^{3'}$
$^{3'}$-- C A A T T G --$_{5'}$

↓

$^{5'}$-- G T T A A C --$^{3'}$
$^{3'}$-- C A A T T G --$_{5'}$

Since no sticky ends are produced, no re - annealing will occur. However, the blunt ends can be joined together by DNA or RNA ligases (Lobban and Kaiser, 1973; Hinton *et al.*, 1978; Hinton *et al.*, 1982). The ligases will also join any other DNA fragments with blunt ends.

A more useful feature can be endowed to blunt ended DNA fragments, such as poly dA homopolymeric extension of the 3' - end of each blunt ended fragment, using the enzyme terminal transferase (poly A

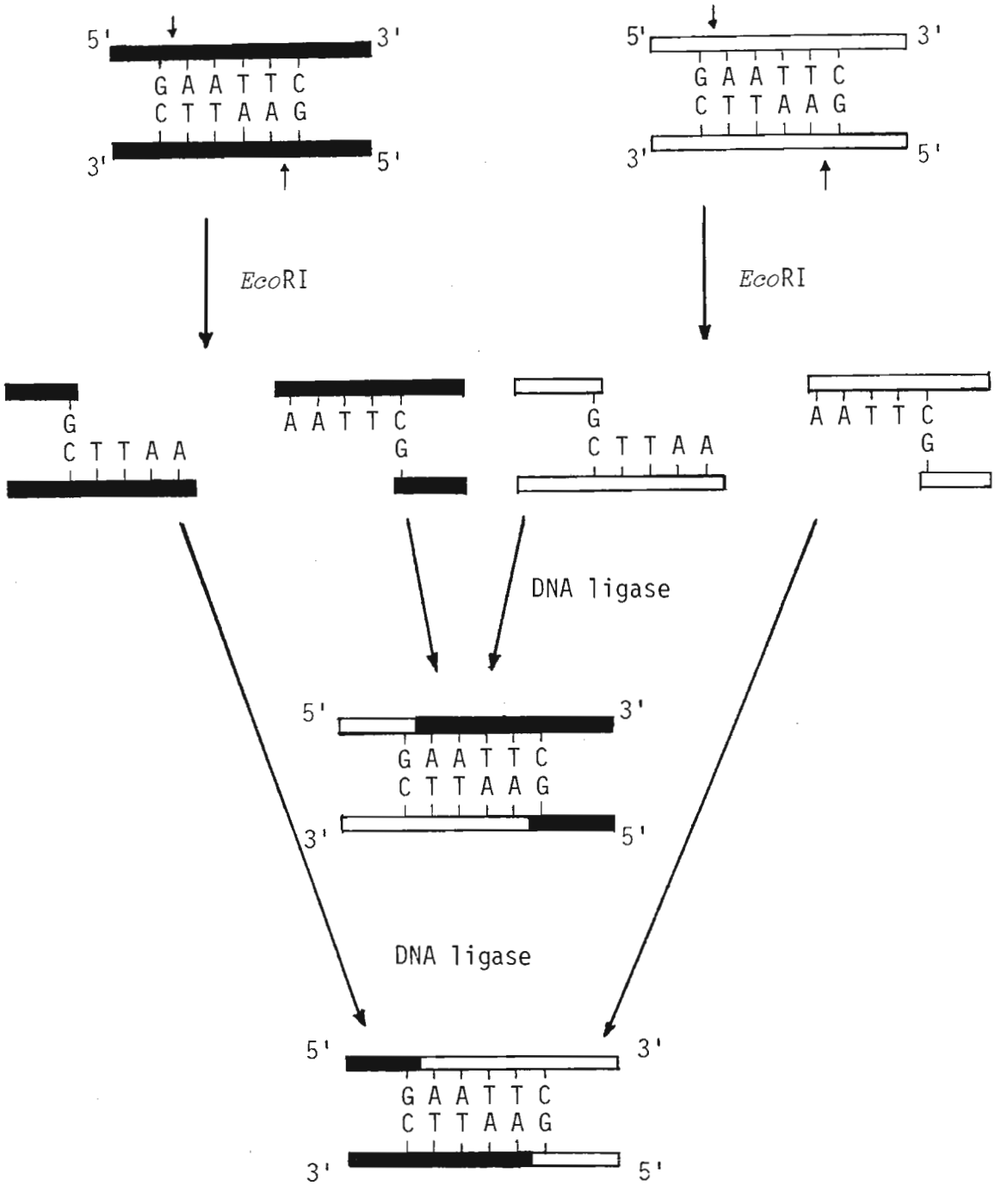


Figure 1.6 : Restriction of two separate fragments of DNA with *Eco*R1 restriction enzyme which makes staggered cuts away from the center of the recognition site (↑) to yield cohesive or "sticky" ends. The sticky ends produced in one DNA fragment can anneal with those produced in the second fragment because they have been produced by the same restriction enzyme.

tailing). Other DNA fragments can be poly dT tailed. The A - T complementary base pairing and DNA ligase can thus be used to join the two types of DNA together. These restriction and recombina - tion methods were employed in the synthesis of recombinant DNA molecules (Mertz and Davis, 1972; Lobban and Kaiser, 1973).

The first practical method for preparing recombinant DNA was used by Cohen and co - workers (1973), who restricted the *E. coli* plasmid pSC101 with *Eco*R1 and converted the double - stranded circular plasmid to a linear molecule containing cohesive ends. They then added foreign DNA which had been subjected to *Eco*R1 cleavage and contained cohesive ends. The nicks were ligated with DNA ligase. Thus a recombinant DNA was created, the plasmid pSC101 being the cloning vector which now carried one or more pieces of foreign DNA. Since pSC101 carried its own origin of replication it could be used to transform *E. coli*. This was followed by a vast amount of research into recombinant DNA construction methods (Section 1.12) and gene transfer methods (Section 1.13). In order to transform prokaryotic and eukaryotic cells the correct origin of replication and promoters had to be inserted, in addition to the gene under study.

A further method for preparing recombinant DNA was also developed. Most eukaryotic mRNA molecules have a number of adenine nucleotides at their 3' - ends (poly A tails). The addition of a short oligo dT chain results in complementary hybridization with the poly A, thus

the poly dT acts as a primer for the enzyme reverse transcriptase, which utilizes the mRNA as a template to synthesize a complementary DNA strand (cDNA). This results in the formation of an RNA - DNA hybrid. The newly synthesized cDNA ends in a hairpin loop which DNA polymerase I utilizes as a primer to copy the newly - synthesized cDNA (Efstratiadis *et al.*, 1976).The result is a double - stranded cDNA molecule with a single - stranded hairpin loop which is subsequently removed by the single - strand specific S1 nuclease (Young and Anderson, 1985; Brown and Stern, 1974). A scheme for the preparation of cDNA is presented in Figure 1.7. The double - stranded cDNA obtained by this method is blunt-ended. The blunt ends can be either tailed (using terminal transferase) or made cohesive by attaching chemically synthesized linkers (using DNA ligase) which have the same single - stranded end sequences as the restricted cloning vector into which the cDNA is to be inserted. The recombinant DNA thus formed can be introduced into a suitable host, such as *E. coli*, and propagated.

The cDNA methods have been used in the cloning of eukaryotic genes such as the globin gene (Efstratiadis *et al.*, 1976), fatty acid binding protein gene (Chan *et al.*,1985), the ovalbumin gene (Monahan *et al.*, 1976) and growth hormone gene (Goedel *et al.*, 1979). Mammalian genes, inserted into plasmid or phage cloning vectors, cannot be expressed unless the correct initiation signals, recognized by *E. coli* ribosomes, are also inserted.

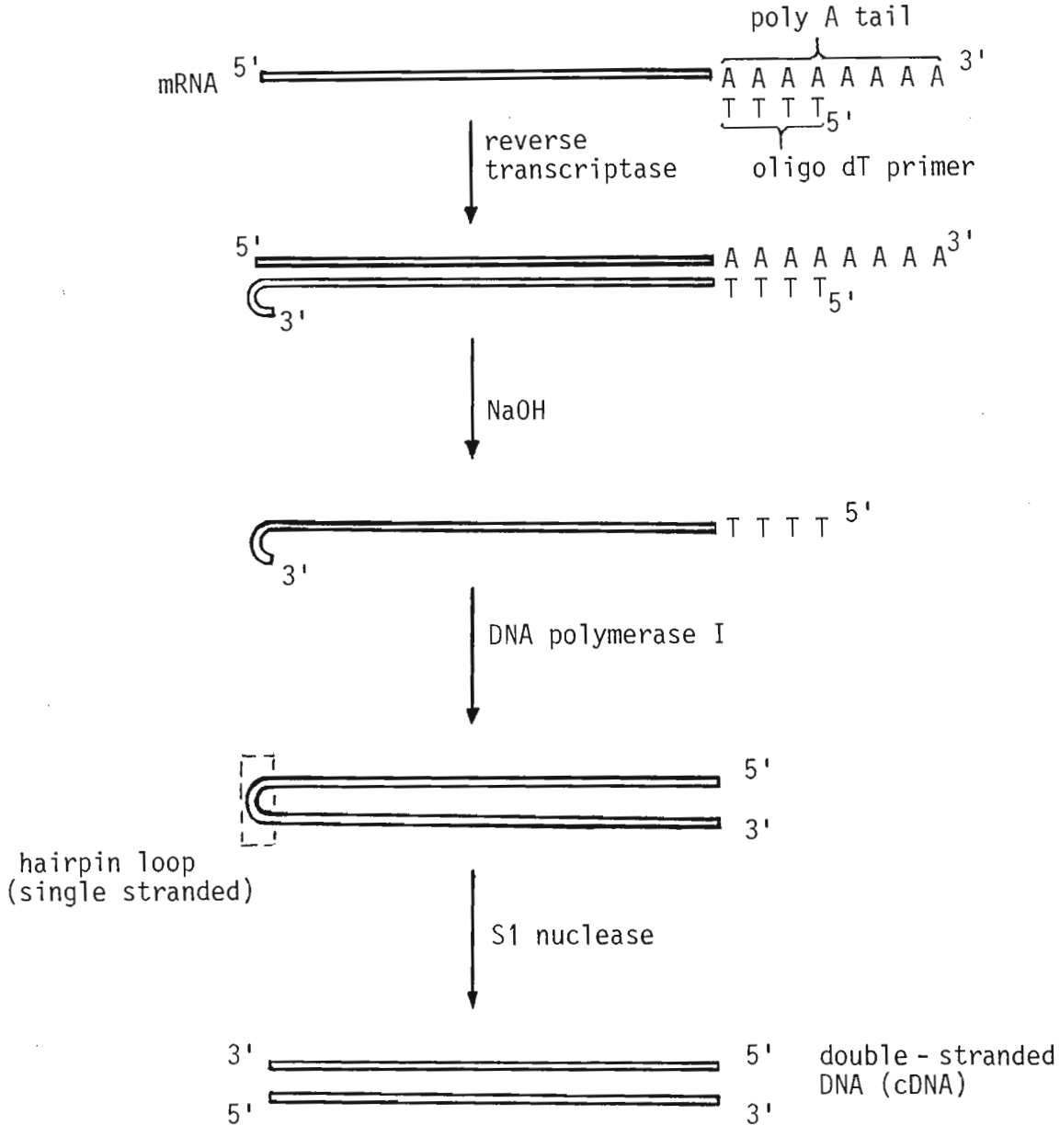


Figure 1.7 : Synthesis of cDNA from mRNA.

Jay and co - workers (1982) chemically synthesized initiation signals, which, when inserted upstream of SV 40 t antigen gene prior to its cloning in the plasmid pBR322, were able to promote the synthesis of the SV 40 t antigen gene. The gene was identical to the gene produced *in vivo*, as determined by immunologic, electrophoretic and proteolytic digestion assays. Since recombinants retain their restricrion sites, their amplified clones can be assayed by restriction analysis.

Recombinant DNA techniques have also been employed to prepare a cDNA clone for bovine adrenal LDL receptor (Russell *et al.*, 1983). Immunoselected poly A - containing RNA were used to construct a cDNA library containing more than 5×10^5 recombinants. One cDNA clone, pLDL R - 1, for bovine LDL receptor, was isolated. The nucleotide sequence of a region of the cDNA insert encoded a protein sequence identical to that of the original bovine LDL receptor, except for one amino acid. The authors attributed this discrepency to technical error or polymorphism in the LDL receptor gene of different animals.

The ability to express eukaryotic proteins in *E. coli* has proved useful in identification and isolation of eukaryotic cDNA clones. An expression vector containing a reconstructed apoprotein E cDNA, a λP_L promoter (regulated by the thermolabile cI repressor) and a ribosomal binding site derived from λcII or *E. coli* β lactamase gene, inserted in the *Pst*I site of pBR322, has been

used to transform *E. coli*. Human apoprotein E has thus been produced successfully in the transformed *E. coli* (Vogel *et al.*, 1985).

Plasmids pBR322 and pDY160 have been utilized in the preparation of recombinants containing human papovavirus (BKV), which have subsequently been amplified in *E. coli* strains HB101 and C600 (Arkigg *et al.*,1981; Harley *et al.*, 1982). Although the BK virus is not associated with clinical diseases in man, it has been shown to have oncogenic potential in some animal cells (Seif *et al.*, 1979). Passage of the virus in human cell lines has resulted in genomic changes, hence amplification of the virus in *E. coli* has also been attempted.

1.12 Recombinant DNA with selectable markers

A number of well defined selectable marker genes have been used in genetic studies in eukaryotic cells. The best characterized and possibly the most widely used is the gene for thymidine kinase (tk), an enzyme used in the salvage pathway of pyrimidine bio - synthesis. During the degradation of DNA, the thymidine released is salvaged and converted to dTMP and then to dTTP which is eventually re - incorporated into DNA. The normal pathway for dTTP synthesis is via dCDP. Thus tk is not an essential component for survival of eukaryotic cells.

Cells lacking the thymidine kinase enzyme are designated tk^-, and can be easily isolated by feeding cells with bromodeoxyuridine (Br dU). Br dU can be phosphorylated by thymidine kinase and incorporated into DNA with lethal effect. The tk^- cells survive as Br dU does not become phosphorylated and hence it cannot be incorporated into host DNA. However, tk^- cells will not survive in HAT medium, which contains hypoxanthine, aminopterin and thymidine, because aminopterin inhibits the normal biosynthetic step : dCDP $\longrightarrow$ dCTP $\longrightarrow$ dTTP (Szybalski *et al.*, 1962). In such a case the only source of dTTP is via the thymidine kinase pathway. Hence only tk^+ cells are likely to survive in HAT medium.

It was found that the tk gene from Herpes simplex virus (HSV) could be incorporated into tk^- mouse cells (by the Ca^{++} - precipitated DNA transfection method described in Section 1.13) to confer resistance to these cells in HAT medium (Wigler *et al.*, 1977; Littlefield, 1974; Munyon *et al.*, 1971). This has meant that the HSV tk gene can be used as a selectable marker for the integration of other genes linked to it and can be detected in culture as surviving colonies.

Mutant tk deficient mouse cells (Ltk^-) have been treated with *Bam*H1 restriction endonuclease - cleaved HSV 1 DNA (Pellicer *et al.*, 1978; Perucho *et al.*,1980). The resultant surviving colonies were shown to stably express the tk^+ phenotype. The tk gene incorporated into the tk^- mouse genome was found to be a 3,4 kilobase DNA fragment. The gene was stably inherited with a frequency of one

copy per chromosome complement; however, the integration was not site - specific.

Enquist and co - workers (1979) constructed a hybrid plasmid by insertion of the tk gene of HSV type I into the *Bam*H I site of the plasmid pBR322 which is found in *E. coli* (Figure 1.8). A restriction map was subsequently prepared for 10 nucleases to confirm its stable insertion in mammalian cells.

Another example of the use of thymidine kinase as a selectable marker for a recombinant DNA was the linking of the HSV type I tk gene to the aminoglycoside 3' - phosphotransferase gene (coded for by the Tn 5 transposon). The Tn5 transposon also codes for the kanamycin - neomycin resistance gene. This makes mammalian cells (murine, human, simian) resistant to the neomycin analogue, G418, which is otherwise toxic to these cells. Both selectable markers were inserted into the plasmid pAG 0. The hybrid was stably expressed in mammalian cells which showed resistance to G418 and could be grown selectively in HAT medium (Colbere - Garapin *et al.*, 1981).

The use of tk gene as a vector for gene transfer into eukaryotic cells has necessitated the conversion of the recipient cells to the tk^- mutant form. The process involved was laborious (Figure 1.9). Therefore a search was made for dominant - acting vectors which could work on normal cells. One such vector constructed was the

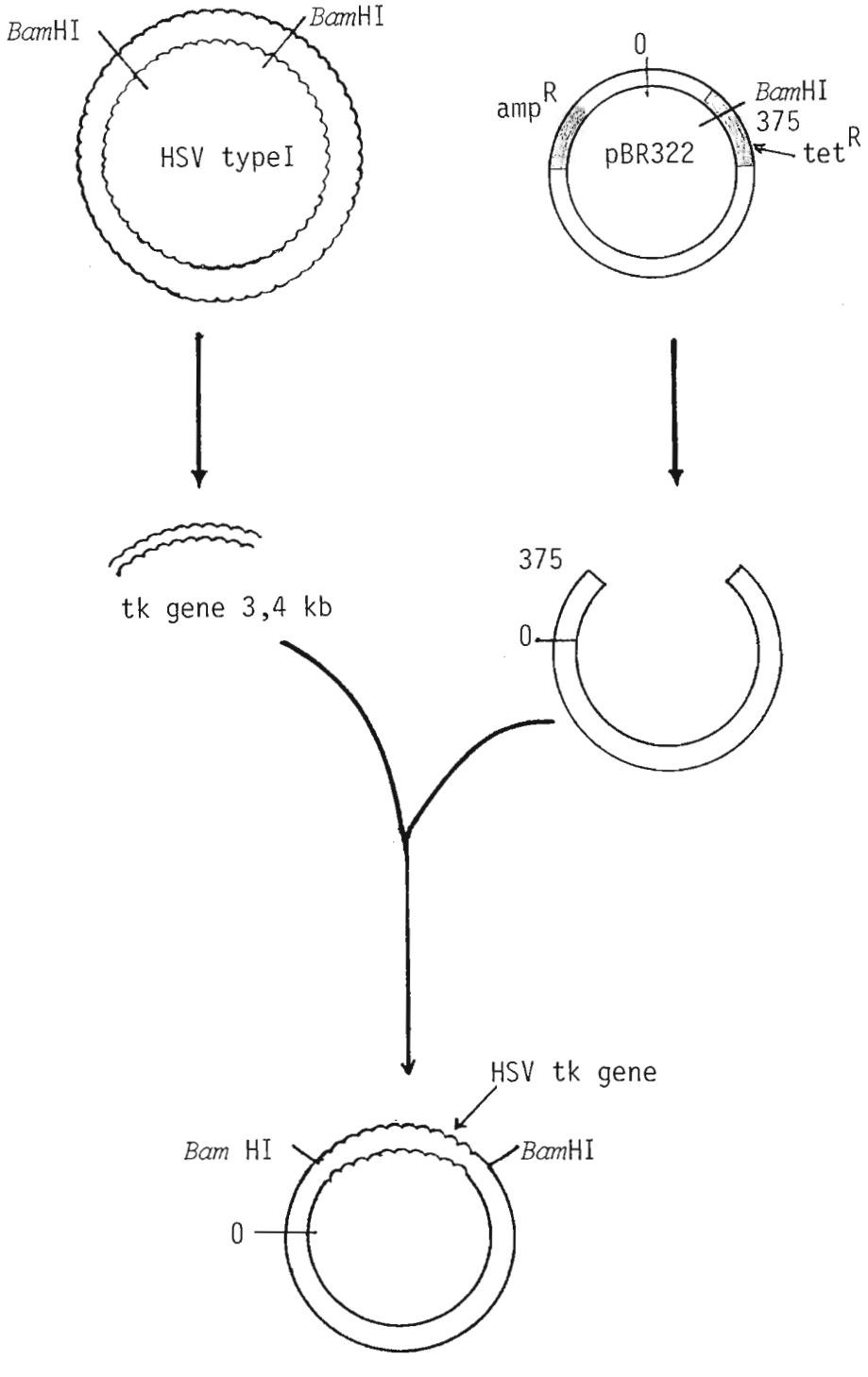
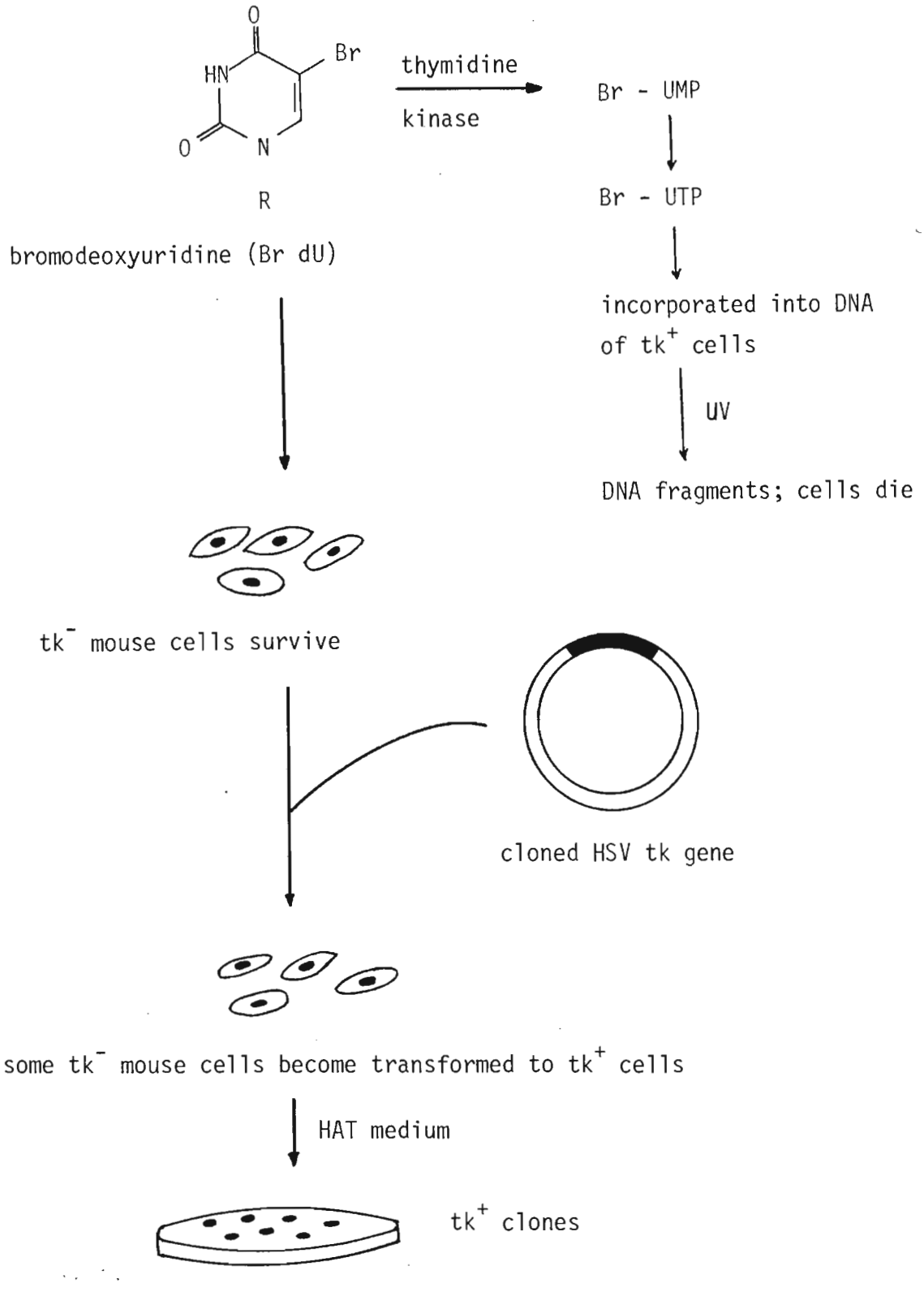


Figure 1.8 : Insertion of thymidine kinase (tk) gene into the *Bam* HI site of pBR322.



Figyre 1.9 : Selection of tk^- cells in the presence of Br dU and subsequent transformation to tk^+ cells.

SVgpt vector, which codes for the enzyme xanthine - guanine phosphoribosyltransferase (XGPT) and allows bacteria to use xanthine as a precursor of purine nucleotides. The corresponding mammalian enzyme hypoxanthine - guanine phosphoribosyltransferase (HGPT) utilizes hypoxanthine as precursor. The SV gpt vector was therefore made by inserting the XGPT coding gene between the promoter and poly A addition sites of the large T antigen gene (Figure 1.10). The SV gpt vector has an efficient SV 40 promoter (Laimins *et al.*, 1982), and can transform $HGPT^-$ mammalian cells to the $HGPT^+$ form. Selection for transformants was carried out in medium containing xanthine and mycophenolic acid. Since mycophenolic acid blocks the HGPT enzyme, only those cells which have taken up the SV gpt vector with the bacterial gene will survive by using xanthine in the medium as a precursor of purine nucleotides (Mulligan and Berg, 1981).

Another dominant - acting vector constructed (SV neo) consists of the prokaryotic neomycin - resistance gene (neo^R gene) inserted into the SV 40 early region. The neo^R gene inactivates neomycin and its analogue, G418, by phosphorylating them. Thus SV neo - transformed cells are resistant to these antibiotics.

Plasmids (especially pBR322) have been widely used as cloning vectors for genes from various sources. The successful insertion of the DNA fragments can be confirmed, usually, by the loss of expression of one of its antibiotic resistance genes. For example, a cDNA copy of the polyadenylated RNA of MS2 bacteriophage was

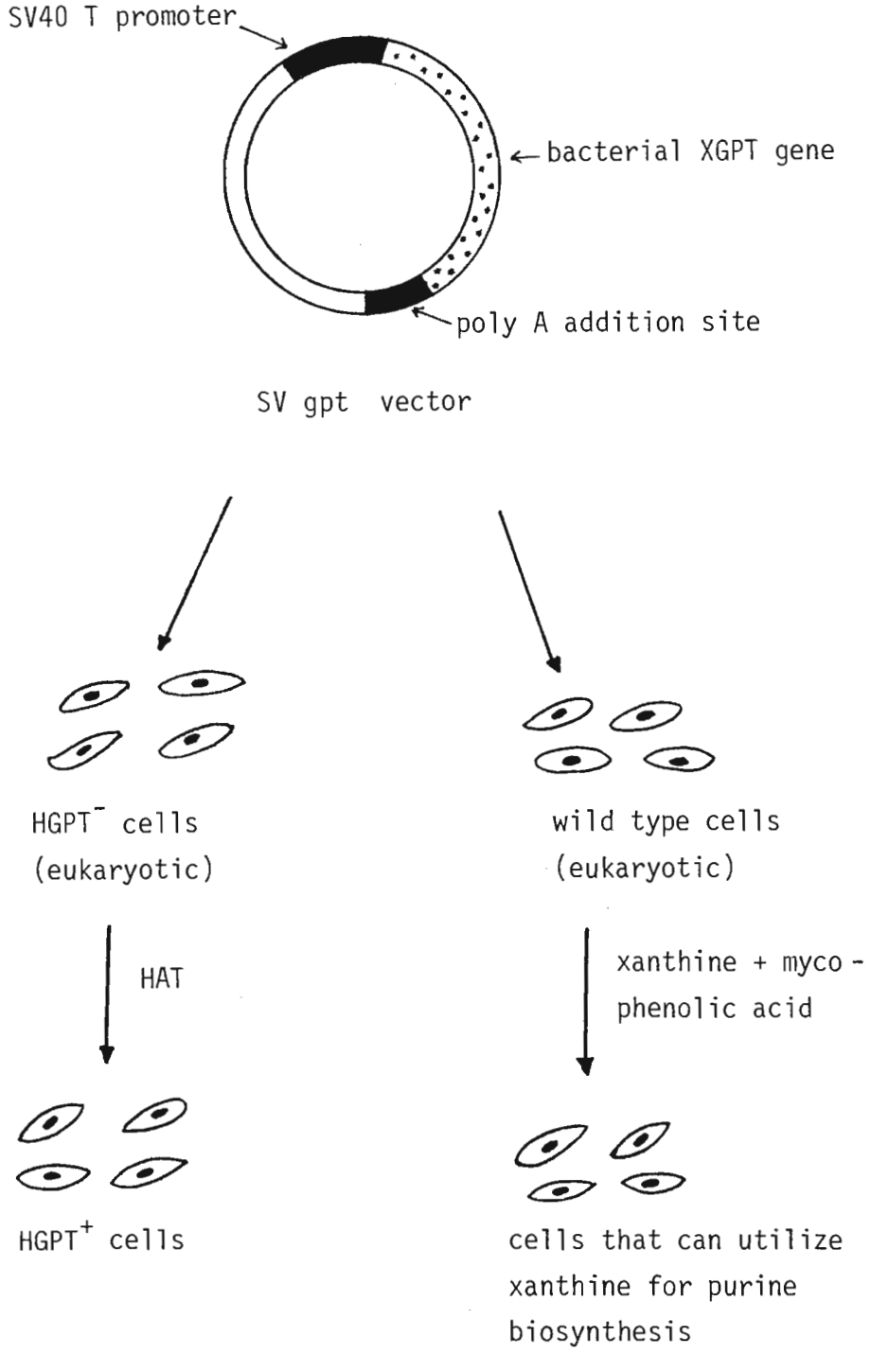


Figure 1.10 : Use of the dominant - acting SV gpt vector for the transfer of genes in eukaryotes. The vector utilizes prokaryotic structural genes under the control of eukaryotic promoters.

synthesized and inserted into the plasmid pBR322, restricted with *Pst*I, by means of the poly dA - poly dT tailing procedure (Devos *et al.*,1979). Since the *Pst*I restriction site is located in the amp^R site of pBR322, successful insertion of the MS2 DNA is confirmed by loss of ampicillin resistance.

The plasmid pBR322 has also been used as a cloning vector for the *Hin*d III restricted APRT gene. The gene coding for the enzyme adenine phosphoribosyl transferase (APRT) has been isolated from hampster and restricted with *Hin*d III. The APRT gene was ligated to pBR322 and used to transform mouse $APRT^-$ cells to the $APRT^+$ phenotype (Lowy *et al.*, 1980). Since plasmid sequences served as biological markers the isolation of the APRT gene from the transformants was possible.

Recombinant genomes which direct the expression of the enzyme chloramphenicol acetyltransferase (obtained from *S. aureus* or *E. coli* episomal factors; Shaw, 1967; Shaw and Brodsky, 1967) confer chloramphenicol resistance to transformed cells, and have been used as selectable markers. The resistance phenotype results from the inactivation of chloramphenicol by acetylation (Shaw, 1975; Goldfarb *et al.*, 1982). Thus the chloramphenicol resistance gene has been used in the preparation and study of recombinants of pBR322 cloning vectors containing SV 40 early region or Adenovirus type 12 (Ad 12) or Rous sarcoma virus (RSV) eukaryotic promoters

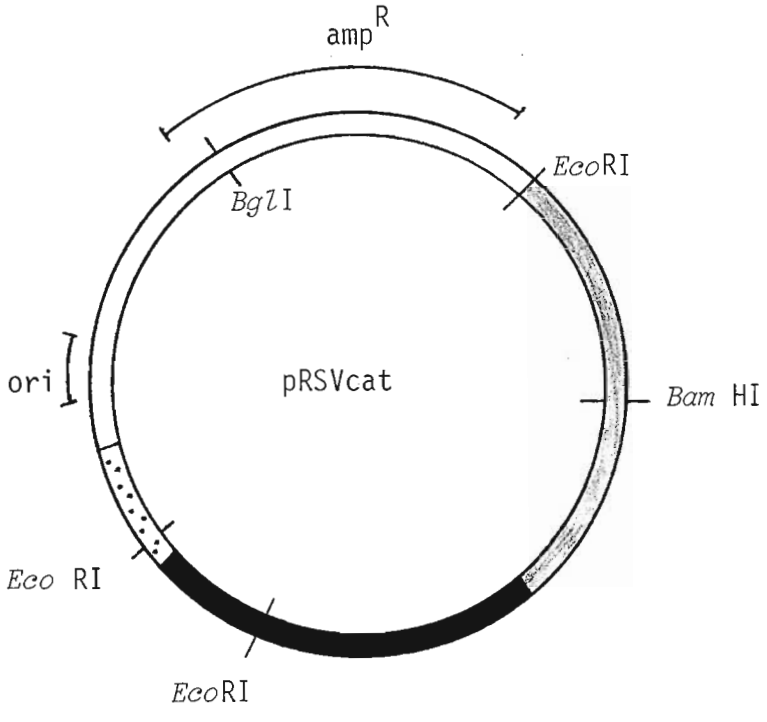


Figure 1.11 : Construction of pRSVcat expression vector. The CAT gene (■) is inserted behind the SV40 early region and poly A addition sites (▩). The RSV long terminal repeat (LTR) is shown by (⚄). Some restriction sites and ori region of pRSVcat are also indicated.

Gorman *et al.*, 1982a, b; Gorman *et al.*, 1983; Kruckzek and Doerfler, 1983; Burke and Mogg, 1985; Darnell and Boime, 1985). The construction of the pSV2cat and pRSVcat recombinants, containing the chloramphenicol resistance gene, are presented in Figure 5.1 and 1.11 respectively. Both expression vectors function well in mammalian cells. The RSV LTR has been utilized as promoter in non - mammalian cells as well (Gorman, 1986).

1.13 Methods for introducing recombinant DNA into eukaryotic cells

Cloning vectors used in gene transfer in eukaryotic systems have several important features. The vectors contain prokaryotic origin of replication and antibiotic resistance sequences which allow for the amplification and selection of the clones in bacterial hosts. They also contain eukaryotic promoter sequences (and regulator) which control initiation of transcription in eukaryotic cells. Polyadenylation sequences are also incorporated in the cloning vectors for efficient expression in mammalian cells. Lastly, the cloning vector contains the test gene or the gene under study.

A number of methods have been employed for the introduction of recombinant DNA into eukaryotic cells. The most widely used method is the calcium phosphate - DNA coprecipitation technique, which was first used to study the infectivity and transforming activity of viral DNA (Graham and van der Eb , 1973; Graham *et al.*,1980; Strain and Wyllie, 1984). The DNA under study is coprecipitated with calcium phosphate at room temperature before being applied

to eukaryotic monolayers in culture. Adsorption of the calcium phosphate - DNA is allowed to occur for 3 - 4 hours at 37°C before replacing the medium. The cells are believed to take up the foreign DNA by a calcium - mediated endocytic process. The foreign DNA molecules have been shown to be incorporated into phagocytic vacuoles (Loyter *et al.*, 1983), but their mechanism of incorporation into the host genome is unknown. This method of gene transfer has been utilized in the introduction of eukaryotic DNA into cultured cells (Pellicer *et al.*, 1980; Gasser and Schimke, 1986). Thymidine kinase has been incorporated into tk^- cells by this method (Pellicer *et al.*, 1978; Colbere - Garapin *et al.*, 1981; Scangos and Ruddle, 1981) and other recombinants into cultured somatic cells (Gorman *et al.*, 1982a, b; Gorman *et al.*, 1983; Kruckzek and Doerfler, 1983; McLauchlan *et al.*, 1985; Burke and Mogg, 1985; Gorman. 1986). Stably inherited clones are visualized as antibiotic resistant colonies within 7 - 14 days while transiently expressed genes are assayed within 48 hours. The efficiency of transformation by the calcium phosphate - DNA coprecipitation method has been enhanced by treatment of transfected cells with glycerol or butyrate (Gorman *et al.*,1982a, b; Gorman *et al.*, 1983; Burke and Mogg, 1985).

In addition to calcium phosphate, other facilitators have been used for the introduction of foreign DNA into cells. These include the polycation DEAE dextran (Vaheri and Pagano, 1965) and poly - ethylene glycol (Chu and Sharpe, 1981). However, the efficiency of transfection with these facilitators is much lower than with the

calcium phosphate method. The calcium phosphate - DNA coprecipita - tion method is regarded as the classical method of gene transfer and has been used for various cell lines such as L cells, NIH 3T3 cells, HeLa cells, CV - 1 cells and fibroblasts. However, this method is not practicable *in vivo*.

Another method for the transfer of foreign DNA into cells is the direct microinjection method. This method makes use of micro - pipettes for the mechanical transfer of macromolecules into somatic cell monolayers and is not restricted to specific cell types (Graessmann and Graessmann,1976; Diacumakos, 1980; Graessmann *et al.*,1980).

Capecchi (1980) has successfully monitored the transfer of the HSV tk gene into LM tk^- mouse nuclei by microinjection with glass micropipettes. The β - globin gene has also been successfully introduced into mutant cells by microinjection and was found to be functional (Anderson *et. al.*, 1980).

DNA can be introduced directly into the cell nucleus or cytoplasm without the use of facilitators. The size of the macromolecules introduced into the cells does not have to be limited. Even single gene copies may be introduced. However, the number of cells transfected becomes a limiting factor and possible cell damage cannot be overlooked. Although transformed cells can be cloned; cell division of injected cells is reduced by 10% (Stacey, 1980). Another disadvantage is that this technique cannot be applied *in vivo*.

Erythrocyte ghost mediation is another technique employed in the transfer of genetic material into eukaryotic cells. The erythrocyte ghosts are loaded with the material under study under hypo - osmotic haemolytic conditions and then fused to somatic cells (Loyter *et al.*, 1975; Schlegel and Mercer, 1980). Methods are also being developed for antibody - directed interaction of erythrocyte ghosts with cell surface antigens. Avidin - coupled erythrocytes have been fused with biotin - labelled cells (Godfrey *et al.*, 1983). However, the introduction of DNA by this method requires the use of EDTA and repeated freeze - thawing to trap DNA in the erythrocyte ghosts (Wiberg *et al.*, 1983). Another disadvantage is that additional unnecessary material is also loaded into the ghosts and introduced into the cells. Moreover, transformation efficiency is lower than that for calcium phosphate - DNA coprecipitation method.

Liposomes have also been used as mediators in transforming cells with foreign DNA. The DNA is trapped in the phospholipid vesicles (liposomes), which then deliver the genetic material (and possibly other substances) to recipient cells (Papahadjopoulus *et al.*, 1974). The mechanism of transfer is still uncertain. However, liposomes tagged with colloidal gold have been shown to bind at coated pits, endosomes and secondary lysosomes (Straubinger *et al.*, 1983; Hardy, 1986). This points to an endocytic transfer rather than by fusion of liposomes with plasma membranes. The efficiency of transformation is lower than methods already described. Liposome - mediated drug targeting has the potential of being more successful than liposome - mediated gene targeting.

DNA may also be trapped in Sendai viral envelopes during reconstitu - tion and subsequently fused to cultured cells by means of the viral F protein (Fusion factor; Vainstein *et al.*, 1983). However, this technique is restricted to specific cell types. This method of gene transfer affords protection against nucleases, thus improving chances of transforming recipient cells. Packaging of concatenated DNA into viral heads (discussed in Section 1.10) may also be employed for the transfer of genes into recipient cells.

The electroporation technique used for gene transfer is a new method of gene transfer. It involves the application of a high voltage to the recipient cells. This induces the formation of pores which allow the entry of foreign DNA into electrically shocked cells (Potter *et al.*, 1984). This technique may be applied to all cell types *in vitro*, but is undesireable as a method of *in vivo* gene transfer.

Protoplast fusion method has also been used to introduce foreign DNA into eukaryotic cells. This technique allows for the transfer of cloned DNA from bacteria to eukaryotes. The DNA is first introduced into bacteria which are subjected to lysozyme digestion of the cell wall to yield bacterial protoplasts (Schaffner, 1980). The fusion of protoplasts with eukaryotic cells is then induced with polyethylene glycol. Although prior purification of the DNA is not essential, the technique is suitable for *in vitro* studies only.

1.14 Assays for the expression of foreign DNA

If recipient cells become transformed by foreign genes, one should be able to detect or assay for the expression of the transferred genes. This can be accomplished by gene screening techniques for stably transformed cells and enzymic assays (usually) for transiently expressed genes.

To screen for foreign DNA in recipient cells, a suitably labelled probe is first prepared by nick translation (Maniatis *et al.*, 1982; Rigby *et al.*, 1977; Hutchison *et al.*, 1982; Manuelidis *et al.*, 1982; Arrand, 1985). The nick translation procedure makes use of DNase I to create single - stranded nicks in double - stranded DNA. *E. coli* DNA polymerase is then utilized to carry out 5'$\longrightarrow$ 3' exonuclease and polymerase reactions. This results in the removal of fragments of single - stranded DNA, starting at the nicks, and replacement with new strands synthesized by the incorporation of labelled deoxyribonucleotides. The nick in each strand therefore moves in a 5' $\longrightarrow$ 3' direction as the strand is being repaired. A diagrammatic representation of the process is given in Figure 1.12. Nick - translation, using a [^{32}P] - labelled deoxynucleotide is most often used in the preparation of the probe. [^{125}I] -, [^{3}H] - labelled and non - radioactive biotinylated nucleosides may also be used (Arrand, 1985). In an alternative and increasingly popular procedure, a restricted probe may be labelled using the Klenow enzyme (Feinberg and Vogelstein, 1983). Probe fragments are heat denatured and single - stranded molecules are labelled,

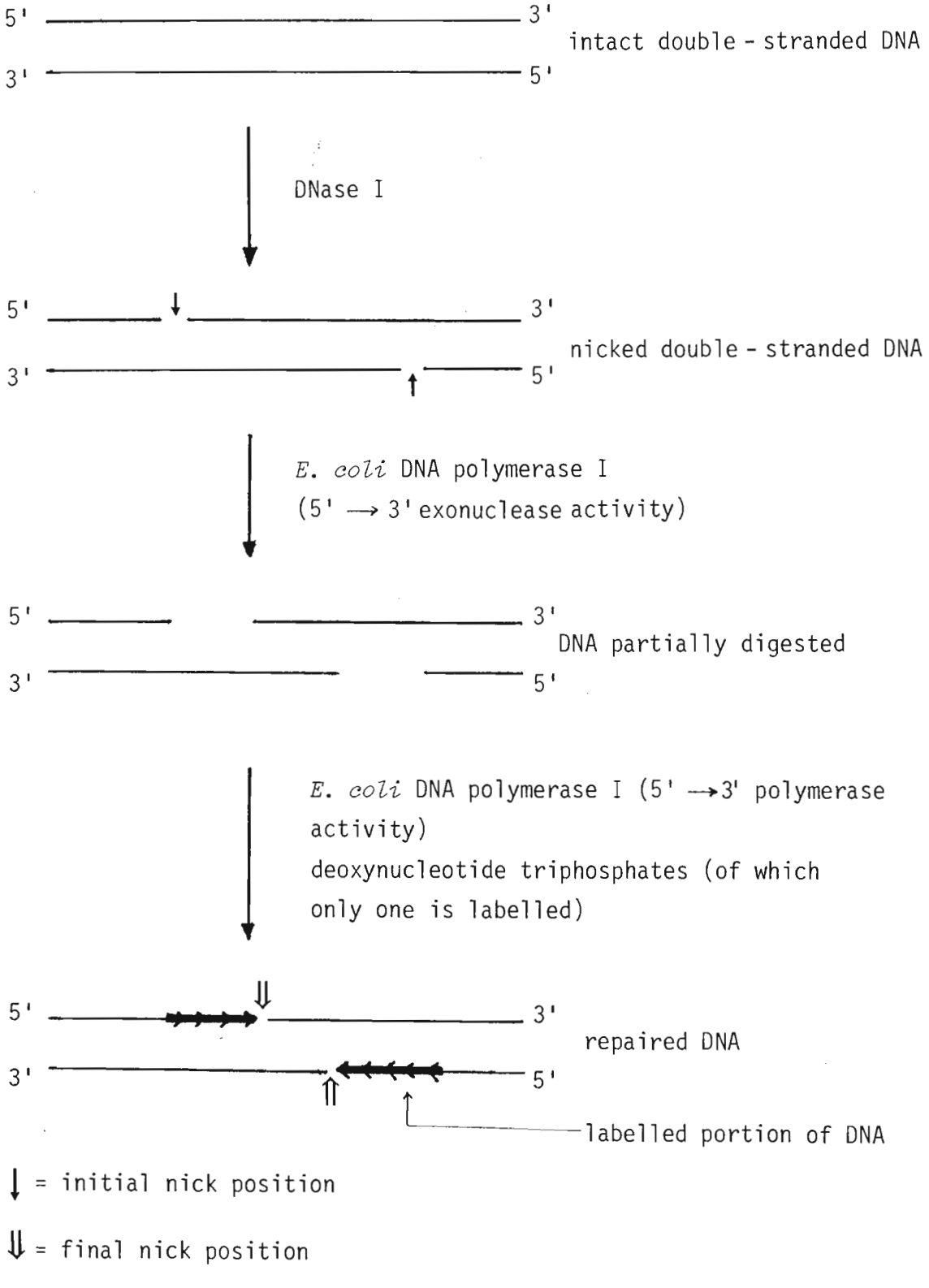


Figure 1.12 : Preparation of DNA probe by nick translation.

using random primers, [α^{32}P] - radiolabelled deoxynucleotide triphosphates and the 5' $\longrightarrow$ 3' polymerase activity of the Klenow fragment of DNA polymerase I.

Cellular DNA is isolated, restricted with an appropriate restriction enzyme , fragments are separated electrophoretically and then hybridized to the labelled probe which has been similarly restricted. Various hybridization strategies may be employed. Cellular DNA may be denatured and transferred onto nitrocellulose filter supports before applying the labelled probe. The techniques are known as Southern blot or dot blot analyses (Southern, 1975; Dubensky *et al.*, 1984; Anderson and Young, 1985; Mackett *et al.*, 1986). Radio - labelled probes hybridized successfully to transformed cellular DNA can be detected by autoradiography (Mason and Williams, 1985).

Hybridization with biotin - labelled probes may be detected with avidin - enzyme or streptavidin - enzyme complexes which elicit a colour reaction and can be assayed colorimetrically (Arrand, 1985) or by electron mocroscopy if *in situ* hybridization techniques are employed (Hutchison *et al.*, 1982; Manuelidis *et al.*, 1982).

Transcription of inserted genes may be established by Northern blotting (Alwine *et al.*, 1977). Here total mRNA is separated on agarose gels in the presence of denaturing agents (methylmercury or formaldehyde) and transferred to chemically treated paper or nitrocellulose (if formaldehyde is used as denaturing agent).

Hybridization of mRNA to labelled probe is detected by autoradiography. Northern blotting is a suitable screening method for transformation of recipient cells with cDNA clones, as this method will reveal whether the cDNAs are full - length or not.

Transiently expressed genes are usually assayed by enzymic methods e.g., the chloramphenicol acetyl transferase (CAT) enzyme produced in mammalian cells transfected with pSV2cat (Gorman *et al.*, 1982; Gorman, 1986) or the dihydrofolate reductase (DHFR) enzyme produced in plants transfected with cauliflower mosaic virus (CaMV) which has the R67 plasmid encoded DHFR gene (Lichtenstein and Draper, 1986).

1.15 LDL receptor - mediation as a possible method of gene transfer in eukaryotic cells and its impact on gene therapy

LDL particles are internalized by the method of receptor - mediated endocytosis (Sections 1.7 and 1.8). It is therefore theoretically possible that LDL complexed with DNA may also be internalized by this method provided that complex formation does not interfere with receptor recognition. This may form the basis for a novel method for introducing foreign DNA into mammalian cells. Moreover, the technique may be assessed using cloning vectors (Sections 1.10 and 1.11) containing selectable markers (Section 1.12). Expression of DNA introduced into the recipient cells may therefore be monitored.

LDL - DNA complexes internalized by receptor - mediated endocytosis would presumably be delivered, via the endosome, to the lysosomes where they would be exposed to a series of hydrolytic enzymes including nucleases (Section 1.9). LDL is degraded largely through the action of proteases and lipases and the DNA could also undergo some degradation. The ultimate success of this method for introducing DNA into mammalian cells would rely on that portion of the DNA which escapes the action of lysosomal nucleases.

The successful transformation of cells in culture by LDL receptor mediation could have a significant impact on gene manipulation *in vitro* and may find application in whole organisms in the field of gene therapy. Abnormal (mutant) genes may be supplemented by stable transformation with normal counterparts. Insertion of normal genes into defective recipient cells may thus correct genetic abnormalities.

Of the various methods of transfection (Section 1.13) of foreign genes into eukaryotic cells, *in vitro*, the use of inactivated Retrovirus (segments coding for viral protein removed) is, at present, being considered as the most favourable vector for the introduction of corrective genes into humans. Enzyme deficiencies such as hypoxanthine phosphoribosyl transferase (HGPT), purine nucleoside phosphorylase (PNP) and adenosine deaminase (ADA) afflict infants. The first of these causes delayed motor develop - ment (Lesch - Nyhan disease), while the latter two cause severe immune deficiency (Kolata, 1984; Miller *et al.*, 1984).

Correction of these genetic diseases is envisaged by removal of the patient's bone marrow (Stem) cells, transfection of the cells by the corrective gene inserted into inactivated Retrovirus, *in vitro*, and subsequent return of the transfected cells into the patient's bone marrow for reproduction. The inactivated Retrovirus, however, can become infective if the patient becomes infected by another (helper) virus. Since Retroviruses have been associated with diseases such as cancer and AIDS (acquired immune deficiency syndrome), the use of the virus in humans would not be entirely safe. Thus, the use of LDL - DNA complexes for the introduction of corrective genes into bone marrow cells, as above, may prove to be a far safer technique in gene therapy, as such procedures can be carried out under physiological conditions, without the risk of viral infection or the use of chemical or mechanical methods of cell transfection. As yet gene expression in transfected cells cannot be controlled. However, it is believed that overproduction of enzymes such as HGPT, PNP and ADA will not be detrimental to the patient.

1.16 Formulation of present work

LDL are composed of protein and several lipid classes and are therefore readily modified (Glomset, 1969; Roberts *et al.*, 1985; Zilversmit *et al.*, 1975; Haberland *et al.*, 1984); moreover, LDL particles may be reconstituted (Krieger *et al.*, 1978; Krieger *et al.*, 1979) to include unnatural or chemically altered components.

The binding, internalization and degradation of such particles has yielded much information with respect to LDL catabolism and the control of LDL anabolism (Brown and Goldstein, 1976; Brown *et al.*, 1976; Goldstein *et al.*,1976; Brown and Goldstein, 1975; Goldstein and Brown, 1974; Brown *et al.*, 1975a, b, c; van der Westhuyzen *et al.*, 1984).

The introduction of DNAs into viable mammalian cells has been induced by methods such as microinjection (Celis *et al.*, 1980; Capecci , 1981; Celis, 1984; Diacumakos, 1980), calcium phosphate - DNA coprecipitation methods (Graham and van der Ebb, 1973; Graham *et al.*, 1979; Wigler *et al.*, 1978; Gorman *et al.*, 1982a, b; Gorman, 1986) and other methods (Section 1.13) for the purpose of using such manipulations to study gene expression in higher eukaryotes. However, the use of low density lipoproteins as carriers of DNA into cells has not been cited in the literature.

Most mammalian cells, normal and transformed, have, on their cell surfaces, receptors for binding low density lipoproteins (Goldstein and Brown, 1977; Brown and Goldstein, 1976; Roensch and Blohm, 1976). This makes the mammalian system an attractive one for the introduction of DNA into cells, *in vitro*, by LDL receptor - mediation. With a view to introducing DNA into eukaryotic cells, LDL was isolated, characterized and then variously modified. The modification of LDL was carried out to enhance its binding to DNA.

One method of modification utilizes the watersoluble carbodiimide, N - ethyl - N' - (3 - trimethylaminopropylammonium) carbodiimide (or ECDI). The carbonyl groups of glutamic and aspartic acid residues of apoprotein B of LDL were modified as shown in Figure 1.13, and resulted in the formation of N - acylureas, under the reaction conditions employed.

The LDL particle was also modified by acetylation (Figure 1.14). Both the protein and lipid portions of LDL undergo acetylation. The major N - acetylations occur at lysine residues and to a lesser extent at asparagine and glutamine residues of apoprotein B. In addition, of the peripheral lipids of LDL, phosphatidylserine and phosphatidylethanolamine are probably also N - acetylated, while cholesterol may be O - acetylated. Thus acetylation of LDL extensively modifies both the lipid and protein components.

Another modification of LDL involved its conjugation to ethidium bromide. The ethidium bromide possibly interacts with the lipid and protein components of LDL (Figure 1.15) to yield the ethidium bromide - LDL conjugate.

The LDL was also labelled by cholesteryl ester exchange as illustrated in Figure 1.16. Two types of reaction were carried out. One required the use of DMSO for the exchange of unlabelled core cholesteryl esters for [^{3}H] - labelled cholesteryl esters. The second method used the enzymatic exchange of cholesteryl esters in lipoprotein deficient serum.

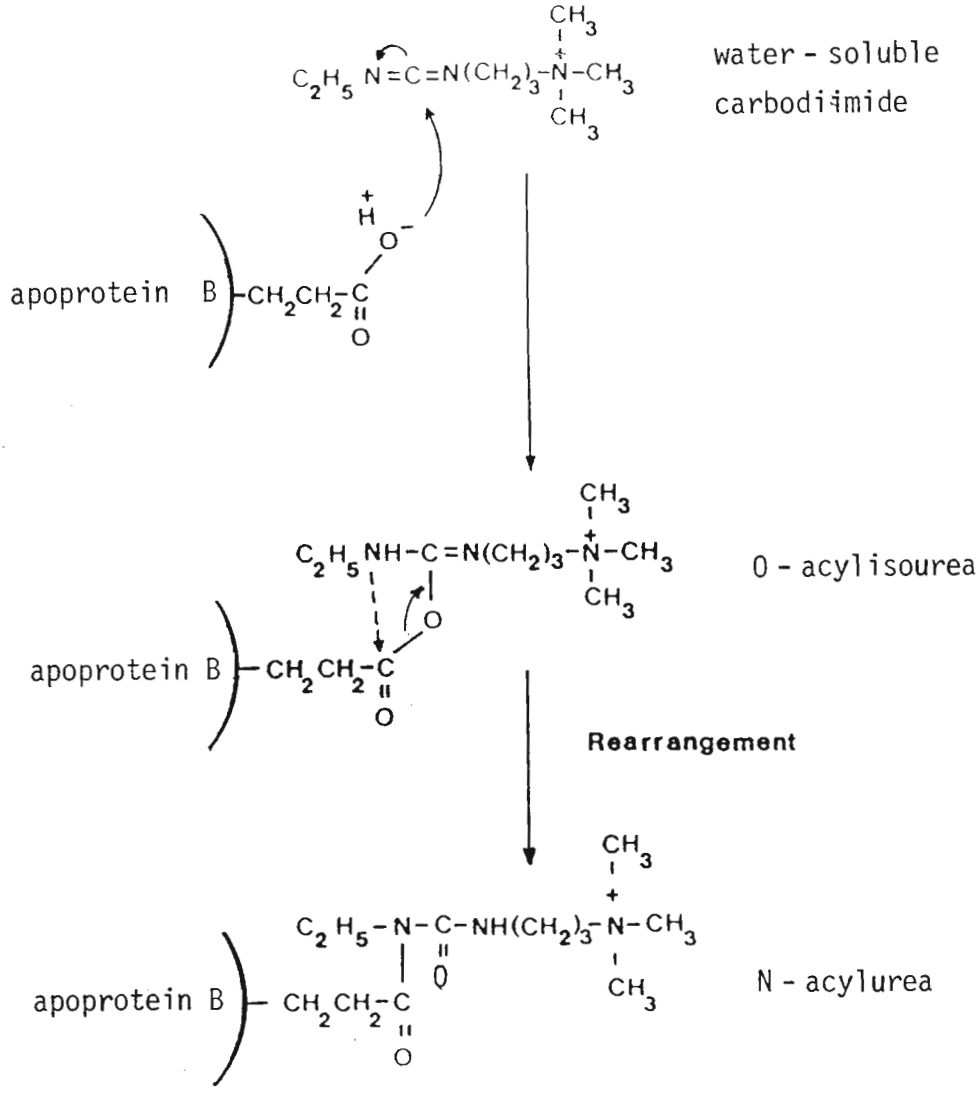
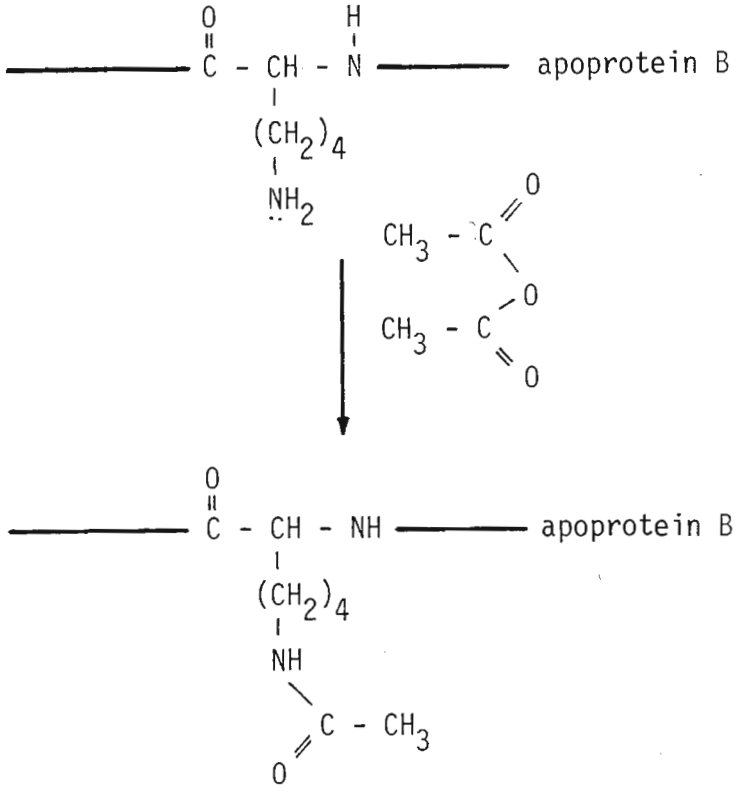


Figure1.13 : Carbodiimide modification of apoprotein B of LDL.

(i)



(ii)

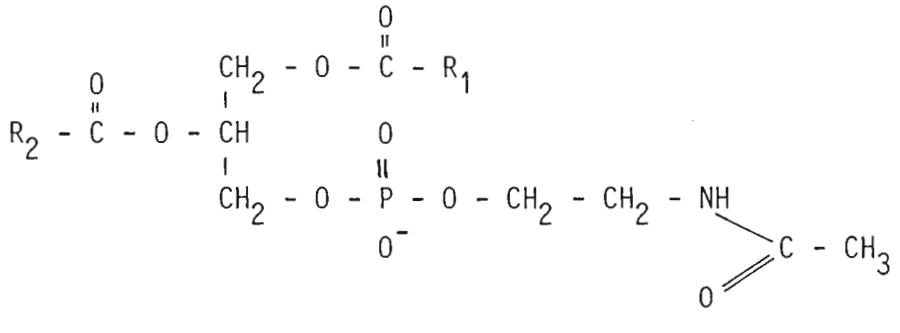


Figure 1. 14 : Acetylation of lysine residues of apoprotein B (i) and of phosphatidylethanolamine (ii) of LDL.

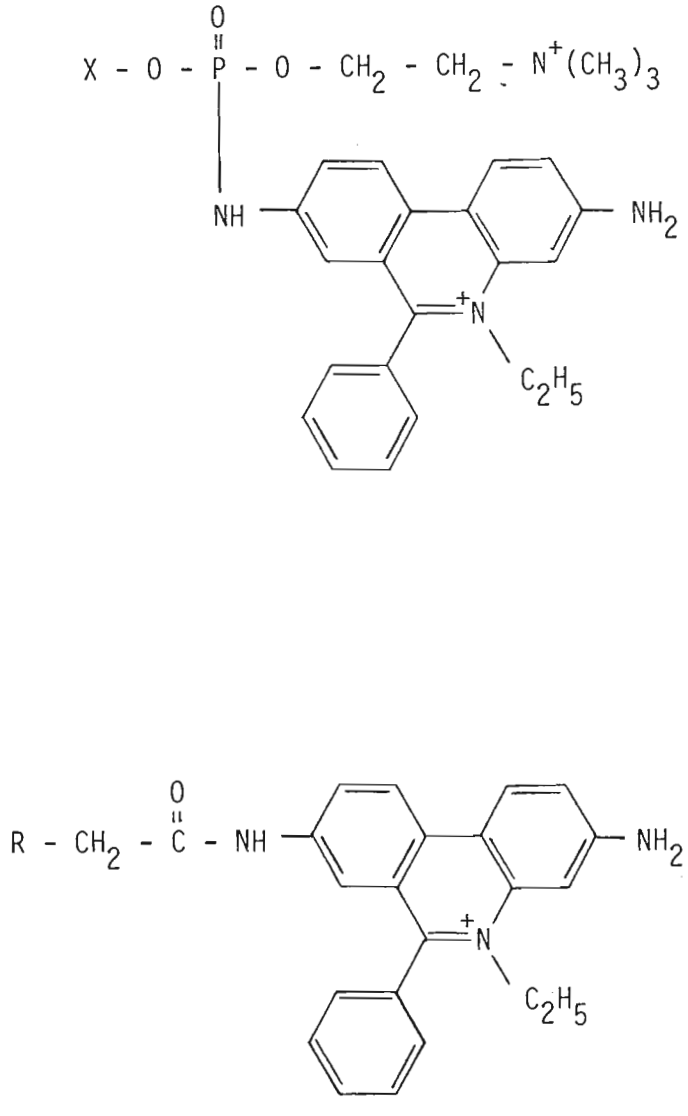


Figure 1. 15 : Ethidium bromide - LDL conjugation in the presence of carbodiimide occurs via the phospholipid portion (a) or protein portion (b) of the LDL particle. The ethidium - LDL conjugation is discussed in detail in Section 2.3.4.

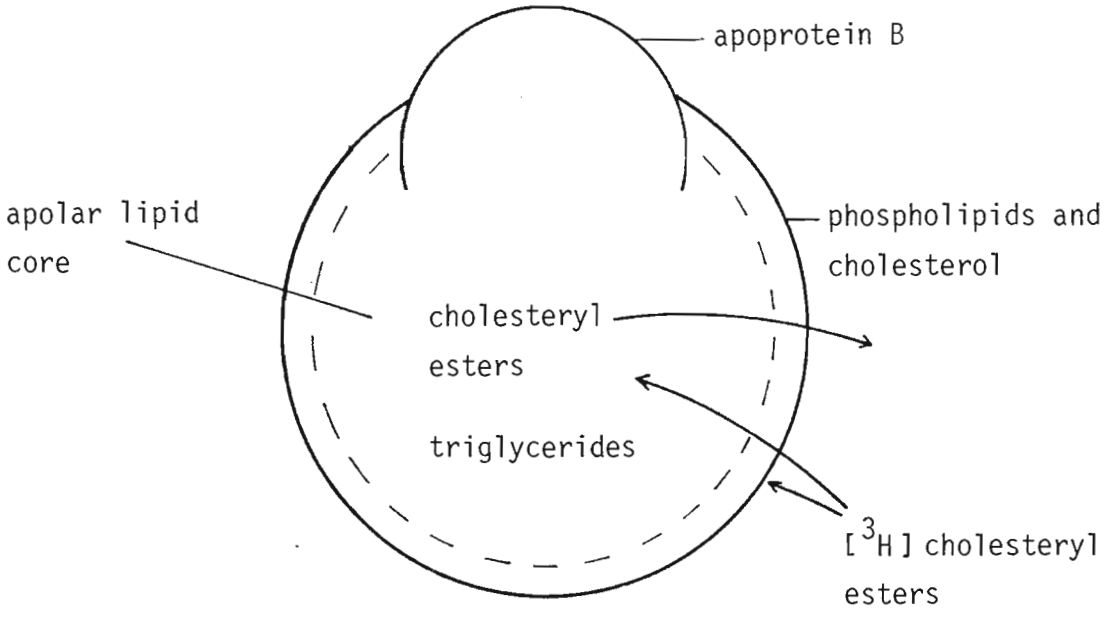


Figure 1.1 6 : Modification of LDL by the cholesteryl ester exchange method. The labelled cholesteryl esters enter the core of the LDL particle and probably also adhere to the external surface.

All the modified LDL were characterized and analysed before attempting to bind them to DNA. The native and modified LDL were complexed to pBR322 DNA or calf thumus DNA. The interaction between native LDL and DNA was found to be mainly electrostatic and a possible mechanism for this interaction is presented in Figure 1.17. Of the modified LDL, the carbodiimide - modified LDL had the highest affinity for DNA. Interaction with the DNA was probably due to both lipid and protein components as illustrated in Figure 1. 18.

The interaction of modified LDL - and native LDL - DNA complexes with cells in culture, was investigated. Since the final aim of the project was to ascertain whether the DNA internalized by LDL receptor mediation could be expressed *in vitro*, both normal and transformed cells were used to carry out studies on the expression of chloramphenicol acetyltransferase (CAT) enzyme. The gene for the CAT enzyme is present in the recombinant pSV2cat. Its expression has been reported in various cell lines, by introducing the DNA by the calcium phosphate - DNA coprecipitation method only. The expression of any gene by LDL receptor - mediated endocytosis has not previously been reported.

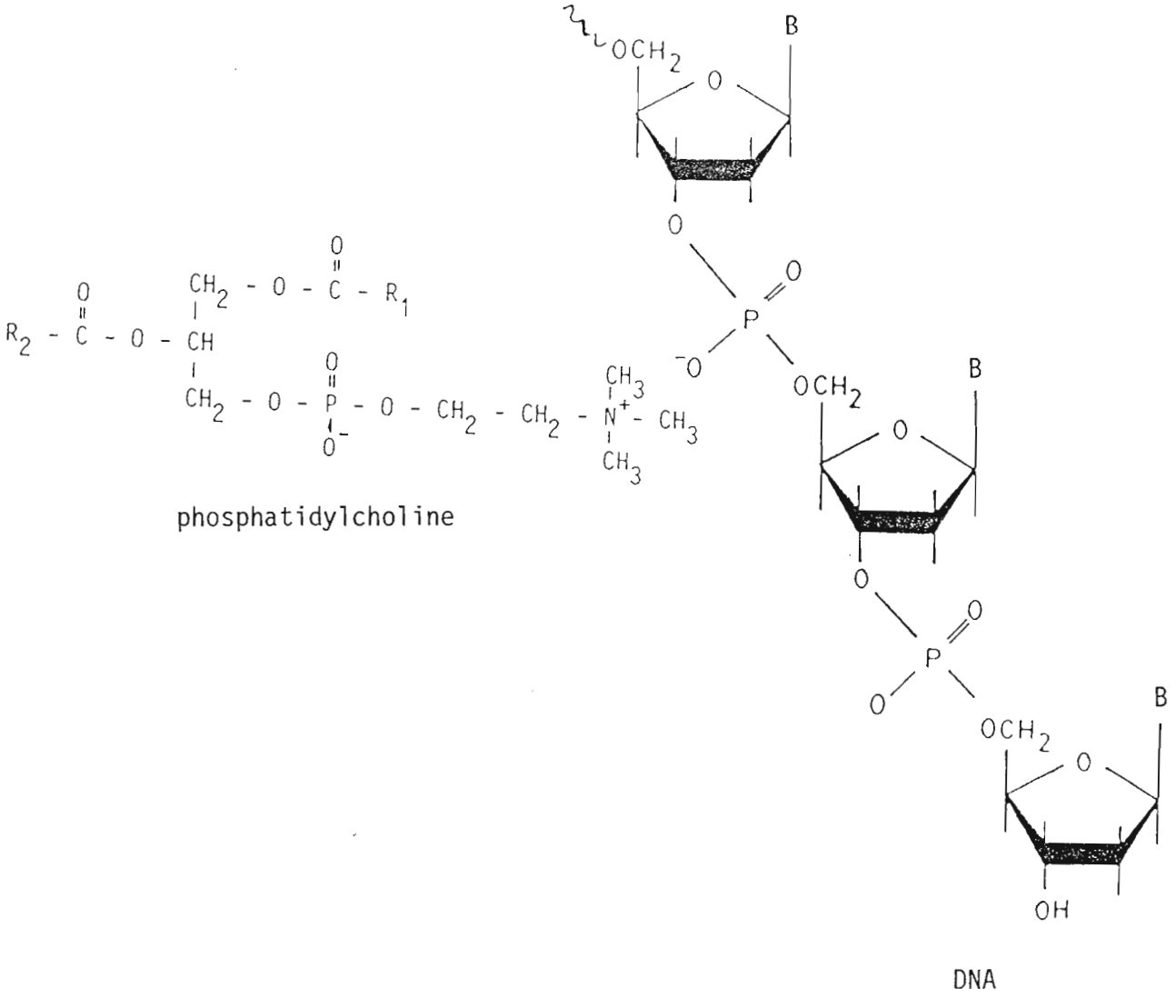


Figure 1.17 : Possible mechanism of interaction of phosphatidylcholine quaternary trimethylammonium group with the negatively charged phosphate groups in DNA. Sphingomyelin and apoprotein B, modified with the carbodiimide also contain the quaternary nitrogen and will therefore react in a similar manner to the above. This is illustrated in the partial CPK model presented in Figure 1.18.

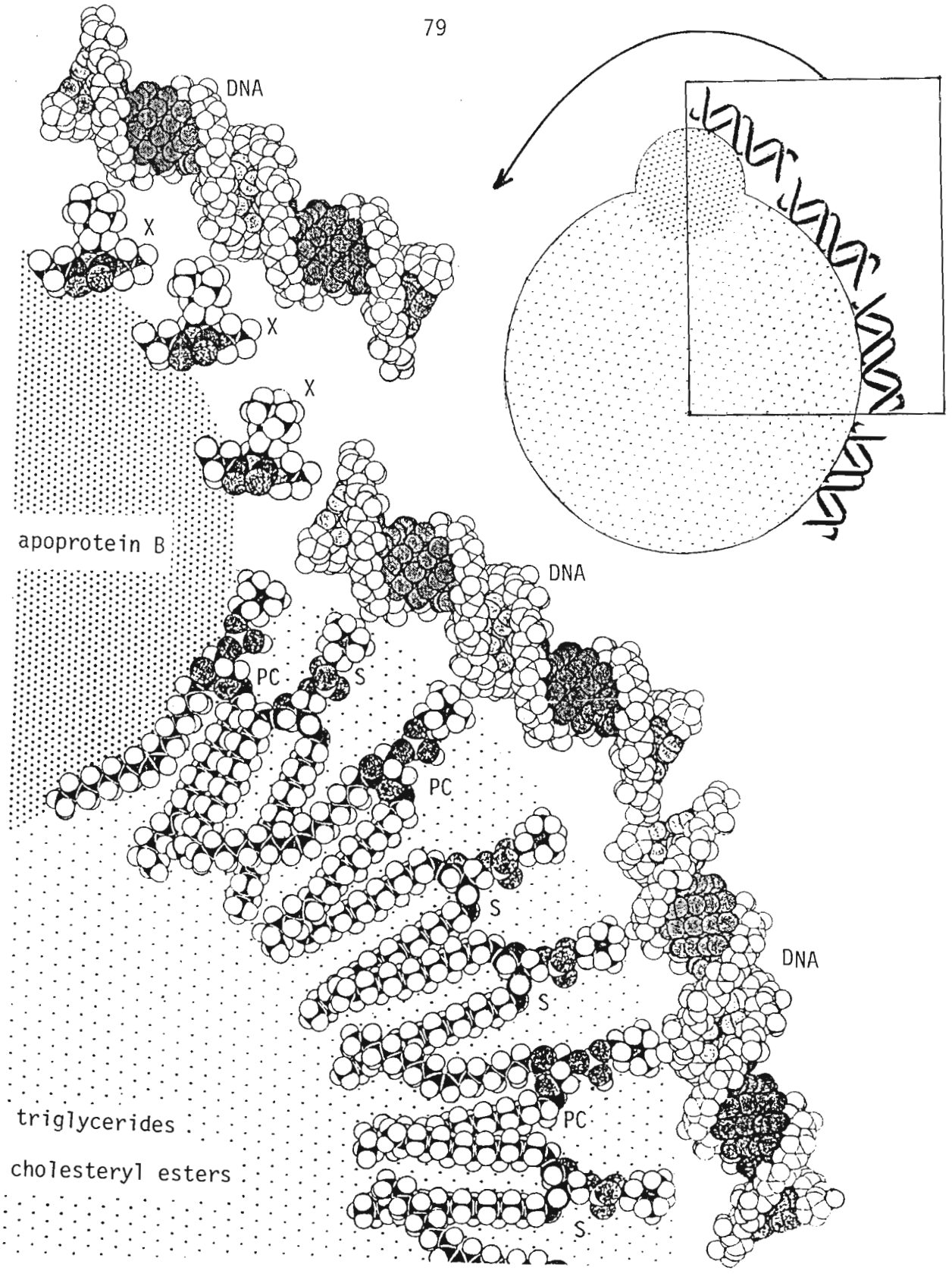


Figure 1.18 : Partial CPK model of part of a carbodiimde-modified LDL particle interacting with fragments of DNA.
PC = phosphatidylcholine, S = sphingomyelin. X = glutamic and aspartic acid residues of apo B modified with carbodiimide to form N - acyl ureas.

C H A P T E R T W O

ISOLATION, MODFICATION AND CHARACTERIZATION OF HUMAN SERUM LOW DENSITY LIPOPROTEINS

2.1 Introduction

In order to study the interaction of LDL with DNA and subsequent interaction of LDL - DNA complexes with eukaryotic cells, the LDL had to be isolated first and characterized to confirm that it was biologcally active. The most frequently used methods, cited in the literature, for the isolation of LDL are the repetitive ultracentri - fugation methods, which employed different types of rotors, speeds and times (Havel *et al.*, 1955; Bragdon *et el.*, 1956; Zechner *et al.*, 1984; Wiklund *et al.*, 1985).

However, all LDL isolations employing sequential ultracentrifugations are time - consuming, although the final product may be fairly pure and concentrated. Isolation of LDL by ultracentrifugation, followed by the separation of the lipoproteins by column chomatography (Rudel *et al.*, 1974) was also time - consuming and the LDL obtained was very dilute and necessitated concentration. The discontinuous ultra - centrifugation method proved to be the most convenient. This method involved a single ultracentrifugation step to separate serum lipo - proteins successfully.

The LDL isolated was also modified to enhance its interaction with DNA. This chapter deals only with the modifications and characteriza -

tion of the LDL particles. The various modifications involved modification of LDL by a water - soluble carbodiimide, conjugation of LDL to ethidium bromide and the acetylation of LDL. The labelling of LDL with [^{3}H] cholesteryl linoleate was also attempted. Two methods for the labelling were employed. The first involved the exchange of cholesteryl esters in the presence of DMSO (Faust *et al.*, 1977; Brown *et al.*,1975; Goldstein *et al.*, 1975; Krieger *et al.*, 1978). The second method used involved an enzymatic cholesteryl ester exchange. A cholesteryl ester exchange enzyme is present in the serum. This enzyme is utilized to carry out the exchange of [^{3}H] cholesteryl esters for the non - labelled core cholesteryl esters present in the LDLs (Glomset, 1959; Zilversmit and Johnson, 1975; Zilversmit *et al.*, 1975; Roberts *et al.*, 1985).

The chemical analysis of native and modified LDLs were done by standardized procedures. The protein content of all preparations was determined by the method of Markwell *et al.* (1981), using bovine serum albumin as standard. This is a modification of the Folin - Lowry method (Lowry *et al.*,1951) but more specific for lipoproteins. The cholesterol (Zak, 1957; Zlatkis and Zak, 1969), inorganic phosphate (Bartlett, 1959; Fiske and SubbaRow, 1925) and triglyceride content were determined for native as well as the modified LDLs. The methods were adapted from those found in the literature, to suit the LDL preparations. The LDL preparations were also subjected to agarose gel electrophresis and immunoassays, as well as to gel exclusion and thin layer chromatography, in an attempt to further characterize them.

2.2 Methods

2.2.1 Isolation of LDL from human serum

Fresh blood was collected from normal, healthy donors who had fasted for twelve hours. The blood was allowed to coagulate and the coagulum was then cut into small pieces with a pair of scissors. Serum was collected from the coagulated blood by centrifugation at low speed (400 x g) for 5 - 10 minutes, at 4°C. The plasma was adjusted to a density of 1,3 g/ ml with solid KBr. The density - adjusted plasma was dispensed into 38,5 ml cellulose nitrate tubes (10 ml / tube) and overlaid with 0,15 M NaCl - 0,27 mM EDTA, pH 7,0 solutions which had been adjusted, with solid KBr, to densities of 1,2 g / ml (5 ml), 1,063 g / ml (10 ml), 1,019 g / ml (8 ml) and 1,006 g / ml (1 - 2 ml) respectively. Ultracentrifugation was carried out in a Beckman ultracentrifuge (Model L5 - 65) for 24 hours at 15°C, using an SW28 rotor, at a speed of 120 000 x g. The separated lipoprotein bands were collected by density gradient fractionation, with optical monitoring at a wavelength of 280 nm. The peak fractions were pooled, dialysed against 0,15 M NaCl - 0,27 mM EDTA (pH 7,0) at 4°C,over a period of 24 hours, with several changes of dialysis buffer,and stored at 4°C. The lipoproteins were analysed by gel exclusion chromatography (Section 2.2.1.5), immunoelectrophoresis (Section 2.2.1.7) and immunodiffusion (Section 2.2.1.6) once the protein content (Section 2.2.1.1) of each lipoprotein had been determined. Once the purity of the LDL preparation had been confirmed, the cholesterol (Section 2.2.1.2), inorganic phosphate (Section 2.2.1.3) and triglyceride content

(Section 2.2.1.4) of each LDL sample was determined.

2.2.1.1 Protein determination

Protein determinations of all lipoprotein samples were carried out by the method of Markwell *et al.* (1981). This is a modification of the Lowry method and more specific for lipoproteins. The protein content of the LDL was used as a measure of LDL concentration in all subsequent experiments, using bovine serum albumin as standard.

2.2.1.2 Cholesterol determination

Cholesterol content of LDL was determined spectrophotometrically using the o - phthalaldehyde colour reagent (Zlatkis and Zak, 1969), and recrystallized cholesterol as standard.

2.2.1.3 Phosphate determination

LDL samples were concentrated to 100 µl (under a stream of N_2 gas) and contained 150 - 300 µg LDL (protein). To each tube was added 100 µl of 10 N H_2SO_4 (final concentration 5,0 N). The LDL samples were incubated at 150 - 160°C in an oil bath for 3 hours, in a fume hood and covered with Parafilm. The tubes were cooled slightly and to each tube was added 2 drops of 30% H_2O_2 with a Pasteur pipette. Digestion was continued for a further 1,5 hours. The digests were cooled and 10% trichloroacetic acid (TCA) was added to give a final volume of 2,5 ml. The inorganic phosphate content was determined according to the method of Fiske and SubbaRow (1925),

using a blank which contained the same amount of EDTA as the LDL samples.

2.2.1.4 Determination of the triglyceride content of LDL samples

The triglyceride content of LDL samples was determined spectrophotometrically with the use of a Boehringer enzymatic kit. The triglyceride content was subsequently calculated, using the molecular weight of trilinolein in the given formula.

2.2.1.5 Gel exclusion column chromatography

A Biogel A5m column (19,5 x 1,0 cm) was prepared and equilibrated with 0,15 M NaCl - 0,01% EDTA (pH 7,0). Each lipoprotein (150 - 200 µg protein) was layered onto the column and eluted with the same solution as above, at a flow rate of 6 ml / hour. The elution was monitored at 280 nm (with the ISCO type 6 optical unit) and the elution profile was recorded with the aid of the UA - 5 absorbance monitor.

2.2.1.6 Immunodiffusion

A 1% agarose solution was prepared in 0,9% saline and poured onto microscope slides (2,5 x 7,5 cm). When solidified and cooled to 4°C (30 - 60 minutes), wells of 2,5 µl capacity were cut into the gel. Anti - human β - lipoprotein (titer 5,9 mg / ml) was applied to the center well and the lipoproteins isolated (Section 2.2.1) were applied to the surrounding wells. The slides were incubated in a

humid box at 4°C for 18 - 24 hours. The precipitin lines formed were either photographed with indirect white light illumination or stained to make permanent records (Section 2.2.1.8).

2.2.1.7 Immunoelectrophoresis

Agarose gels (1%) were prepared with barbital buffer (0,05 M), pH 8,0 , and poured onto microscope slides (2,5 x 7,5 cm). Wells and troughs were cut prior to electrophoresis. Lipoprotein samples were applied to the wells and electrophoresed in the Shandon electrophoresis apparatus, using Whatman No. 1 filter paper wicks dipped into the above buffer. Electrophoresis was carried out at 150 - 300 volts (approximately 35 mA) for 2,5 hours. Anti - human antiserum (100 µl per trough; titer 5 mg / ml) was applied to each trough and allowed to diffuse over a period of 24 hours at room temperature. Precipitin lines were recorded before or after staining.

2.2.1.8 Permanent recording of immunoprecipitation or immunodiffusion plates

The immunodiffusion and immunoelectrophoresis plates were washed in 0,9% saline for 10 minutes followed by distilled water washes of approximately 30 minutes. The washed gels were pressed dry with Whatman No. 1 filter paper, after eliminating all air bubbles, then oven - dried at 90°C for 5 - 10 minutes. Slides were stained with 0,5% Coomassie brilliant blue in ethanol - acetic acid - water (5 : 1 : 5, by volume) for 10 minutes. The slides were then de - stained in ethanol - water - acetic acid (5 : 5 : 1, by volume)

for 10 minutes, blotted dry and oven - dried. Unstained plates were photographed under oblique white light illumination, using a high - contrast monochromatic film.

2.2.1.9 Agarose gel electrophoresis

Agarose (0,19 g) was dissolved in 13,5 ml distilled water by boiling. The agarose was then cooled to 75°C and to it was added 1,5 ml of a 10 - fold concentrated electrophoresis buffer (0,36 M Tris HCl, pH 7,5 , 0,3 M $NaHPO_4$, 0,1 M EDTA). The gel was cast on a UV - transparent plastic gel plate, of dimensions 60 x 90 mm, when cooled to 60°C. The gel was allowed to set for one hour before applying samples (10 µg protein per well) to the wells. Electro - phoresis was carried out in the same buffer as above, in a BioRad DNA subcell electrophoresis apparatus, at a constant voltage of 40 V, for 3,5 hours. A peristaltic pump was used to circulate the buffer. The gels were subsequently stained by one of two methods after electrophoresis: (a) Gels were stained overnight in 0,25% Coomassie brilliant blue in methanol - water - acetic acid (5 : 5 : 1, by volume) and destained in methanol - water - acetic acid (5 : 5 : 1, by volume) for 48 hours. (b) Gels were stained in Coomassie brilliant blue (0,003%) in 10% TCA and did not require destaining. The gels were subsequently photographed. Results for gels stained by methods (a) and (b) are presented in Figure 2.7 (a) and (b) respectively.

2.2.1.10 Thin layer chromatography

LDL samples were subjected to thin layer chromatography to separate the lipid components. The following solvent systems were used (all ratios are by volume) : solvent A : $CHCl_3$ - MeOH - 7 N NH_3 (65 : 24 : 4); solvent B : $CHCl_3$ - MeOH - AcOH - H_2O (25 : 15 : 4 : 2); solvent C : hexane - diethylether - acetic acid (70 : 30 : 1); solvent D : $CHCl_3$ - MeOH (2 : 1); solvent E : petroleum ether (60 - 80°C) - diethylether - acetic acid (80 : 20 : 1); solvent F : $CHCl_3$ - MeOH - petroleum ether (60 - 80°C) - diethylether - acetic acid - water (11 : 7 : 16 : 4 : 2 : 1). Spots were located with iodine vapour or with 50% H_2SO_4 and charring. The choline - or ethanolamine-containing phospholipids were sometimes located with ninhydrin, prior to the H_2SO_4 spray and charring.

2.2.1.11 Separation and solubilization of apoprotein B from the lipid components of LDL

Intact LDL (1,2 mg / ml) was precipitated with heparin (final concentration 1 mg / ml) and $MnCl_2$ (final concentration 92 mM). Precipitation was allowed to occur at room temperature for 15 minutes. The LDL, pelleted at 9 000 x g (2 minutes, room temperature), was redissolved in 0,5 ml of 0,15 M NaCl - 0,05 M Tris - HCl (pH 7,4) - 0,01% EDTA (STE buffer) or 20 mM Tris - HCl (pH 8,2) - 0,1 M SDS (sodium dodecyl sulfate) - 1% mercaptoethanol (TSM buffer). The precipitation of LDL with ammonium sulfate was also carried out, at pH 7,0. Solubilization of the pellicle formed was again attempted with STE or TSM. In a third method, LDL was precipitated with Mg^{++}

(final concentration 0,25 M). The pellicles and precipitates formed (above) were washed with 0,1% NaCl and dissolution was attempted with STE and TSM. In addition, attempts were made to dissolve precipitated LDL in 5% NaCl or 10 % $NaHCO_3$.

In an attempt to obtain the apoprotein in a soluble non - denatured form, without the lipid components, the lipids were extracted with $CHCl_3$ - MeOH - AcOH - H_2O (25 : 15 : 4 : 2, by volume) from the LDLs precipitated by the above methods. Solubilization of the protein residues was then attempted in all the solutions described above.

In addition, lipids were extracted from unprecipitated LDL with $CHCl_3$ - MeOH - AcOH - H_2O (25 : 15 : 4 : 2, by volume). This resulted in precipitation of the apoproteins. The solubilization of the precipitated protein was again attempted by the methods described above. The solubilization of LDL with 5% SDS was also attempted.

2.2.2 Preparation of carbodiimide - modified LDL (ECDI - LDL)

LDL (1,5 mg, 3,0 nmoles, dialysed against 0,15 M NaCl, pH 6, at 4°C) was made up to 1,6 ml with 0,15 M NaCl. To this was added 8,91 mg (30 µmoles) of the water-soluble carbodiimide, [^{3}H] 1-ethyl -3- [3 - (trimethylpropylammonium] carbodiimide (abbreviated ECDI), dissolved in 140 µl distilled water and adjusted to pH 6. Incubation, in the dark, was carried out at room temperature for a duration of 48 hours. The [^{3}H] ECDI - LDL was dialysed against 0,15 M NaCl -

0,01% EDTA (pH7,0), with several changes of dialysis buffer over 24 hours, to remove unreacted carbodiimide. The protein content of the ECDI - LDL was determined, and the carbodiimide - modified LDL was stored at 4°C. The ECDI - LDL was also electrophoresed on a 1,25% agarose gel (Section 2.2.1.9) to ascertain whether carbodiimide modification of LDL had occurred. In addition, the lipid and protein components of [^{3}H] - ECDI - LDL were separated (as described in Section 2.2.1.11) and counted in an attempt to locate the components of LDL which had been modified by the carbodiimide.

2.2.2.1 Gel exclusion chromatography of ECDI - LDL

[^{3}H] ECDI - LDL (150 µg protein content) was applied to a 19 x 1 cm Biogel A5m column and eluted as described in Section 2.2.1.5. The elution of ECDI - LDL was monitored at 280 nm. Fractions (0,5 ml) were collected and their radioactivity was determined on a Beckman liquid scintillation counter (Model LS 1305T), using Beckman HP / b liquid scintillation fluid.

2.2.2.2 Reaction of LDL and carbodiimide at different mole ratios

LDL (1,84 mg protein, 0,37 nmole) in 0,15 M NaCl - 0,01% EDTA, was adjusted to pH 6 with 0,01 M HCl. [^{3}H] ECDI (8,1 mg, 27,27 µmoles) was dissolved in 135 µl distilled water and adjusted to pH 6 with 0,01 M HCl. The LDL and carbodiimide were mixed in mole ratios of 1 : 500, 1 : 1 000, 1 : 5 000, 1 : 10 000 and 1 : 20 000, and incubated at room temperature, in the dark, for 48 hours. Each reaction mixture was precipitated with 10% cold (4°C) TCA and

transferred to a separate GF / C filter. Each filter was washed with cold 5% TCA (5 x 5 ml), air - and oven - dried (90°C, 15 minutes) and counted (as in Section 2.2.2.1) to determine the acid - precipitable radioactivity.

2.2.2.3 Digestion of [^{3}H] ECDI - LDL with phospholipase C and phospholipase D

ECDI- LDL (750 µg) was digested with 45 units of phospholipase D (from *Streptomyces chromofuscus*) in a reaction mixture containing 20 mM Tris - HCl, pH 8,2, 0,2 mg Triton X - 100 and 4 mM $CaCl_2$. For the phospholipase C digestion 750 µg of ECDI - LDL was incubated in 20 mM Tris - HCl, pH 7,5, 0,2 mg Triton X - 100, 4 mM $CaCl_2$ and 45 units of phospholipase C (from *Bacillus subtilis*) in a total volume of 500 µl. Both reaction mixtures were incubated at 37°C. Timed aliquots were withdrawn from each reaction mixture and transferred to nitrocellulose filters. The filters were washed twice with 500 µl of 0,1 M NaCl - 0,05 M Tris - HCl, pH 7,6 - 0,0025 M EDTA. The radioactivity on each dried filter was subsequently determined. A parallel digestion of another carbodiimide - modified protein, transferrin, was carried out to establish that the [^{3}H] trimethylpropylammonium residues could be hydrolysed off the carbodiimide - modified proteins. The reason for carrying out these experiments was to establish that the carbodiimide residues are also removed by the phospholipase D, which removes choline groups from phosphatidylcholine (discussed in Section 2.3.2.2).

2.2.2.4 Thin layer chromatography of ECDI - LDL digested with phospholipase D

[^{3}H] ECDI - LDL (750 µg) was digested with phospholipase D under the same conditions as described in section 2.2.2.3. The reaction was stopped and the lipid components extracted with $CHCl_3$ - MeOH - AcOH - H_2O (25 : 15 : 4 : 2, by volume). The apoprotein B precipi - tated at the aqueous - organic interface. Both the aqueous and and organic phases were reduced in volume under a stream of N_2 gas, and spotted on a silica gel $60F_{254}$ TLC plate. The compound N,N,N, - trimethyldiaminopropane was spotted as standard. The plate was developed in $CHCl_3$ - MeOH - AcOH - H_2O (25 : 15 : 4 : 2, by volume) and the spots located by iodine vapour. The radioactivity of the spots was subsequently determined.

2.2.2.5 Attempts to conjugate ECDI to phospholipids

Phosphatidylserine dipalmitoyl (1 mg) was suspended in 0,5 ml dimethyl formamide (DMF). To this was added 1,55 mg ethyl trimethylpropyl - ammonium carbodiimide (ECDI), dissolved in 50 µl DMF. The reaction mixture was incubated at room temperature for 48 hours, in the dark. The reaction mixture was analysed by thin layer chromato - graphy. The chromatograms were developed in solvent A (Section 2.2.1.10). Similar reactions were carried out with other phospho - lipids, namely, phosphatidylcholine, phosphatidylethanolamine and also with sphingomyelin.

2.2.3 Preparation of acetylated LDL

LDL (1 mg protein content) was dialysed against 0,15 M NaCl, pH 7,0. The LDL was cooled in an ice bath, and to it was added 0,6 ml saturated sodium acetate, dropwise, with continuous stirring. To this was added 12 µl (0,13 mmole) acetic anhydride, 2 µl at a time, with continuous stirring. The acetylation reaction was allowed to proceed in an ice bath at all times and the additions were made over a period of 1 hour. The reaction mixture was stirred for an additional 30 minutes. The acetylated LDL was dialysed against 0,15 M NaCl - 0,27 mM EDTA (pH 7,0), over a period of 24 hours, at 4°C, with several changes of dialysis buffer.

2.2.3.1 Analysis of acetylated LDL

Protein, cholesterol, inorganic phosphate and triglyceride content of the acetylated LDL were determined as described in Sections 2.2.1.1, 2.2.1.2, 2.2.1.3 and 2.2.1.4 respectively. Acetylated LDL was also subjected to immunodiffusion and agarose gel electrophoresis (Sections 2.2.1.6 and 2.2.1.9 respectively).

2.2.4 Preparation of LDL - ethidium bromide conjugate

LDL (3,3 mg, 6.6 nmoles), 5,9 mg (14,96 µmoles) ethidium bromide (2,7 diamino - 10 - ethyl - 9 - phenylphenanthridium bromide) and 9 mg (99.12 µmoles) of carbodiimide (N - ethyl - N´- (3 dimethyl - aminopropyl)- carbodiimide hydrochloride) were dissolved separately and adjusted to pH 6,0 with 0,1 N HCl, in a total volume of 1,2 ml

each. The three solutions were then mixed together and conjugation of the ethidium bromide was allowed to proceed at room temperature, in the dark, for 48 hours. The ethidium bromide LDL conjugate (EB - LDL) was dialysed against 0,15 M NaCl - 0,27 mM EDTA (pH 7,0), at 4°C, for 24 hours, with several changes of dialysis buffer. The preparation was analysed by agarose gel electrophoresis as described in Section 2.2.1.9.

2.2.4.1 Chemical and spectral analysis of the ethidium bromide - LDL conjugate (EB - LDL)

The EB - LDL was subjected to spectral analysis (UV and visible) and agarose gel electrophoresis (Section 2.2.1.9). The protein content was determined as described in Section 2.2.1.1.

2.2.5 Preparation of [^{3}H] - cholesteryl linoleoyl LDL

2.2.5.1 Preparation of [^{3}H] - cholesteryl linoleate

The preliminary preparation of cholesteryl linoleate was unlabelled. Linoleic anhydride (10 mg, 18,4 mmoles) was placed in a dry pre - weighed amber round - bottomed flask. Cholesterol (4,373 mg, 12,2 mmoles) was dissolved in 75 µl pyridine and added to the linoleic anhydride. Esterification was allowed to proceed at 37 - 40°C for 24 hours. The reaction mixture and standard cholesteryl linoleate were spotted onto a silica gel 60F_{254} TLC plate (5 x 20 cm) and developed in solvent C (Section 2.2.1.10). Spots were located by spraying with 10% perchloric acid and charring. To prepare the

labelled cholesteryl linoleate, 250 µl of [^{3}H] cholesterol (specific activity 42,62 Ci / m mole; 7,32 n moles) was evaporated to dryness. The residue was redissolved in 250 µl dry pyridine and evaporated to dryness again. The residue was redissolved in 20 µl dry pyridine, under a stream of N_2 gas. To this was added 5 µl (100 n moles) linoleic anhydride and mixed by swirling. The esterification was carried out at 37°C, over a period of 24 hours.

2.2.5.2 Preparative thin layer chromatography

The [^{3}H] cholesteryl linoleate was isolated by preparative thin layer chromatography on a silica gel 60F_{254} plate (10 x 20 cm). Standard, unlabelled cholesteryl linoleate and cholesterol were chromatographed on the same plate, as markers. The chromatogram was developed in solvent C (Section 2.2.1.10). The labelled cholesteryl linoleate was extracted from the plate with 4 x 1 ml $CHCl_3$. A 5 µl aliquot was counted to determine the radioactivity of the labelled cholesteryl linoleate. The preparation was stored at -15°C, in a teflon - lined, screw - capped vial until required.

2.2.5.3 Preparation of labelled cholesteryl linoleoyl - LDL in the presence of DMSO

[^{3}H] cholesteryl linoleate (7,11 µCi, 0,167 n moles) in $CHCl_3$ was evaporated to dryness under a stream of N_2 gas. The residue was redissolved in 25 µl of dimethylsulfoxide (DMSO). To this was added 1 mg of LDL, in 20 mM Tris HCl (pH 8,2) - 0,15 M NaCl -

0,01% EDTA. The reaction mixture was incubated at 37°C for 2,5 hours. The final DMSO concentration was 2,5%. A second reaction was set up in a separate tube, as described above, except that the [^{3}H] - cholesteryl linoleate residue was not redissolved in DMSO, but in the above buffer. Each reaction mixture was dialysed separately against 5 x 400 ml buffer (20 mM Tris HCl, pH 8,2 - 0,15 M NaCl - 0,01% EDTA) at 4°C for 24 hours. The dialysed [^{3}H] cholesteryl linoleoyl LDL (CL - LDL) was centri - fuged at 9 000 x g for 3 minutes, to remove denatured material. A 10 µl aliquot of each preparation was counted in a liquid scintilla - tion counter. Protein determination (Section 2.2.1.1), cholesterol determination (Section 2.2.1.2) and agarose gel electrophoresis (Section 2.2.1.9) of the preparations were carried out.

2.2.5.4 Preparation of [^{3}H] cholesteryl linoleoyl LDL by an enzymatic cholesteryl ester exchange method

The experimental procedure was adapted from the method of ROberts *et al.*, (1985). [^{3}H] cholesteryl linoleate (200 µCi, 42,6 Ci / m mole) in $CHCl_3$ was evaporated to dryness under a stream of N_2 gas. The [^{3}H] cholesteryl linoleate was redissolved in 100 µl acetone and added to 5 ml of lipoprotein - deficient serum. The acetone was evaporated with N_2 gas applied to the solution for 45 minutes. To this was added 11 mg of LDL (protein content) in 5,0 ml of 0,15 M NaCl - 0,01% EDTA, pH 7,0. The cholesteryl ester exchange was allowed to proceed at 4°C for one hour, with gentle swirling every 10 minutes. The reaction mixture was then adjusted to a density of

1,3 g / ml and the labelled LDL was recovered by discontinuous density gradient ultracentrifugation as described in Section 2.2.1. The [^{3}H] cholesteryl linoleoyl LDL (CL - LDL) was collected by density gradient fractionation, with optical minitoring at 280 nm. An aliquot was removed from each fraction to determine the radio - activity. The CL - LDL peak fractions were pooled, dialysed against 0,15 M NaCl - 0,01% EDTA, pH 7,0 and stored at 4°C. Chemical and electrophoretic analyses were also carried out on this preparation.

2.3 Results and discussion

2.3.1 Isolation of LDL

LDL was isolated between the densities 1,019 - 1,063 g / ml. Various methods were attempted for the isolation of low density lipoproteins. Sequential multiple ultracentrifugation proved to be time - consuming. The method of Rudel *et al.*, (1974) required a single ultracentrifugation step to separate the lipoproteins from the serum proteins. This was followed by the separation of the lipoproteins on a Biogel A5m column (90 x 2,5 cm). The separation took 54 hours at a flow rate of 6 ml / hour (Figure 2.1 a). However the lipoproteins were very dilute and necessitated concentra - tion. Concentration of LDL by pressure dialysis with the Amicon ultrafiltration apparatus or the Millipore immersible Cx10 ultra - filter resulted in partial degradation of LDL. Although the concentration of LDL was effective, lipids were deposited on the

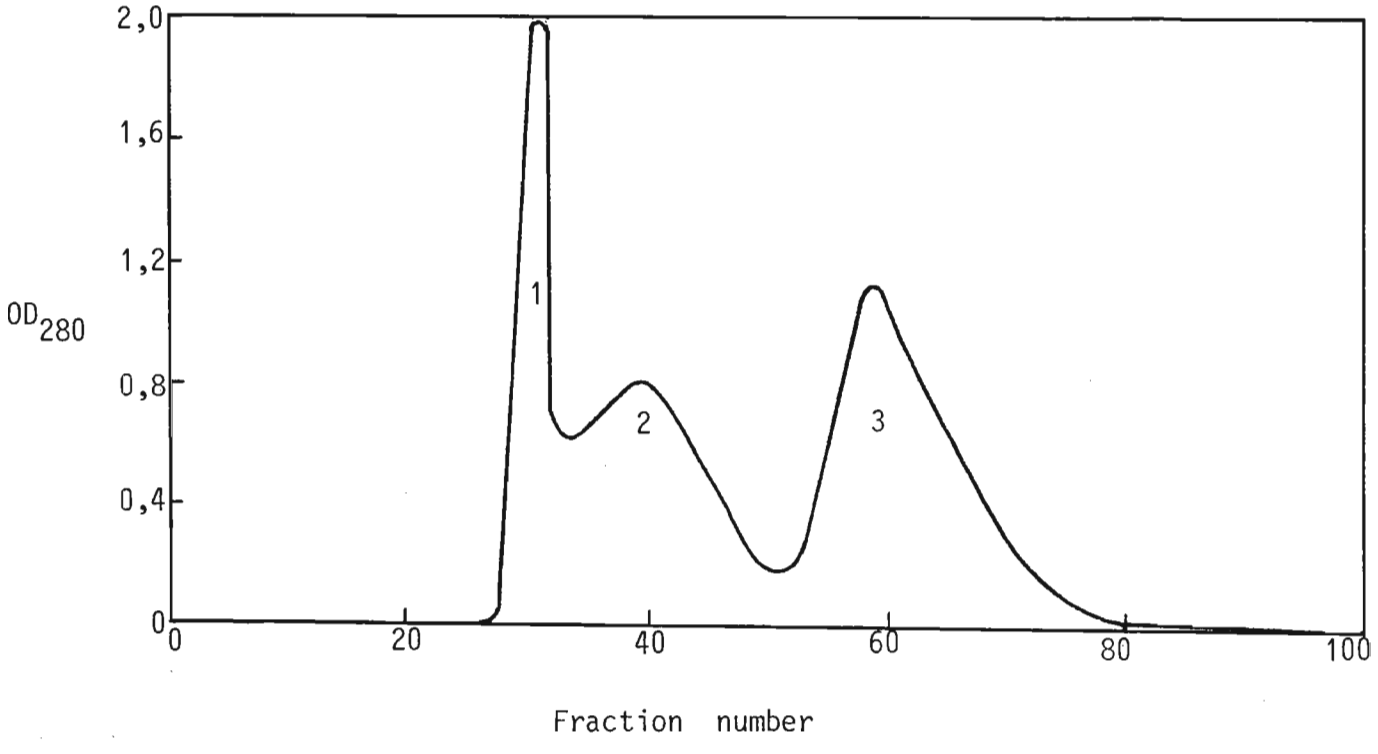


Figure 2.1 a: Elution profile of lipoproteins separated on a Biogel A5m column. Peak 1 = VLDL, peak 2 = LDL and peak 3 = HDL.

filters and some LDL protein was precipitated. Thin layer chromatography of the LDL lipids before and after concentration are presented in Figure 2.1 b. LDL isolation was also attempted by the method of Zechner *et al.*,(1984). This also involved sequential ultracentrifugation. The LDL preparations showed contamination with VLDL (Figure 2.2). The use of a Pasteur pipette for the removal of the VLDL, after the first ultracentrifugation step, was not efficient. A tube slicer was not available.

The isolation of LDL by a single spin discontinuous gradient ultracentrifugation method (Section 2.2.1) was found to be the best with respect to both the time taken for the isolation and the purity of the LDL. Initially blood was collected into vessels containing EDTA (Hinton *et at.*, 1973; Hatch and Lees, 1968). It was subsequently found that better LDL preparations were obtained when blood was allowed to coagulate before LDL isolation. The density gradient fractionation of the lipoprotein bands resulting from the ultracentrifugation yielded 3 peaks (Figure 2.3). The HDL band overlapped with serum proteins (peak 3). The density of the isolated LDL band, prior to dialysis, was 1,04 g / ml. Dialysis of LDL samples was limited to 24 hours as extensive dialysis resulted in loss of cholesterol from the LDL particles. The protein : cholesterol ratio of dialysed LDL preparations was between 1 : 1,5 and 1 : 1,6, and decreased to 1 : 1,2 upon further dialysis.

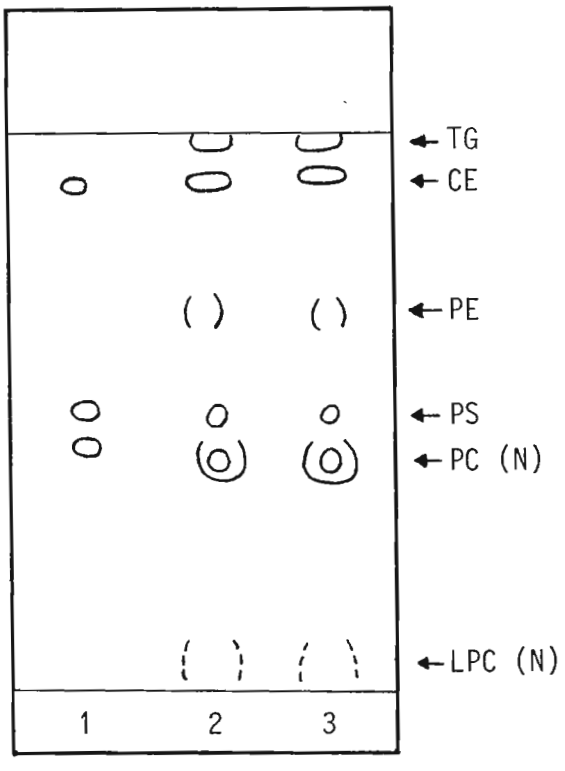


Figure 2.1 b: Thin layer chromatography of LDL samples before concentration (1) and after concentration, by pressure dialysis (2) and ultrafiltration (3). Chromatograms were developed in solvent B (Section 2.2.1.10). Spots were located by spraying with 50% H_2SO_4 and charring. Prior location of amino group-containing lipids was carried out with ninhydrin (N). TG = triglycerides; CE = cholesteryl esters; PE = phosphatidylethanolamine; PS = phosphatidylserine; PC = phosphatidylcholine; LPC = lysophosphatidylcholine.

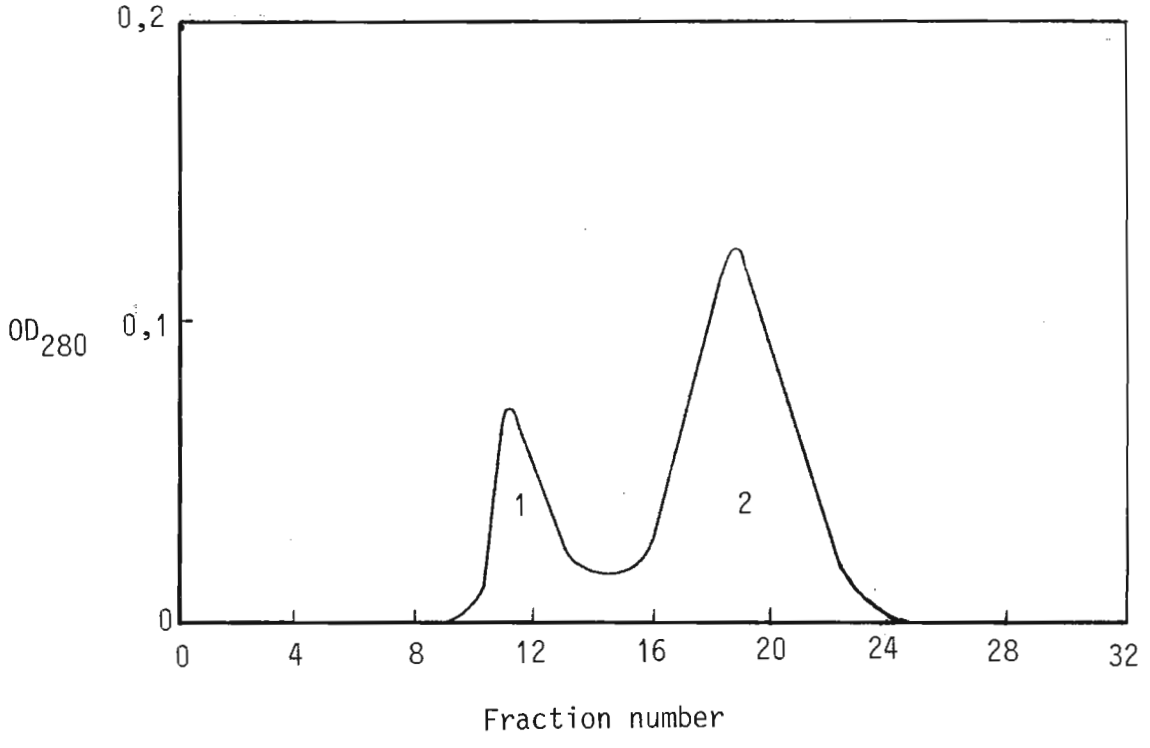


Figure 2.2 : Elution of LDL from Biogel A 5m column. The LDL (2) was isolated by the method of Zechner *et al.*(1984) and was contaminated with VLDL (1).

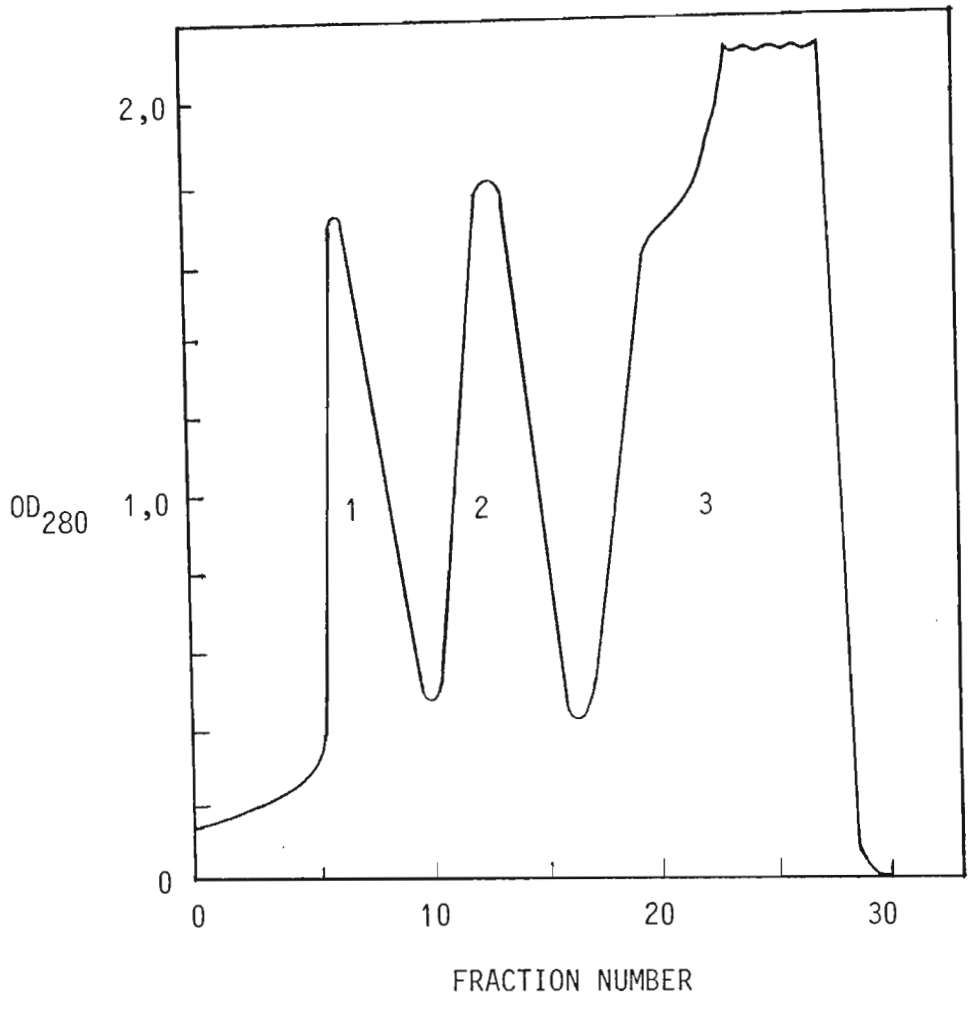


Figure 2.3 : Density gradient fractionation of serum lipoproteins separated by discontinuous gradient ultracentrifugation. The VLDL (peak 1), LDL (peak 2) and HDL (peak 3) were subsequently analysed by column chromatography and electrophoresis.

2.3.1.1 Gel exclusion chromatography of isolated LDL

Compared to previous methods of LDL isolation (Figures 2.1 a and 2.2) the discontinuous gradient ultracentrifugation method yielded purer samples of LDL, which were more concentrated. A 200 µl sample (150 - 200 µg protein content) from each lipoprotein band was eluted from a Biogel A5m column (Figure 2.4). The VLDL (peak 1) and LDL (peak 2) bands yielded single peaks, while the HDL band (peak 3) showed slight contamination with albumin (peak 4).

2.3.1.2 Immunoassays

Immunoassays to determine the purity of LDL samples isolated by different methods, were done on agarose gels. The antiserum used was anti - β lipoprotein antiserum. The immunoglobulin used here was polyclonal. The LDL and VLDL gave positive results, while HDL yielded negative results (Figure 2.5). VLDL and LDL have common antigenic determinants which are different from HDL (Shore and Shore, 1972). Both LDL and VLDL contain apoprotein B (Morrisett *et al.*, 1975; Lees, 1976; Goldstein and Brown, 1977), hence a precipitin line was obtained for VLDL also. Lipoprotein deficient serum was obtained after ultracentrifugation and floatation of the lipoproteins. As expected, it yielded negative results in the immunoassays. Immunoelectrophoresis of the lipo - proteins confirmed that LDL was pure. The antiserum used here was anti - whole human antiserum. In some preparations HDL was not contaminated with albumin (Figure 2.6).

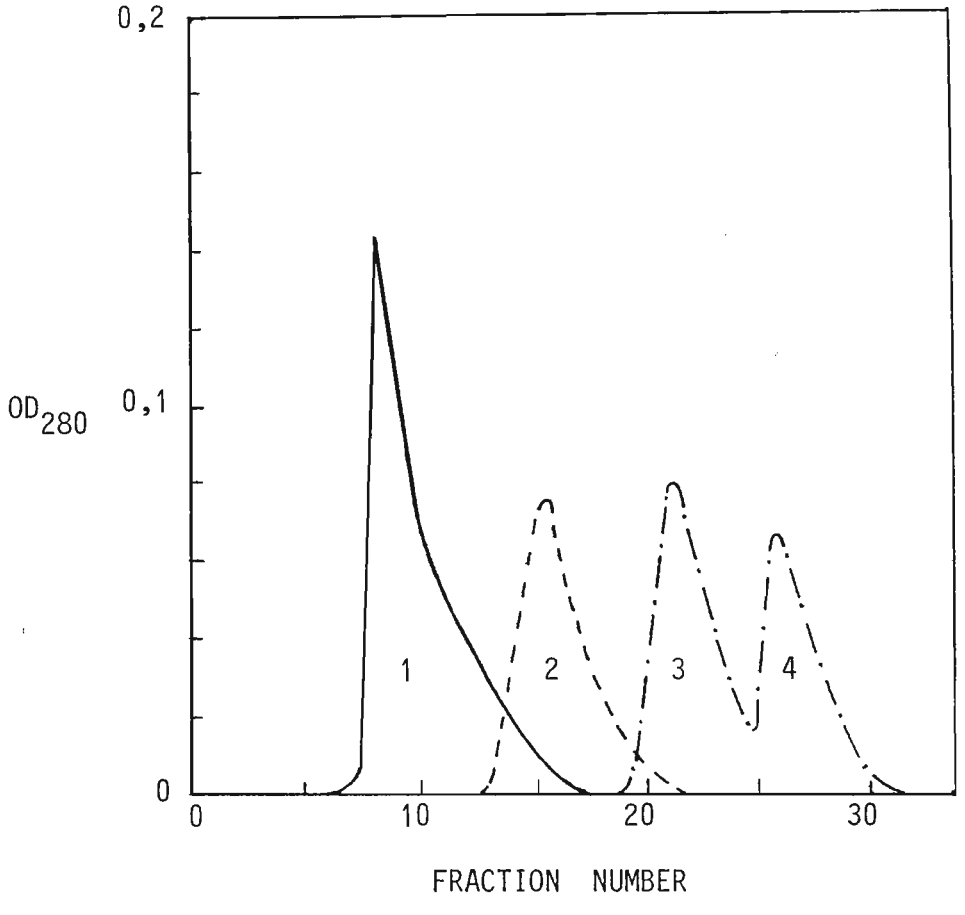
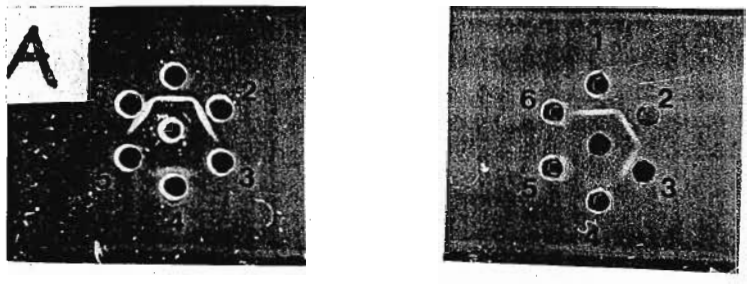


Figure 2.4 : Elution of lipoproteins from a Biogel A 5m column. VLDL (1) and LDL (2) yielded single peaks while HDL (3) was contaminated with albumin (4).



A B

Figure 2.5 : Ouchterlony immunodiffusion assay of human serum lipoproteins. Center wells contained anti human β - lipoprotein antiserum. In A, wells 2, 3, 4, and 5 contained LDL, HDL, VLDL and LPDS respectively, isolated by the discontinuous gradient method. Wells 6 and 1 contained LDL samples isolated by the methods of Rudel *et al.*(1974) and Zechner *et al.*(1984) respectively. In B, the LDL isolated by the discontinuous gradient method was serially double diluted, from 1 - 6. Well 1 contained 2,5 μg LDL.

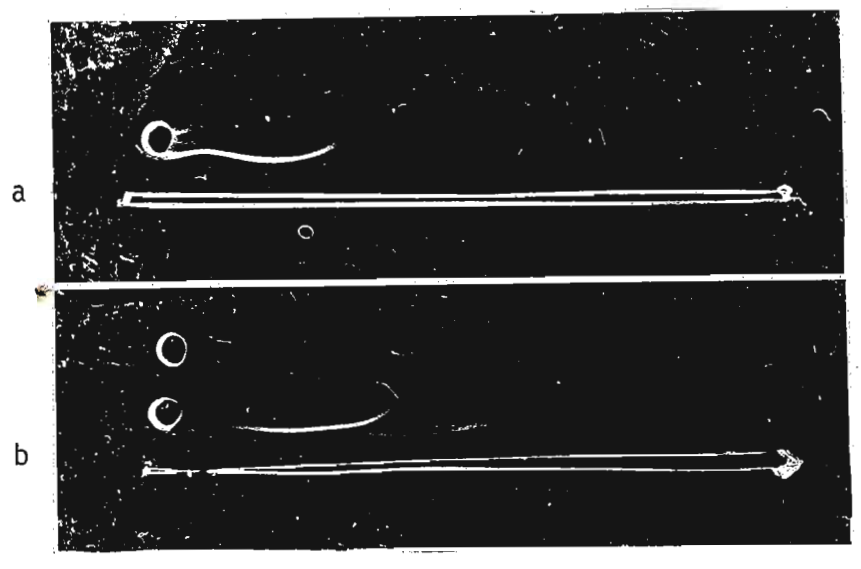


Figure 2.6 : Immunoelectrophoresis of human LDL (a) and combined lipoproteins (b) isolated by the discontinuous gradient method. The precipitin lines formed were against whole anti - human serum antiserum.

2.3.1.3 Agarose gel electrophoresis

Each lipoprotein sample (VLDL, LDL and HDL) was mixed with bromophenol blue tracking solution or stop solution (4 M urea, 50% (w / v) sucrose, 0,01% bromophenol blue, pH 7,0). Electrophoresis was carried out until the tracking dye reached the end of the agarose gels. This took about 3,5 hours and resulted in a good separation of the lipoproteins. Staining of the gels by method a (Section 2.2.1.9) was long and gave fainter spots (Figure 2.7 a). Method b was more rapid. Results were obtained within 1 hour and the staining of the spots was enhanced (Figure 2.7 b) by this method.

2.3.1.4 Separation and solubilization of apoprotein B

All methods employed resulted in the precipitation of LDL. Extraction of intact LDL with organic solvents ($CHCl_3$ - MeOH - AcOH - H_2O, 25 : 15 : 4 : 2, by volume) also resulted in precipitation of the LDL. The amount of heparin utilized for the precipitation of LDL varies from 0,1% to 0,5% (Warnick and Albers, 1977; Burnstein and Scholnick, 1974; Burnstein *et al.*, 1970). LDL was successfully precipitated with 0,1% heparin, leading to a drop in pH to 5. The precipitated LDL was white and had a homogeneous distribution. $(NH_4)_2SO_4$ and Mg^{++} precipitation methods resulted in pellicle formation. The pellets and pellicles were insoluble in low salt (0,1% NaCl) and could therefore be washed before attempting resolubilization. The heparin - Mn^{++} precipitated LDL was successfully solubilized in STE and TSM (Table 2.1), but not with

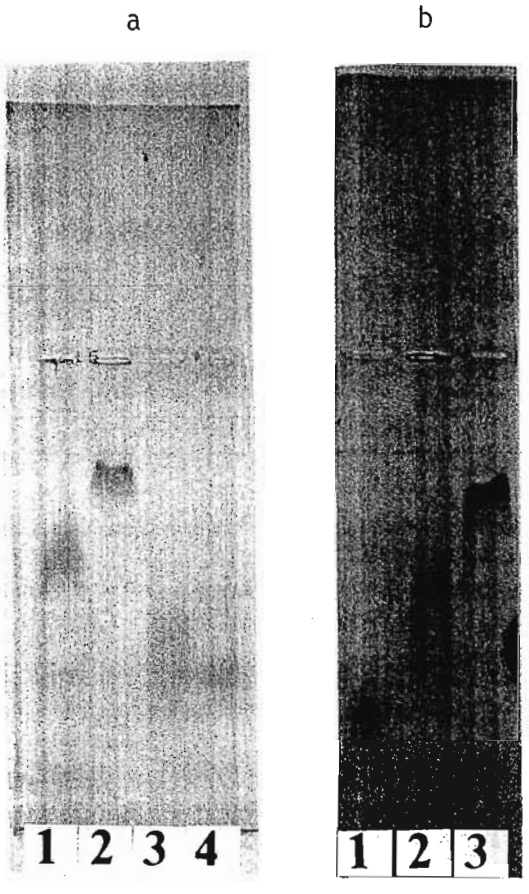


Figure 2.7 : Agarose gel electrophoresis of human serum lipoproteins isolated by the discontinuous gradient ultracentrifugation method. In (a) lanes 1, 2 and 3 contain VLDL, LDL and HDL respectively. Lane 4 contains a human serum albumin standard. In (b) lanes 1, 2 and 3 contain HDL, LDL and VLDL respectively. Staining methods for (a) and (b) were as described in Section 2.2.1.9.

10 % $NaHCO_3$ (Burnstein *et al.*, 1970). Addition of $NaHCO_3$ increased the pH to 9. Decreasing the pH to 7, with HCl, did not improve the solubility. The $(NH_4)_2SO_4$- and Mg^{++} - precipitated LDL was soluble in TSM only. The apoprotein B, separated by extraction of lipid did not dissolve in any solution except TSM. Since the apoprotein B could not be solubilized without the use of a denaturing solution (TSM) it was not suitable for use in the study of apo B - DNA interactions. A table of the precipitation and solubilization results are presented in Table 2.1. The separa - tion of apoprotein B in the presence of albumin (Srinivasan *et al.*, 1979) or starch (Gustafson, 1965) was undesireable, as this would necessitate subsequent removal of such stabilizers.

2.3.2 Carbodiimide modification of LDL

The modification of proteins with carbodiimides has been employed by many researchers as a tool to study the properties of these molecules (Basu *et al.*, 1976; Timkovich, 1977; Dailey and Strittmatter, 1978; Geren *et al.*, 1984; Miller *et al.*, 1983; Lambeth *et al.*, 1984; Huckett *et al.*, 1986; Gordhan *et al.*, 1986). Khorana (1961) was first to use carbodiimides as reagents for conjugating proteins and other molecules together. By varying the conditions of reaction one can form the N - acylureas of the carbodiimides, instead of conjugating them (Figure 1.13). Thus, instead of forming peptide bonds, a watersoluble carbodiimide, [^{3}H] ECDI, was utilized to form the N - acylureas. Unlike the reaction which brings about peptide bond formation, with subsequent

Solubilization	LDL precipitation method			
	Heparin-Mn^{++}	$(NH_4)_2SO_4$	Mg^{++}	Solvent extraction
STE	Y	N	N	N
TSM	Y	Y	Y	Y
5% NaCl	N	N	N	N
10% $NaHCO_3$	N	N	N	N
5% SDS	Y	Y	Y	Y

Table 2.1 : Precipitation of intact LDL and subsequent attempts at solubilization. LDL samples were precipitated with heparin - Mn^{++}, $(NH_4)_2SO_4$ or Mg^{++}. The precipitated LDLs were then subjected to solvent extraction to remove the lipids. Extraction of lipids from intact LDL, with solvent, also resulted in precipitation of the proteins (last column). Solubilization was then attempted as indicated. Y = precipitate dissolved; N = precipitate did not dissolve.

release of the urea, here the carbodiimide moiety underwent internal rearrangement and became covalently attached to the glutamic and aspartic acid residues of the apoproteins. The modification of LDL proteins can yield two types of N - acylureas (Figure 2.8 a).

Gel exclusion chromatography (Biogel A5m) confirmed the formation of [^{3}H] ECDI - LDL. The radioactivity peak (due to labelled ECDI) followed the optical density peak (the column was first standardized with native LDL). Results are presented in Figure 2.8 b. Carbodiimide modifications of LDL were also carried out at different mole ratios (Figure 2.9). From the results obtained it was decided that, for subsequent preparations of ECDI - LDL, a mole ratio of 1 : 10 000 would be used. Agarose gel electrophoresis has shown that ECDI - LDL is positively charged (Figure 2.10 a) and moves in the opposite direction to native LDL. Immunodiffusion assays (Figure 2.10 b) show that despite the modification of glutamic and aspartic acid residues by the carbodiimide, some antibody - binding sites on the apoprotein B were unaffected. Hence positive precipitin lines were obtained for the carbodiimide - modified LDL as well as for native LDL.

2.3.2.1 Investigating the possibility of carbodiimide modification of lipid components of LDL

Thin layer chromatography (TLC) of native and carbodiimide - modified LDL was carried out to ascertain whether any of the lipid components were modified with ECDI. Lipids were extracted with $CHCl_3$ - MeOH -

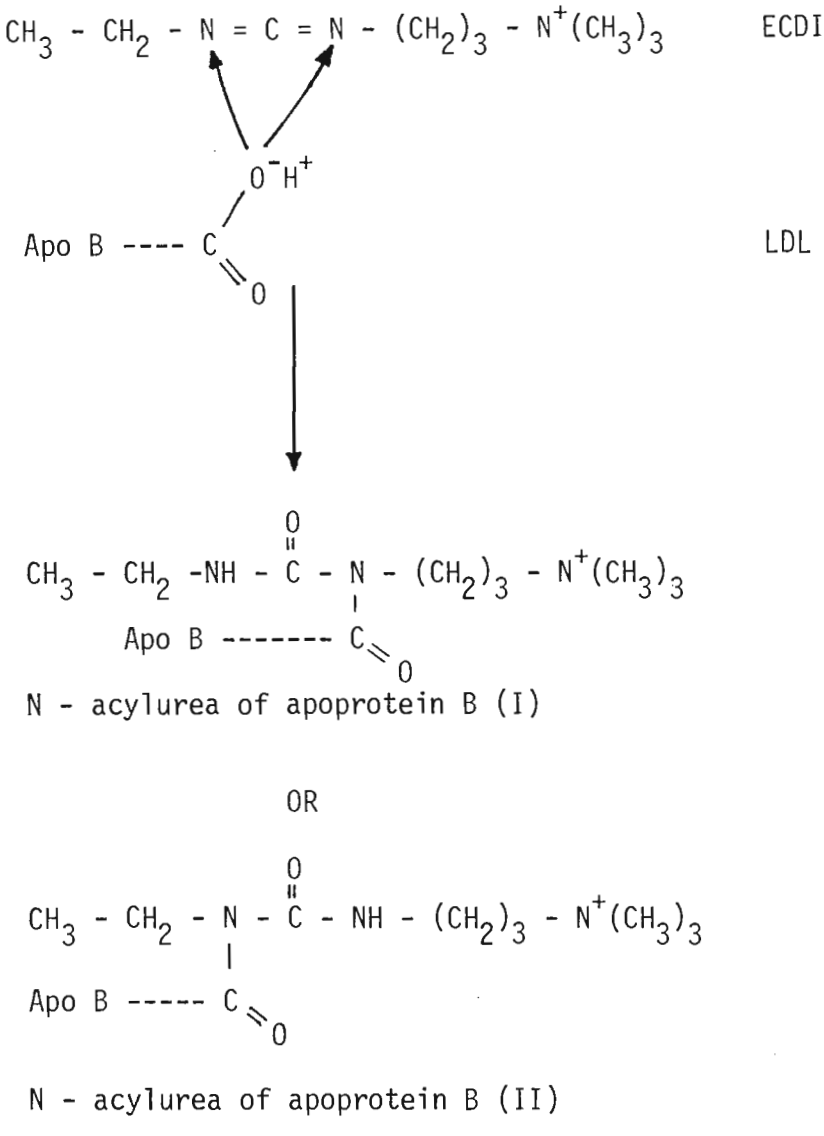


Figure 2.8 a: Carbodiimide modification of glutamic and aspartic acid residues of LDL yields either N - acylurea (I) or (II), depending on the mode of addition.

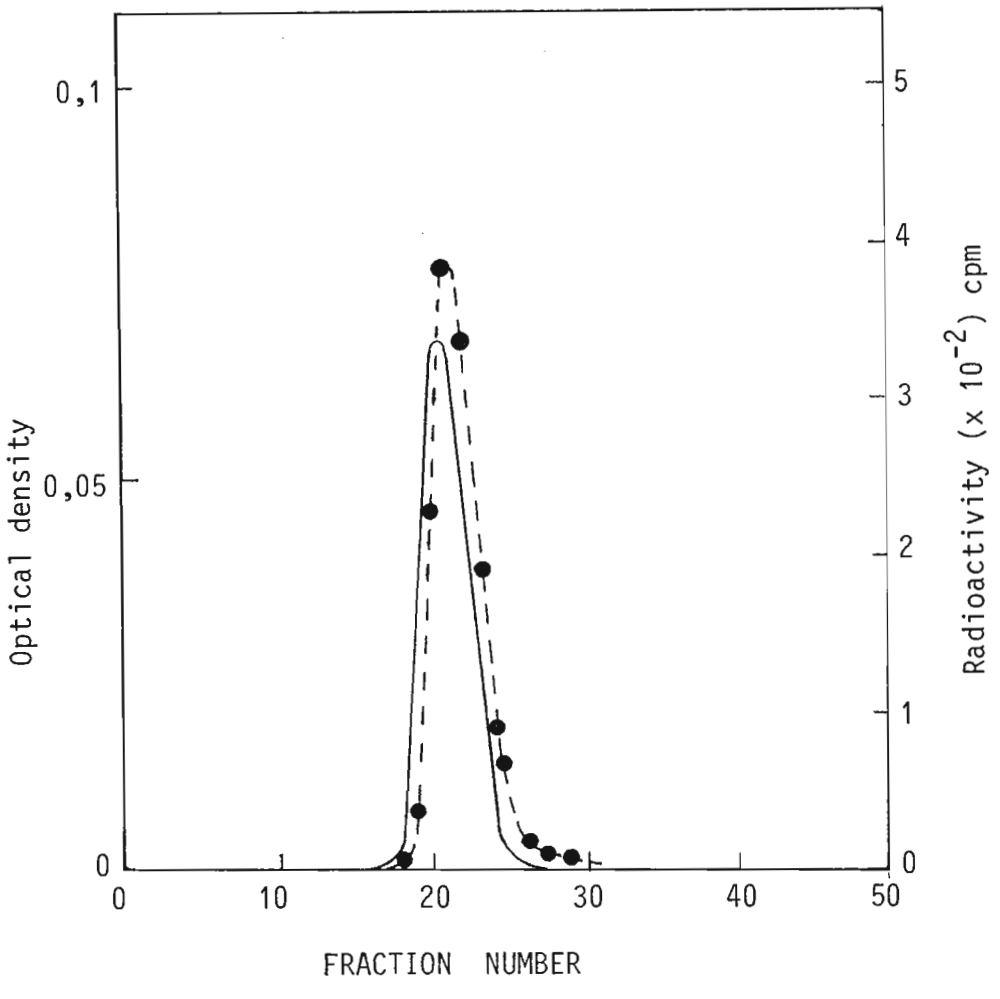


Figure 2.8 b : Elution profile of carbodiimide - modified LDL from a Biogel A 5m column, monitored at 280 nm (——). The radioactivity of peak fractions was determined and plotted on the same graph (----).

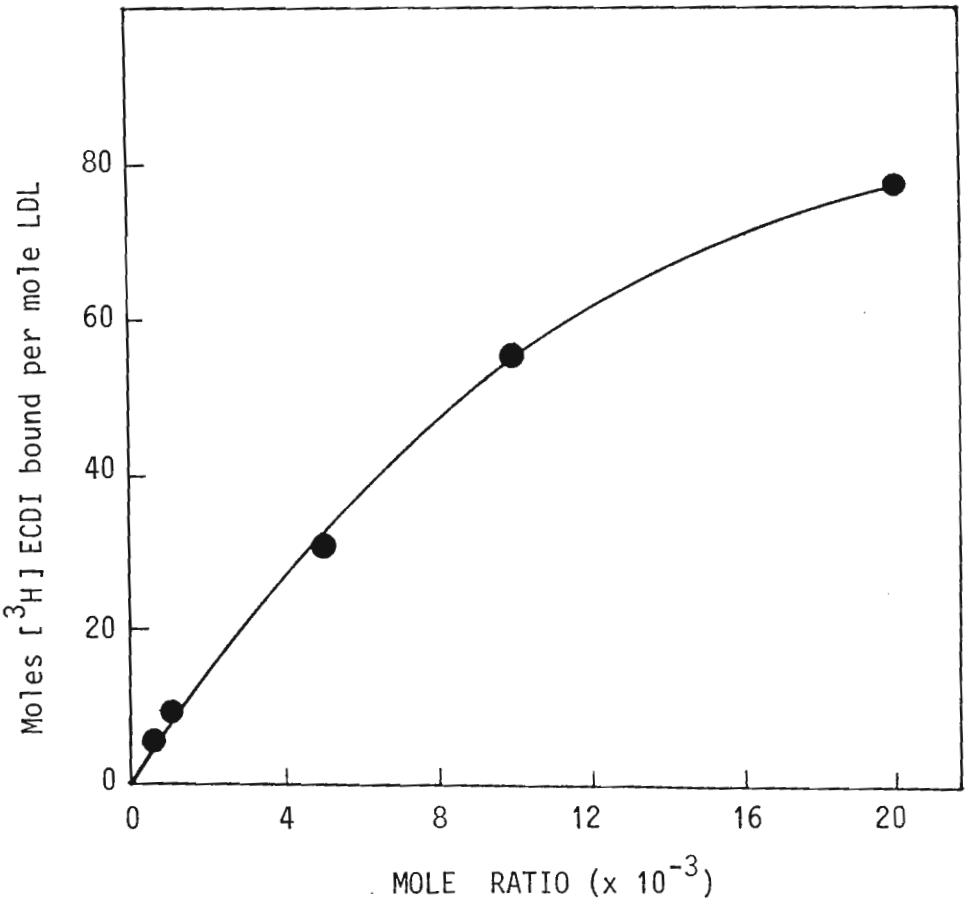


Figure 2.9 : Mole ratios of [^{3}H] ECDI bound per mole of LDL, with increasing carbodiimide concentrations.

Figure 2.10 a : Agarose gel electrophoresis of LDL (lane 1) and ECDI - LDL (lane 2).

Figure 2.10 b : Immunodiffusion assay of ECDI - LDL (left) and native LDL (right) against anti human β - lipo - protein (center wells). A serial double dilution was done (clockwise); well 1 contained 2,4 µg LDL protein in each case.

AcOH - H_2O (85 : 15 : 10 : 4, by volume). Different solvent systems were used in order to resolve different lipid components (Section 2.2.1.10). Solvent A (Figure 2.11 a) gave different R_f values for the different phospholipids, but did not resolve the cholesteryl esters and triglycerides. Solvent B (Figure 2.11 b) was also suited to phospholipid separation. Solvent C (Figure 2.11 c) was utilized for the separation of neutral lipids. However, the resolution was poor (no difference in R_f values of the different cholesteryl esters and triglycerides). Solvents D and E (Figures 2.11 d and 2.11 e) did not resolve the neutral lipids either. Solvent F (Figure 2.11 f) yielded the same R_f values for the cholesteryl esters and the triglycerides. Since there was no difference in the R_f values of the separated lipid components of LDL and ECDI - LDL, it was assumed that the lipid components of ECDI - LDL had not been modified by the carbodiimide. However, to give additional support to this assumption, [^{3}H] ECDI - LDL lipids were separated by TLC on silica gel $60F_{254}$ plates (Figure 2.12), scraped, extracted with $CHCl_3$ - MeOH - AcOH - H_2O (85 : 15 : 10 : 4, by volume) and counted. The only radioactivity detected was in the standard, [^{3}H] ethyl trimethylpropylammonium carbodiimide. Since the phospholipids are situated peripherally on the LDL particle (Deckelbaum *et al.*,1977; Brown and Goldstein, 1984), they are more likely to undergo carbodiimide modification than the neutral (core) lipids. For this reason individual phospholipids were reacted with carbodiimide, to modify them under the conditions described in Section 2.2.2.5. TLC of reaction mixtures yielded no change in R_f values, hence there was no indication of carbodiimide modification of LDL lipids.

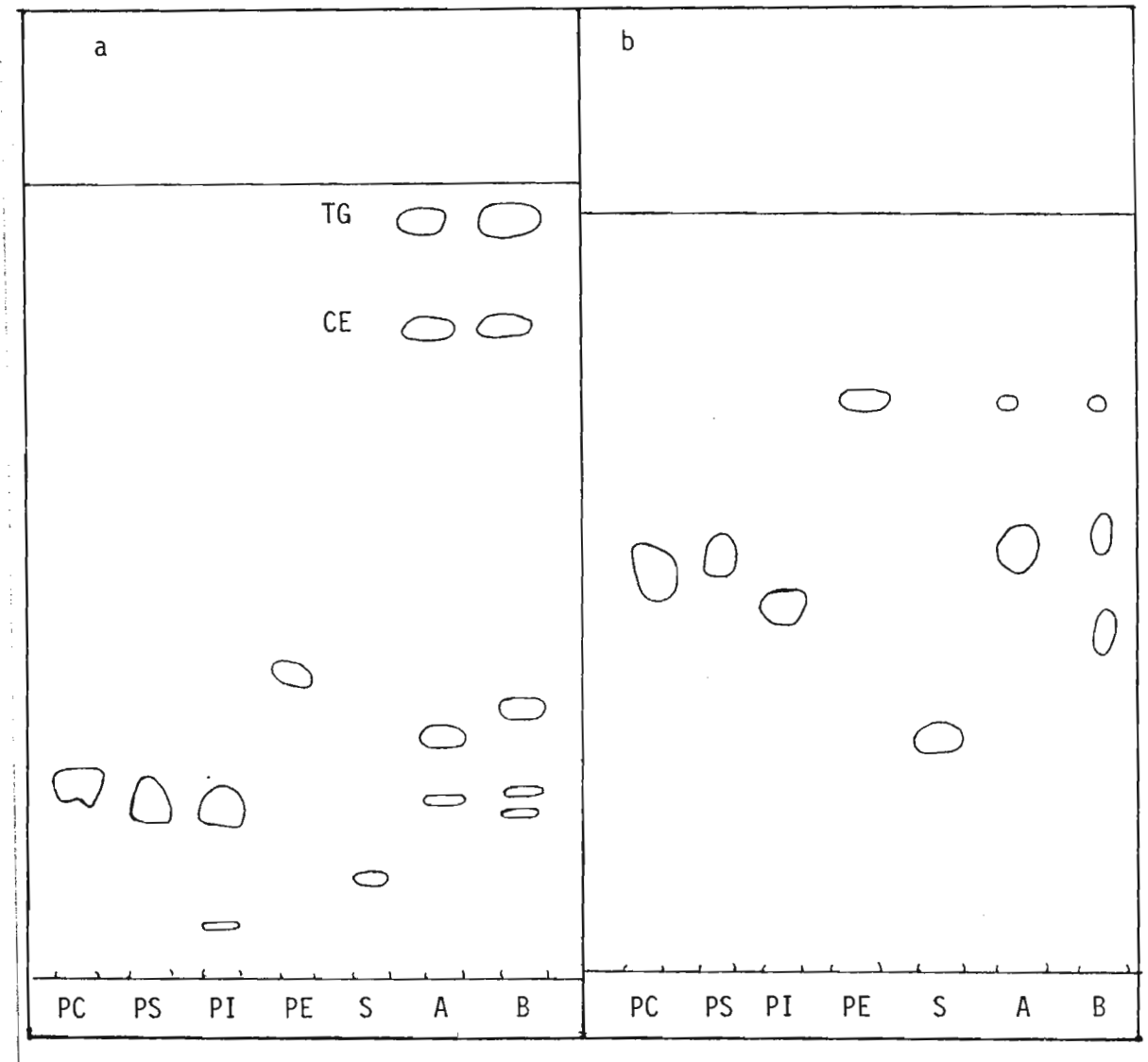


Figure 2.11 a and b : Thin layer chromatography of LDL (A) and ECDI - LDL (B) lipids on silica gel 60 F_{254} plates. Plate a was developed in solvent A and plate b in solvent B. Both solvents were suited to the separation of phospholipids. Abbreviations are defined in the legend of Figure 2.1 b. PI = phosphatidylinositol.

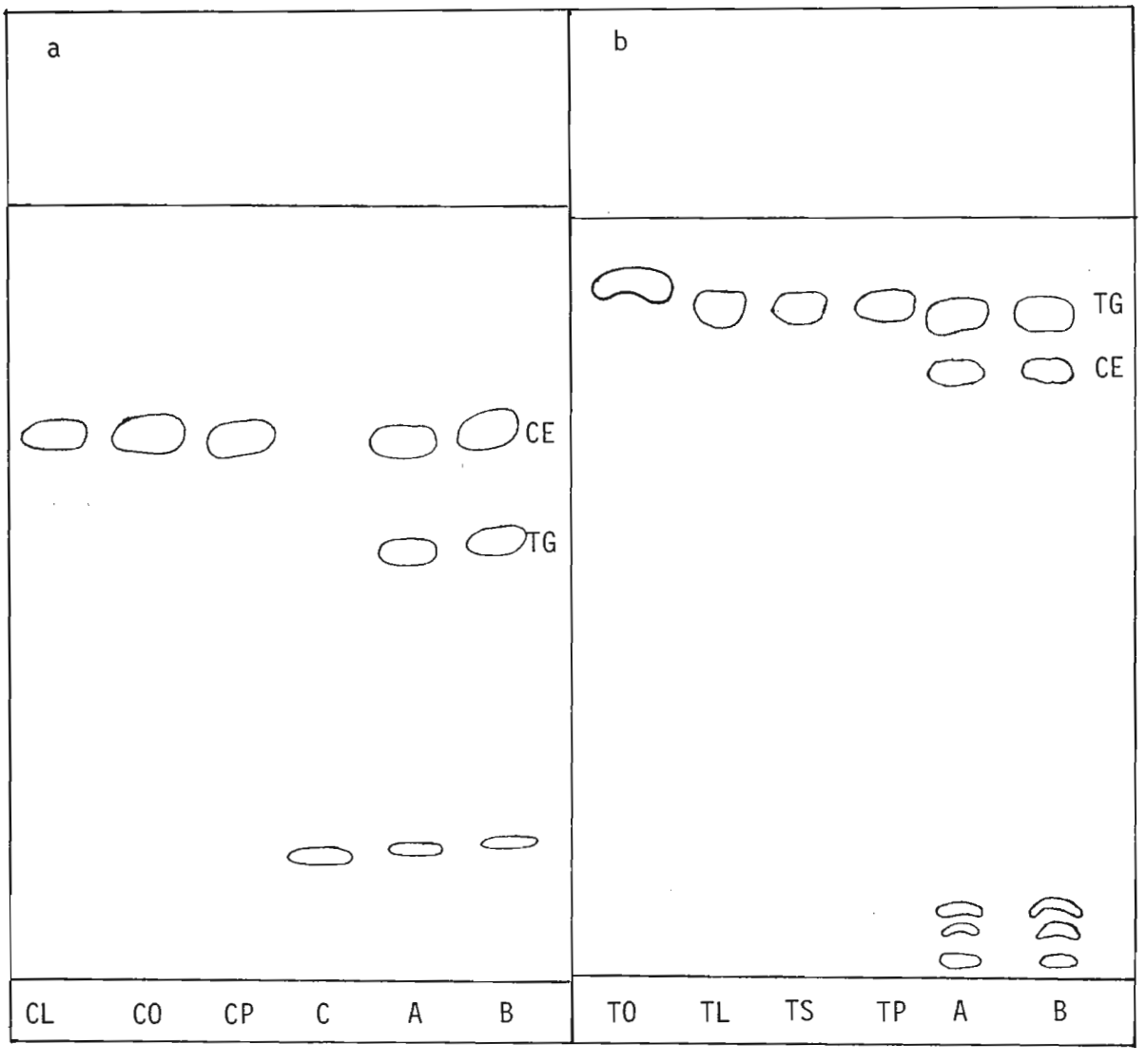


Figure 11 c and d : Thin layer chromatography of LDL (A) and ECDI - LDL (B). Plates were developed in solvents C (a) and D (b). CL = cholesteryl linoleate; CO = cholesteryl oleate; CP = cholesteryl palmitate; C = cholesterol; TO = triolein; TL = trilinolein; TS = tristearin; TP = tripalmitin.

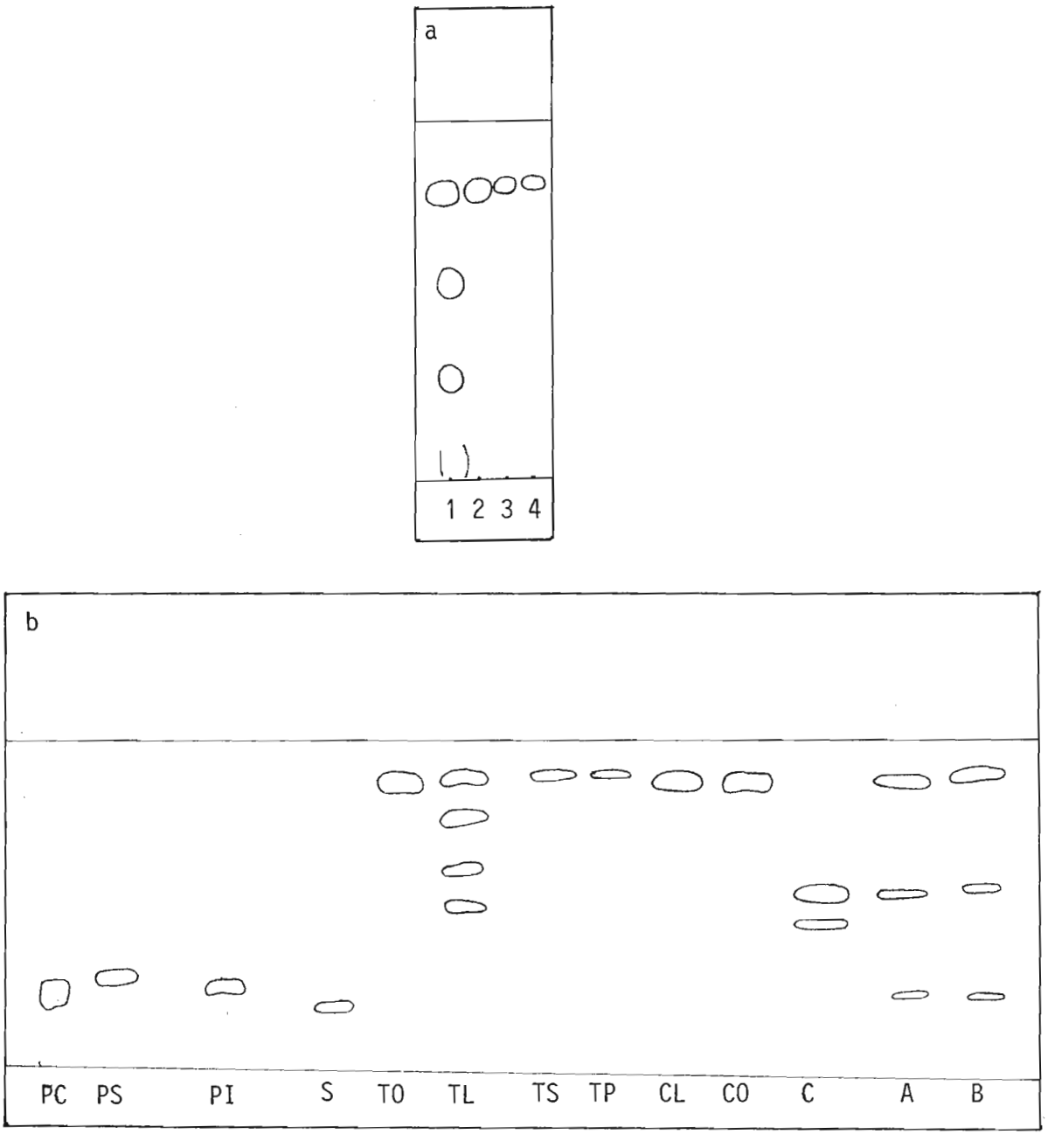


Figure 2.11 e and f : Thin layer chromatography of LDL (A) and ECDI - LDL (B). Plate a was developed in solvent E and plate B in solvent F. 1, 2, 3 and 4 are TL, TO, TS and TP respectively. Lipids are abbreviated as per Figures 2.1 b, 2.11 c and 2.11 d.

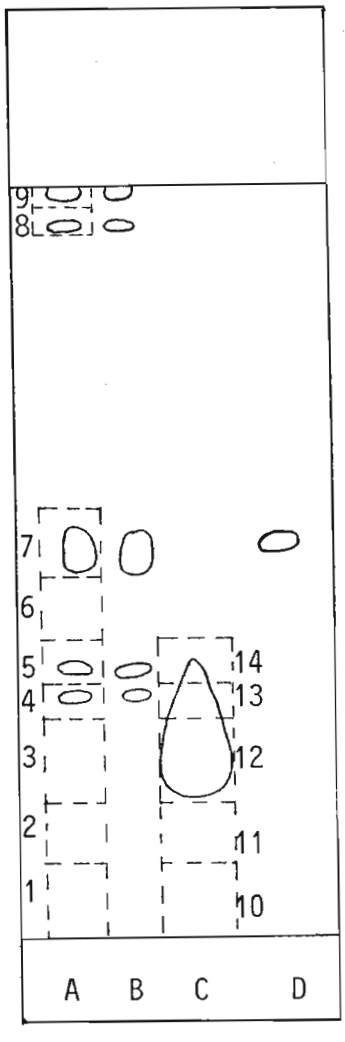


Figure 2.12 : Thin layer chromatography of [^{3}H] ECDI - LDL developed in $CHCl_3$ - MeOH - AcOH - H_2O (85 : 15 : 10 : 4, by volume). Spots were located with I_2 vapour and marked. The plate was then sectioned (dotted lines), scraped, extracted as described in Section 2.3.2.1 and counted. A = [^{3}H] ECDI - LDL; B = LDL; C = standard [^{3}H] ECDI; D = phosphatidylcholine.

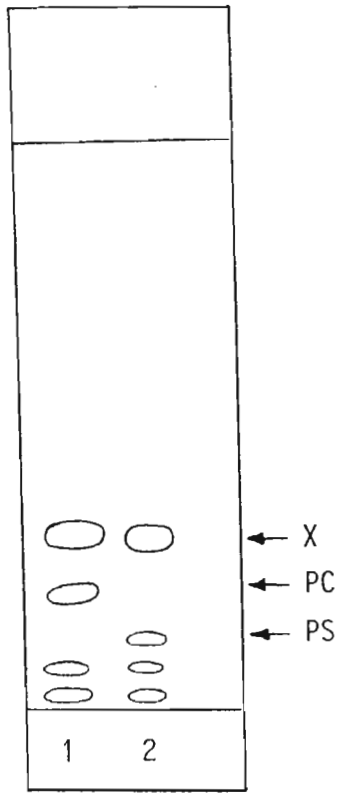


Figure 2.13 : Thin layer chromatography of phosphatidylcholine (1) and phosphatidylserine (2) reacted with ECDI. Spots were located with I_2 vapour and identified with the use of standards. X = ECDI.

Figure 2.13 shows phosphatidylcholine and phosphatidylserine reacted with ECDI and chromatographed on silica gel $60F_{254}$ plate and developed in solvent B (Section 2.2.1.10). Thin layer chromato - grams are presented because R_f values of the lipid components vary with humidity from day to day (Skipski *et al.*, 1964; Skipski and Barclay, 1969).

When lipids are extracted from LDL, the protein component precipitates out, as the protein cannot exist in solution without the lipid (Lee and Alaupovic, 1974). The precipitated proteins of [^{3}H] ECDI - LDL were solubilized in 5% SDS and counted. It was found that 99,5% of the total radioactivity was located in the solubilized proteins, thus establishing that carbodiimide modification of the protein component and not the lipid component had occurred.

2.3.2.2 Phospholipase C and D digestion of ECDI - LDL

[^{3}H] ECDI - LDL did not undergo cleavage at the N - acylurea when digested with phospholipase C. However, the trimethylpropylammo- nium residues were hydrolysed by phospholipase D (Figure 2.14 a, b). The results also show that [^{3}H] carbodiimide modified transferrin also undergoes phospholipase D digestion. The phospholipase D enzyme will hydrolyse choline and ethanolamine residues from phosphatidylcholine, lysophosphatidylcholine, sphingomyelin and phosphatidylethanolamine, respectively. Phospholipase C, on the other hand, cleaves phosphorylcholine, phosphorylethanolamine and phosphorylserine residues from the respective phospholipids

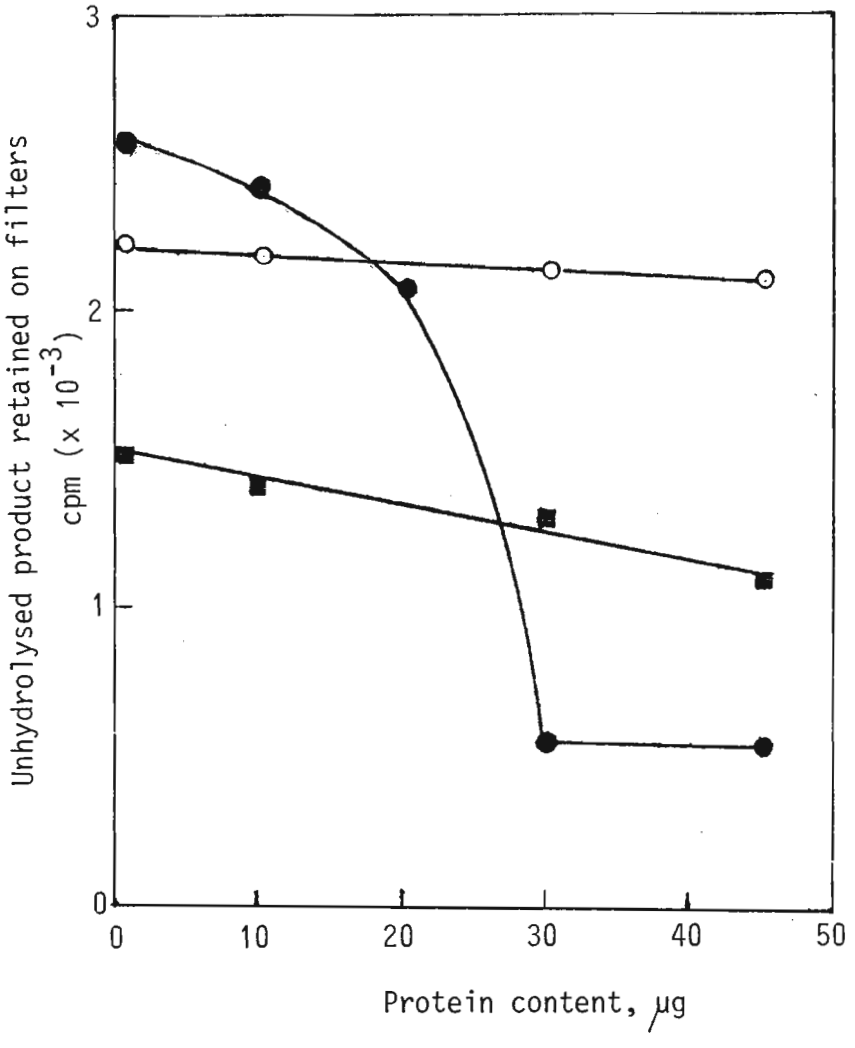


Figure 2.14 a : Digestion of [^{3}H] ECDI - LDL with phospholipase D (●—●) and phospholipase C (○—○), and ECDI - transferrin with phospholipase D (■—■).

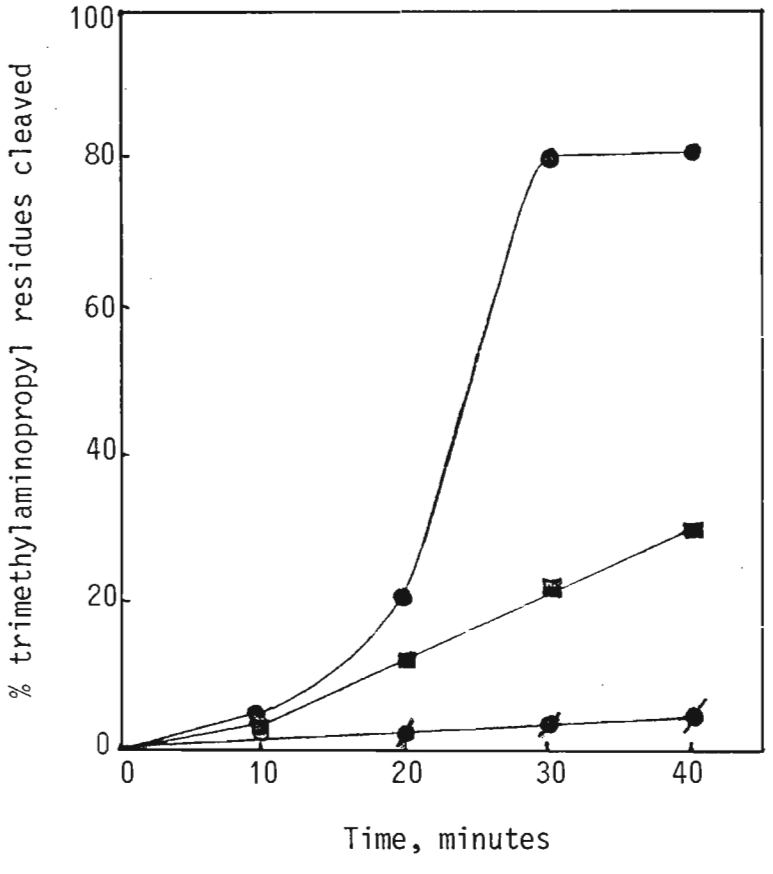


Figure 2.14 b : Digestion of [^{3}H] ECDI - LDL with phospholipase D (●) and phospholipase C (◐), and ECDI - transferrin with phospholipase D (■). The data from Figure 2.14 a were expressed as percentages.

(Lehninger, 1975). The action of phospholipase C and D, on the above lipids, is presented in Figure 2.15 a. Since the trimethylpropyl ammonium group of the carbodiimide has similarities in structure and spatial arrangement to that of phosphatidylcholine and sphingomyelin (Figure 2.15 b, Figure 2.15 c) it is possibly recognized by phospholipase D and is therefore hydrolysed as shown in Figure 2.15 d. Two confugurations of the N - acylurea are possible as indicated in Figure 2.8 a. With structure I, phospholipase C cleavage is possible but not phospholipase D cleavage, due to steric hinderance by apoprotein B of LDL. With structure II, phospholipase C cleavage is sterically hindered by the apoprotein, whilst the phospholipase D cleavage is feasible. Since the N - trimethyl group was labelled, the phospholipase C and D digestions of ECDI - LDL could be monitored. Since the phospholipase C digestion of ECDI - LDL was low and phospholipase D cleavage was high, indications are that the N - acylureas formed by the reaction of apoprotein B with the carbodiimide, is more favourable with the trimethylpropylammonium nitrogen (80%) than with the ethyl nitrogen (20%) (Figure 2.14 b).

The ability of phospholipase D to effectively remove choline groups from the lipoproteins was confirmed with the use of a Boehringer assay kit. The removal of choline groups was determined spectrophotometrically, using phosphatidylcholine as standard (Takayama *et al.*, 1977). For equivalent amounts of LDL and ECDI - LDL utilized for the assay, ECDI - LDL yeilded a 4% higher value. This additional value is attributed to the trimethylpropylammonium group

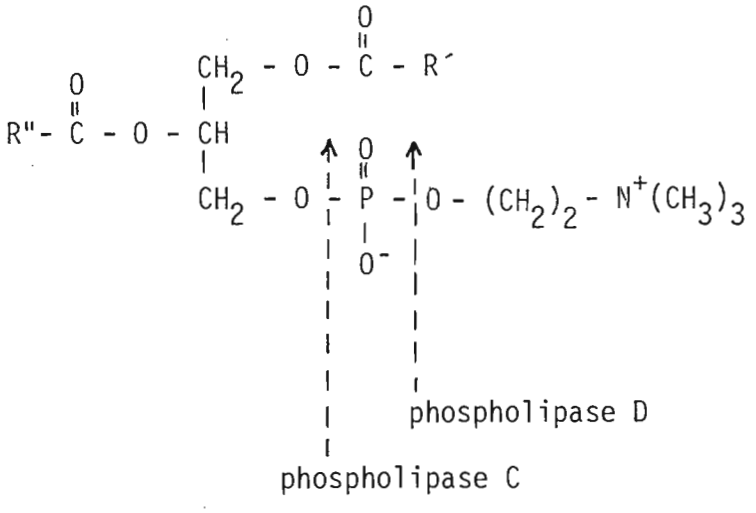
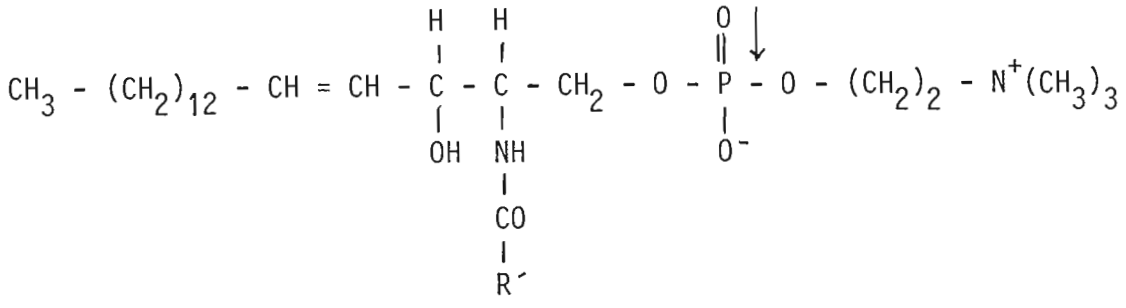
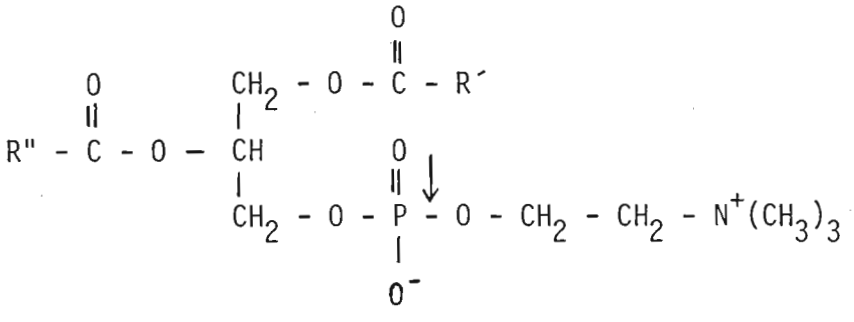


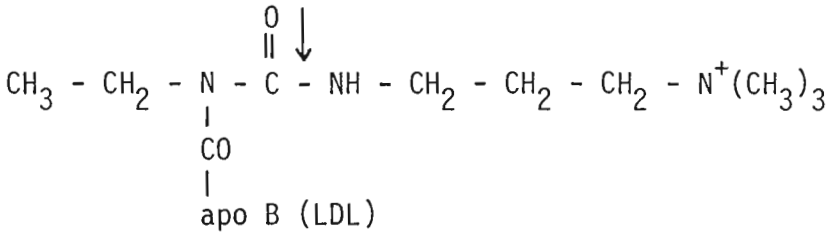
Figure 2.15 a : Sites of cleavage by phospholipase C and phospholipase D on phosphatidylcholine. Phospholipase C cleaves off phosphorylcholine residues and phospho - lipase D cleaves off choline residues.



Sphingomyelin (I)

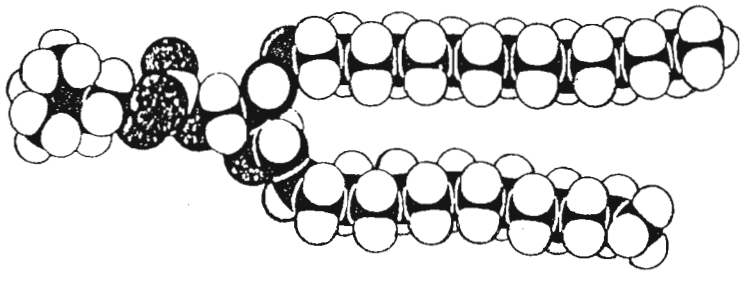


Phosphatidylcholine (II)

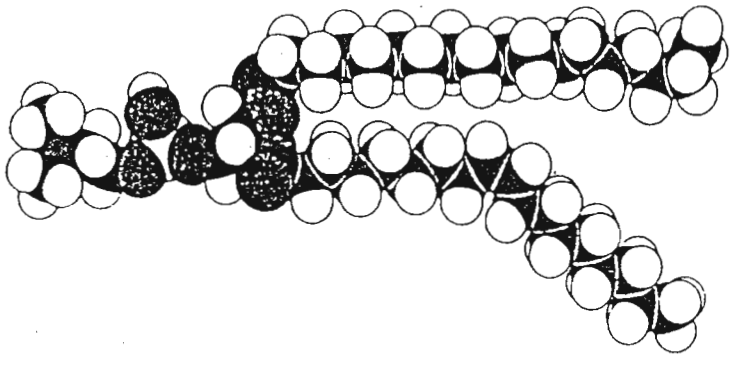


N - acylurea of LDL (III)

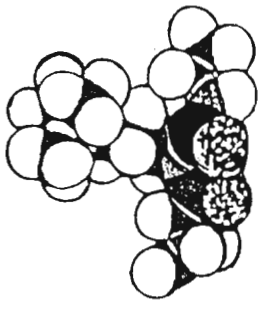
Figure 2.15 b : Phospholipase D digestion of sphingomyelin (I), phosphatidylcholine (II) and the N - acylurea of carbodiimide - modified LDL (III) indicated by arrows.



(I)



(II)



(III)

Figure 2.15 c : CPK models of sphingomyelin (I), phosphatidylcholine (II) and the trimethylpropyl-ammonium group of ECDI (III) showing the same spatial arrangement of the quaternary nitrogen covalently bound to three methyl groups.

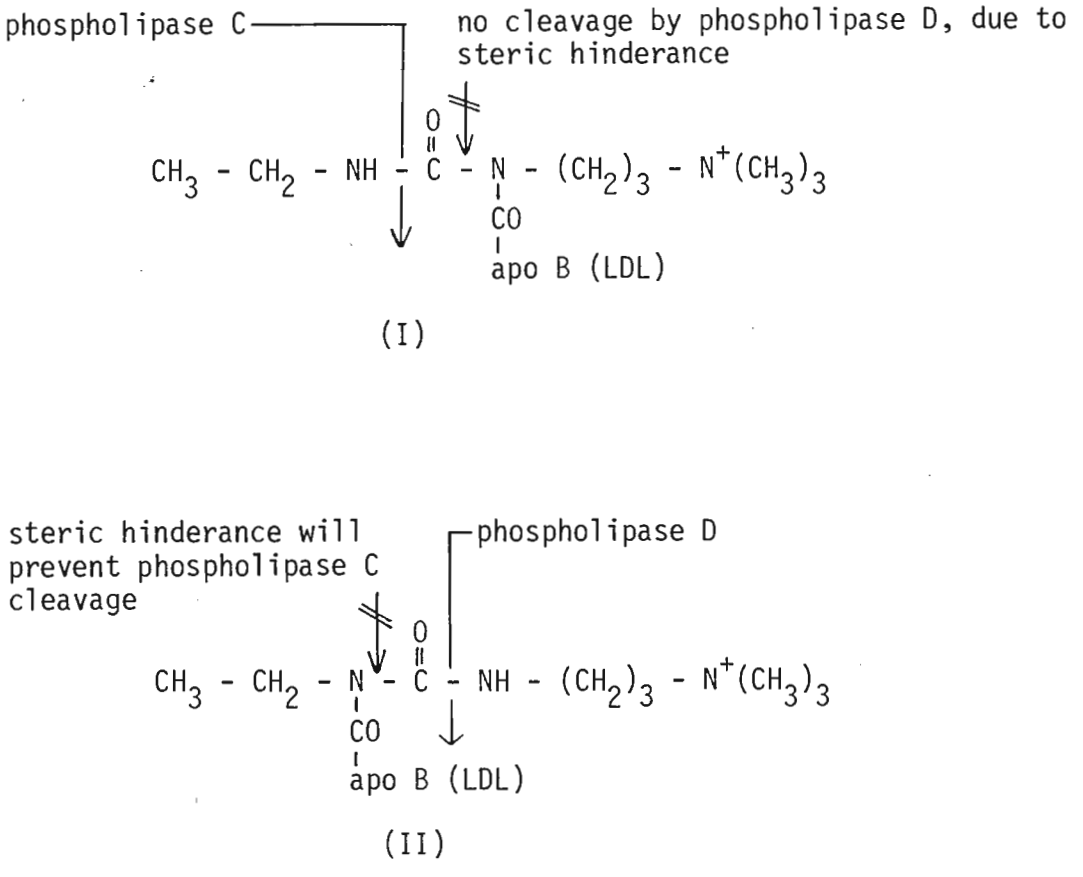


Figure 2.15 d : Possible mode of action of phospholipase C and phospholipase D on the N - acylureas (Figure 2.8 a) of carbodiimide - modified LDL apoprotein B. Since no phospholipase C digestion of [^{3}H] ECDI - LDL was detected, II is the possible N - acylurea hydrolysed by phospholipase D.

removed from the carbodiimide - modified LDL proteins. However, the possibility of protease activity in the commercial preparation of phospholipase D (Boehringer Mannheim) cannot be ruled out.

2.3.2.3 Analysis of phospholipase D - digested ECDI- LDL

Since there was a significant decrease in radioactivity of [^{3}H] ECDI - LDL, following phospholipase D digestion, the reaction mixture was also subjected to thin layer chromatography to confirm that the trimethylpropylammonium group was cleaved. A TLC and histogram, presented in Figure 2.16, of the spots scraped off the TLC and counted, indicate that the counts may be due to trimethylpropylammonium groups. The action of phospholipase D is, therefore, the same as on phosphatidylcholine or sphingomyelin. Hence the structural similarity to these phospholipids results in its cleavage by phospholipase D. Since only the carbodiimide moiety was labelled, it suggests that the trimethylpropylammonium residue was indeed susceptible to phospholipase D digestion.

2.3.3 Acetylation of LDL

Acetylation of LDL was confirmed by agarose gel electrophoresis (Figure 2.17 a). The acetylation method was adapted from published procedures (Basu *et al.*, 1976; Goldstein *et al.*, 1979; Fraenkel - Conrat, 1975). The mobility of acetylated LDL was greater than that of native LDL and of other modified LDLs. Despite extensive acetylation of the protein component, the immunoassay (Figure 2.17 b) was positive. This indicates that the antibody recognition sites

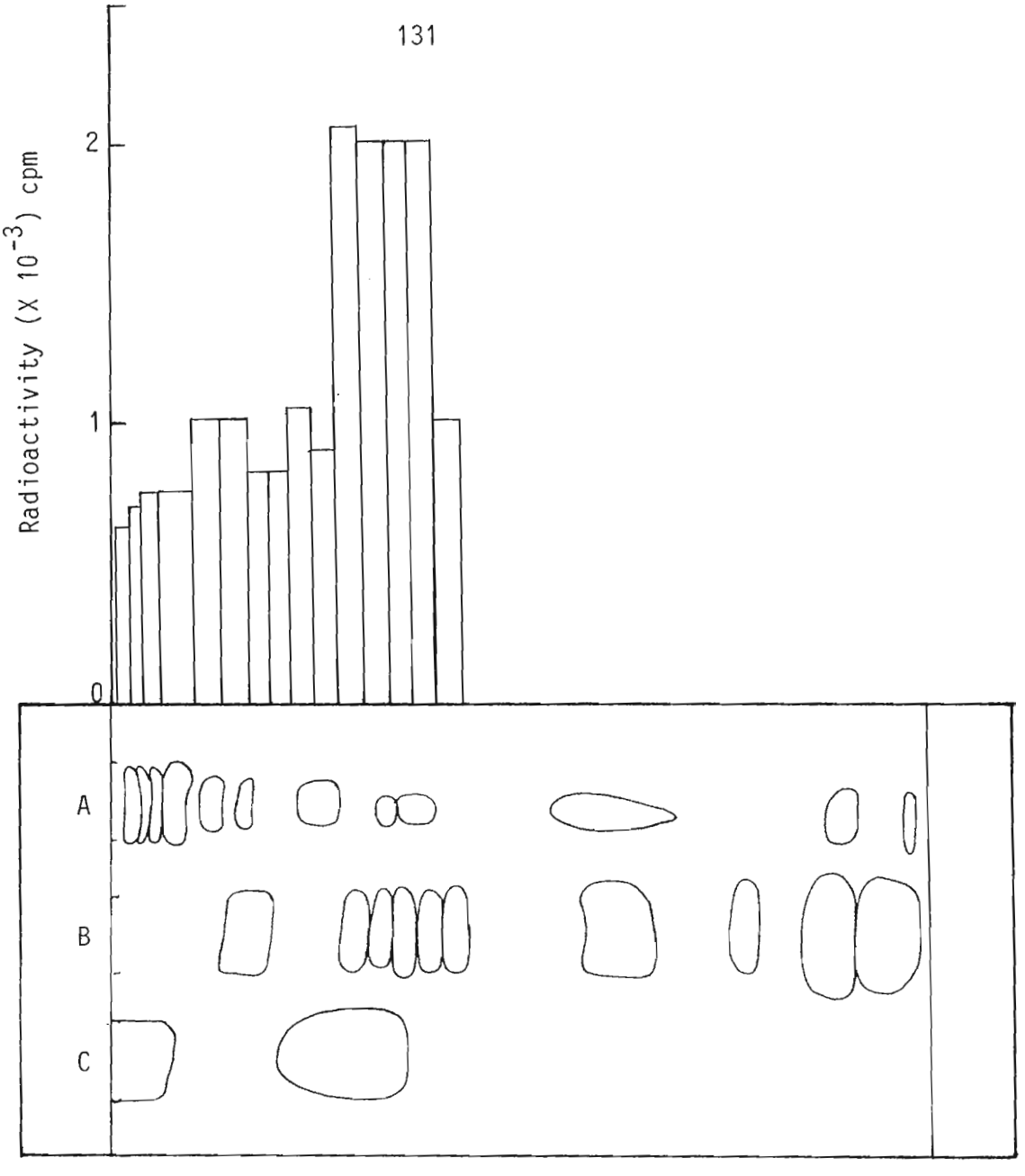
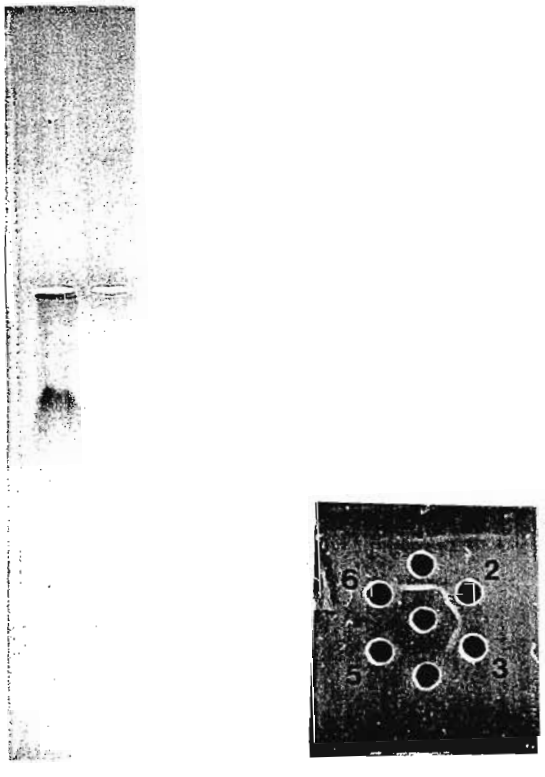


Figure 2.16 : Thin layer chromatogram and histogram of phospholipase D digested [^{3}H] ECDI-LDL. The aqueous (A) and organic (B) phases were chromatographed separately. Highest counts were in the region of N,N,N - trimethyldiamino - propane (C).



1 2

a b

Figure 2.17 : a : Agarose gel electrophoresis of native LDL (lane 1) and acetylated LDL (lane 2).

b : Immunodiffusion assay of acetylated LDL, serially double diluted (clockwise, starting at top well), against anti human β - lipoprotein antiserum (center well).

were unaffected by the acetylation. The anti β - lipoprotein antiserum has multiple antigenic sites for the LDL apoprotein B.

2.3.4 Modification of LDL with ethidium bromide

For the ultraviolet (UV) spectral analysis 8,33 µg / ml of ethidium bromide (EB, standard) was used, and gave a λmax value of 386 nm while the EB - LDL conjugate had a concentration of 184 µg / ml (LDL protein) and yielded a λmax value of 288 nm (Figure 2.18 a). For the visible spectra 100 µg / ml EB and 760 µg LDL / ml were utilized. The λ max values obtained were 482 nm and 465 nm, respectively (Figure 2.18 b). The extinction for EB was calculated at 482 nm. The mole ratio of LDL : EB was calculated to be 1 : 38 in the EB - LDL conjugate. The conjugation of EB to LDL was confirmed by agarose gel electrophoresis (Figure 2.19 a). The EB - LDL conjugate was slightly retarded in the electrophoretic field because of the positively charged nitrogen of EB. Carbodiimide modification of LDL is ruled out here because such modification causes ECDI - LDL to move towards the negative electrode (as shown in Figure 2.10 a).

Conjugation of EB to the LDL can occur via the LDL protein side chain carboxyl groups of glutamic acid and aspartic acid residues (Figure 2.19 b) or via the phosphate functions of the peripherally located lipids. Conjugation of EB to the protein is covalent and results in the formation of an amide bond. The reaction of phospholipid with EB results in the formation of covalent phosphoramidate bonds (Figure 2.19 c).

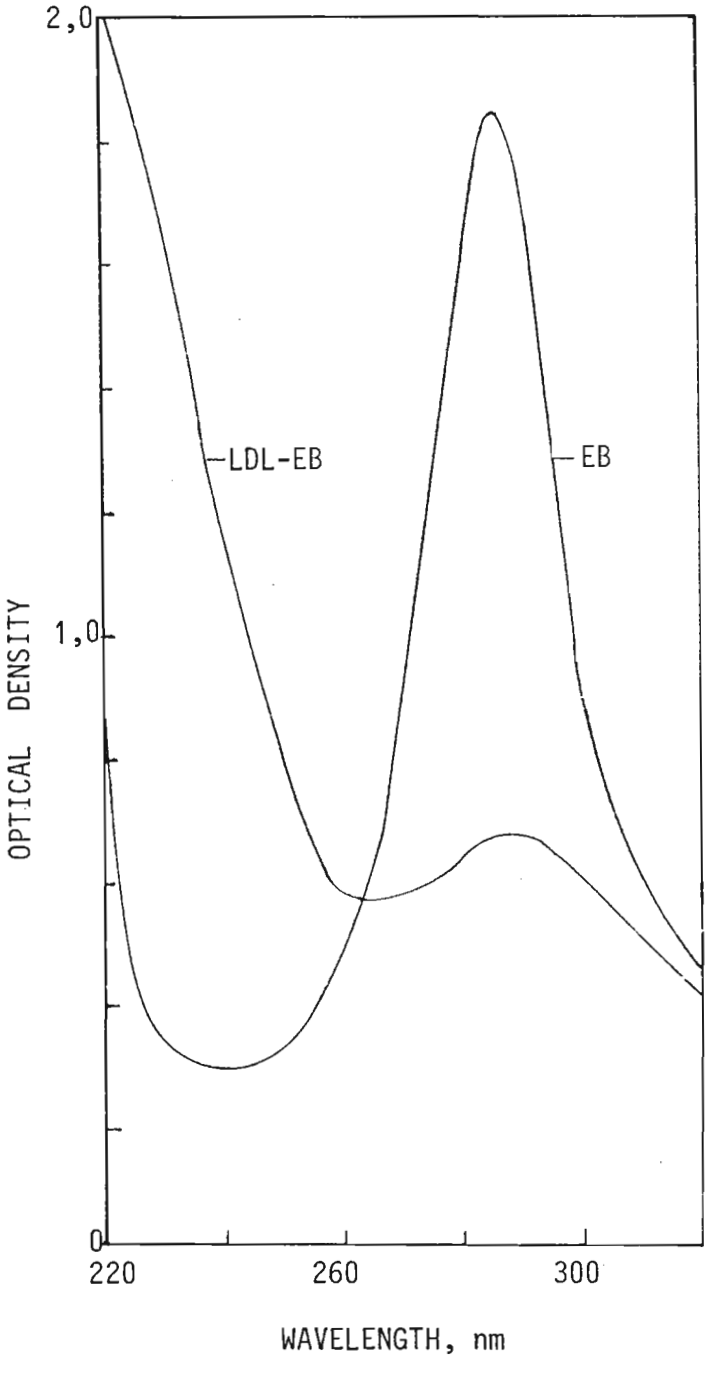


Figure 2.18 a : UV spectra of LDL - EB conjugate and EB standard in 0,05 M NaCl - 0,002 M EDTA, pH 7,0.

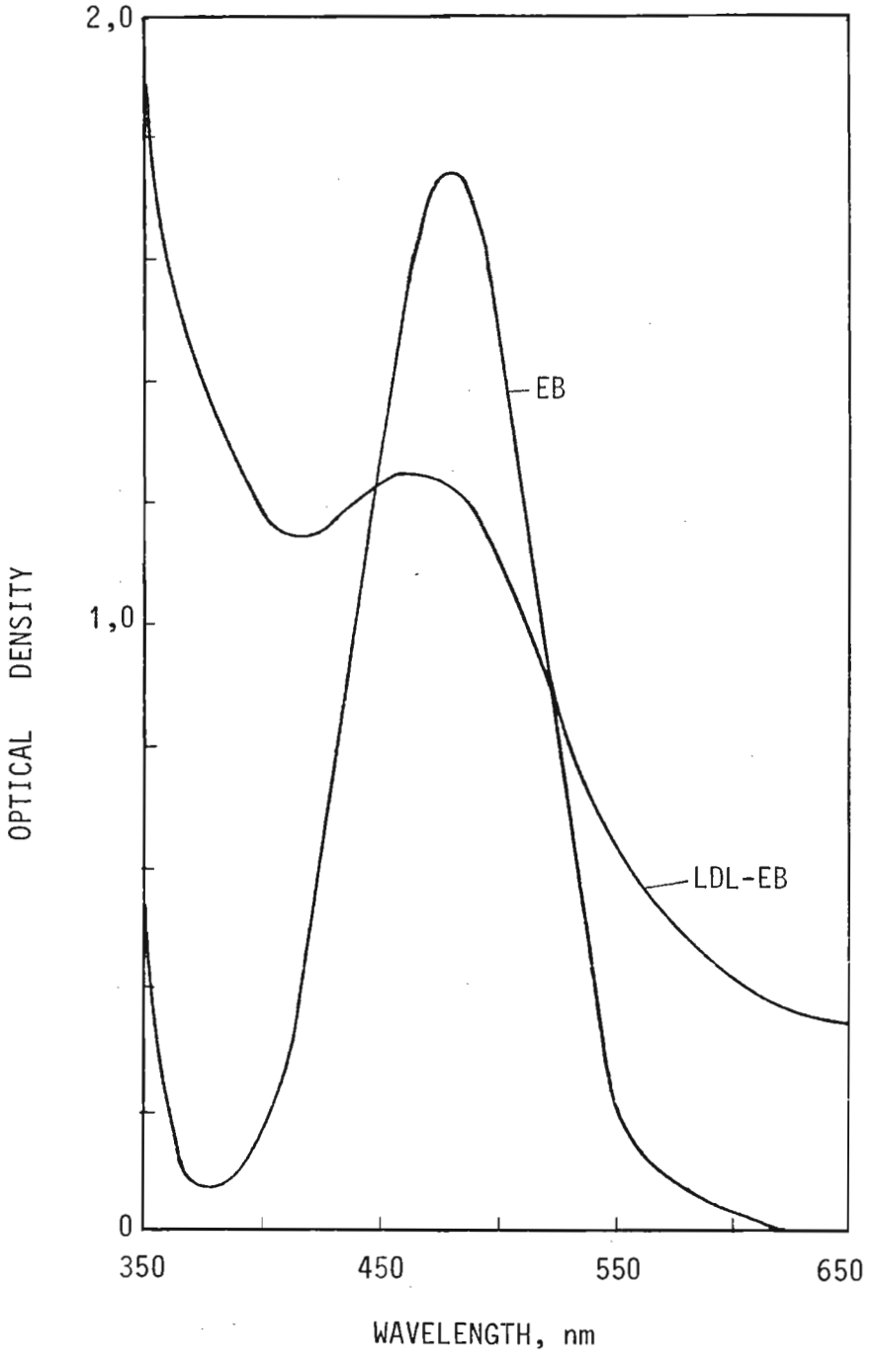


Figure 2.18 b : Visible spectra of LDL - EB conjugate and EB standard in 0,05 M NaCl - 0,002 M EDTA, pH 7,0.

Figure 2.19 a : Agarose gel electrophoresis of native LDL (A) and EB - LDL (B) stained with 0,5% Coomassie brilliant blue.

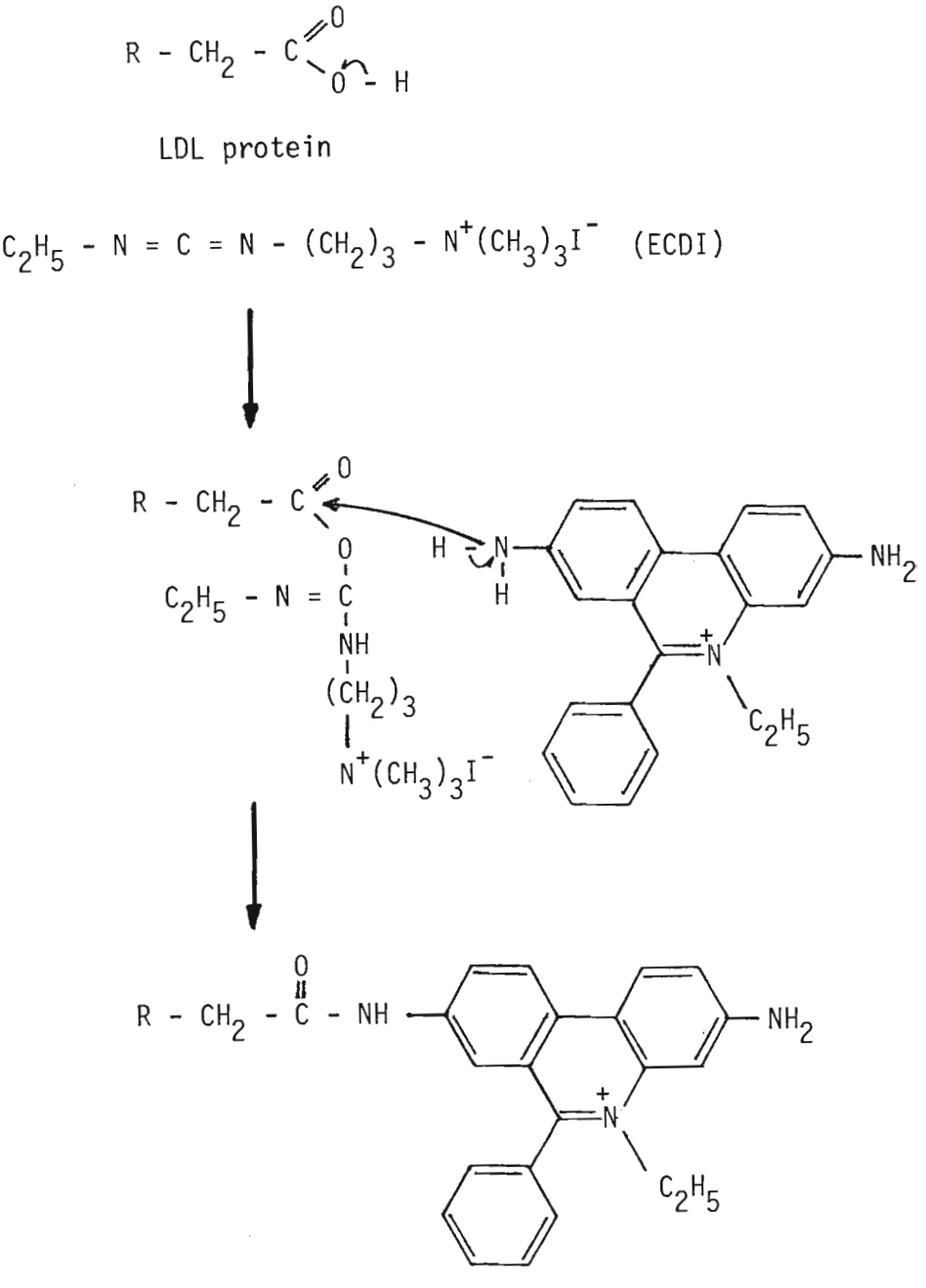


Figure 2.19 b : Conjugation of EB with LDL protein carboxyl groups, in the presence of carbodiimide.

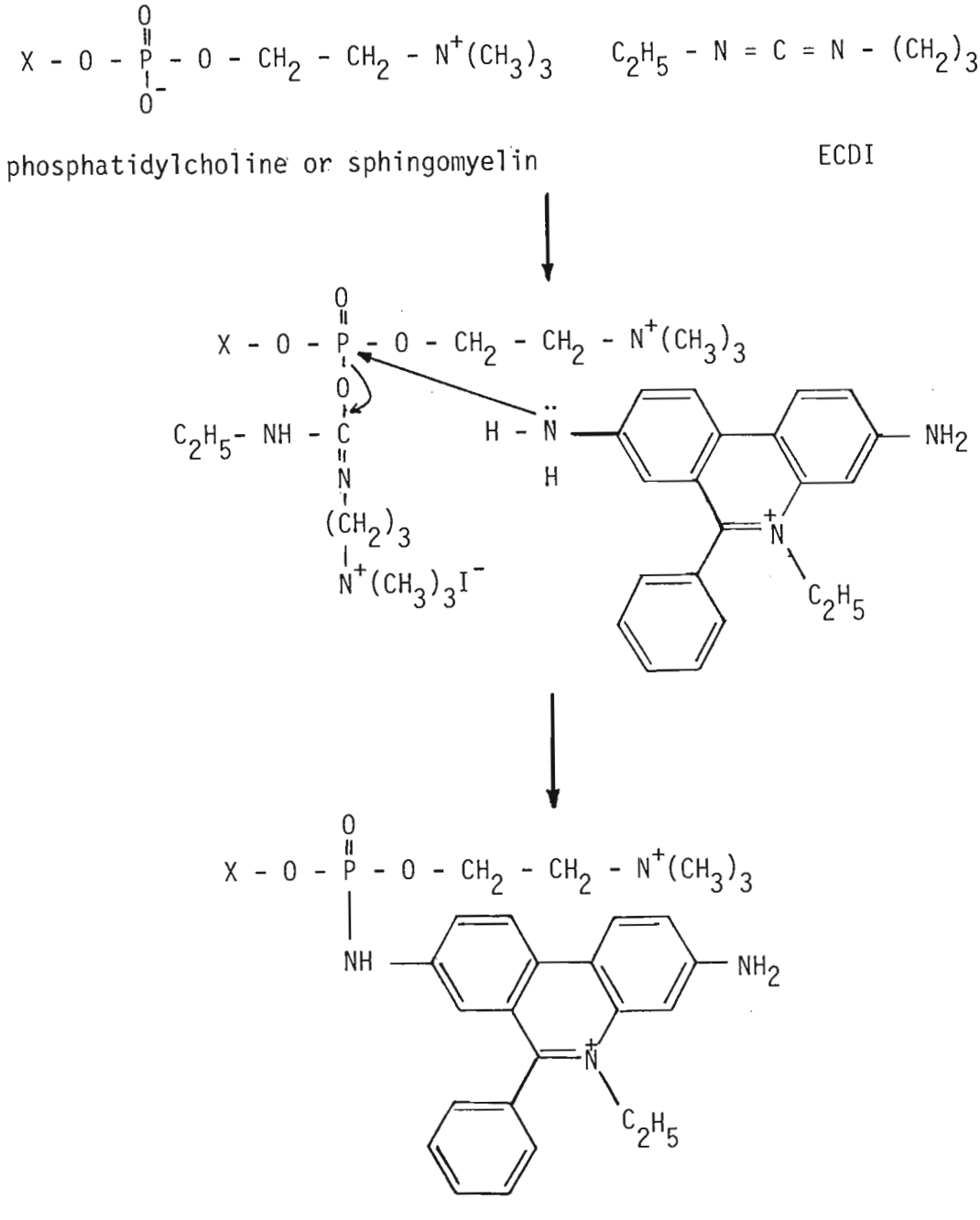


Figure 2.19 c : Interaction of ethidium bromide with LDL lipids in the presence of carbodiimide.

2.3.5 Cholesteryl ester exchange in LDL

2.3.5.1 Preparation of cholesteryl linoleate

Preliminary preparation of cholesteryl linoleate showed that the desired product would be obtained in reasonable yield (Figure 2.20 a). Cholesterol and cholesteryl linoleate yielded purple spots when sprayed with sulfuric acid and charred. The [^{3}H] cholesteryl linoleate was subsequently prepared as described in section 2.2.5.1 and purified by preparative TLC (Figure 2.20 b). The [^{3}H] choles - teryl linoleate had an activity of 42 µCi / m mole and the yield was 36,5%.

2.3.5.2 Preparation and analysis of cholesteryl linoleoyl LDL

The purpose of carrying out the cholesteryl ester exchange reaction was to devise a method of labelling LDL without altering its properties drastically. The labelled LDL would then be utilized in studying LDL - DNA interactions. Two methods were employed for the preparation of [^{3}H] cholesteryl linoleoyl LDL (CL - LDL). The chemical method of preparation necessitated the use of DMSO. A final concentration of 2,5% was used. Up to 10% DMSO has been used for such reactions (Faust *et al.*, 1975; Goldstein *et al.*, 1975; Krieger *et al.*, 1978). The [^{3}H] CL - LDL prepared by this method had an activity of 15 000 cpm / nmole. Thirty pmoles of cholesteryl linoleate was incorporated per nmole LDL. The protein : cholesterol ratios of these preparations were 1 : 1,4 for the [^{3}H] CL - LDL prepared in DMSO and 1 : 1,6 for [^{3}H] CL - LDL prepared without DMSO.

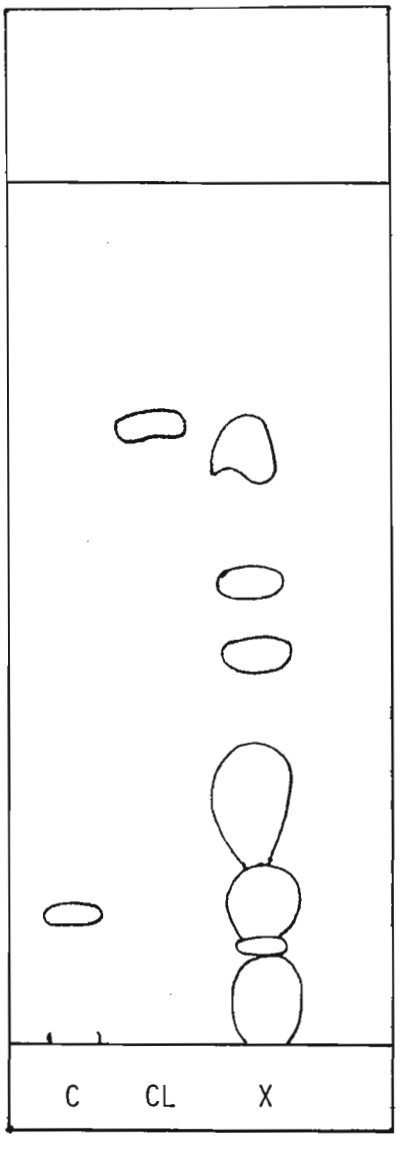


Figure 2.20 a : Thin layer chromatography of unlabelled cholesteryl linoleate (CL) reaction mixture (X). Cholesterol (C) and CL were located in the reaction mixture with the use of standards.

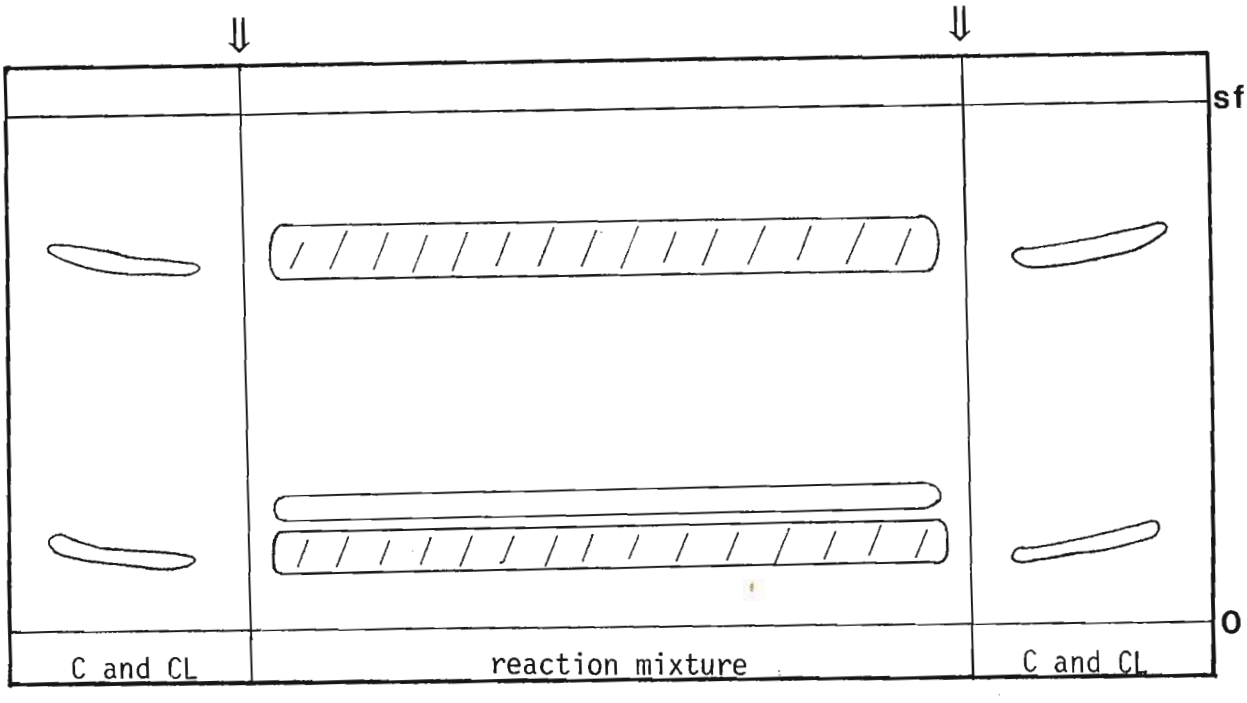


Figure 2.20 b : Preparative thin layer chromatography employed for separation of [^{3}H] cholesteryl linoleate from its reactants. The plate was cut (arrows) and the standards were located with 10% $HClO_4$ and charring. Areas matching the standards, in the center piece of the TLC plate, were scraped, extracted and counted. C = cholesterol; CL = cholesteryl linoleate.

Immunodiffusion assays showed the same reactivity of [^{3}H] CL - LDL to anti human β - lipoprotein antiserum, as native LDL, or DMSO - treated LDL and [^{3}H] CL - LDL prepared in the absence of DMSO (Figure 2.21). Agarose gel electrophoresis of these preparations had a different mobility from that of native LDL (Figure 2.22). A second method of introducing [^{3}H] cholesteryl linoleate into LDL was by the enzymatic cholesteryl ester exchange method (Roberts *et al.*,1985; Zilversmit and Johnson, 1975; Glomset, 1959; Zilversmit *et al.*, 1975). A cholesteryl ester exchange enzyme is found in the $\rho > 1{,}21$ g / ml fraction of plasma (Bachorik, 1979). This transfer enzyme exchanges lipids from one lipoprotein type to another and was utilized for the exchange of cholesteryl esters of LDL for the labelled cholesteryl esters. Figure 2.23 shows the distribution of radioactivity of the fractions collected, after ultracentrifugation. Dialysed, labelled preparations had an activity of 13 000 cpm / nmole LDL. This was slightly lower than the CL - LDL prepared by the DMSO method. The protein : cholesterol ratio was 1 : 1,24.

2.3.6 Chemical analysis of native and modified LDLs

In addition to determining the protein content (Section 2.2.1.1) of all the LDL samples, cholesterol (Section 2.2.1.2), inorganic phosphate (Section 2.2.1.3) and triglyceride content (Section 2.2.1.4) were also determined. Various methods for the determination of protein content have been documented (Lowry *et al.*, 1951; Miller, 1959; Mokrasch, 1975; Peterson, 1977). The method of Markwell *et al.*,(1981), for protein determination, is a modification of the

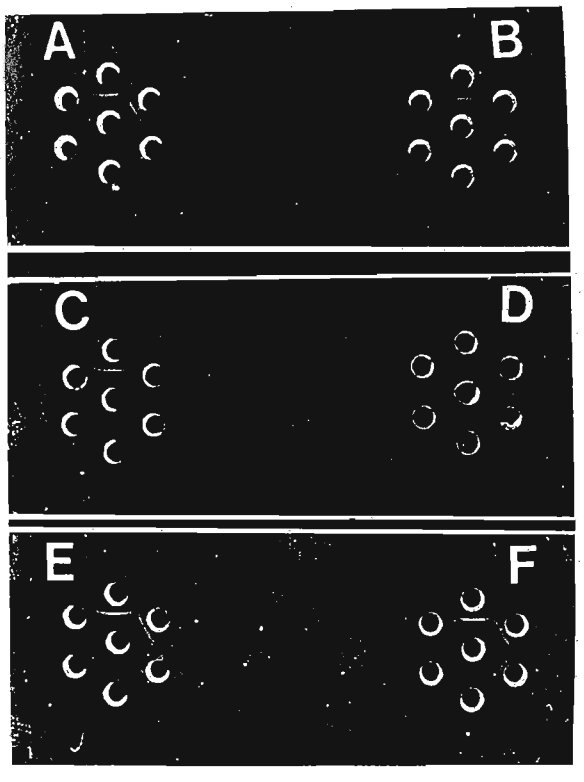


Figure 2.21 : Immunodiffusion assay of native LDL (A), [^{3}H] CL - LDL prepared in DMSO (B), [^{3}H] CL - LDL prepared in the absence of DMSO (C), unlabelled CL - LDL prepared with 180 ng CL (D), unlabelled CL - LDL prepared with 1800 ng CL (E) and LDL treated with 2,5% DMSO.

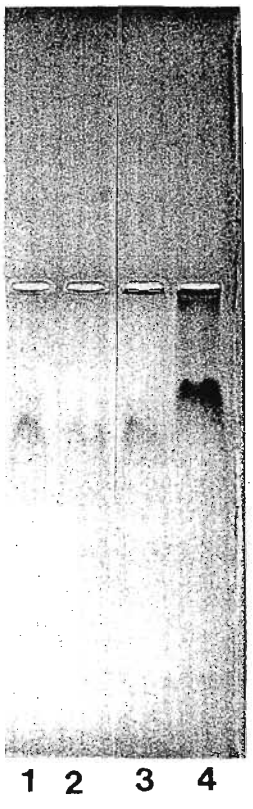


Figure 2.22 : Agarose gel electrophoresis of CL LDL prepared in DMSO (1), CL - LDL prepared in the absence of DMSO (2), LDL treated with DMSO (3) and native LDL (4).

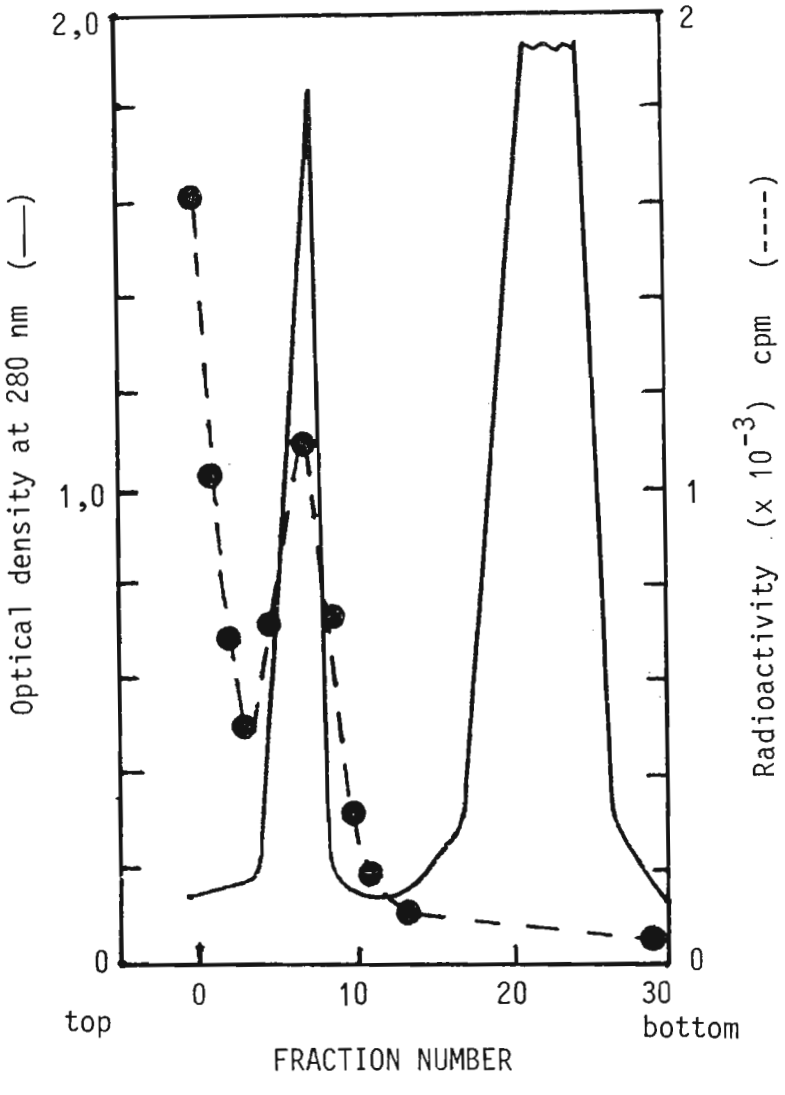


Figure 2.23 : Fractionation of [^{3}H] CL - LDL prepared by the enzymatic cholesteryl ester exchange method and subsequent ultracentrifugation. Aliquots from each fraction were counted. The [^{3}H] CL - LDL eluted in fractions 5 - 10. Radioactivity in fractions 1 - 4 was due to the unincorporated [^{3}H] cholesteryl linoleate.

Folin - Lowry method and is more specifically designed for the determination of protein content in lipoproteins. It gave more consistent results and was a more rapid method than the Mokrasch method, which is also designed for lipoproteins.

Cholesterol determination of native and modified LDLs, was determined by the method of Zlatkis and Zak (1969). This method was found to be superior to other methods (Zak, 1957; Bartlett, 1969; Kabara, 1962; Mann, 1961; Rudel and Morris, 1973), in that the results were far more consistent and colour development was good. The method was also rapid, in contrast to other methods which necessitated extraction and quantitative transfer of cholesterol, leading to inconsistencies in the results.

The inorganic phosphate content of the native and modified LDL samples was carried out (Section 2.2.1.3) by the method of Fiske and SubbaRow (1925). Prior to the determination, the inorganic phosphate was released from the phospholipids at 150 - 160°C, in the presence of H_2SO_4 and H_2O_2 (Bartlett, 1969; Chen *et al.*, 1956). The final concentration of H_2SO_4 had to be maintained below 0,5 N. Above this concentration the phosphomolybdate colour did not develop. Final concentrations of NaCl and EDTA were 3 mM and 6 µM respectively, and did not affect the determinations. These were, however, corrected for with the blank. Phosphate determination without salt was not possible as dialysis against distilled water resulted in flocculation of the LDL. Thus all LDL samples subjected to the 4,5 hour digestions, prior to phosphate analysis, contained NaCl (0,15 M) and EDTA (0,27 mM). To convert lipid phosphorus to phospholipids, the results obtained (inorganic phosphate) were

multiplied by a factor of 25 (Lee and Alaupovic, 1974), since inorganic phosphates constitute approximately 4% of the phospholipids. The spectrophotometric determination of phospholipids, using malachite green as the colour reagent (Teichman *et al.*, 1974; Itaya and Ui, 1966) gave inconsistent results and was abandoned. The enzymatic determination of phospholipids (Boehringer) was not attempted because the reagents were not available.

The triglyceride content of native and modified LDLs was carried out by the enzymatic method, using the Boehringer kit. Other methods (Witter and Whitner, 1972) were not attempted as they required triglyceride extraction and / or saponification. These were time - consuming and could lead to erroneous results as they entailed quantitative transfers as well.

The results of the above analyses of native and modified LDLs are presented in Table 2.2. The theoretical values were determined, using the following parameters : proteins : 22% of total LDL; cholesterol : 45%; triglycerides : 7,5 - 10%; inorganic phosphates : 0,9%; carbohydrates : 3 - 5% (Goldstein and Brown, 1977; Shore and Shore, 1972).

The isolation of LDL was always accompanied by a loss in lipids, especially cholesterol. Thus a protein : cholesterol ratio of 1 : 1,5 to 1 : 1,6 was a measure of the integrity of the LDL particle (Anderson *et al.*, 1981; Brown and Goldstein, 1976;Brown *et al.*, 1976; Brown and Goldstein, 1975). Ratios below these values indicate

LDL samples	Protein	Cholesterol	Inorganic Phosphate	Triglyceride	Protein : Cholesterol Ratio
native unmodified LDL	100	164,0	4,5 (112,5)	35,0	1 : 1,64
[^{3}H] ECDI - LDL	100	155,9	2,8 (70,25)	33,5	1 : 1,56
[^{3}H] CL - LDL (prepared in DMSO)	100	167,0	4,3 (107,5)	33,9	1 : 1,67
[^{3}H] CL - LDL (prepared without DMSO)	100	151,0	4,6 (115,0)	37,2	1 : 1,51
LDL treated with DMSO	100	151,5	4,8 (120,0)	43,0	1 :1,52
Ac - LDL	100	91,0	4,4 (110,0)	42,6	1 : 0,91
[^{3}H] CL - LDL (prepared enzymatically)	100	124,0	3,6 (90,0)	43,6	1 : 1,24
Theoretical values	100	200,0	4,1 (102,5)	31,8-45,5	1 : 2,00

Table 2.2 : Chemical analysis of native and modified LDLs. Samples containing 100 µg each of LDL protein were used for the determination of cholesterol, inorganic phosphate and triglycerides. All results are expressed in µg and are the average of two or more determinations. Figures in parenthesis are values of phospholipids in the samples, calculated from phosphate determinations.

a considerable loss of cholesterol from the LDL particle, for example, Ac - LDL and [^{3}H] CL - LDL prepared by the enzymatic cholesteryl ester exchange method (Table 2.2). However, it is possible that the peripheral cholesterol, and not the core cholesteryl esters, were lost during LDL isolation, modification and dialysis. There was little loss of triglycerides, which, like the cholesteryl esters, are located in the core of the LDL particle (Cardin *et al.*, 1982; Brown and Goldstein, 1984). The CL - LDL prepared in the presence of DMSO showed a higher protein : cholesterol ratio, as expected, since it was prepared in the presence of excess cholesteryl esters (namely, cholesteryl linoleate). A possible reason for the loss of cholesterol from the enzymically prepared [^{3}H] CL - LDL is the second ultracentrifugation step to recover the labelled LDL. It is possible that the labelled cholesteryl esters adhere to the outer surface of the LDL particles and are `stripped off´ when subjected to ultracentrifugation.

The levels of phospholipids (Table 2.2) were a little higher than the theoretical values except in the case of [^{3}H] ECDI - LDL and [^{3}H] CL - LDL (prepared enzymically). Losses were attributed to prolonged dialysis and the second ultracentrifugation step, respectively. The phospholipids are situated peripherally on the LDL particles and soluble in aqueous medium.

2.4 Concluding remarks

Low density lipoproteins are heterogeneous particles containing proteins (apo B, apo A and apo C), lipids (triglycerides, cholesteryl esters, cholesterol and phospholipids) and carbohydrates (attached to proteins), with molecular weights ranging from 2×10^6 to $3,5 \times 10^6$ daltons. The LDL particles were successfully and rapidly isolated by bouyant density gradient ultracentrifugation, at densities 1,019 to 1,063. LDL was surprisingly easy to modify, notwithstanding its complexity, with respect to its protein (carbodiimide modification, acetylation, EB modification) and its lipid components (acetylation, EB conjugation, CE exchange). Important findings were that extensive dialysis and multiple ultracentrifugations resulted in decreased protein : cholesterol ratios (Table 2.2).

Modification of the LDL particles did not affect their immunogenic properties. All the modified LDLs gave positive precipitin lines The detection of modification in specific regions was not possible by Ouchterlony double diffusion assays, using a polyclonal antiserum.

The modified LDLs showed different electrophoretic mobilities on agarose gels. The carbodiimide - modified LDL was retarded whilst CL - LDL and EB - LDL were slightly more advanced than native LDL. The acetylated LDL showed the highest mobility towards the anode because of its high negative charge, after acetylation. Thus, despite some losses in cholesterol content during modification

the LDL particles maintained biological activity.

Attempts were made to separate the protein and lipid components of LDL. The lipids were successfully extracted in organic solvents and separated by thin layer chromatography to determine whether lipids had been modified. All attempts to isolate and resolubilize the protein component of LDL, without the use of denaturing aqueous solutions, failed. This solubility problem arises from the hydrophobicity of that part of the protein which is embedded in the apolar core of the LDL, which consists of neutral lipids. To date, solubilization of the apoprotein B, without the use of stabilizers (such as starch or albumin) or denaturing solutions has not been documented. However, an aqueous solution of native apoprotein B, without stabilizers or denaturants, was required for the purpose of studying its interaction with DNA, in addition to the interaction of the whole LDL particle with DNA (dealt with in Chapter 3).

C H A P T E R T H R E E

BINDING AND INTERACTION OF NATIVE AND MODIFIED LDL WITH DNA

3.1 Introduction

LDL particles were modified and labelled as described in Chapter 2. The native and modified LDLs were then reacted with either calf thymus DNA or plasmid pBR322 DNA, which had been labelled by nick translation (described in Section 1.14; Maniatis *et al.*,1982; Rigby *et al.*, 1977). The interaction of the LDL particles with DNA was determined by the nitrocellulose filter - binding assay (Hinkle and Chamberlin, 1972; Zubay, 1980; Johnson *et al.*, 1980). The principle behind this assay is that uncomplexed or double - stranded DNA is not retained by the filters. Hence the double - stranded DNA may be retained by nitrocellulose filters if complexed to protein, due to protein retention by nitrocellulose filters. The retention of protein - DNA complexes can be detected if the DNA is radiolabelled.

Binding of DNA was suspected to occur through the lipid portion of the LDL particle. To investigate this, the binding of DNA to individual lipids was also studied. In addition, the type of interaction between LDL particles and DNA molecules was investigated. Such interactions of native and modified LDLs with DNA have not been previously investigated.

3.2 Methods

3.2.1 Preparation of labelled DNA by nick translation

The plasmid pBR322 DNA (99 ng) was nick - translated in a reaction volume of 40 µl, containing 40 µCi [^{3}H] dTTP, 0,05 M Tris - HCl, pH 7,6, 1 mM mercaptoethanol, 10 mM $MgCl_2$, 20,5 µM dATP, 19,5 µM dGTP, 21,5 µM dCTP, 121 pg (2×10^{-4} units) DNase and 10 units DNA polymerase No. 1. The reaction mixture was incubated at room temperature for 40 minutes and nick translation was stopped by the addition of 10µl 0,1 M EDTA, pH 7,7. The volume of reaction mixture was increased to 110 µl by the addition of 60 µl STE buffer (100 mM NaCl - 10 mM Tris - HCl, pH 7,6 - 1 mM EDTA). The total reaction mixture was layered onto a prepared mini - spun column of Sephadex G 50 (70mm x 5 mm I.D.) equilibrated with STE and centrifuged at room temperature in a 15 ml MSE centrifuge tube. The eluant containing the nick - translated pBR322 DNA was collected in a 1,5 ml Eppendorf tube. Two aliquots (2 µl each) were withdrawn to determine the direct count and TCA precipitable count, respectively, of the [^{3}H] pBR322 DNA. The remaining plasmid DNA was stored at -20°C until required.

3.2.2 Binding of plasmid DNA to unmodified LDL

Native (unmodified) LDL was reacted with nick - translated [^{3}H] pBR322 DNA at room temperature for 20 minutes, in STE_1 buffer (0,025 M Tris - HCl, pH 7,6 - 0,05 M NaCl - 0,27 mM EDTA). The concentration of LDL was varied from 0 to 1,0 µg while that of

the plasmid DNA per reaction mixture was kept constant at 18 ng. The formation of the LDL - DNA complex was assayed by the nitro - cellulose filter - binding method. Each reaction mixture was transferred to a 25 mm (diameter) nitrocellulose filter (presoaked in STE_1) and washed with 4 x 500 µl of STE_1, to remove DNA that had not complexed with LDL. The filtration rate was adjusted to approximately 20 µl / second. The filters were air - dried for 30 minutes and oven - dried at 90°C for 15 minutes. The nitrocellulose filters were then placed in counting vials. Beckman HP / b liquid scintillation fluid (5 ml) was added to each vial, and the radio - activity retained on the filters was determined on a Beckman (Model LS - 3150T) liquid scintillation counter.

3.2.3 Interaction of ECDI - LDL with plasmid pBR322 DNA

The carbodiimide modified LDL (prepared as described in Section 2.2.2) was reacted with [^{3}H] pBR322 DNA (preparation : Section 3.2.1) under the same conditions as described for native LDL (Section 3.2.2), except that the ECDI - LDL concentrations utilized were in the range of 0 - 0,1 µg and 0,1 - 1,0 µg . The amount of [^{3}H] pBR322 DNA used per reaction mixture was the same as that for native LDL.

3.2.4 Binding of native LDL and ECDI - LDL to plasmid pBR322 DNA at varying salt concentrations

LDL (0,4 µg) or ECDI - LDL (0,05 µg) was first reacted with 18 ng [^{3}H] pBR322 DNA at room temperature for 20 minutes in a series of Eppendorf tubes. Increasing amounts of NaCl were added to the tubes,

ranging from 0,05 to 0,5 M. The reaction mixtures were incubated for a further 5 minutes and lipoprotein - DNA binding was determined by the nitrocellulose filter - binding assay (Section 3.2.2).

3.2.5 Binding of [^{3}H] pBR322 DNA to LDL and ECDI - LDL treated with phospholipase D

Thirty µg each of LDL and ECDI - LDL were digested with phospholipase D (0,45 units) from *Streptomyces chromofuscus*, in separate reaction vessels containing 0,1 mg / ml Triton X - 100, 50 mM Tris - HCl, pH 8,2, 10 mM $CaCl_2$. The reaction mixtures were incubated at 37°C for 20 minutes. DNA - binding studies were then carried out, using 0 - 1,0 µg of LDL or ECDI - LDL in STE_1. Incubation of the phospholipase D - treated lipoproteins with DNA was then carried out at 20°C. Lipoprotein - DNA binding was determined by the nitrocellulose filter - binding assay (Section 3.2.2). Phosphatidylcholine di - palmitoyl and a carbodiimide - modified protein, CDI - transferrin, were also digested with phospholipase D under the same conditions, as a check for phospholipase D activity.

3.2.6 Binding of [^{3}H] pBR322 DNA to acetylated LDL (Ac - LDL)

The assay was the same as for native LDL (Section 3.2.2), using a range of 0 - 10 µg Ac - LDL (protein content), and a constant concentration (18 ng) of the plasmid DNA. Results are presented in figure 3.5.

3.2.7 Binding of DNA to ethidium bromide - LDL conjugate (EB - LDL)

The EB - LDL conjugate was prepared as described in Section 2.2.4. The EB - LDL (100 µg) was reacted with 18 ng [^{3}H] pBR322 DNA, which was isotope - diluted with 0,3 µg of unlabelled pBR322 DNA, at room temperature for 20 minutes. The reaction mixture was layered onto a 5 - 20% continuous sucrose gradient containing 0,1 M NaCl - 0,05 M Tris - HCl, pH 7,5 - 0,005 M EDTA. Ultracentrifugation was carried out at 18°C for 4,5 hours in an SW 50 rotor at 45 000 rpm. Fractions were drop - collected by piercing the bottom of the tubes. The optical density of each fraction was determined at 280 nm. The radioactivity of each fraction was also determined. Complex - formation between EB - LDL (200 µg LDL protein) and DNA (100 µg calf thymus DNA) was also analysed by glycine - sucrose density gradient ultracentrifugation. A 5 - 20% continuous gradient was prepared in glycine - NaOH, pH 8,8 - 0,005 M EDTA - 0,025 M NaCl. Ultracentrifugation was carried out as above, but for a shorter time (3 hours). Fractions were collected as before. Since the calf thymus DNA was not labelled, the optical density of each fraction was determined at 280 nm and at 260 nm.

3.2.8 Binding of [^{3}H] pBR322 DNA to cholesteryl linoleoyl LDL

To [^{3}H] CL - LDL prepared by the DMSO method and the cholesteryl ester exchange method (Sections 2.2.5.3 and 2.2.5.4, respectively), in 0,025 M Tris - HCl, pH 7,6 - 0,05 M NaCl - 0,27 mM EDTA, was added a constant amount (18 ng) of [^{3}H] pBR322 DNA. Incubation

was carried out at room temperature for 20 minutes. The binding of the DNA was assayed by the nitrocellulose filter - binding method (Section 3.2.2).

3.2.8.1 Binding of [^{3}H] pBR322 DNA to LDL treated with increasing amounts of unlabelled cholesteryl linoleate in DMSO

Cholesteryl linoleate (0 - 180 ng) in $CHCl_3$ was evaporated to dryness in each reaction tube and redissolved in 2,5 µl DMSO by vortexing. To the cholesteryl linoleate was added a constant amount (1 µg) of LDL in STE_1 buffer (Section 3.2.2). The reaction mixtures were incubated at 37°C for 1 hour. [^{3}H] pBR322 DNA was then added to each reaction mixture and incubated at room temperature for 20 minutes. The binding of the DNA to the cholesteryl linoleate - treated LDL was assayed by the nitrocellulose filter - binding method (Section 3.2.2).

3.2.9 Binding of [^{3}H] pBR322 DNA to lipid - impregnated filters

Whatman No.1 filter discs (15 mm diameter) were impregnated with different lipids, 100 µg each, dissolved in 10 µl $CHCl_3$, according to Ariatti *et al.*, 1986. The blank consisted of a filter without lipids. A chloroform - impregnated filter was used as a control. The filters were air - dried for 1 hour at room temperature to ensure that all traces of solvent ($CHCl_3$) had been removed. Each filter was placed in a clean glass vial and overlaid with a 100 µl solution of [^{3}H] pBR322 DNA (9 ng each) in STE_1. The vial lids were replaced to ensure that a moist chamber had formed, for the

binding of the DNA to the lipids, The reaction vessels were incubated for 18 hours at room temperature. Each disc was placed on a separate 25 mm nitrocellulose filter, in a Millipore filtration apparatus and washed with 5 x 3 ml aliquots of STE_1. The Whatman filter discs were air - dried for 30 minutes and oven - dried for 15 minutes at 90°C. The radioactivity retained on each lipid - impregnated Whatman filter disc was then determined.

3.3 Results and Discussion

3.3.1 Nick translation of plasmid pBR322 DNA

The mini - spun column used for the purification of [^{3}H] pBR322 DNA was prepared according to the procedure of Maniatis *et al.*(1982). The [^{3}H] dTTP was evaporated to dryness and then redissolved in 6,5 µl distilled water. The incubation time employed for the nick - translation was found to be optimum. An increase in time did not significantly increase the extent of labelling of the plasmid DNA. The direct counts were in the region of 200 000 cpm while the TCA - precipitable counts were between 140 000 cpm and 180 000 cpm / 36 ng. A lower TCA - precipitable count was expected as losses are expected to occur during filtration. Also, any [^{3}H] dTTP present in the nick - translated DNA preparation would be removed when filters were washed.

3.3.2 Binding of native LDL and ECDI - LDL to [^{3}H] pBR322 DNA

There was a definite increase in binding of [^{3}H] pBR322 DNA with

increasing amounts of LDL (Figure 3.1). Subsequent assays showed improved binding and reached saturation at an LDL concentration of 0,4 µg (Figure 3.2). In contrast, the binding of ECDI - LDL to DNA in the same range (i.e. 0,1 - 1,0 µg protein content) showed a decrease in the amount of [^{3}H] pBR322 DNA bound to increasing amounts of ECDI - LDL (Figure 3.2). However, there was increased DNA binding when ECDI - LDL was used in the range of 0 - 0,1 µg LDL protein content (Figure 3.3), with saturation at 0,05 µg (50 ng) of ECDI - LDL. Thus ECDI - LDL has a 10 - fold higher affinity for the DNA than unmodified (native) LDL.

3.3.3 Studies on the binding and interaction of DNA with LDL and carbodiimide - modified LDL particles at varying salt concentrations

Since LDL and ECDI - LDL binding of [^{3}H] pBR322 DNA reached saturation at 0,4 µg and 0,05 µg LDL protein content, respectively, these amounts of native and carbodiimide - modified LDL were reacted with constant concentrations of [^{3}H] pBR322 DNA (18 ng) and subsequently treated with increasing amounts of NaCl. Since the LDL - DNA complexes bind to the nitrocellulose filters, while uncomplexed, double - stranded DNA passes through (Hinkle and Chamberlin, 1972), the amount of radioactivity retained on the filters is an indication of the lipoprotein - DNA complex formation, as proteins are re - tained on the filters. Maximum binding of LDL to [^{3}H] pBR322 occurred between 0,05 - 0,1 M NaCl (Figure 3.4), whilst that of ECDI - LDL with the DNA occurred at 0,2 M NaCl. Above these concentrations of NaCl there was a rapid `fall off ´ of radioactivity, indicating

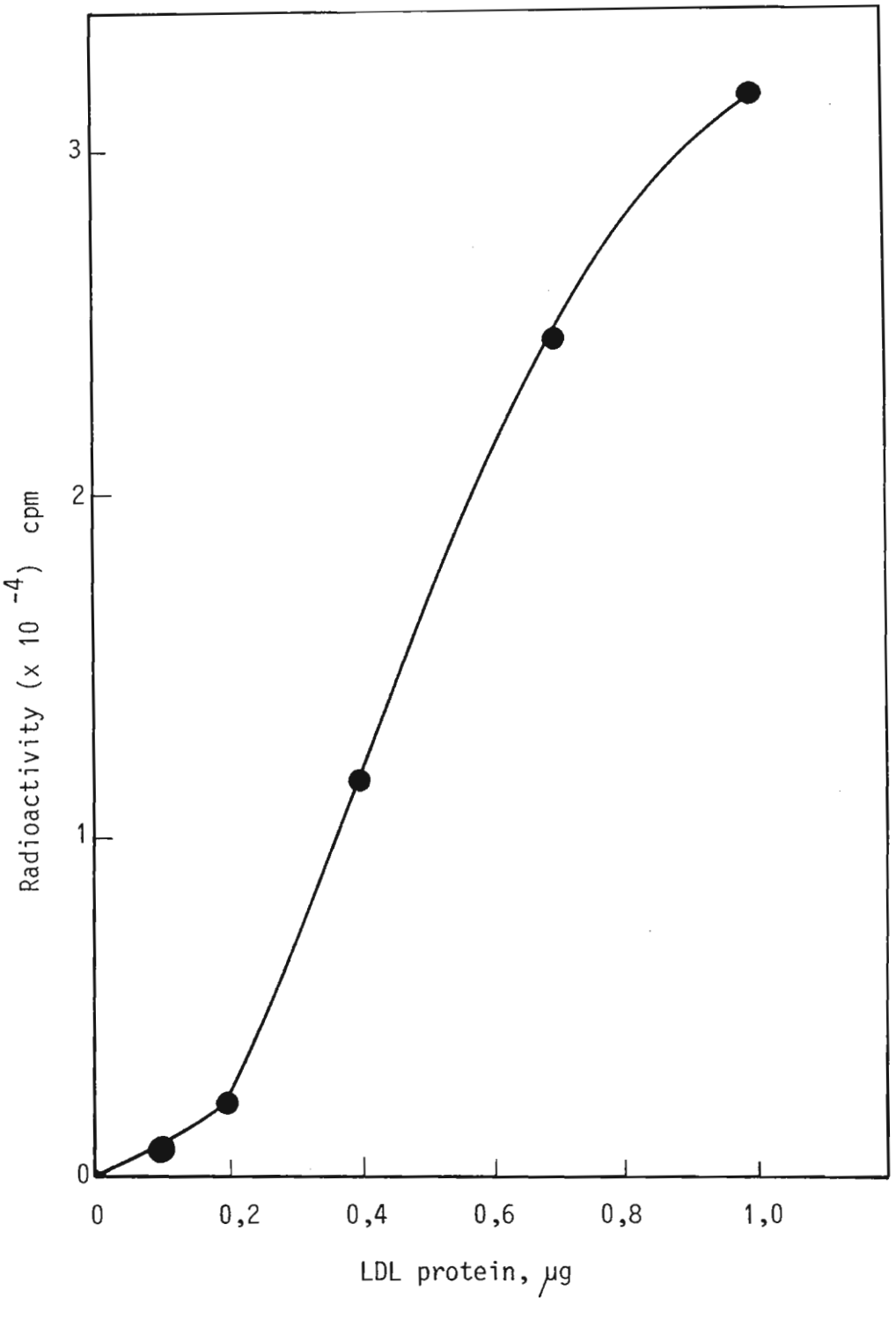


Figure 3.1 Binding of [^{3}H] pBR322 DNA to native LDL.

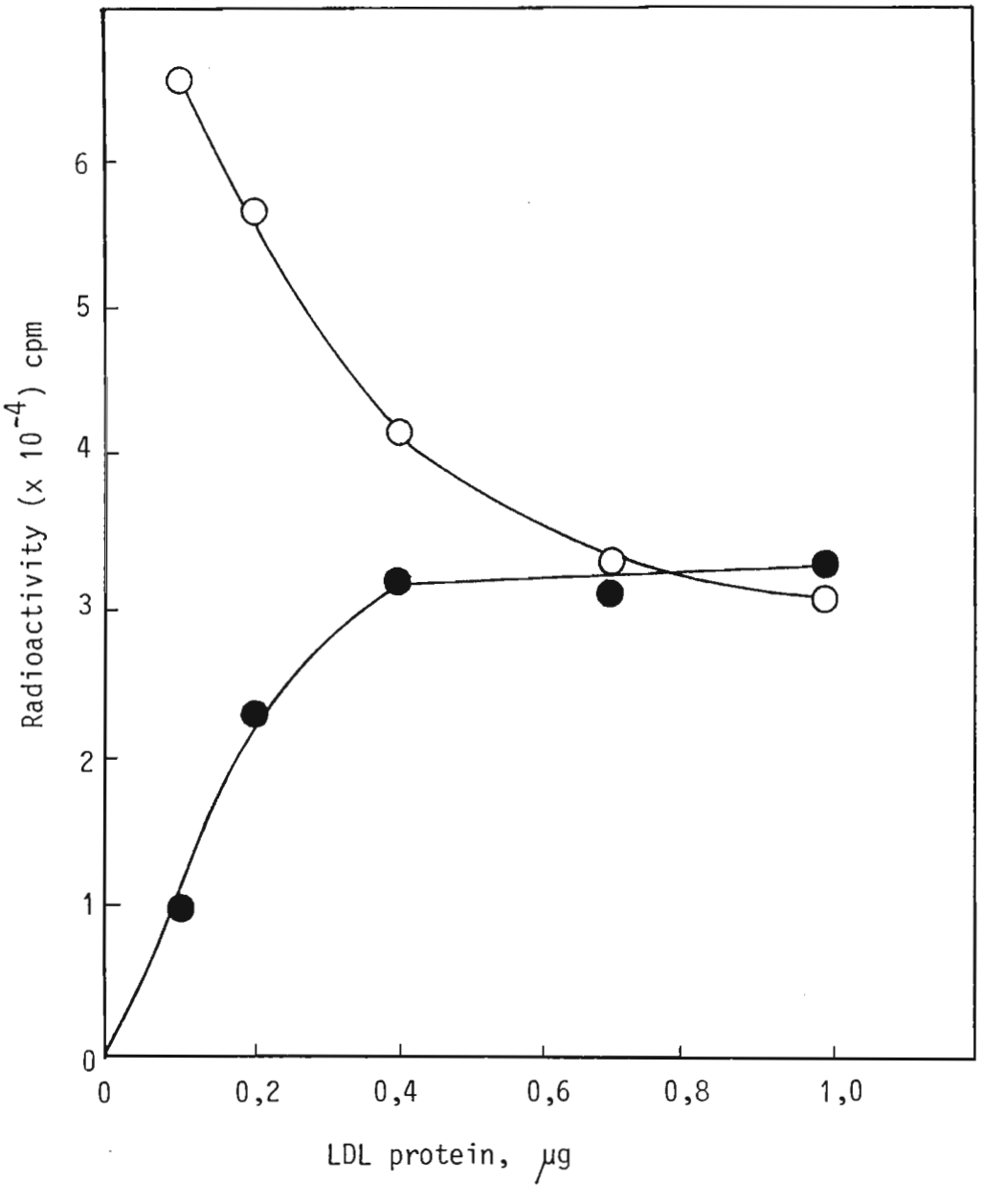


Figure 3.2 : Binding of LDL (●—●) and ECDI - LDL (○—○) to [^{3}H] pBR322 DNA at a range of 0,1 - 1,0 LDL protein concentration.

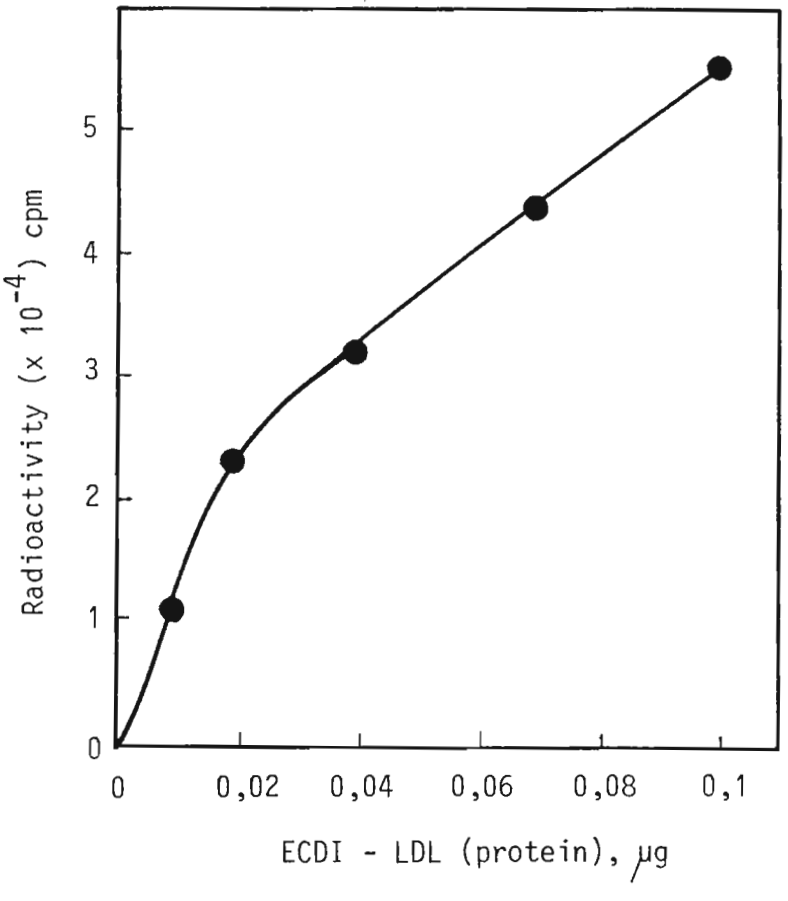


Figure 3.3 : Binding of [^{3}H] pBR322 DNA to ECDI - LDL at a concentration range of 0 - 0,1 µg of protein. ECDI - LDL showed a 10 - fold greater affinity for the DNA than did the native LDL.

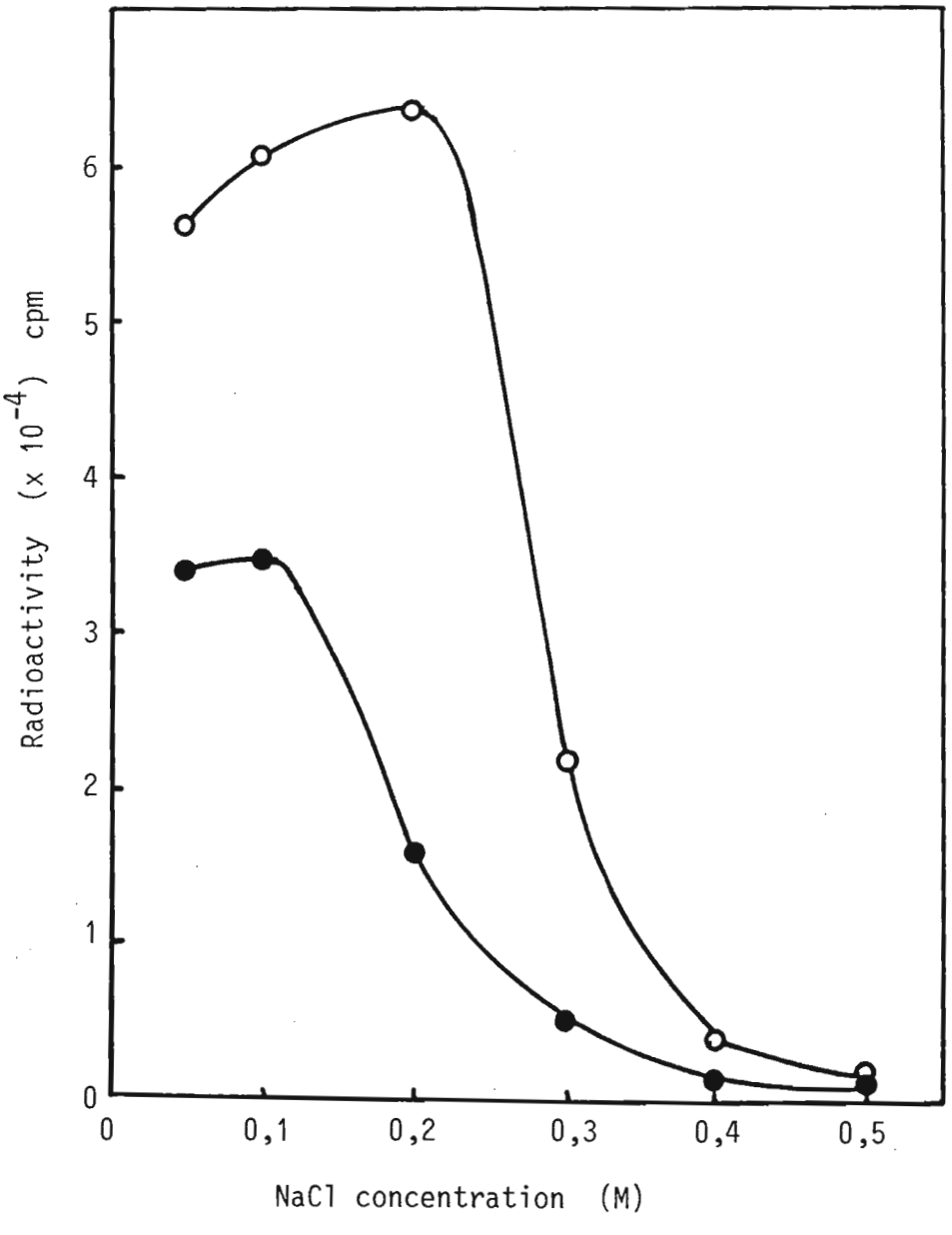


Figure 3.4 : Binding of native LDL (●) and ECDI - LDL (○) to [^{3}H] pBR322 DNA at increasing salt concentrations.

that there was less binding at higher salt concentrations. Binding of LDL or ECDI - LDL with [^{3}H] pBR322 DNA is therefore electrostatic at low salt concentrations. As the salt concentration was increased, the electrostatic attraction between the lipoproteins and the DNA was disrupted, causing dissociation of the lipoprotein - DNA complexes and hence a decreased retention on nitrocellulose filters. Since the binding of DNA to LDL and ECDI - LDL has been shown to be electrostatic, such attractions must occur between the negative phosphate groups of DNA and the positively - charged quaternary nitrogen groups of the phospholipids on the lipoproteins (as described in Chapter 1, Figure 1.17). Higher affinity of the DNA for ECDI - LDL must therefore be due to the additional positively - charged quaternary amino groups introduced into the protein moiety, by the carbodiimide modification of the LDL (Figure 1.18).

3.3.3.1 Phospholipase D digestion of ECDI - LDL

The peripheral lipids of native or unmodified LDL probably interact with the negatively - charged phosphate groups of DNA as illustrated in Figure 1.17. Carbodiimide modification of LDL results in the acquisition of additional quaternary amino groups by the protein moiety of LDL and hence a higher affinity of the DNA for the ECDI - LDL is attained (Figures 3.2 and 3.3). To support the contention that increased affinity was indeed due to the carbodiimide modification, ECDI - LDL was subjected to digestion with phospholipase D, which removed the N - trimethylpropylammonium groups from the N - acyl - ureas (Figure 3.5 a). The activity of phospholipase D was confirmed

as described in Sections 2.3.2.2 and 2.3.2.3. Phospholipase D - digested ECDI - LDL showed reduced affinity for DNA (Figure 3.5a) at lower concentrations (0 - 0,6 µg). The same was found to be true for the unmodified phospholipase D - digested LDL. This reduced affinity could be attributed to the removal of the positive - ly charged quaternary nitrogen groups, which reduced the electro - static attraction between the DNA and the lipoproteins (Figure 3.5b). The affinity for both lipoproteins was increased at concentrations greater than 0,6 µg protein content. This could be attributed to transition to a different type of interaction between the DNA and the lipoproteins. This latter interaction was probably non - electrostatic and due to the exposed fatty acyl groups of the phospholipase D - digested lipids. This hypothesis was investigated further and is discussed in Section 3.3.7.

3.3.4 Binding assay of [^{3}H] pBR322 DNA to acetylated LDL

The binding of the plasmid DNA to acetylated LDL (Ac - LDL) was compared with that of native LDL, and assayed by the nitrocellulose filter binding method. Since LDL was extensively acetylated, it was expected to have a lower affinity for the DNA. It has been reported that acetylation of LDL alters the particles drastically, such that the acetylated LDL is no longer recognized by the LDL receptors of fibroblasts (Goldstein *et al.*, 1979; Basu *et al.*, 1976). However, receptor recognition sites on the LDL occur on the protein moiety, therefore non - recognition must be due to N - acetylation of lysine residues (ε-amino groups; Figure 3.6 b).

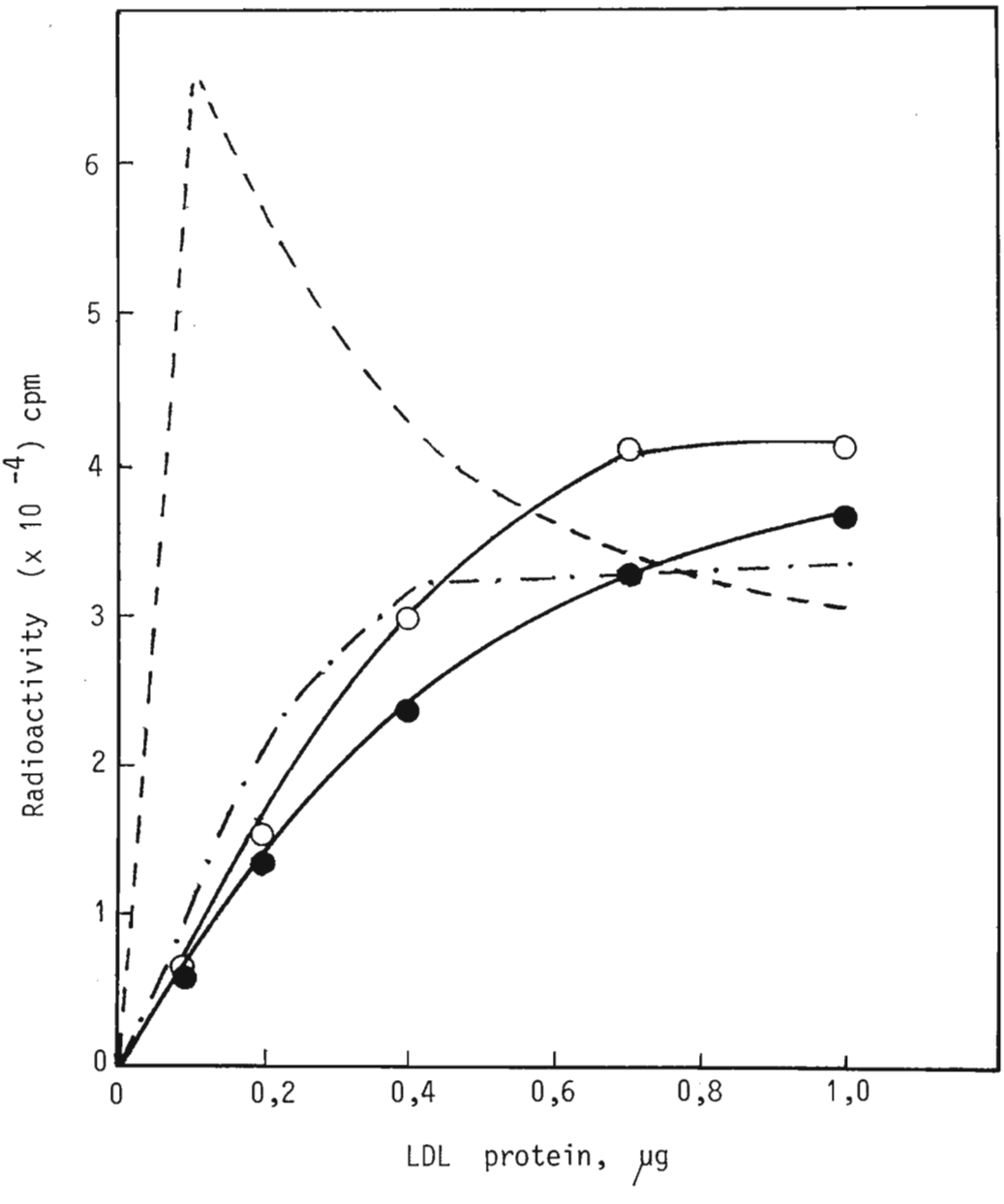
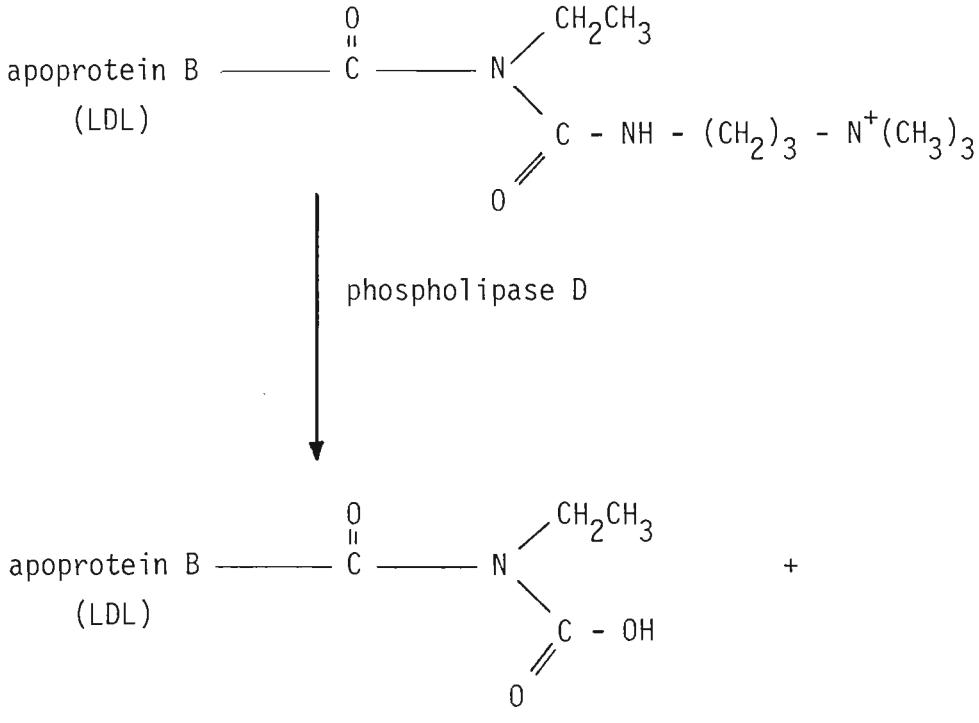


Figure 3.5 a : Binding of [^{3}H] pBR322 DNA to phospholipase D - treated LDL (●) and phospholipase D - treated ECDI - LDL (○). The binding of ECDI - LDL to DNA (----) was much higher at lower concentrations of the carbodiimide modified LDL. The binding of untreated LDL to DNA (-•-•-) is also indicated.



$H_2N - (CH_2)_3 - N^+(CH_3)_3$

N,N,N - trimethylaminopropylamine

Figure 3.5b : Possible mechanism of phospholipase D digestion of carbodiimide - modified LDL protein

In contrast, the reduced affinity of Ac - LDL for DNA (Figure 3.6 a) is probably due to quaternary amino - acetyl group interactions (Figure 3.6 b). Hence the acetylation of LDL effectively reduces the availability of positively - charged quaternary nitrogens which can react with the negatively - charged phosphate groups of DNA (Figure 3.6 b III). However, the small amount of Ac - LDL - DNA binding could possibly be due to the interaction of hydrophobic fatty acyl groups of the phospholipids with DNA.

3.3.5 Binding of DNA to EB - LDL conjugate

The Tris - sucrose density gradient analysis of EB - LDL - DNA complex formation at pH 7,5 is presented in Figure 3.7. The OD_{280} peak indicates the presence of LDL protein (curve 3). However, the radioactivity of these fractions (fractions 29 - 31) was negligible. Most of the radioactivity (curve 2), matched that of uncomplexed [^{3}H] pBR322 DNA (curve 1). Results appear to indicate that the formation of EB - LDL - DNA was negligible. The complex formation was subsequently analysed by glycine - sucrose density gradient ultracentrifugation at pH 8,8 (Figure 3.8). Curves A (1) and A (2) are density gradient fractionation profiles of EB - LDL and ct - DNA, respectively. Curves B (1) and B (2) indicate the formation of EB - LDL - DNA complex in much higher yield than that of the previous experiment (Figure 3.7). A possible reason for this may be the higher amount of DNA utilized for complex formation, or the difference in pH. High OD_{260} readings obtained from the bottom fractions (Curve B 1) were due to fragmented DNA. High OD_{280}

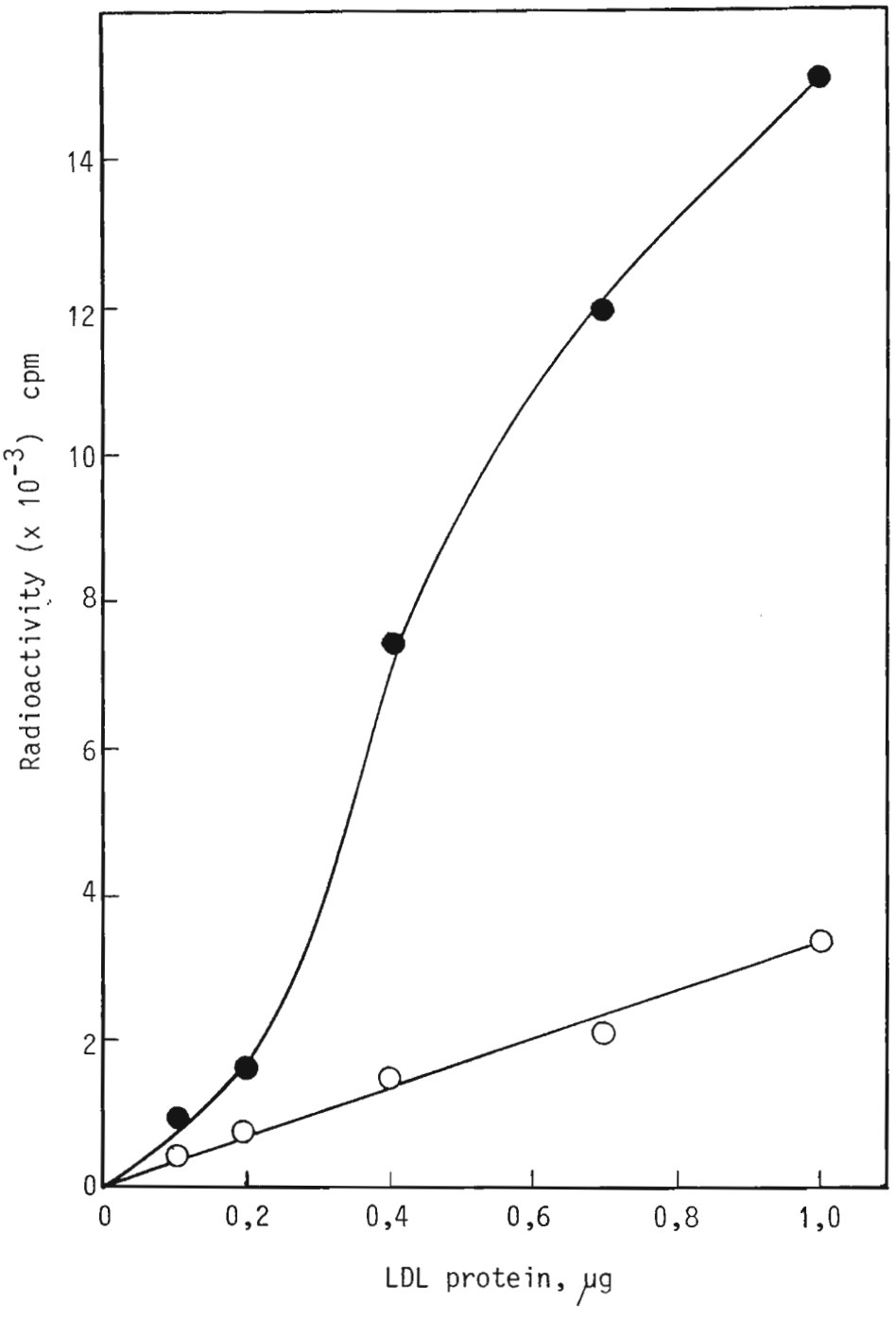


Figure 3.6 a : Binding of [^{3}H] pBR322 DNA to native LDL (●) and acetylated LDL (○).

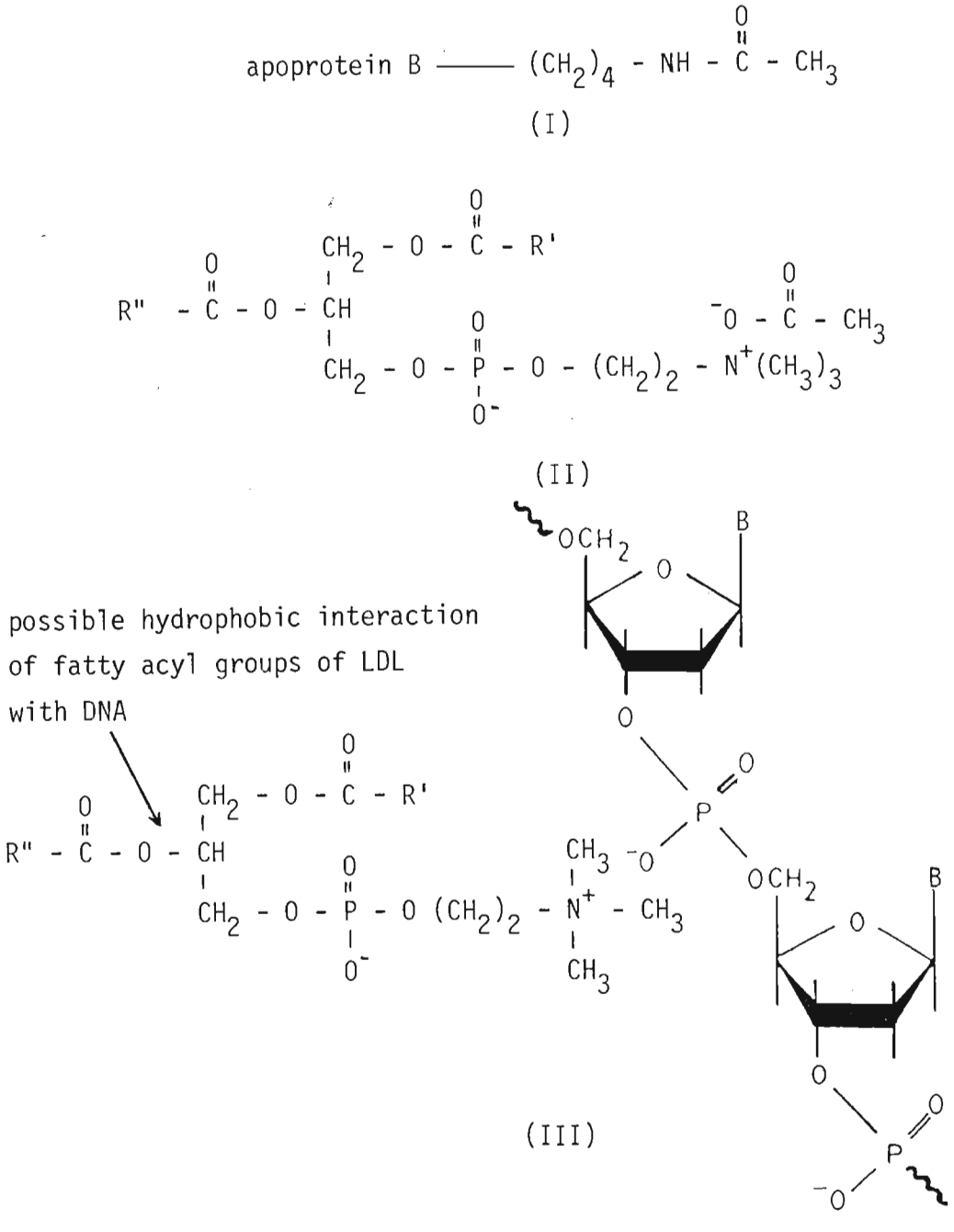


Figure 3.6 b : Acetylation of LDL modifies lysine residues of apo - protein B as shown (I). Since the major peripheral lipid components of LDL are phospholipids, these can possibly react with acetate ions as illustrated in (II). This would eliminate the interaction of the phospholipid quaternary nitrogen with DNA (III).

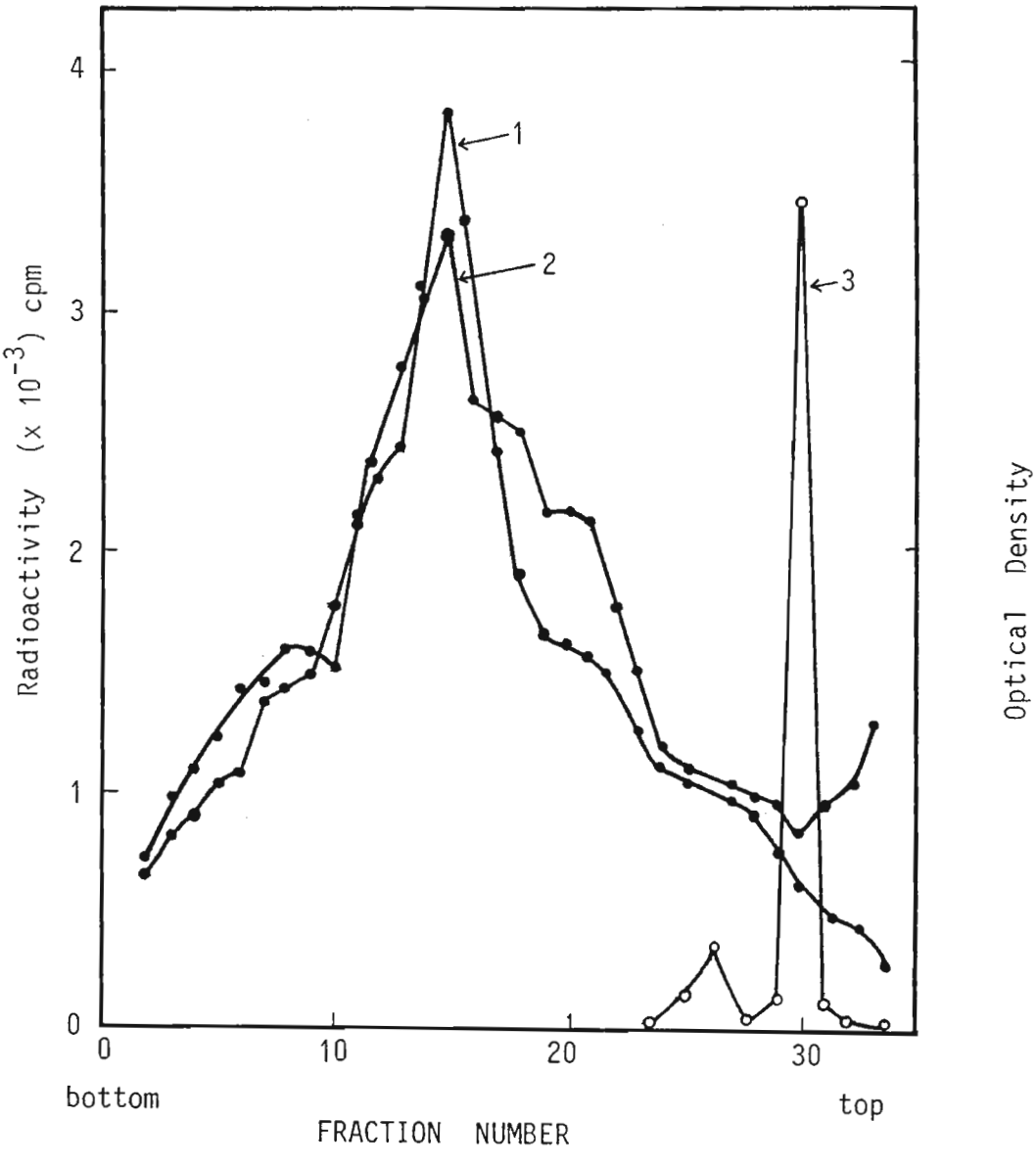


Figure 3.7 : Tris - sucrose density gradient profiles of EB - LDL + pBR322. 1 : pBR322 DNA (cpm); 2 : EB - LDL + pBR322 (cpm); 3 : EB - LDL + pBR322 DNA (OD_{280}).

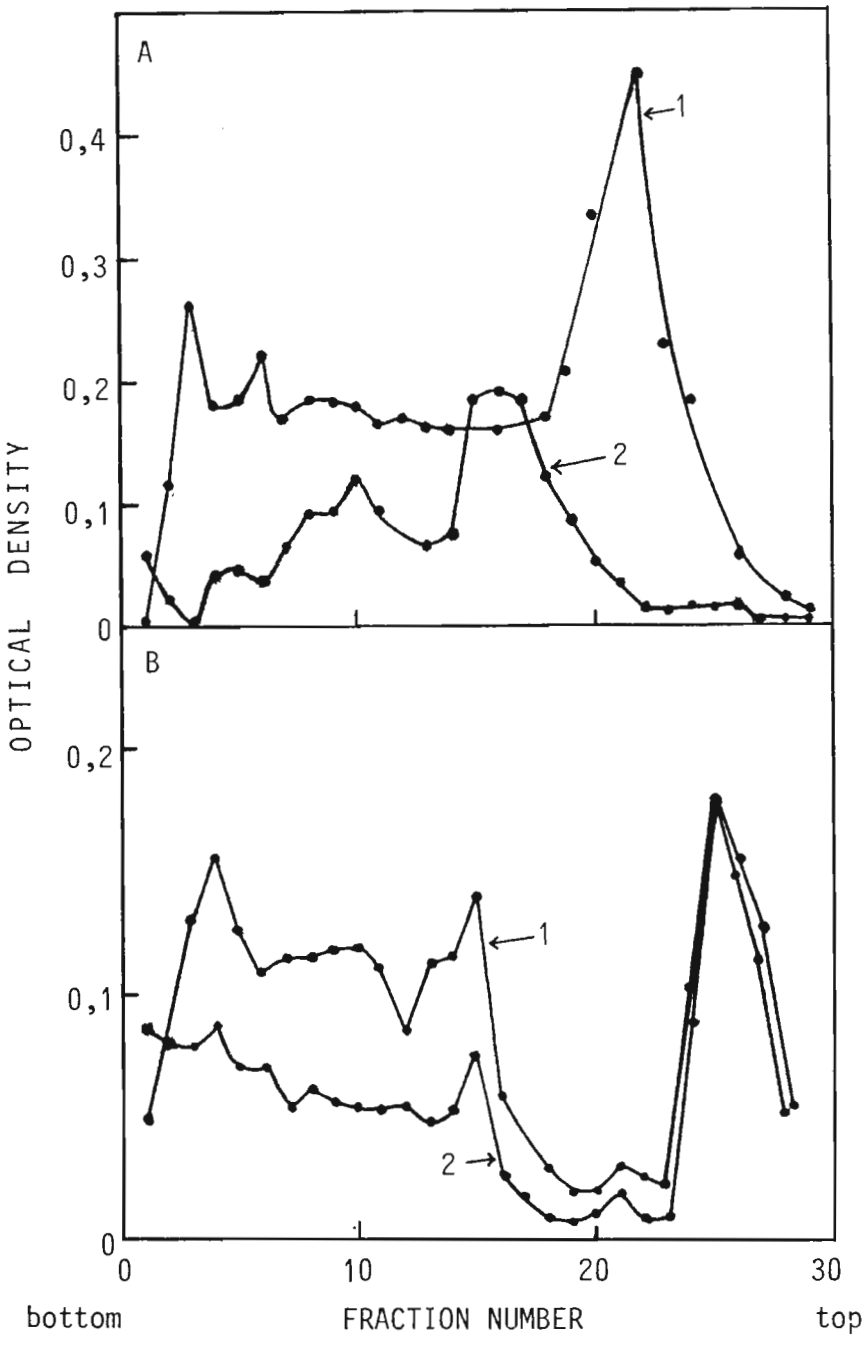


Figure 3.8 : Glycine-sucrose density gradient analysis of EB-LDL and EB-LDL-ct DNA reaction mixtures. A1: EB-LDL (OD_{280}); A2: ct DNA (OD_{260}); B1: EB-LDL-ct DNA (OD_{280}); B2: EB-LDL-ctDNA (OD_{280}).

readings of bottom fractions (Curve A 1) is likely to be denatured protein components of the LDL particles.

Conjugation of the EB - LDL to the DNA occurs by the intercalation of the EB phenanthridium moiety to DNA (Le Pecq, 1971; Berman and Neidle, 1979; Alden and Kim, 1979; Ariatti *et al.*,1986; Hawtrey *et al.*, 1986). This intercalation occurs at hydrophobic sites (Le Pecq, 1979) at the low ionic strengths employed in the experiments. A possible mechanism for the intercalation of ethidium bromide - LDL complex to DNA is presented in Figure 3.9.

3.3.6 Binding of [^{3}H] CL - LDL to pBR322 DNA

There was no binding of [^{3}H] pBR322 DNA to [^{3}H] CL - LDL prepared in the presence of DMSO (Section 2.2.5.3). This was probably due to the use of DMSO (final concentration 2,5%) in the preparation of the modified LDL. DMSO is known to make lipid bilayers of cell membranes more permeable. DMSO probably disrupts the LDL structure, thus allowing an exchange of cholesteryl esters, but also allowing a substantial loss of core lipids.

DNA has been shown to react with the intact, unmodified LDL by an ionic interaction (Section 3.3.3), probably between the negatively - charged phosphate groups of DNA and positively - charged quaternary nitrogens of peripheral lipids, namely phosphatidylcholine and

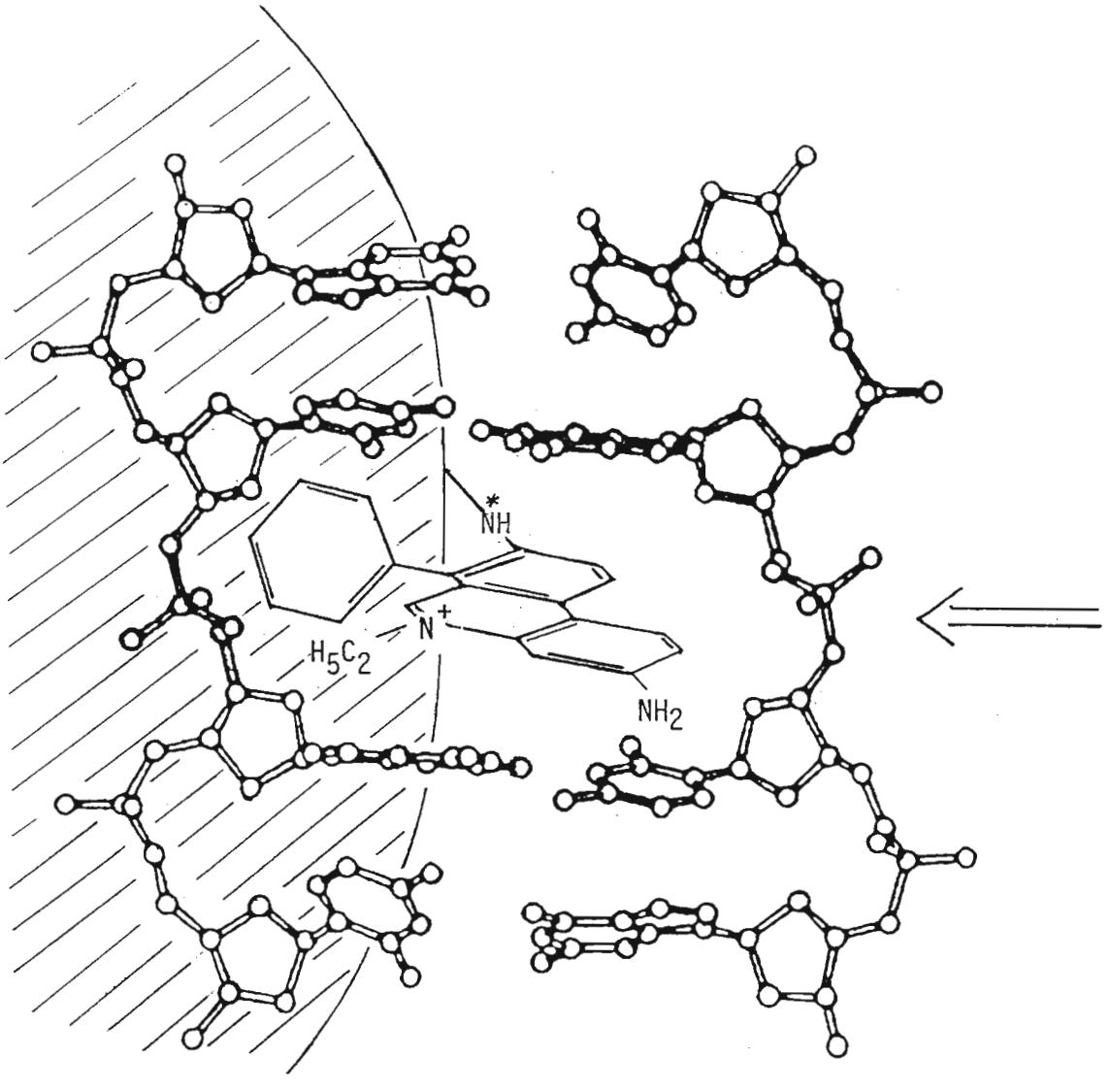
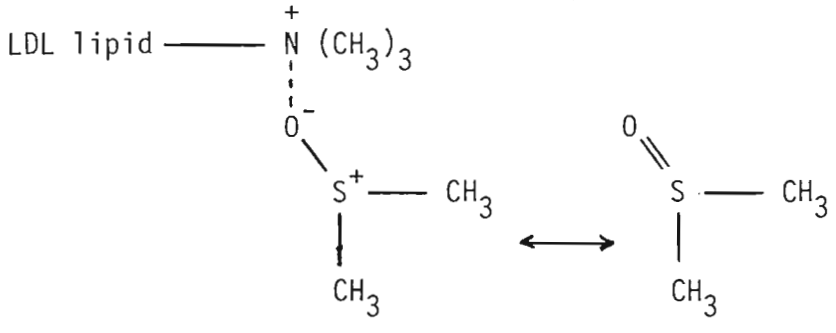


Figure 3.9 : Possible model for the intercalation of EB (in EB - LDL) to double - stranded DNA. A part of the DNA is presented as a `ball and stick model´ to differentiate it from the EB (solid lines). The sequence of the double stranded DNA is $5'$ - GpCpGpCp - $3'$. The phenanthridium moiety is perpendicular to the phenyl ring and intercalates with the DNA as if it were a base - pair. The attachment of the EB to the LDL (▨) occurs via one of its reactive amino groups (*), as described in Section 2.3.4. The DNA unwinds partially at the intercalation site (arrow) to accomodate the EB.

sphingomyelin. However, if the quaternary amino groups become involved in interaction with the electronegative oxygen atoms of DMSO, as shown below, the interaction of the modified LDL with DNA might possibly be eliminated.



The binding of [^{3}H] pBR322 DNA to [^{3}H] CL - LDL prepared by the cholesteryl ester exchange method (Section 2.2.5.4) was successful. Results are presented in Figure 3.10, after correcting for the radioactivity due to the [^{3}H] cholesteryl linoleate in the modified LDL. Maximum binding occurred at 7 μg LDL protein content. The binding from 0 - 2 μg was linear, followed by a sharp increase in affinity at higher concentrations.

A further DNA binding study was carried out with native LDL treated with increasing amounts of cholesteryl linoleate in DMSO (Section 3.2.6.1). There was increased binding of [^{3}H] pBR322 DNA with increasing amounts of [^{3}H] cholesteryl linoleate added (Figure 3.11), reaching a maximum at 130 ng cholesteryl linoleate. Thus the effects of DMSO on the DNA - binding ability of LDL particles is not clear.

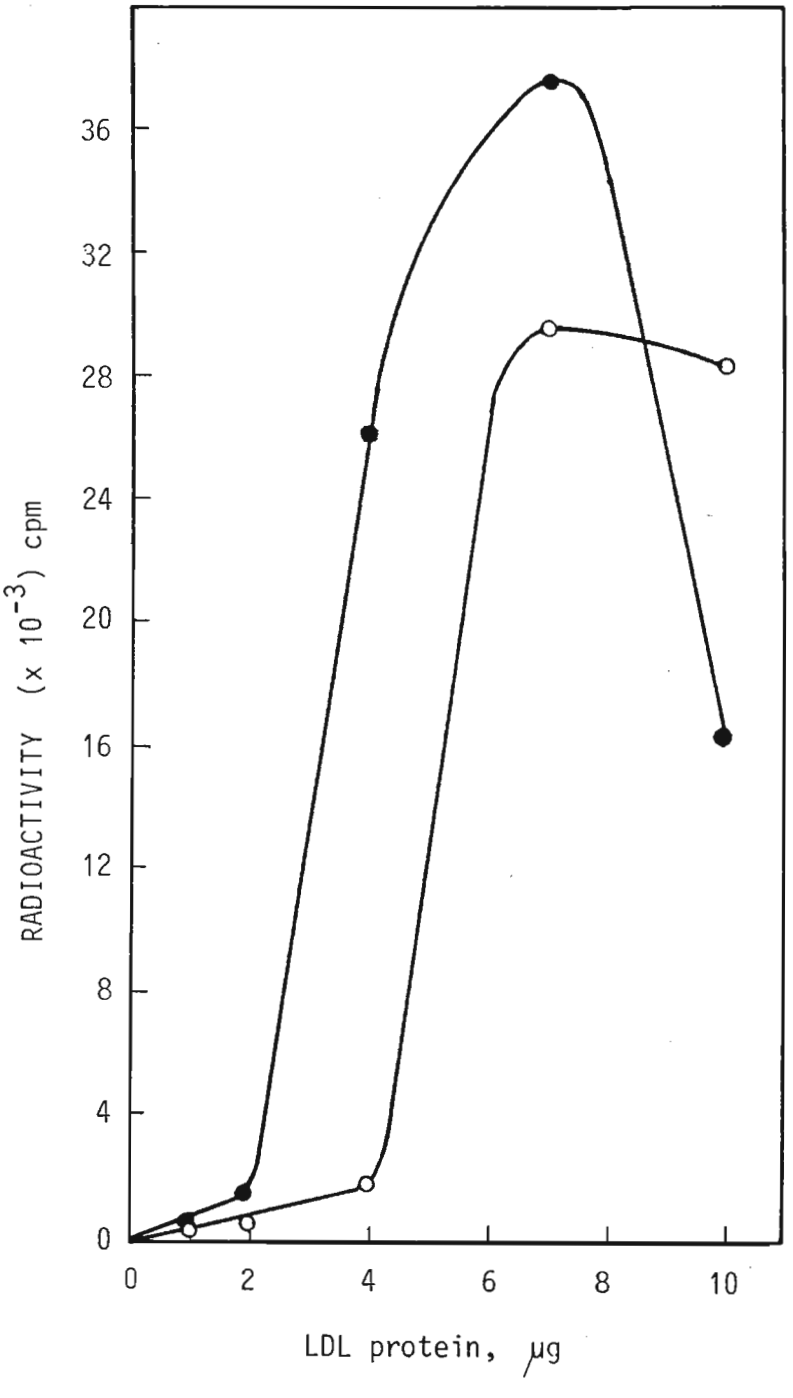


Figure 3.10 : Binding of native LDL (○) and [^{3}H] CL - LDL (●) to [^{3}H] pBR322 DNA. Results presented here have been corrected for the activity due to the [^{3}H] CL-LDL.

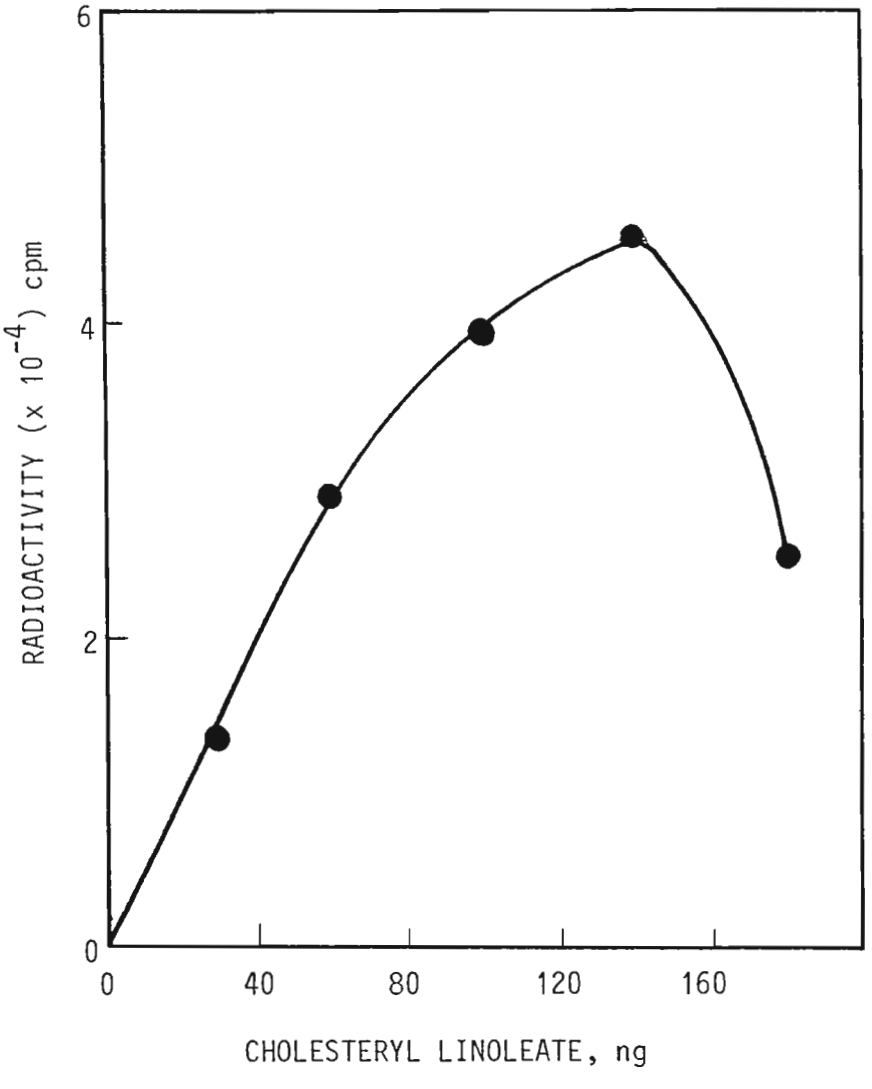


Figure 3.11 : Binding of [^{3}H] pBR322 DNA to LDL treated with increasing amounts of cholesteryl linoleate in DMSO.

Modifying the LDL particle with cholesteryl linoleate does enhance its affinity for DNA to a small extent, but it also provides a method of labelling the LDL particle. Such a modification does not alter the immunological properties of LDL (Figure 2.21), nor does it affect the receptor binding properties of the LDL (Faust *et al.*, 1977; Brown *et al.*, 1975; Roberts *et al.*, 1985) but it has been found to enhance its affinity for DNA to a small extent (Figures 3.10 and 3.11). This increased affinity may be attributed to the adsorption of the cholesteryl linoleate to the exterior of the LDL particle, and has been investigated further (Section 3.3.7).

3.3.7 Interaction of [^{3}H] pBR322 DNA with individual lipids

To study further the interaction between pBR322 DNA and lipid components of LDL, the binding of [^{3}H] pBR322 DNA to individual lipids on Whatman filters was carried out (Section 3.2.9). Results presented in Table 3.1 showed that the DNA binds to phospholipids. (The LDL particle consists of peripheral phospholipids, which interact with DNA.) It was also found that cholesteryl esters interacted with DNA. The triglycerides, however, showed a far greater affinity for the DNA than did the other lipids. Since the peripherally situated lipids present polar head groups to the aqueous exterior, in the intact LDL, DNA interaction will occur with the hydrophilic portions of these peripheral lipids (as discussed in Section 1.16). Although cholesteryl esters and triglycerides have shown a far greater affinity than the phospho - lipids have, these lipids are located in the interior (core) of

LIPID	cpm / micromole
phosphatidylcholine dilinoleoyl	19 894
phosphatidylcholine dioleoyl	3 947
phosphatidylcholine dipalmitoyl	4 024
phosphatidylcholine distearoyl	17 929
cholesteryl linoleate	27 338
cholesteryl palmitate	22 406
cholesteryl oleate	30 292
cholesterol	616
trilinolein	32 593
triolein	12 224
tripalmitin	47 076
tristearin	63 448

Table 3.1 : The binding of [^{3}H] pBR322 DNA to filters impregnated with lipid. Results are averages of duplicate studies.

the LDL. Thus, due to their hydrophobicity and interior location in LDL, these lipids cannot participate in interactions with the DNA.

Of considerable interest is the finding that the cholesteryl esters showed a greater affinity for DNA than the phospholipids. This lends support to the proposal that modification of LDL with cholesteryl linoleate results in peripheral or surface adsorption of the cholesteryl esters, subsequently enhancing the affinity of the LDL particles for the DNA (Section 3.3.6).

Since cholesterol bound DNA very poorly, the affinity of DNA for cholesteryl esters must be due to hydrophobic interactions between the DNA and the fatty acid chains of cholesteryl esters. To support this hypothesis, further DNA - lipid binding experiments were carried out, using increasing amounts of cholesteryl esters or triglycerides (both lipids contain fatty acid chains). Results in Figure 3.12 show that binding to DNA was highest with triglycerides (tristearin), which contain three fatty acid chains, compared to phospholipids, which contain two fatty acid chains per molecule. Even the straight chain hydrocarbons, hexadecane and octadecane, showed an affinity for DNA. This indicates that DNA participates in hydrophobic interactions with fatty acids and straight chain paraffins. Results appear to indicate that the degree of binding of lipids to DNA could possibly be related to the degree of unsaturation of the fatty acid chains.

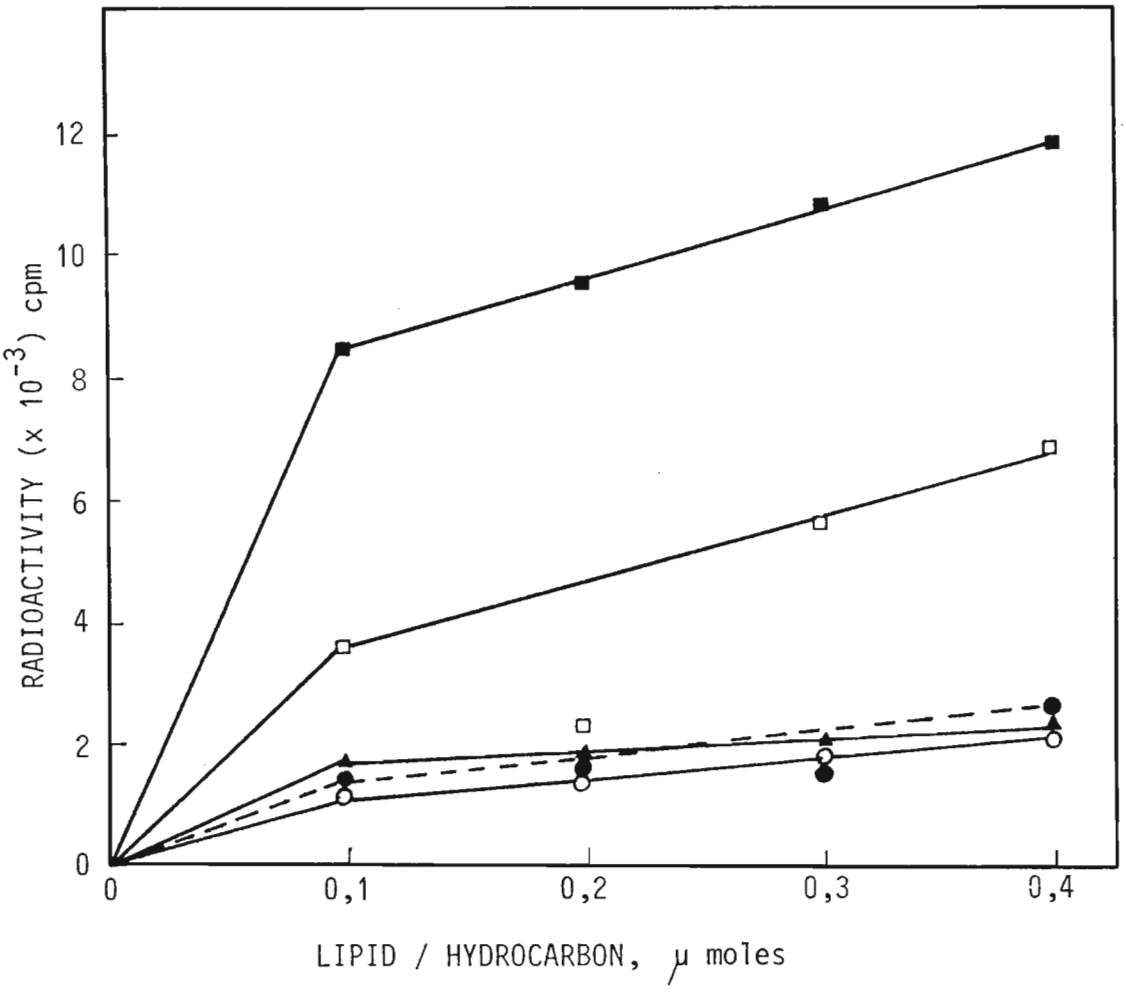


Figure 3.12 ; Binding of [^{3}H] pBR322 DNA to lipid - / hydrocarbon - impregnated Whatman No.1 filter discs. Exactly 9 ng DNA was applied to each disc. The lipid or hydrocarbon used were tristearin (■), phosphatidyl - choline distearoyl (□), phosphatidylcholine di- palmitoyl (●), octadecane (○) and hexadecane (▲).

3.4 Concluding remarks

The carbodiimide - modified LDL showed a 10 - fold greater affinity for [^{3}H] pBR322 DNA than native LDL. However, both lipoproteins showed a similar electrostatic interaction with DNA. This interaction, in the case of native LDL, is presumed to be between the positively - charged quaternary nitrogen groups of the peripheral phospholipids of LDL and the plasmid DNA.

Carbodiimide modification of LDL confers additional positive charges in the form of trimethylpropylammonium groups, which also contain quaternary nitrogens. The interaction between ECDI - LDL and DNA is also electrostatic, but additional affinity for the DNA is attributed to the trimethylpropylammonium groups. The electrostatic attraction between the lipoproteins and DNA is greater at low salt concentrations (Figure 3.4), that is, between 0,1 and 0,25 M NaCl; and is destroyed at higher salt concentrations.

The removal of trimethylpropylammonium residues with phospholipase D suggests that the higher affinity of ECDI - LDL for DNA was due to its carbodiimide modification, since removal of this group yielded ECDI - LDL - DNA binding curves which were almost equivalent to those of native LDL - DNA.

Acetylated LDL showed little affinity for DNA as it was extensively modified, with respect to its protein and lipid components (discussed in Chapter 2). Acetylation is likely to remove the positive charges

on the LDL and thus reduce the electrostatic attraction between LDL and DNA.

Of the two methods of modification of LDL with cholesteryl linoleate, the chemically modified LDL (Section 2.2.5.3) showed negligible affinity for the DNA (pBR322). The DMSO possibly interacts with positively charged phospholipid groups thus preventing their association with DNA. However, the [^{3}H] cholesteryl linoleoyl LDL prepared by the enzymatic cholesteryl ester exchange method (Section 2.2.5.4) showed significant binding of pBR322 DNA, as assayed by the nitrocellulose filter binding method (Figure 3.10).

LDL was also modified by conjugation with ethidium bromide. Its affinity for DNA was assayed by Tris - sucrose and glycine - sucrose density gradient ultracentrifugation. The latter assay, carried out at pH 8,8 , showed binding of EB - LDL conjugate to DNA. Le Pecq (1971) proposed that the interaction between ethidium bromide and DNA was by intercalation of the dye to the DNA at hydrophobic sites. A model for the interaction of EB - LDL conjugate, with plasmid pBR322 DNA is presented in Figure 3.9.

As confirmation of the interaction of lipid components with DNA, individual lipids were reacted with plasmid DNA (Section 3.3.7). A surprising finding was that triglycerides showed the greatest affinity for the DNA, followed by cholesteryl esters and phospho - lipids. Cholesterol did not react with the DNA. The interaction between the neutral lipids and DNA are most likely to be hydrophobic

and will not occur in the intact LDL as neutral lipids are embedded in the core of the LDL particle. The phospholipids, however, are located on the periphery of the LDL particle (Goldstein and Brown, 1984) and can participate in electrostatic interactions with the DNA phosphate backbone.

Perebityuk and Bronin (1984) reported the association of plasmid NR1 DNA with membranes at high salt concentrations of 0,5 M, at initiation and termination of replication. However, the membrane - DNA complex was susceptible to disruption by high salt concentrations in the elongation stage of replication. It is possible that the former associations with the membrane lipid bilayers were hydro - phobic, or with membrane proteins. The latter interactions were possibly electrostatic and similar to the associations of LDL peripheral phospholipids with plasmid pBR322 DNA.

X - ray diffraction data has suggested that the interaction between phosphatidylcholine and fatty acid occurs in the vicinity of their carboxyl groups, that is, the carboxyl group of the fatty acid chain is located in the region between the carbonyl at C2 and the glycerol moiety of phosphatidylcholine. This suggests a possible hydrophobic interaction. A similar hydrophobic interaction (Figure 3.6b) may be postulated for the interaction of neutral lipids with DNA. Such interactions may be investigated by NMR and PMR spectroscopy, ESR, circular dichroism and X - ray diffraction techniques, which have thus far been utilized in the study of lipid - lipid and lipid - protein interactions (Lee, 1977; Papahadjopoulus,1973; Chapman, 1973).

CHAPTER FOUR

RECEPTOR - BINDING ACTIVITIES OF NATIVE AND CARBODIIMIDE - MODIFIED LDL AND OF NATIVE AND CARBODIIMIDE - MODIFIED LDL - DNA COMPLEXES IN EUKARYOTIC SYSTEMS

4.1 Introduction

Mammalian cells contain, on their plasma membrane surfaces, receptors for low density lipoproteins (Goldstein *et al.*,1979a; Young *et al.*, 1986; Aulinskas *et al.*,1983; St. Clair *et al.*, 1986; Brown *et al.*, 1975 a, b, c; Brown and Goldstein, 1974; Bradley *et al.*, 1984). Thus, as long as the receptor site on the LDL is not modified, the LDL should bind to the cell surface receptor and be internalized as described in Chapter 1 (Figure 1.2). Of all the modifications of LDL attempted (Chapter 3) only acetylated LDL and cholesteryl linoleoyl LDL prepared in the presence of DMSO, did not bind to the DNA. Although the immunological properties of these modified LDL particles were not altered (Figures 2.17 b and 2.21) the receptor - binding properties of acetylated LDL were altered (Goldstein *et al.*, 1979 b). For these reasons the acetylated LDL and cholesteryl linoleoyl LDL (prepared in DMSO) could not be utlilzed for studying LDL - DNA interactions with eukaryotic systems.

The ethidium bromide - conjugated LDL - DNA complex was also not used for eukaryotic studies for several reasons. The formation of EB - LDL DNA complexes necessitated the use of high pH. A deviation

from physiological pH for studying receptor binding activities would not be conducive to *in vitro* studies. The EB - LDL conjugate reacts with DNA by intercalation of the phenanthridium moiety with the DNA (Figure 3.9; Le Pecq, 1971). This intercalation results in partial unwinding of the DNA, which is undesireable as supercoiled plasmid constructs carrying specific genes are to be introduced into cells in culture (Chapter 5). In addition, the ethidium bromide is a known carcinogen, which can cause cellular mutation and possibly mask the expression of the internalized DNA.

Thus, of all the modified LDLs, the cholesteryl linoleoyl LDL (CL - LDL)prepared by the cholesteryl ester exchange method (Section 2.2.5.4) and ECDI - LDL (Section 2.2.2), which enhanced LDL - DNA binding could be utilized for receptor - binding studies in eukaryotic cells. Of these, the latter was chosen for the studies because it showed a higher affinity for DNA.

Native LDL and ECDI - LDL were labelled with $[^{125}I]$ I_2 in order to follow the receptor - binding activity of LDL and ECDI - LDL and LDL - DNA and ECDI - LDL - DNA in eukaryotic systems. In parallel experiments the DNA was labelled. The purpose of these experiments was to establish that DNA, complexed with LDL or ECDI - LDL was internalized by LDL receptor - mediated endocytosis.

4.2 Methods

4.2.1 Preparation of culture medium

Eagle's minimum essential medium (MEM) was prepared in 20 mM Hepes buffer, pH 7,2 , and supplemented with 10% fetal calf serum, penicillin (10 mg / ml), streptomycin (10 mg / ml), fungizone (2,5 mg / ml) and sodium bicarbonate (25 mM). Eagle's MEM without fetal calf serum was also prepared for use with lipoprotein deficient serum. MEM was filter - sterilized and stored at 4°C.

4.2.2 Preparation of lipoprotein - deficient serum

Lipoproteins from freshly collected human serum were isolated at densities below 1,21 , as described in Section 2.2.1. The lipopro - tein - deficient serum was collected at densities greater than 1,21 g / ml, dialysed against 0,15 M NaCl - 0,01% EDTA at 4°C, over a period of 24 hours, with several changes of buffer and stored at -20°C after filter sterilization (Millipore Millex GS filter). The protein content of the lipoprotein deficient serum was determined by the method of Markwell *et al.* (1981).

4.2.3 Culture of normal skin fibroblasts

Fibroblasts were obtained from a skin biopsy of a clinically normal adult male donor. Fibroblasts were used between the fifth and fifteenth passage. Culturing techniques were adopted from the methods of Brown and Goldstein (1976). Cells were grown in 75 cm^2

stock flasks at 37°C. To subculture, the fibroblast monolayers were treated with 0.25% trypsin (1 ml) in Hank's balanced salt solution (Freshney, 1986), without Ca^{++} and Mg^{++}, for 2 - 5 minutes at 37°C. The action of trypsin was stopped with 2 ml growth medium containing 10% fetal calf serum (FCS). The dissociated cells were pelleted at 900 x g, washed once with phosphate buffered saline (PBS) and resuspended in medium containing 10% FCS. Cells were plated in 25 cm^2 flasks containing 3,0 ml MEM supplemented with FCS. The cells attached to the flasks within 24 hours. On the third day after seeding, the medium was replaced with fresh medium. On the sixth day cells were washed with PBS and 3,0 ml of fresh medium containing 5% lipoprotein - deficient serum was added. Experiments were initiated 24 hours after the cells had been incubated with lipoprotein - deficient serum (LPDS).

4.2.4 Iodination of native LDL and carbodiimide - modified LDL

N - chlorobenzene sulfonamide derivatized non - porous polystyrene beads (Pierce), of diameter 3,175 mm , were used in the preparation of [^{125}I] LDL and ECDI - [^{125}I] LDL. Two beads were washed twice with 5 ml 0,05 M sodium phosphate buffer, pH 7,4 , and dried on Whatman No. 1 filter paper. The beads were added to a solution of [^{125}I] NaI (5 µl, specific activity 10 mCi / ml) and 0,05 M sodium phosphate buffer (400 µl; pH 7,4) and allowed to stand for 5 minutes at room temperature. To this solution was added 5 - 10 µg of native LDL or ECDI - LDL in 0,05 M phosphate buffer, pH 7,4. The reaction mixture was incubated at room temperature for 7,5

minutes and the iodinated compound was purified on a Sephadex G 25 (medium) column (19 x 1 cm). The radioactivity of 5 µl aliquots of alternate fractions (0,5 ml fractions) was determined on a gamma counter (Model ANSR - 7040) in addition to monitoring optical density at 280 nm. Peak fractions, containing the highest activity were pooled and the protein content was determined according to the method of Markwell *et al.* (1981; Section 2.2.1.1).

4.2.5 Preparation of LDL - DNA complexes and carbodiimide - modified LDL - DNA complexes

[^{125}I] - labelled native and carbodiimide - modified LDL were complexed with six - time sheared calf thymus DNA (ct DNA). Unlabelled native and carbodiimide - modified LDL were complexed with nick - translated [^{3}H] pBR322 DNA. The preparation of [^{125}I] LDL - ct DNA and ECDI - [^{125}I] LDL - ctDNA were carried out in a similar manner: To 3,75 µg [^{125}I] - labelled lipoprotein was added 0,25 µg DNA. Each reaction was incubated at room temperature for 20 minutes, in 0,05 M Tris - HCl, pH 7,5 - 0,025 M NaCl - 0,27 mM EDTA in a total volume of 200 µl. Each preparation was utilized without further treatment, for receptor - binding studies in normal skin fibroblasts. For the preparation of LDL - [^{3}H] pBR322 and ECDI - LDL - [^{3}H] pBR322, 1,0 µg LDL or ECDI - LDL was reacted with 18 ng [^{3}H] pBR322 at room temperature for 20 minutes, in the same buffer as above. The complexes were subsequently added to skin fibroblast monolayers and assayed for receptor recognition and internalization by receptor - mediated endocytosis.

4.2.6 Total binding of [^{125}I] LDL and ECDI - [^{125}I] LDL

Monolayers of skin fibroblasts (about 70 - 80% confluent) were pre - chilled at 4°C for 30 minutes. The growth medium was removed and 3,0 ml MEM containing 5% lipoprotein deficient serum (LPDS) and varying amounts of [^{125}I] LDL or ECDI - [^{125}I] LDL (0 - 80 µg) was added to the culture flasks. The monolayers were incubated at 4°C for 1 hour without shaking. The medium from each flask was removed and retained for assay of degradation products (Section 4.2.7). The monolayers were washed according to the method of Goldstein *et al.* (1976) and Brown *et al.* (1976), using buffer containing bovine serum albumin. Cells were finally washed with 50 mM Tris - HCl (pH 7,4) - 0,15 M NaCl and solubilized in 1 ml 0,1 N NaOH. A 500 µl aliquot of the cell suspension was withdrawn to determine total cell associated radioactivity, while a 50 µl aliquot was utilized to determine cellular protein content.

4.2.7 Assays for [^{125}I] - labelled degradation products

The assay method was adapted from the method of Goldstein and Brown (1974). Following incubation of monolayers at 4°C for 1 hour, the medium was removed and precipitated with an equal volume of cold 20% TCA. After standing at 4°C for 30 minutes the TCA precipitated material was pelleted by centrifugation at 3 000 rpm and discarded. To a 1 ml aliquot of the supernatant was added 100 µl KI (1%) and 30 µl 30% H_2O_2. Free iodine was

extracted with 2 ml $CHCl_3$. A 500 µl aliquot of the aqueous phase was counted to determine the amount of TCA - soluble [^{125}I] - labelled degradation product released into the medium by the fibroblasts.

4.2.8 Determination of specific binding of iodine - labelled LDL and ECDI - LDL

Binding assays were carried out as described in Section 4.2.6. Increasing amounts of [^{125}I] LDL and ECDI - [^{125}I] LDL (0 - 600 ng) were added to normal skin fibroblast monolayers in the presence or absence of a 200 - fold excess of unlabelled native LDL. The specific binding was then determined as described in Section 4.3.5.

4.2.9 Binding of [^{125}I] LDL and ECDI [^{125}I] LDL by normal skin fibroblasts in the presence of heparin

Monolayers were chilled for 30 minutes. To the cells were added 0 - 600 ng of [^{125}I] LDL or ECDI - [^{125}I] LDL. Flasks were incubated at 4°C for 1 hour. After incubation the medium was removed and the monolayers were washed as described in Section 4.2.6. To each flask was added 1 ml of fresh medium containing 1 mg heparin, and the cells were incubated at 37°C for 1 hour. The medium was then removed and an aliqout was counted to determine the heparin-released or receptor - bound lipoprotein. The cells were solubilized in 0,1 N NaOH and an aliquot of the cell suspension was counted to determine the heparin - resistant counts. The total cellular

protein content for each flask was also determined.

4.2.10 Binding studies at 4°C and 37°C

One set of flasks containing normal skin fibroblast monolayers were incubated at 4°C and a second set at 37°C, after addition of [^{125}I] LDL or ECDI - [^{125}I] LDL. Both sets were processed in an identical manner. The medium from each flask was assayed for TCA - soluble degradation products as described in Section 4.2.7. The monolayers were washed (Section 4.2.6), solubilized in 0,1 N NaOH and the radioactivity determined. The binding and degradation were thus compared at 4°C and 37°C.

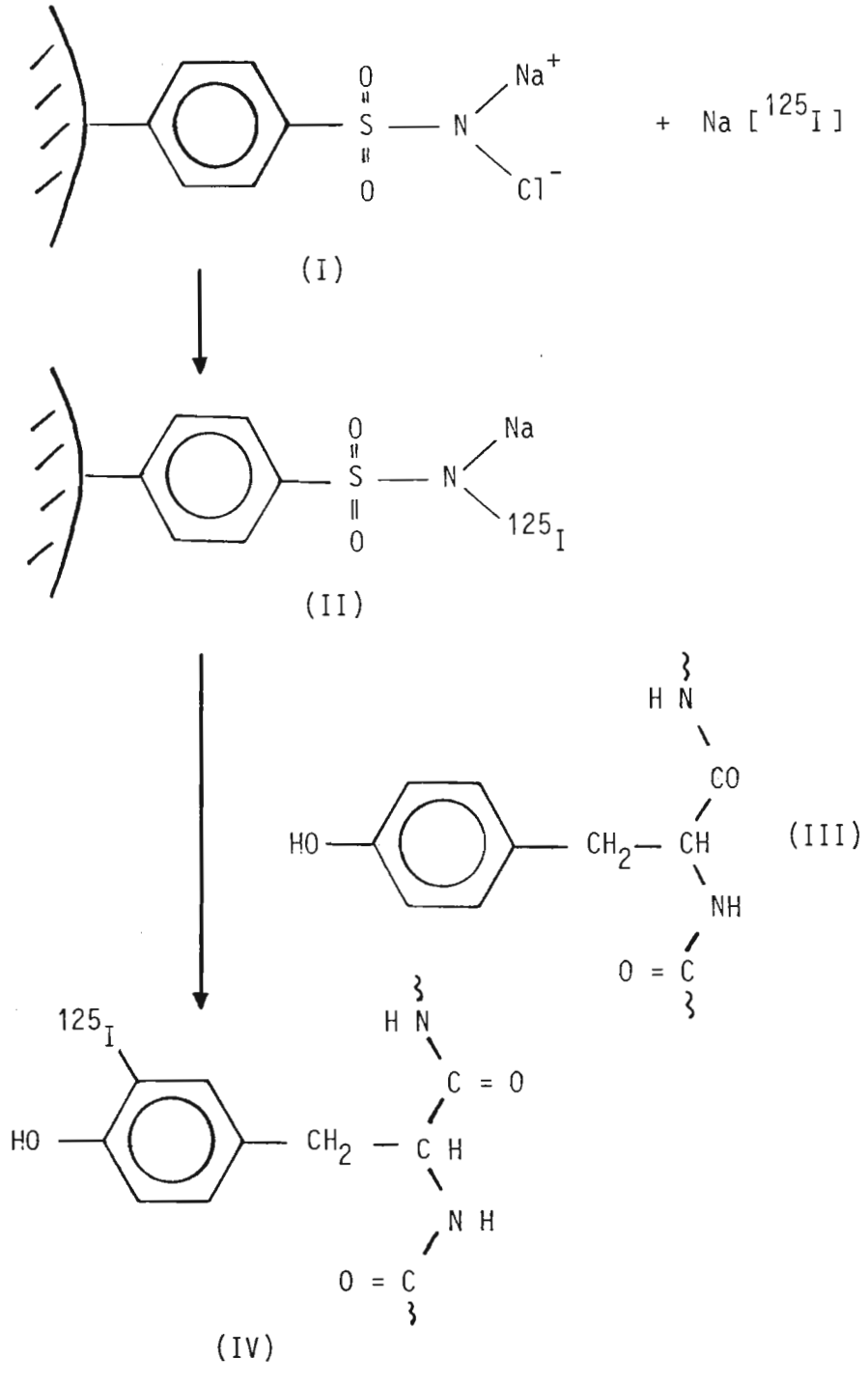
4.2.11 Binding, internalization and degradation of LDL - DNA and carbodiimide - modified LDL - DNA complexes

Binding, internalization and degradation assays of [^{125}I] LDL - ctDNA, ECDI - [^{125}I] LDL - ct DNA, LDL -[^{3}H] pBR322 and ECDI - LDL - [^{3}H] pBR322 (prepared as described in Section 4.2.5) in normal skin fibroblasts, *in vitro*, at 4°C were carried out as outlined in Section 4.2.6 and 4.2.7 except for one variation : All monolayers were pre - treated with 10 µg of six - times sheared calf thymus DNA for 10 minutes prior to the addition of the above complexes to the cell cultures. Heparin - release experiments were carried out as described in Section 4.2.9.

4.3 Results and Discussion

4.3.1 Iodination of native and carbodiimide - modified LDL

Both LDL and ECDI - LDL were successfully iodinated by using the N - chlorobenzene sulfonamide derivatized polystyrene beads. A scheme for the synthesis is presented in Figure 4.1. The iodine monochloride method of McFarlane (1958), used by Bilheimer *et al.* (1972) and other researchers (Trezzi, 1984; Brown and Goldstein, 1974a; Bradley *et al.*, 1984; Anderson *et al.*, 1981; Goldstein *et al.*, 1976; Brown and Goldstein, 1975), for the iodination of LDL was not employed as the method of preparation required conditions of high pH, which must affect the integrity and solubility of the LDL. The iodination of the LDL apoprotein occurs at tyrosine residues (Brown and Goldstein, 1976) without drastically altering the receptor binding properties of the particles. The iodinated LDL and ECDI - LDL were purified by gel filtration on a Sephadex G 25 column. Peak fractions were counted on a γ - counter in addition to being monitored at OD_{280}. The elution profiles presented in Figure 4.2 shows that the radioactivity peak followed the optical density peak closely. [^{125}I] LDL had a specific activity of 4 500 cpm / ng, whilst that of ECDI - [^{125}I] LDL was 5 000 cpm / ng. The number of moles of I per LDL calculated was $< 1{,}0$. Similar rates of iodination of native LDL have been reported for iodination by the iodine monochloride method (Brown and Goldstein, 1974a). The iodination procedure proved to be satisfactory since recoveries of LDL and ECDI - LDL were between 90 and 95%.



Digure 4.1 : Iodination of apoprotein B of LDL (III) with the use of N - chlorobenzene sulfonamide - derivatized polystyrene (I), which is first iodinated (II) and which subsequently iodinates a tyrosine residue of apoprotein B (IV).

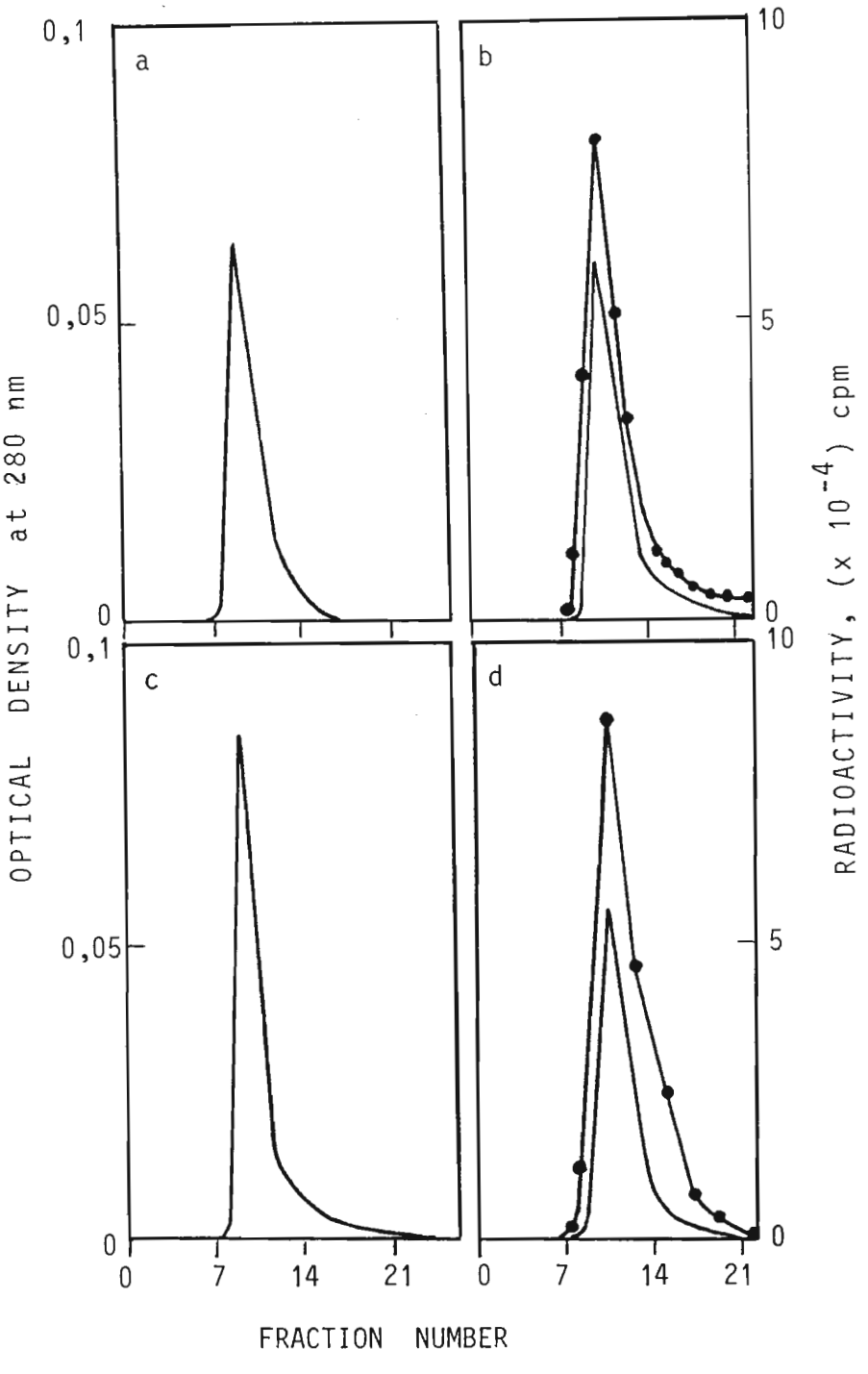


Figure 4.2 : Gel filtration profiles of native LDL (a), [^{125}I] LDL, (b), ECDI - LDL (c) and ECDI - [^{125}I] LDL (d) recorded on an ISCO monitor, at OD_{280} (——). The radioactivity (●——●) of peak fractions (1µl aliquots) was determined on a ɣ - counter. Both the standard and labelled components eluted between fractions 8 and 13.

4.3.2 Preparation of [^{125}I] LDL - DNA and ECDI - [^{125}I] LDL - DNA complexes

The initial activities of [^{125}I] LDL - DNA and ECDI - [^{125}I] LDL - DNA were 1 500 cpm / ng and 2 500 cpm / ng respectively. The activities of LDL - [^{3}H] pBR322 and ECDI - LDL - [^{3}H] pBR322 were 4 000 cpm / ng and 6 000 cpm / ng respectively. The activity of the iodine labelled complexes were determined for each experiment. [^{3}H] - labelled LDL - DNA and ECDI - LDL - DNA complexes were utilized to monitor the metabolism of the DNA in the above complexes.

4.3.3 Receptor - binding activities of [^{125}I] LDL and ECDI - [^{125}I] LDL in cell cultures of normal skin fibroblasts

[^{125}I] LDL and ECDI - [^{125}I] LDL bound to receptors of normal skin fibroblasts in culture to different extents. The binding of ECDI - [^{125}I] LDL at 80 ng was 100 - fold greater than that of [^{125}I] LDL (Figure 4.3). Results presented here constitute both bound and internalized [^{125}I] - labelled LDL and ECDI - LDL (i.e., total binding assays).

4.3.4 Degradation of [^{125}I] LDL and ECDI - [^{125}I] LDL

Degradation products were obtained within 1 hour of addition of [^{125}I] LDL and ECDI - [^{125}I] LDL to normal skin fibroblast monolayers in culture. Compared to the [^{125}I] LDL, the ECDI - [^{125}I] LDL was degraded to a far greater extent, as shown in Figure 4.4. However, the ratio of bound : degraded [^{125}I] LDL (data used from

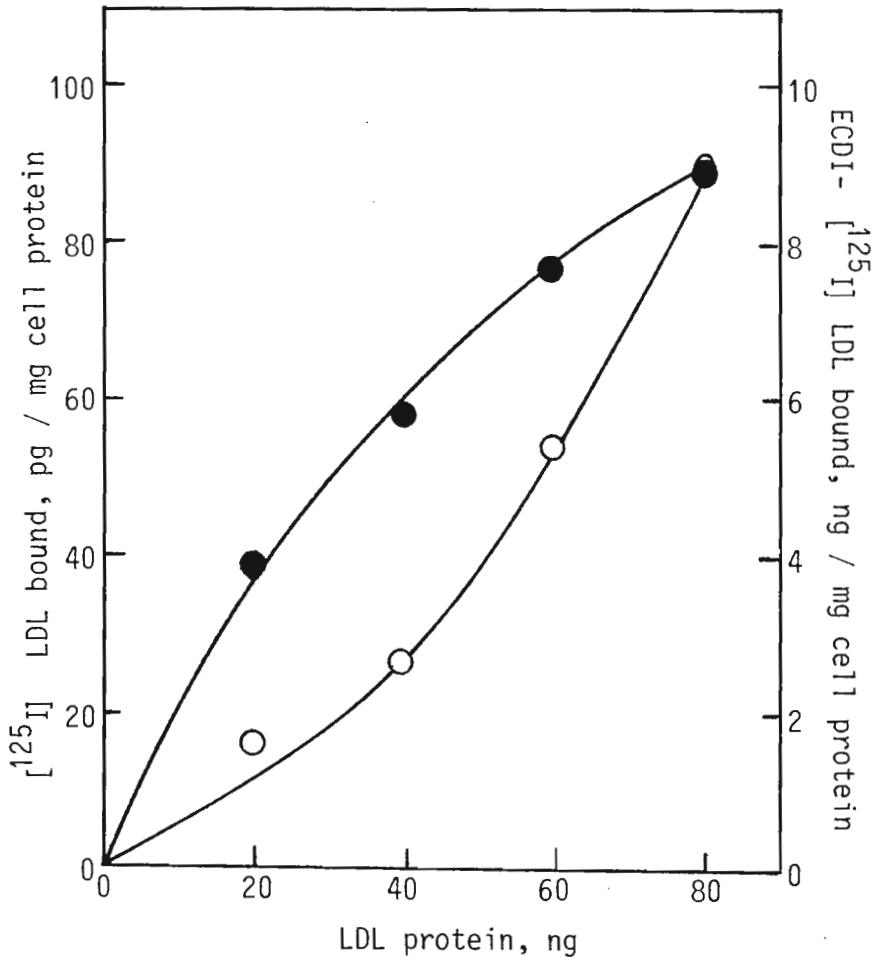


Figure 4.3 : Binding of [^{125}I] LDL (●) and ECDI-[^{125}I] LDL (○) in normal skin fibroblasts at 4°C. [^{125}I] LDL had a specific activity of 179 cpm / ng while ECDI - [^{125}I] LDL had a specific activity of 75 cpm / ng. The average cellular protein values were 250 µg and 325 µg respectively.

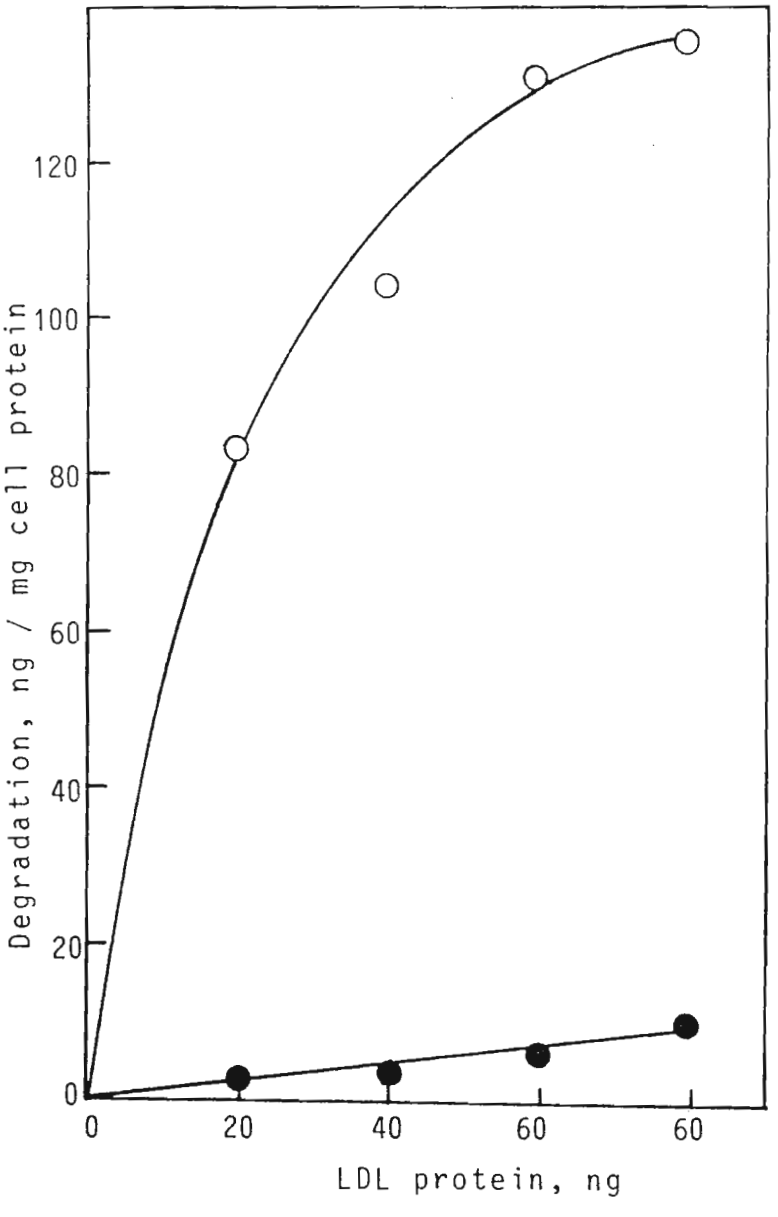


Figure 4.4: Degradation of [^{125}I] LDL (●) and ECDI -[^{125}I] LDL (○) by normal skin fibroblasts at 4°C. The specific activity and protein content were the same as for Figure 4.3.

Section 4.4.3) was 1 : 100, whilst the ratio of ECDI - [^{125}I] LDL bound : degraded was 1 : 1,4. This indicates that although a greater amount of ECDI - [^{125}I] LDL was bound (and internalized), and a larger amount was degraded, the turnover of native LDL was greater than that of carbodiimide - modified LDL (i.e., 1 : 100 as opposed to 1 : 1,4). This suggests that in addition to binding specifically to the LDL receptors on the cell surfaces, the carbodiimide - modified LDL binds non - specifically to the plasma membrane. If these non - specifically bound ECDI - LDL are subsequently internalized, they would escape lysosomal degradation, whereas the native LDL, internalized by receptor mediated endocytosis, duly undergoes lysosomal degradation, yielding a 67 - fold greater turnover.

4.3.5 Specific binding of [^{125}I] LDL and ECDI - [^{125}I] LDL

Binding studies were carried out as described in Section 4.2.8. The specific binding was subsequently calculated by subtracting the binding of [^{125}I] LDL or ECDI - [^{125}I] LDL, obtained in the presence of 200 - fold excess of unlabelled native LDL, from that obtained in the absence of unlabelled native LDL (total binding). The binding data was processed as described by Brisette and Noël (1986). Results presented in Figures 4.5 and 4.6 show that there was reduced binding of both [^{125}I] LDL and ECDI - [^{125}I] LDL in the presence of excess LDL. This illustrates that unlabelled LDL replaces the [^{125}I] LDL or ECDI - [^{125}I] LDL at the receptor sites.

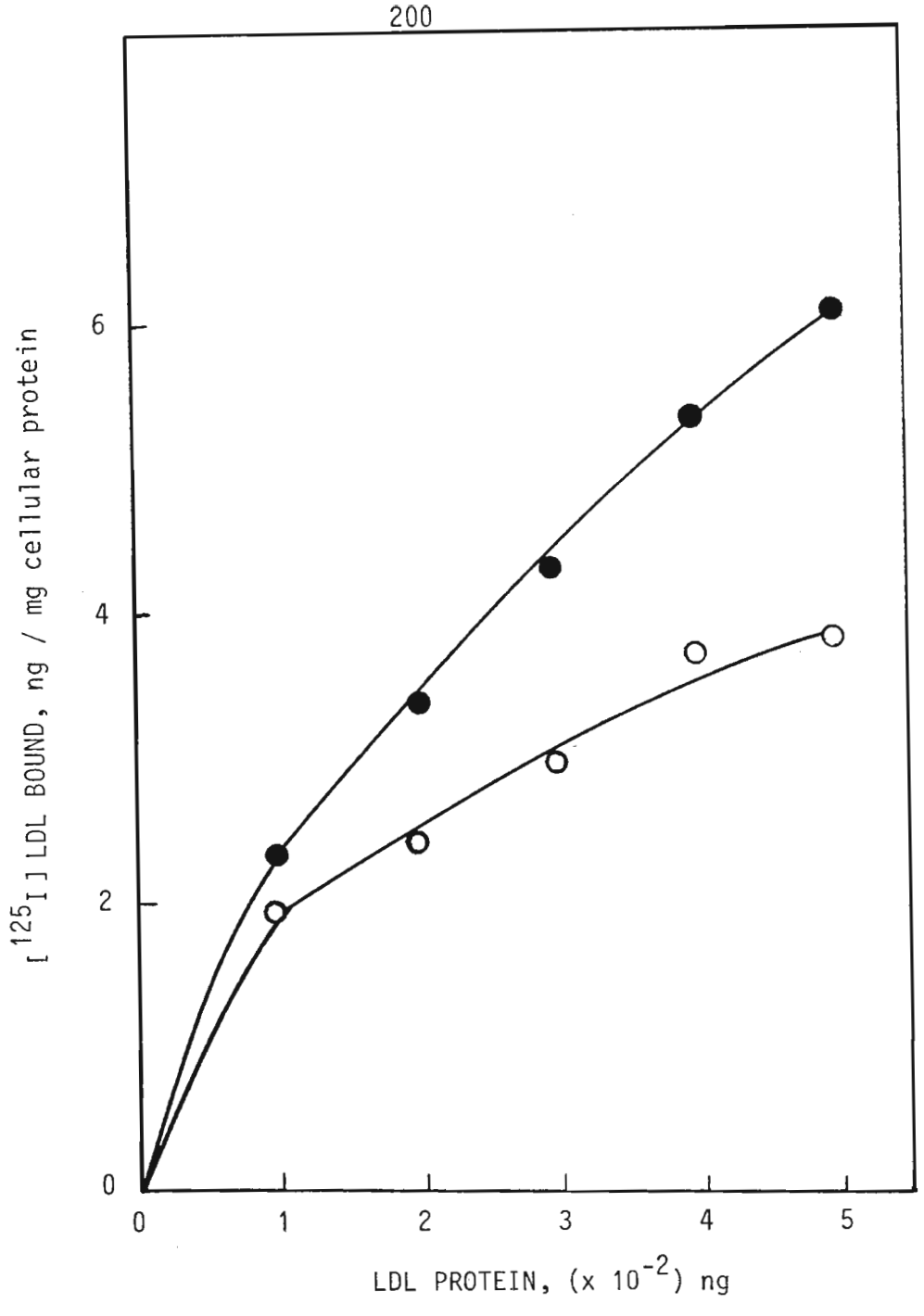


Figure 4.5 : Binding of [^{125}I] LDL to normal skin fibroblasts in the presence (○) and absence (●) of 200 - fold excess unlabelled LDL. The specific activity of [^{125}I] LDL was 250 cpm / ng and the total cellular protein per flask averaged 425 µg.

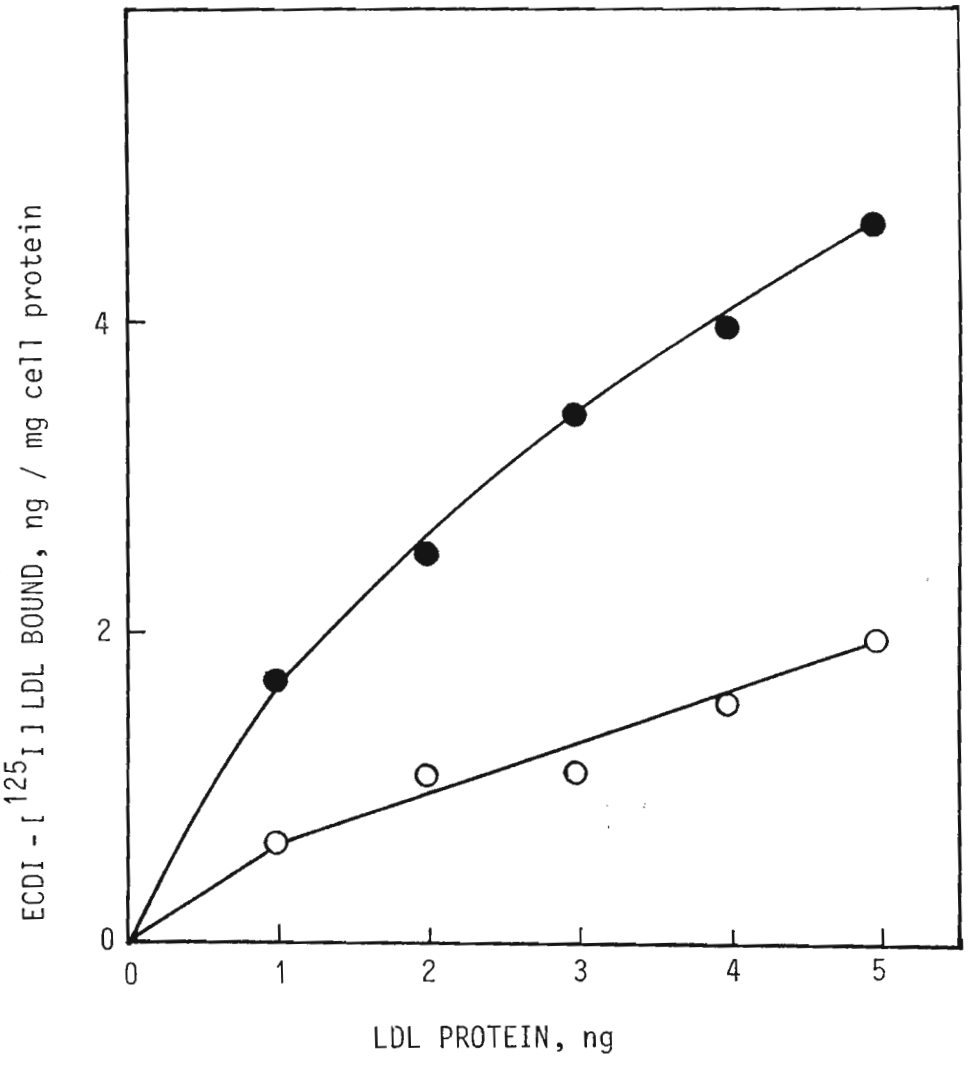


Figure 4.6 : Binding of ECDI - [^{125}I] LDL to normal skin fibroblasts in the presence (○) and absence (●) of 200 - fold excess unlabelled LDL.

The specific binding data obtained from Figures 4.5 and 4.6 were transformed into plots of the ratio of receptor - bound to free lipoprotein versus the receptor - bound lipoprotein. Results on the Scatchard plot (Figure 4.7) showed that the [^{125}I] LDL bound specifically to the cell surface receptors as reported by Brown and Goldstein (1974). The ECDI - [^{125}I] LDL, on the other hand, bound both specifically to the LDL receptors, and by a second type of binding. This additional binding possibly occurs at `residual´ receptor sites (Brown and Goldstein, 1975). Nevertheless, the experimental results (Figures 4.5, 4.6 and 4.7) illustrate that the carbodiimide - modified LDL does enjoy receptor recognition and is internalized by adsorptive endocytosis.

4.3.6 Competitive binding of ECDI - [^{125}I] LDL to normal skin fibroblasts in the presence of native LDL

In order to confirm that carbodiimide - modified LDL was subject to receptor recognition, a competitive binding experiment was carried out (as described in Section 4.2.6) in the presence of increasing amounts of native, unlabelled LDL. There was a progressive decrease in the binding of ECDI - [^{125}I] LDL to the normal skin fibroblasts (Figure 4.8) with increasing amounts of native LDL, up to 10 μg (LDL protein). Additional amounts of native LDL (exceeding 10 μg) did not displace any more ECDI - [^{125}I] LDL, hence the ECDI - [^{125}I] LDL remaining bound to the cells was probably non - specifically bound. This experiment illustrates that carbodiimide - modified LDL competes with native LDL for the same receptor sites

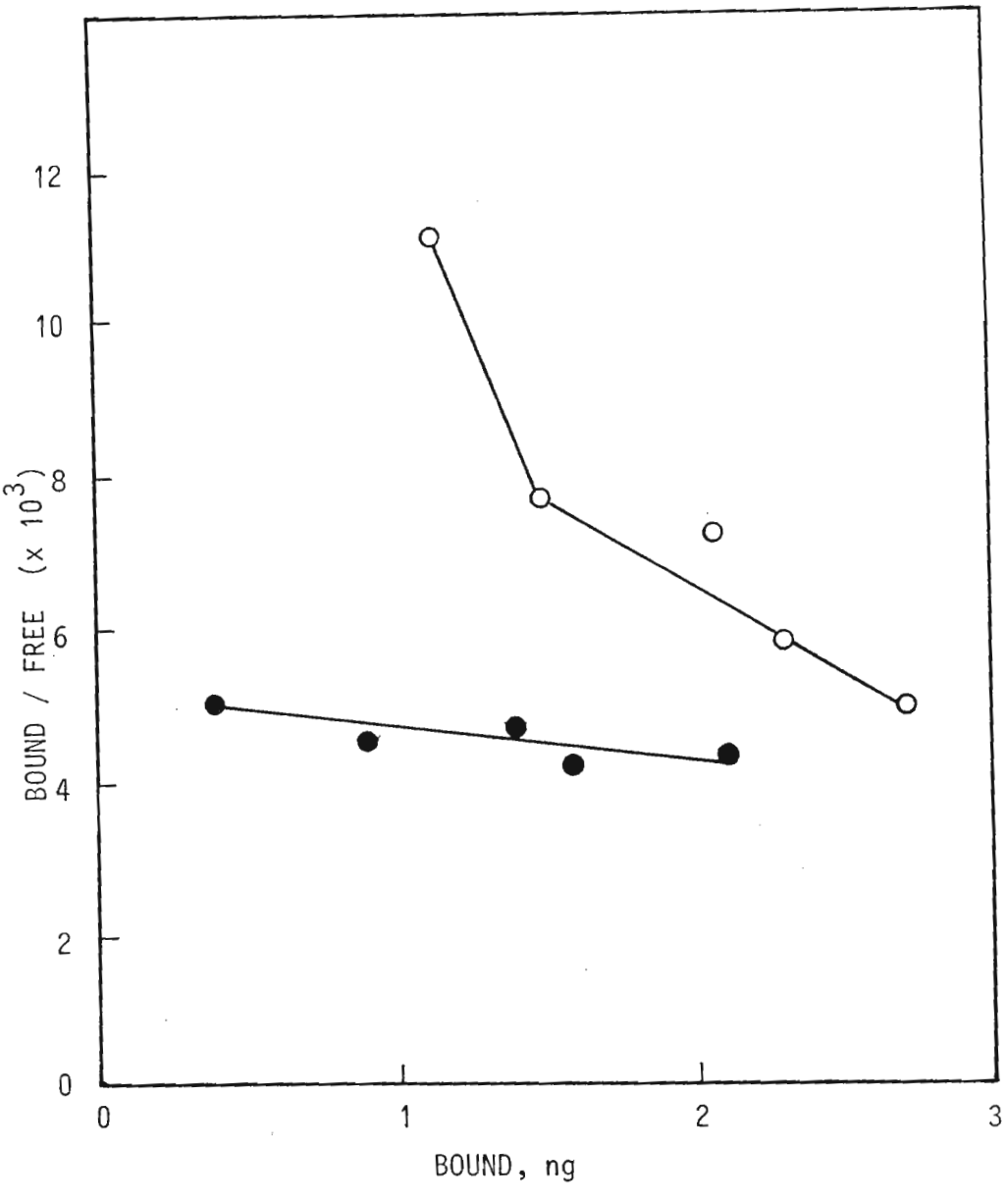


Figure 4.7 : Scatchard transformations for the binding of [^{125}I] LDL (●) and ECDI - [^{125}I] LDL (○) from Figures 4.5 and 4.6. `Bound / free´ on the ordinate of the Scatchard plot represents the amount of specifically bound [^{125}I] LDL and ECDI - [^{125}I] LDL (ng / mg protein) divided by the concentration of unbound [^{125}I] LDL or ECDI - [^{125}I] LDL (ng) remaining in the culture medium.

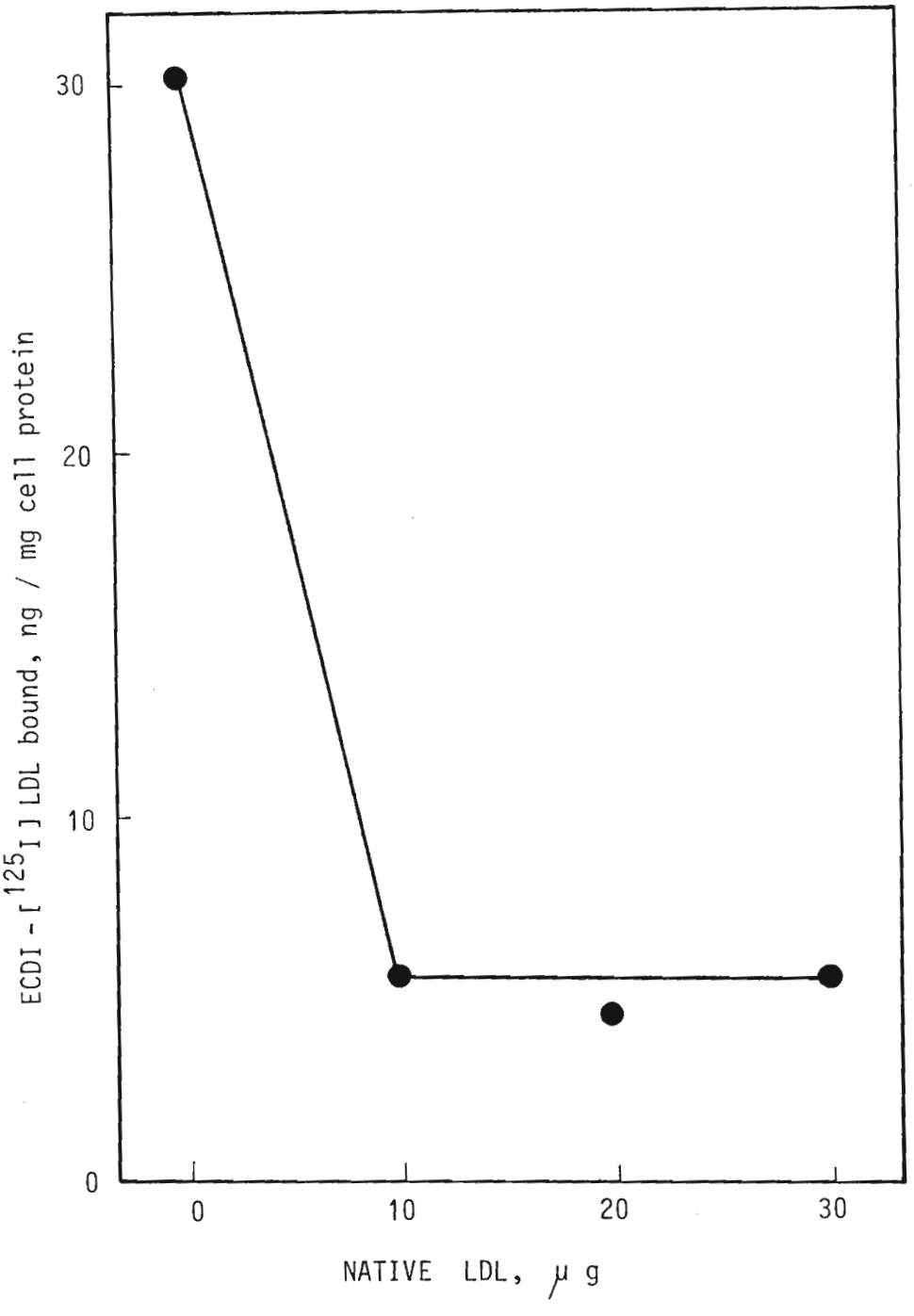


Figure 4.8 : Competitive binding of ECDI - [^{125}I] LDL to normal skin fibroblasts . Four hundred ng of ECDI - [^{125}I] LDL was applied to each flask. The specific activity of the ECDI - [^{125}I] LDL was 432 cpm / ng. The total protein content per flask averaged 398 μg. Native LDL was added as indicated.

and is therefore subject to receptor - recognition for binding, internalization by receptor - mediated endocytosis and lysosomal degradation. Faust *et al.*(1977) have shown similar competitive reductions in the binding of labelled LDL in cultured mouse adrenal cells. The addition of ACTH, however, enhanced receptor - binding of the labelled LDL in the presence of unlabelled LDL.

4.3.7 Heparin chase experiments as a means of monitoring receptor - binding activities of [^{125}I] LDL and ECDI - [^{125}I] LDL

Surface - bound [^{125}I] LDL and ECDI - [^{125}I] LDL were released with heparin. Results (Figure 4.9) show that 3 - fold more ECDI - [^{125}I] - LDL was released with heparin than [^{125}I] LDL at 600ng (protein content). The binding of native LDL reached saturation at approximately 300 ng, while the carbodiimide - modified LDL binding increased with increasing amounts of ECDI - [^{125}I] LDL. This indicates that the native LDL bound specifically to the cell surface receptors while ECDI - [^{125}I] LDL bound non - specifically to the plasma membrane in addition to binding to the LDL receptors, hence the heparin - released ECDI - [^{125}I] LDL did not reach saturation (Figure 4.9).

The heparin resistant counts represent the [^{125}I] LDL and ECDI - [^{125}I] LDL internalized by LDL receptor mediated endocytosis (Brown and Goldstein, 1976b). Similar amounts of [^{125}I] LDL and ECDI - [^{125}I] LDL were internalized (Figure 4.10). This indicates

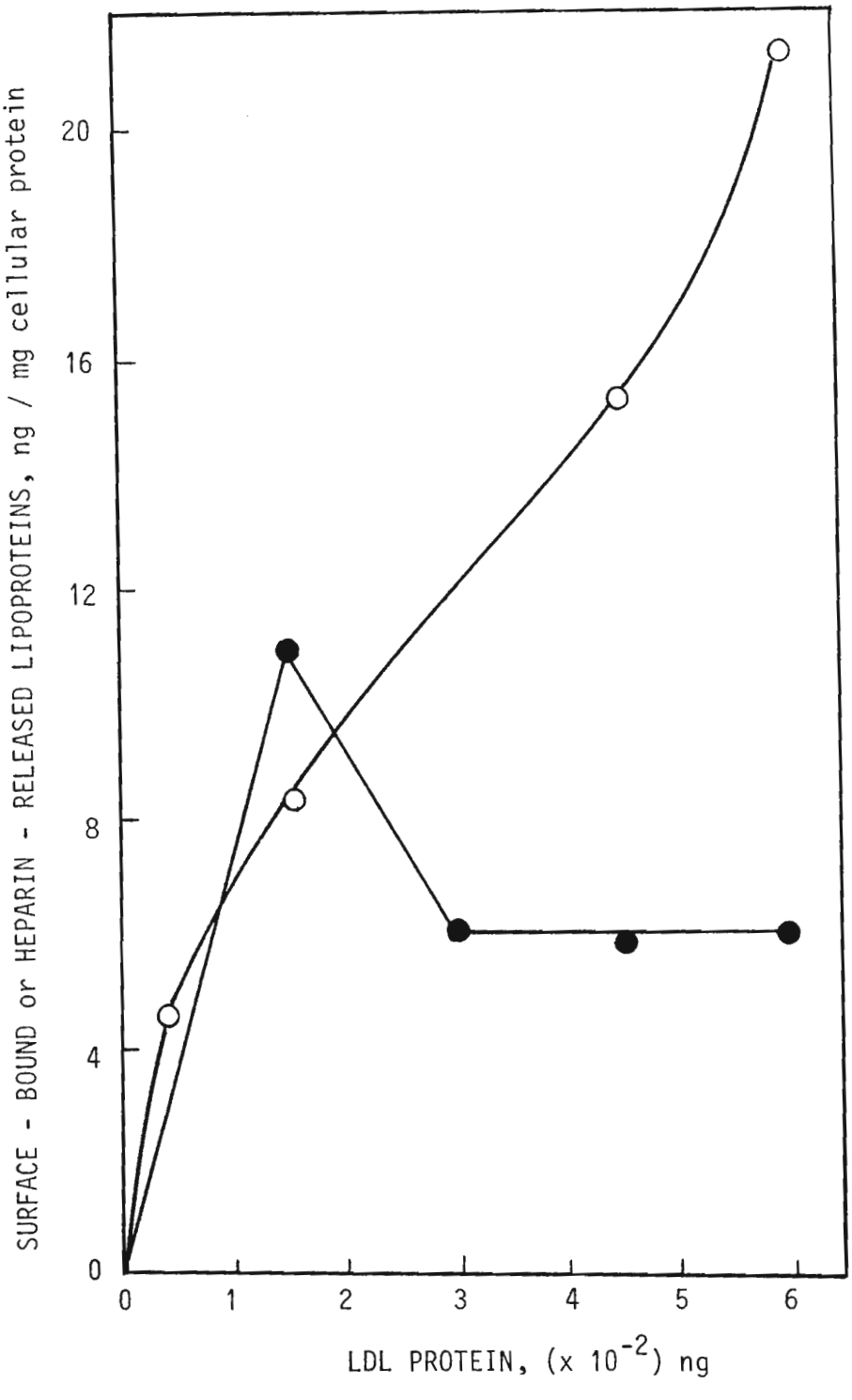


Figure 4.9 : Heparin - release of surface - bound [^{125}I] LDL (●) and ECDI - [^{125}I] LDL (O) from skin fibroblasts. Specific activities were 1481 cpm / ng and 75 cpm / ng, and cellular protein contents were 423 µg and 418 µg, respectively.

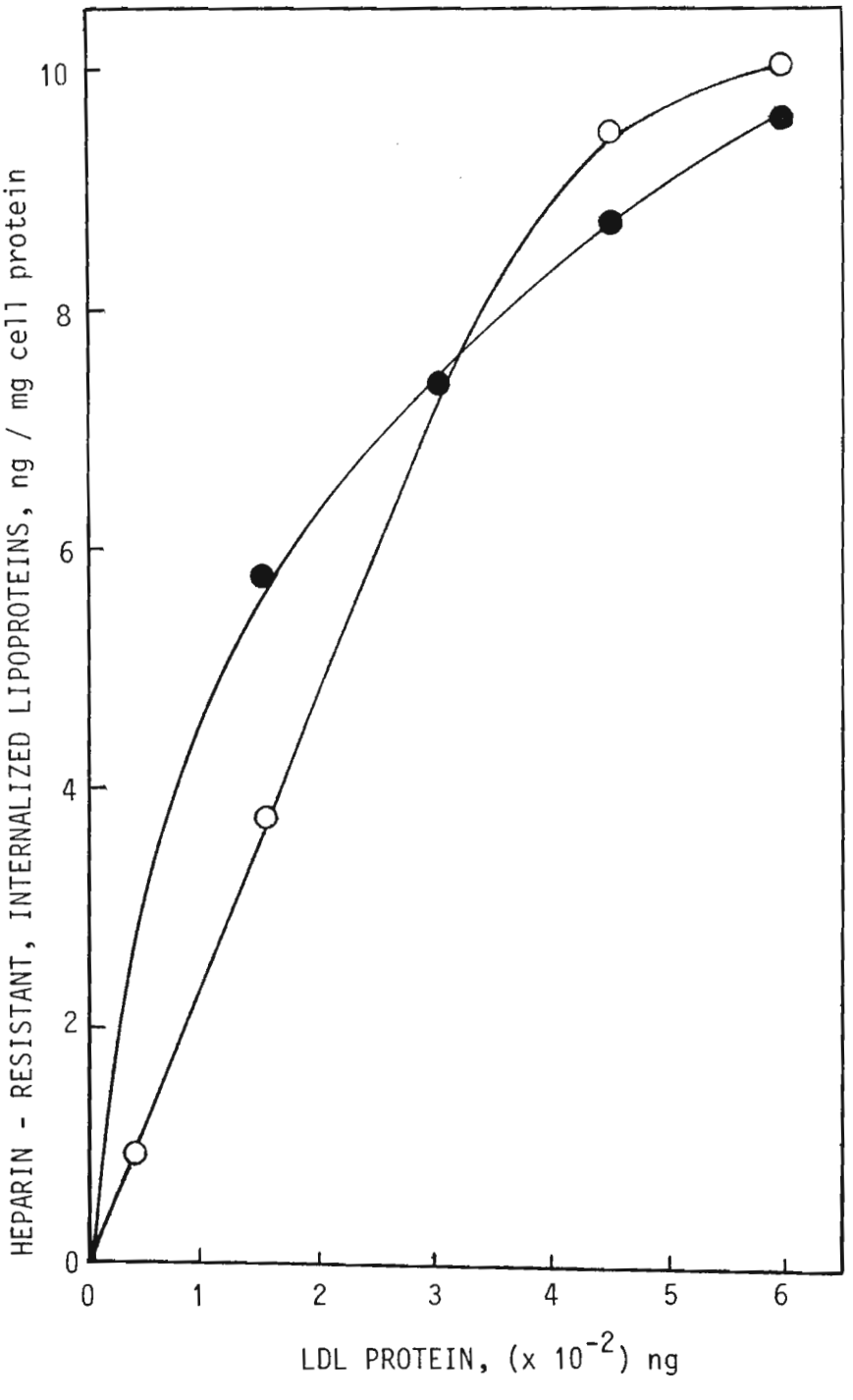


Figure 4.10 : Internalization of [^{125}I] LDL (●) and ECDI - [^{125}I] - LDL (○), in normal skin fibroblasts, which were heparin - resistant.

that only the receptor - bound LDL and carbodiimide - modified LDL was internalized, by LDL receptor - mediated endocytosis, since the degradation of both lipoproteins (Figure 4.11) was also almost equivalent.

4.3.8 Temperature dependence of binding and degradation of LDL and ECDI - LDL

Binding studies were carried out at both 4°C and 37°C. Results presented in Table 4.1 indicate that binding and degradation of [^{125}I] LDL and ECDI - [^{125}I] LDL were greater at 37°C than at 4°C. A repeat of the experiments at higher concentrations of LDL protein also yielded greater binding and degradation at 37°C. Enhanced binding of native LDL to the receptors at 4°C and greater internalization at 37°C has been reported (Brown and Goldstein, 1976 a). However, the purpose of the experiment was to establish that both binding and internalization were feasible at 4°C. Results in Sections 4.3.3, 4.3.4 and 4.3.9 show that binding, internalization and degradation were successfully carried out at 4°C.

4.3.9 Binding and degradation of [^{125}I] LDL-ct DNA and ECDI - [^{125}I] - LDL - ct DNA *in vitro*

The binding of native and carbodiimide - modified LDL - DNA complexes to normal skin fibroblasts in culture was studied at 4°C. At 2 µg of LDL or ECDI - LDL protein content approximately 500 ng of [^{125}I] LDL - ct DNA was bound and internalized while at the same

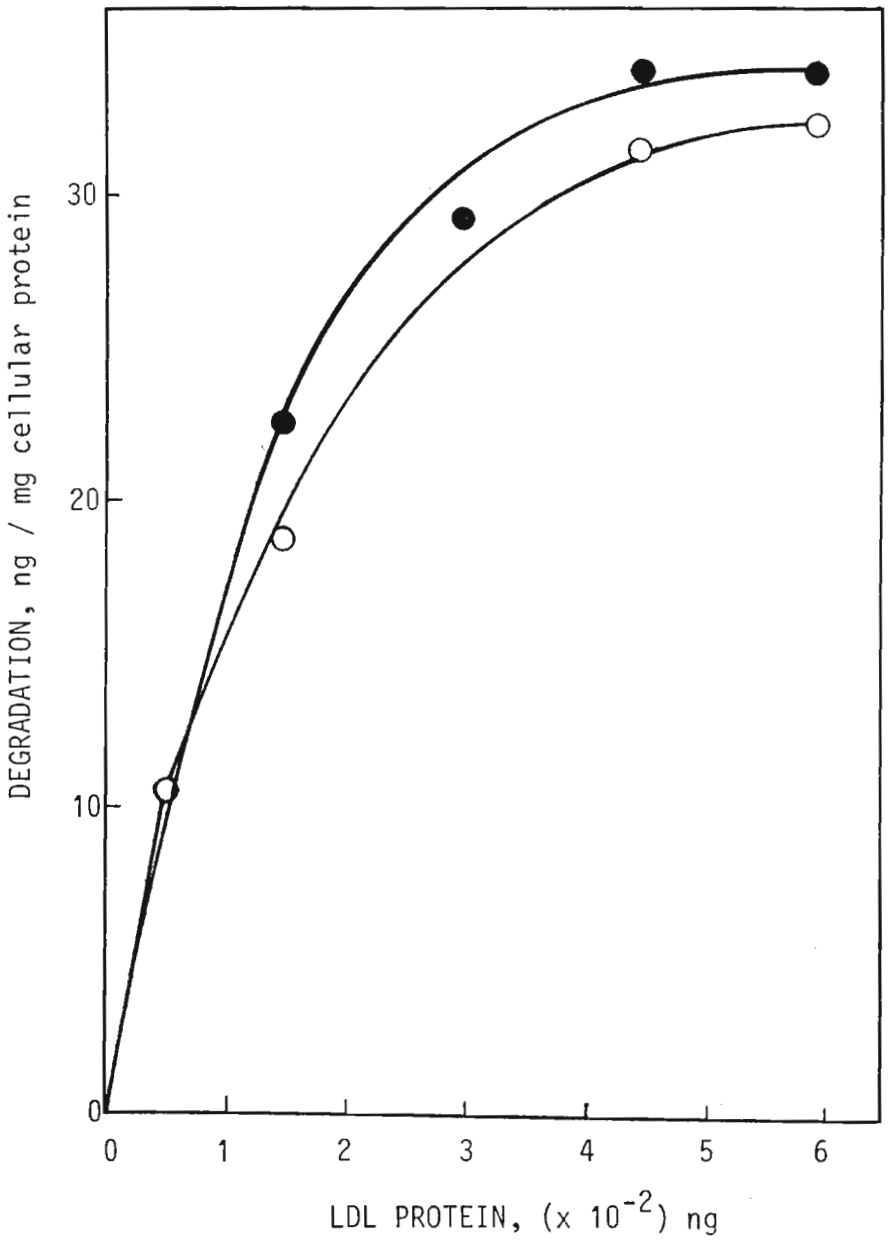


Figure 4.11 : Degradation of [^{125}I] LDL (●) and ECDI - [^{125}I] - LDL (○) by normal skin fibroblasts.

	Binding, ng / mg protein		Degradation, ng / mg protein	
	4°C	37°C	4°C	37°C
[^{125}I] LDL				
40 ng	0,06	0,77	3,39	73,2
80 ng	0,10	2,33	9,08	241,7
ECDI - [^{125}I] LDL				
40 ng	2,6	4,68	84,3	188,5
80 ng	9,05	12,74	136,1	207,3

Table 4.1 : Binding and degradation of [^{125}I] LDL and ECDI - [^{125}I] - LDL at 4°C and 37°C. The activities of [^{125}I] LDL and ECDI - [^{125}I] LDL were 1250 cpm / ng and 575 cpm / ng respectively. The average cellular protein values were 278 µg for the former and 458 µg for the latter. Total binding included both surface - bound and internalized LDL and ECDI - LDL.

concentration approximately 100 ng of ECDI - [^{125}I] LDL - ct DNA was bound and internalized, that is, 5 - fold less binding occurred with the carbodiimide - modified LDL - DNA complexes (Figure 4.12). However, ECDI - LDL has a 10 - fold greater affinity for DNA (Section 3.3.2) than native LDL, hence these particles carry twice the DNA carried by native LDL because they bind far more DNA.

The degradation of native LDL (complexed with DNA) was 10 - fold greater than for carbodiimide - modified LDL (complexed to DNA) (Figure 4.13). Although this experiment indicated that ten times more [^{125}I] LDL was degraded than ECDI - [^{125}I] LDL, one could not estimate the extent of binding, internalization and degradation of the DNA complexed to these lipoproteins. For this reason, a further experiment was carried out, using the same lipoprotein - DNA complexes, but this time with labelled DNA (Section 4.3.10).

4.3.10 Assay for surface - bound and internalized LDL - DNA and ECDI - LDL - DNA complexes using heparin

To determine whether the LDL and ECDI - LDL complexed to DNA did carry the DNA to the LDL receptors and were subsequently internal - ized, binding assays were carried out with lipoprotein - DNA complexes which contained labelled DNA (i.e, [^{3}H] pBR322 DNA). Surface - bound LDL - [^{3}H] pBR322 and ECDI - LDL -[^{3}H] pBR322 were released with heparin (as described in Section 4.2.9). Results show that far more ECDI - LDL - [^{3}H] pBR322 complexes were surface - bound than LDL - [^{3}H] pBR322 (Figure 4.14). This was attributed to non -

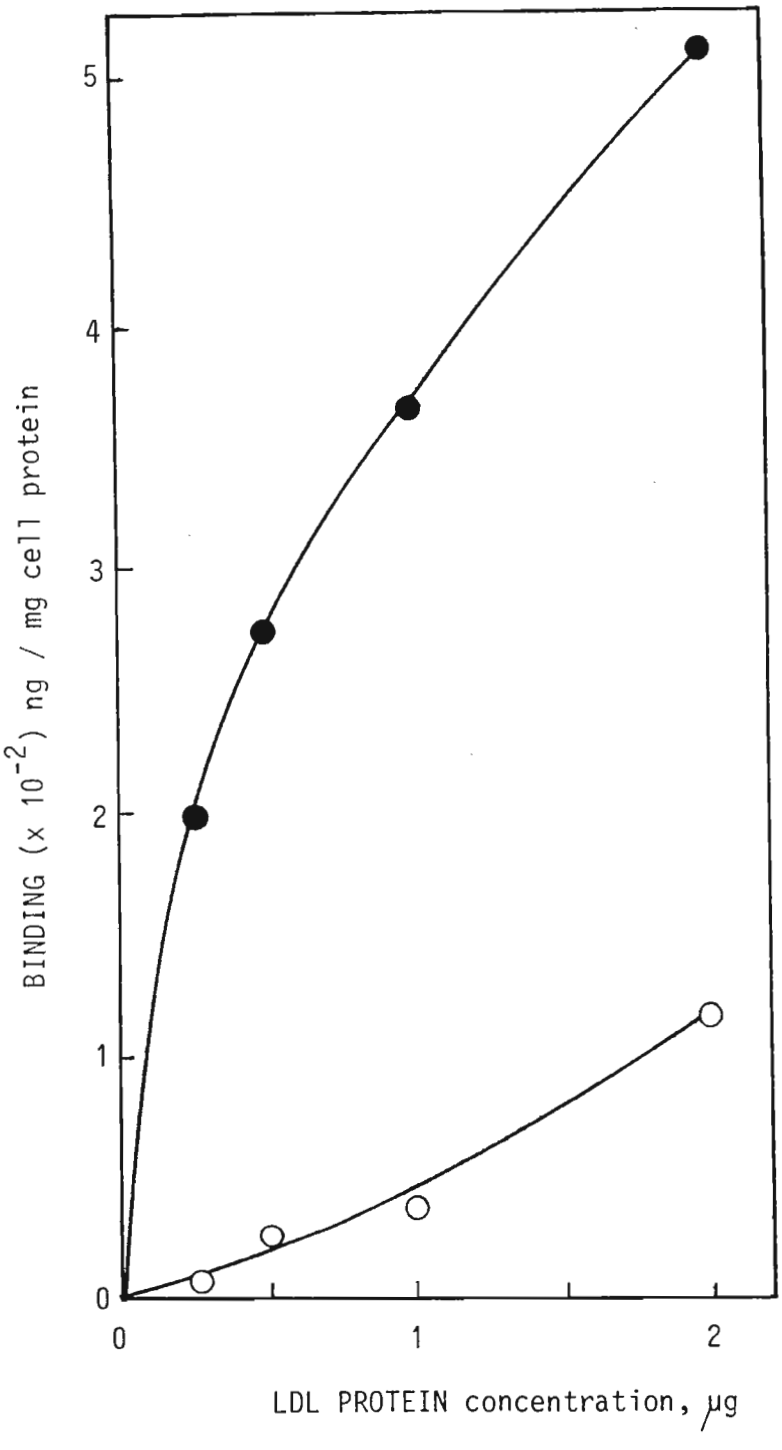


Figure 4.12 : Binding of [^{125}I] LDL - ct DNA (●) and ECDI - [^{125}I] - LDL - ct DNA (○) to normal skin fibroblasts. The specific activities of the [^{125}I] - labelled lipoproteins were 960 cpm / ng and 65 cpm / ng respectively. The average value for total cellular protein was 229 µg.

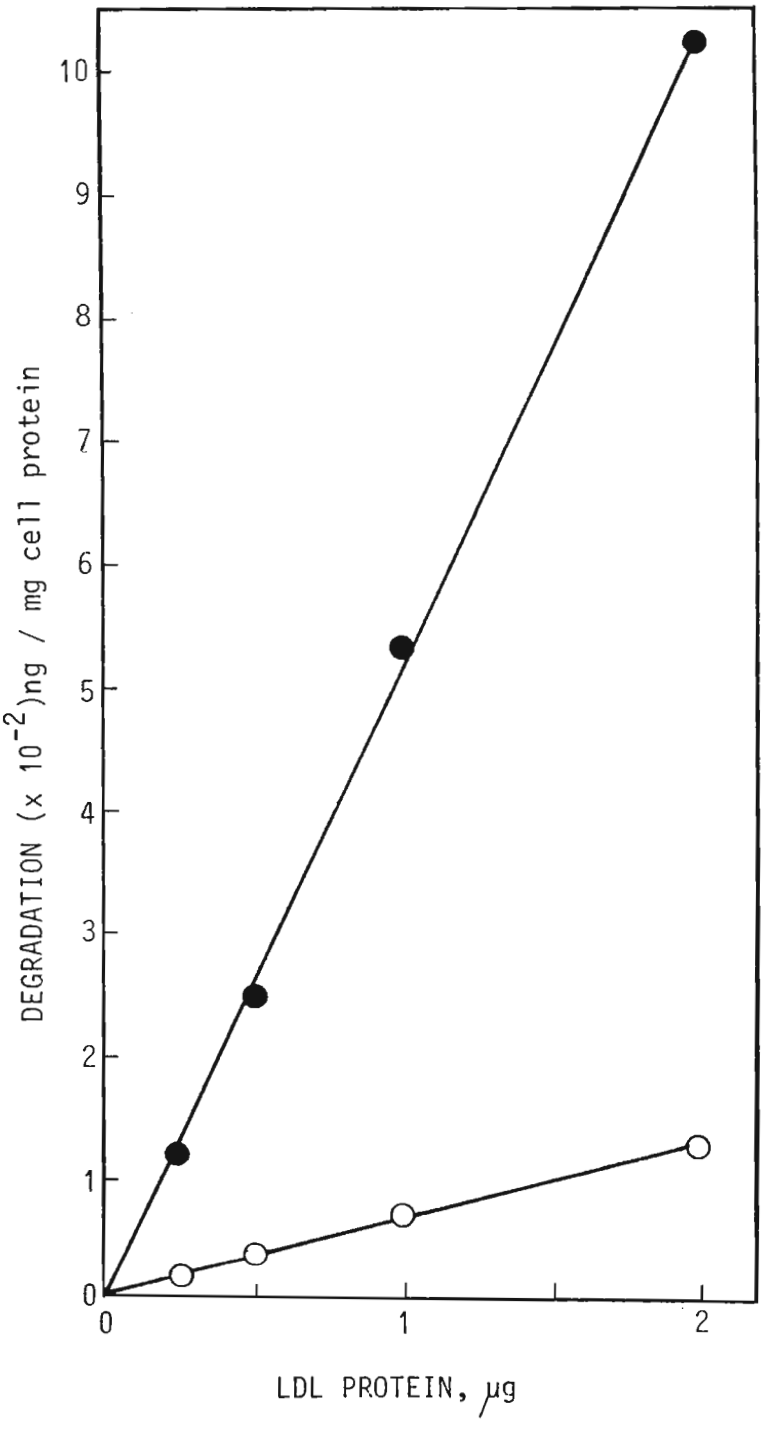


Figure 4.13 : Dedradation of [^{125}I] LDL - ct DNA (●) and ECDI -[^{125}I]- LDL - ct DNA (○) by normal skin fibroblasts.

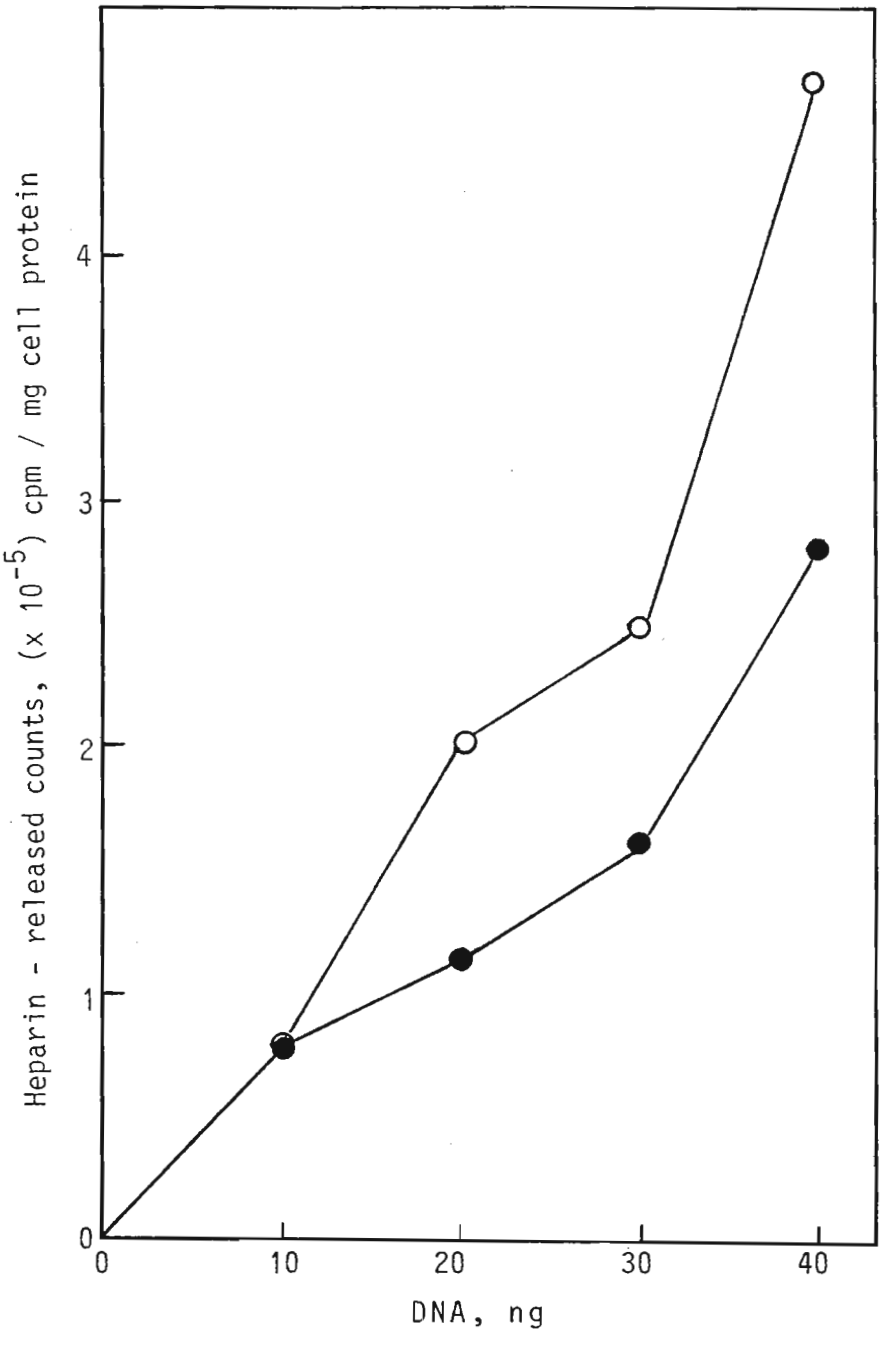


Figure 4.14 : Surface - bound LDL [^{3}H] pBR322 (●) and ECDI - LDL - [^{3}H] pBR322 (○) released from normal skin fibroblasts with heparin. The average cellular protein value per flask was 250 µg. The ratio of lipoprotein to DNA in the lipoprotein - DNA complexes was 100 : 1.

specific binding of the modified LDL to the surface of the skin fibroblasts (as described in Section 4.3.7). The amounts of LDL - [^{3}H] pBR322 and ECDI - LDL - [^{3}H] pBR322 internalized (heparin resistant counts) were equivalent (Figure 4.15). Thus, although more of the modified LDL (complexed to DNA or uncomplexed) bound to the cell surface, only those that were bound to LDL receptors were internalized (Figure 4.6). Non - specifically bound ECDI - LDL - [^{3}H] pBR322 was, therefore, not internalized. There was a slight difference (Figure 4.16) in the degradation of LDL - [^{3}H] pBR322 and ECDI - LDL - [^{3}H] pBR322, the former being a little higher. Thus both lipoprotein - DNA complexes were internalized by LDL receptor - mediation and degraded in the cell lysosomes. However, the amount of degradation was more than 50% lower than the internalization . Compared with the iodine - labelled lipoprotein - DNA complexes internalized and degraded (Figures 4.12 and 4.13), far less DNA was degraded than the lipoproteins in these complexes.

4.3.11 Competitive binding of ECDI - LDL - [^{3}H] pBR322 complex to normal skin fibroblast LDL receptors in the presence of native LDL

The reaction was analogous to that described in Section 4.3.6. Results in Figure 4.17 show that there was decreased binding of ECDI - LDL - [^{3}H] pBR322 to normal skin fibroblast monolayers when increasing amounts of unlabelled LDL were added. This experiment also shows that, like the ECDI - [^{125}I] LDL, the ECDI - LDL - [^{3}H] pBR322 competes with native LDL for the same receptor sites. Thus the

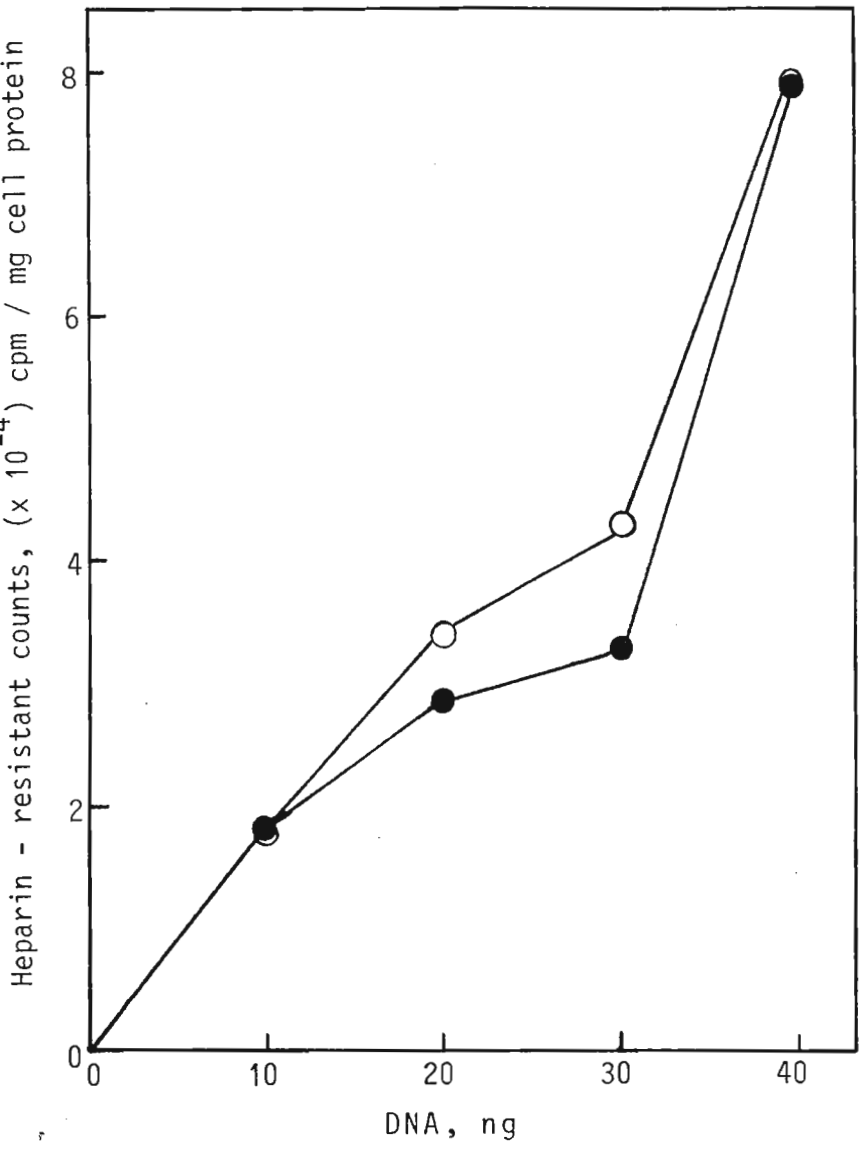


Figure 4.15 : Internalization of LDL - [^{3}H] pBR322 (●) and ECDI - LDL [^{3}H] pBR322 (O) complexes by normal skin fibroblasts.

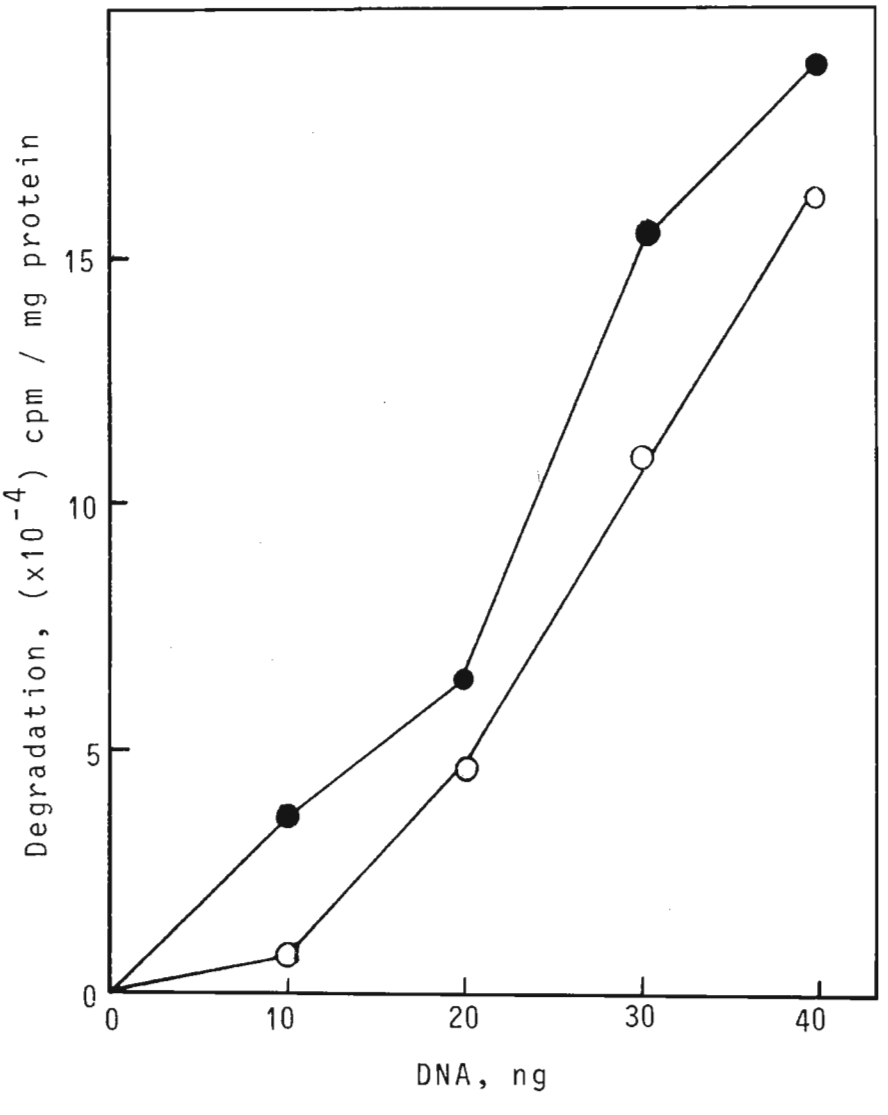


Figure 4.16 : Degradation of LDL - [^{3}H] pBR322 (●) and ECDI - LDL - [^{3}H] pBR322 (○) by normal skin fibroblasts.

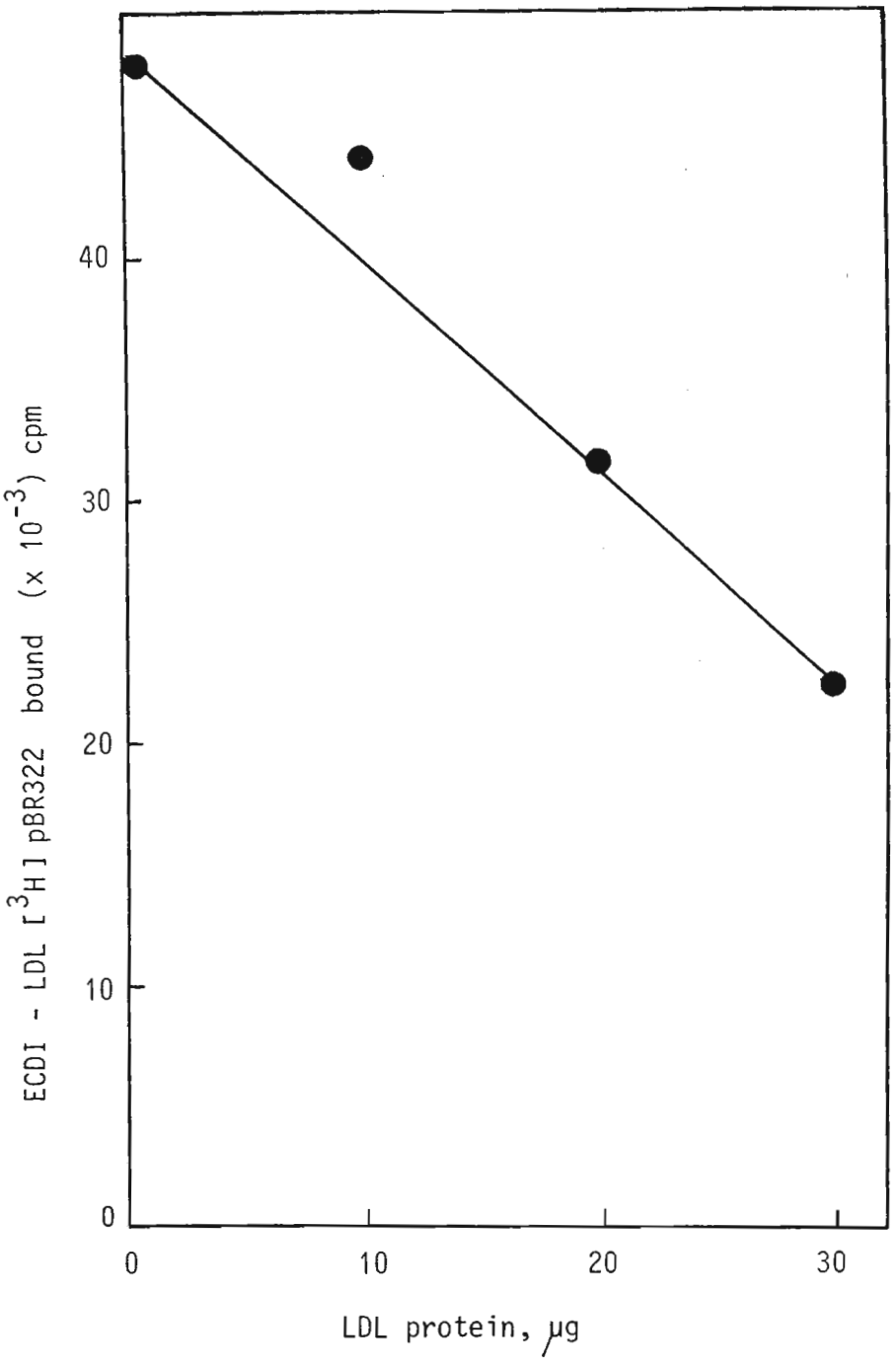


Figure 4.17 : Competitive binding of LDL and ECDI-LDL - [^{3}H] pBR322 complex to normal skin fibroblasts. A 100 ng of ECDI - LDL - [^{3}H] pBR322 (LDL protein content) was added to each flask. Native LDL was added as indicated.

carbodiimide - modified LDL - DNA complex is also subject to recognition by the LDL receptors for binding and internalization.

4.4 Concluding remarks

The total binding of ECDI - [^{125}I] LDL (LDL bound to receptors and internalized) was 100 - fold greater than that of native LDL. However, a study of the turnover (binding versus degradation) of the two lipoproteins showed that the ratio of bound : degraded [^{125}I] LDL was 1 : 100. This was 67 - fold greater than that of ECDI - [^{125}I] LDL, suggesting that some carbodiimide - modified LDL bound non - specifically to the fibroblast membranes. This was confirmed by results of the specific binding of [^{125}I] LDL and ECDI - [^{125}I] LDL (Section 4.3.5, Figures 4.5 and 4.6) and the Scatchard transformation (Figure 4.7) data. The additional type of binding by ECDI - [^{125}I] LDL may be analogous to the binding to `residual receptor sites´ described by Brown and Goldstein (1975 a). Nevertheless, a competitive binding of ECDI - [^{125}I] LDL to the same receptors as native LDL (i.e., their displacement by native LDL) was shown to occur (Figure 4.8). This confirmed that ECDI - [^{125}I] LDL enjoyed recognition by the same receptors as native LDL.

Heparin - chase experiments also served to illustrate that both [^{125}I] LDL and ECDI - [^{125}I] LDL enjoyed LDL - receptor recognition and were internalized and degraded to the same extent. ECDI - [^{125}I] LDL bound to non - receptor sites on the plasma membrane

were not internalized by LDL receptor mediation or degraded in the lysosomes.

For receptor - binding studies involving lipoprotein - DNA complexes, either the lipoprotein or the DNA component was radiolabelled. The lipoprotein to DNA ratios varied from 15 : 1 to 55 : 1. Binding assays showed an overall 2 - fold greater binding of [^{125}I] LDL - DNA than ECDI - [^{125}I] LDL - DNA (figures 4.12 and 4.13). However, heparin - chase experiments showed that although more of the carbodiimide - modified LDL - DNA complex was surface - bound, there was not a significant difference in the internalization and degradation of the DNA component of the two lipoprotein - DNA complexes (Figures 4.14 - 4.16). A decrease in the binding of ECDI - LDL - [^{3}H] pBR322, to normal skin fibroblasts, in the presence of native LDL showed that the internalization of the carbodiimide - modified LDL - DNA complex was LDL receptor - mediated.

C H A P T E R F I V E

TRANSFECTION OF CELLS IN CULTURE BY RECEPTOR - MEDIATED ENDOCYTOSIS

5.1 Introduction

The classical and most frequently used method for the introduction of foreign DNA into cultured cells is by the calcium - precipitated DNA technique (Graham and van der Eb , 1973; Pellicer *et al.*,1980; Colbere - Garapin *et al.*, 1981; Scangos and Ruddle, 1981; Gorman *et al.*, 1982 a, b; Laimins *et al.*, 1982; Kruckzek and Doerfler, 1983; Gorman *et al.*, 1983; Mc Lauchlan *et al.*, 1985; Burke and Mogg,1985; Darnell and Boime, 1985; Gorman, 1986). Several other methods for the introduction of foreign DNA into eukaryotic cells have also been employed (Section 1.13). Few of these techniques, however, can be applied *in vivo*, especially to larger organisms, including mammals.

A method of transfecting eukaryotic cells with a view to its applicability *in vitro* and *in vivo* was sought. The use of serum and serum carriers for drug targeting has been employed. However, the use of ligands such as LDL, insulin, α_1 - acid glycoprotein, growth hormone and transferrin, for the transfer of genetic material into eukaryotic cells is a novel idea which has not been tested previously. Since these ligands contain receptors on most cell surfaces of mammalian organisms, such ligands complexed with foreign DNA should be able to transfer the DNA into the cells by

their normal internalization process of receptor - mediated endocytosis.

The ligand utilized for this study was human serum low density lipoprotein (LDL) which contains receptors on all normal somatic cell types throughout the body. The foreign DNA utilized for the study was the recombinant pSV2cat DNA. This is an expression vector which contains the beta - lactamase gene and origin of replication from plasmid pBR322, coupled to the SV40 early transcription region. The SV 40 early region contains the 72 base - pair (bp) repeated sequences which function as activators or enhancers of early viral gene expression in eukaryotic systems (Bernoist and Chambon, 1981; Gruss *et al.*, 1981). The pSV2cat recombinant DNA also contains the chloramphenicol acetyltransferase (CAT) gene (Gorman *et al.*, 1982, a, b; Gorman *et al.*,1983; Gorman, 1986). The construction of the pSV2cat recombinant vector is presented in Figure 5.1.

Chloramphenicol acetyltransferase (CAT) activity is not an endogenous characteristic of mammalian cells (Gorman *et al.*, 1982b), thus the successful expression of CAT activity in mammalian cells is indicative of successful transfection of the cells. The CAT gene is transiently expressed in mammalian cells in culture, thus the pSV2cat recombinant DNA should provide a convenient method for assaying the expression of the vector, introduced into mammalian cells, *in vitro*, by LDL receptor - mediated endocytosis.

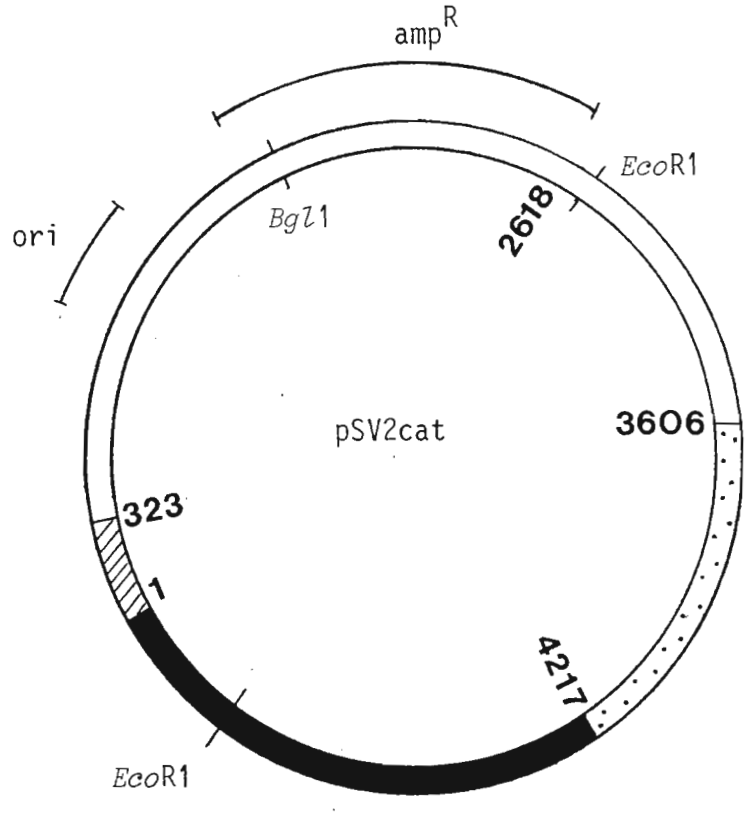


Figure 5.1 : The pSV2cat expression vector. The recombinant DNA contains the SV 40 early region promoter (▨) sequences and the polyadenylation site and small t intron from SV 40 (⚄). It also contains the prokaryotic ori region and the amp^R gene from pBR322. The cat gene is indicated by (■). The pSV2cat plasmid contains 5 003 base pairs.

In order to carry out the transfection of mammalian cells by LDL receptor - mediated endocytosis, LDL was first prepared as described in Section 2.2.1. A portion of the LDL was also modified with carbodiimide as described in Section 2.2.2. The expression vector pSV2cat was amplified in *E. coli* HB 101 according to published methods (Maniatis *et al.*, 1982; Gorman, 1986). Lipoprotein - DNA complexes were applied to sub - confluent monolayers of several mammalian cell lines. Successful transfection of these cells by LDL receptor - mediated endocytosis was detectable as the expression of chloramphenicol acetyltransferase activity.

5.2 Methods

5.2.1 Preparation of pSV2cat plasmid DNA (Method 1)

5.2.1.1 Growth of pSV2cat plasmid in *E. coli*

A tryptose agar base (TAB) streak - plate was prepared using *E. coli* HB 101 containing pSV2cat plasmid grown on an agar slope. The TAB agar plate was incubated overnight at 37°C. A single colony from the streaked plate was used to inoculate a 15 ml liquid culture of Penassay broth, prepared according to the manufacturer's instructions. This constituted the first inoculum. The inoculum was then added to a 75 ml aliquot of Penassay broth to allow further growth of the plasmid - containing bacteria, overnight, at 37°C, without shaking. The overnight culture was used to inoculate 750 ml of Penassay broth, which was subsequently incubated at 37°C, with

vigorous shaking, for 6 hours or until the turbidity had increased (OD_{600} = 0,6). To the plasmid - *E. coli* culture was added 170 µg chloramphenicol / ml broth and amplification was allowed to proceed overnight, with vigorous shaking, at 37°C. All culture media contained 50 µg ampicillin per ml of medium.

5.2.1.2 Harvesting of *E.coli* containing the pSV2cat plasmid DNA

E. coli cells containing the pSV2cat plasmid DNA were harvested by centrifugation at 4 000 x g for 30 minutes, at 4°C. The pelleted bacterial cells were washed by suspension in buffer (0,1 M NaCl - 0,01 M Tris - HCl, pH 7,8 - 1 mM EDTA) and centrifugation. The bacterial cells were resuspended in 10 ml STET (0,1 M NaCl - 10 mM Tris - HCl, pH 8,0 - 0,1 mM EDTA - 0,5% Triton X 100).

5.2.1.3 Lysozyme treatment of pSV2cat DNA - containing *E. coli*

Lysozyme (20 mg) was dissolved in 1 ml 10 mM Tris - HCl (pH 8,0) and added to the pSV2cat plasmid - containing *E. coli* cells in STET. The bacterial cells were incubated with lysozyme for 10 minutes at room temperature. The resultant viscous solution was boiled rapidly over a flame, plunged into boiling water for 40 seconds and then into an ice - bath for 5 minutes. The bacterial cell debris was pelleted at 80 000 x g, in an SW 50 rotor, for 30 minutes, at 4°C and discarded. The supernatant constituted the crude plasmid preparation.

5.2.1.4 Purification of plasmid DNA by cesium chloride density gradient ultracentrifugation

The crude plasmid DNA preparation was mixed with CsCl to give a refractive index of 1,3862. Ethidium bromide (10 mg / ml) was added to the preparation, and dispensed into 3,5 ml nitrocellulose tubes. Each tube was overlayed with paraffin oil and placed in an SW 50 rotor. Ultracentrifugation was carried out at 10 000 x g at 20°C for 35 hours. The upper and lower plasmid bands were located under UV illumination and collected with a finely drawn out Pasteur pipette. The ethidium bromide was extracted twice with water - saturated butanol. The top and bottom plasmid bands were dialysed against 0,01 M Tris - HCl, pH 8,0 - 1 mM EDTA at 4°C, with six changes of buffer over a period of 36 hours. The plasmid preparation was analysed by agarose gel electrophoresis and UV spectroscopy. The plasmid DNA was also subjected to restriction analysis (Section 5.2.3). The pSV2cat DNA was stored at -20°C.

5.2.2 Alternate method for the preparation of pSV2cat plasmid DNA (Method 2)

Plasmid - containing *E. coli* were grown in "superbroth" (Gorman, 1986), prepared as follows : 24 g tryptone, 48 g yeast extract, 10 ml glycerol and 1 800 ml distilled water were mixed together; to this mixture was added 25 g K_2HPO_4 and 7,6 g KH_2PO_4 dissolved in 200 ml

distilled water. A 5 ml inoculum in "superbroth" was prepared from a single colony picked off a streaked TAB plate by incubation at 37°C for six hours, and added to 2 l of "superbroth". The bacterial growth and amplification was then carried out as described in Section 5.2.1.1. The plasmid - containing bacterial cells were harvested as described in Section 5.2.1.2. The *E. coli* HB 101 cells were either stored at -20°C at this stage of plasmid preparation or resuspended in TES (40 mM Tris - HCl, pH 7,5 - 40 mM EDTA - 25 % sucrose) and subjected to lysozyme treatment. For the lysozyme treatment, the bacterial cells were resuspended in 8,0 ml TES and placed on ice. To the cell suspension was added 0,6 ml lysozyme solution (20 mg dissolved in TES), and incubated on ice for 5 minutes. EDTA (2,4 ml, 0,25 M, pH 8,0) was added and incubation on ice was continued for a further 5 minutes. To the resultant viscous solution was added 9,7 ml Triton solution (0,1% Triton X - 100 - 8 mM EDTA - 50 mM Tris - HCl, pH 7,9) and incubation was continued on ice for an additional 10 minutes. Bacterial cell debris was pelleted at 75 000 x g, in an SW 28 rotor, for 30 minutes, at 4°C. The plasmid and cellular DNA - containing supernatant was subjected to CsCl density gradient ultracentrifugation as described in Section 5.2.1.4. The ethidium bromide was extracted with isopropanol saturated with CsCl solution at a density of 1,574 g / ml. The plasmid DNA was dialysed against several changes of 0,01 M Tris - HCl, pH 8,0 - 0,001 M EDTA at 4°C, analysed by restriction endonuclease digestion, and stored at - 20°C.

5.2.3 Restriction endonuclease digestion of pSV2cat DNA

Plasmid DNA prepared by methods 1 and 2 was subjected to digestion with *Eco*R1 and *Pst*1 restriction enzymes, according to the manufacturers' recommendations, at medium salt strengths. In a total volume of 42 µl, pSV2cat DNA (1,725 µg) was incubated at 37°C for 1 hour, in a reaction mixture containing 50 mM NaCl, 10 mM Tris - HCl (pH 7,6), 10 mM $MgCl_2$ and 1 mM mercaptoethanol, with 3,7 units of *Eco*R1 restriction enzyme. For the *Pst*1 digestion, 5 units of enzyme was used to digest 2 µg pSV2cat DNA, in a reaction mixture containing 55 mM NaCl, 10 mM Tris - HCl (pH 7,2), 10 mM $MgCl_2$ and 0,5 mg / ml albumin.

5.2.4 Ethanol precipitation of plasmid DNA

To the plasmid DNA dialysed in 10 mM Tris - HCl, pH 8,0 - 0.001 M EDTA was added 0,1 volume 4 M NaCl and 2,5 volumes redistilled ethanol. The DNA was precipitated at -20°C for 1 hour and then centrifuged at 9 000 x g, at 4°C, to pellet the DNA. The DNA was dissolved overnight in 10 mM NaCl for use in the transfection of cells in culture.

5.2.5 Transfection of cells in culture with pSV2cat DNA

5.2.5.1 Transfection of cells in culture by the calcium phosphate - DNA coprecipitation technique

Cells in culture were trypsinized and plated in 25 cm^2 flasks, to a density of 5 x 10^5 cells, 24 hours prior to transfection.

Three hours prior to transfection fresh complete medium was added to each flask. The calcium phosphate - DNA complex preparation for each flask was as follows : To the pSV2cat DNA (1 - 10 µg) was added $CaCl_2$ to give a final concentration of 250 mM in a volume of 250 µl. In experiments where low concentrations of DNA were added, 10 µg calf thymus DNA was added as carrier. The Ca^{++}- DNA solution was then added dropwise with agitation (sterile N_2 gas or air bubbled through) to 250 µl Hepes buffered saline which consisted of 1,64% (w / v) NaCl, 1,2% (w / v) 2 - [4 - (2 - hydroxyethyl) - 1 - piperazinyl] - ethane - sulfonic acid and 0,04% (w / v) Na_2HPO_4 adjusted to pH 7,1. The calcium phosphate - DNA coprecipitation was allowed to occur at room temperature for 30 minutes and then added to the cells. Monolayers were incubated at 37°C for 4 - 18 hours post - transfection before being washed and fed with complete medium. Incubation was continued until 48 hours post - transfection. The cell lines utilized for transfection studies were normal skin fibroblasts, HeLa cells, fetal fibroblasts, CV - 1 cells and human lung fibroblasts.

5.2.5.2 Transfection of cells in culture by LDL receptor mediation

Cells in culture were trypsinized and plated in 25 cm^2 flasks 24 hours prior to transfection, to a density of 5 x 10^5 cells. About 10 minutes prior to transfection the complete medium was removed and replaced with 3 ml Eagle's MEM containing 10% lipoprotein - deficient serum. Ten µg calf thymus DNA was also added to each flask prior to transfection. Each flask received

lipoprotein - DNA solutions prepared as follows : To 50 - 100 µg LDL or ECDI - LDL (protein) in STE_1 (25 mM Tris - HCl, pH 7,6 - 50 mM NaCl - 2,7 mM EDTA) was added 1 - 25 µg DNA (in 10 mM NaCl), in a total volume of 500 µl. The solution was mixed and incubated at room temperature for 20 minutes before being added to each flask. Cells were incubated for 24 hours post - transfection before complete medium was added. Incubation was continued for up to 48 hours post - transfection. The cell lines used in the experiments were human skin fibroblasts, HeLa cells, fetal fibroblasts, CV - 1 cells and human lung fibroblasts.

5.2.5.2.1 Preparation of cell - free extract

Transfected cells (in 25 cm^2 flasks) were washed with phosphate - buffered saline (PBS) pH 7,5 (2 washes, 3 ml each). Cells from individual flasks were incubated on ice for 5 minutes with 1 ml each of TEN (40 mM Tris - HCl, pH 7,5 - 1,0 mM EDTA - 0,15 M NaCl). Monolayers were dislodged with a cell scraper and transferred to Eppendorf tubes and placed on ice. Cells were pelleted at 9 000 x g for 1 - 2 minutes and resuspended in 100 µl 0,25M Tris - HCl, pH 7,9 and then disrupted by freeze - thawing. Cell debris was pelleted at 9 000 x g and discarded. The cell - free supernatant was assayed for chloramphenicol acetyltransferase (CAT) activity.

5.2.5.2.2 Assay for the expression of the CAT gene

The cell - free extracts (prepared as described in Section 5.2.5.2.1)

were assayed for chloramphenicol acetyl transferase activity. Fifty µl of cell extract in a final Tris - HCl (pH 7,9) buffer concentration of 0,25 M was incubated with 0,2 µCi [^{14}C] chloram - phenicol (D - threo - [dichloroacetyl - 1 - ^{14}C] chloramphenicol, 54 mCi / m mole) and 0,8 mM acetyl coenzyme A in a total volume of 100 µl. The reaction mixtures were incubated at 37°C for exactly one hour. Cell extracts from flasks to which either only DNA or lipoprotein were added were used as controls. An enzyme control was also included with each set of assays. CAT enzyme activity was stopped by the addition of 1 ml ethylacetate to each reaction mixture. Thorough mixing ensured that the chloramphenicol (CM) and its acetylated derivatives were extracted into the ethyl acetate. The organic layer was retained and evaporated to dryness under a stream of N_2 gas. The residue was redissolved in 30 µl ethylacetate and spotted onto silica gel 60 F_{254} plastic - backed thin layer plates. Each plate was developed in freshly prepared $CHCl_3$ - MeOH (95 : 5, v / v). The acetylated products were located by auto - radiography.

5.2.5.2.3 Autoradiography

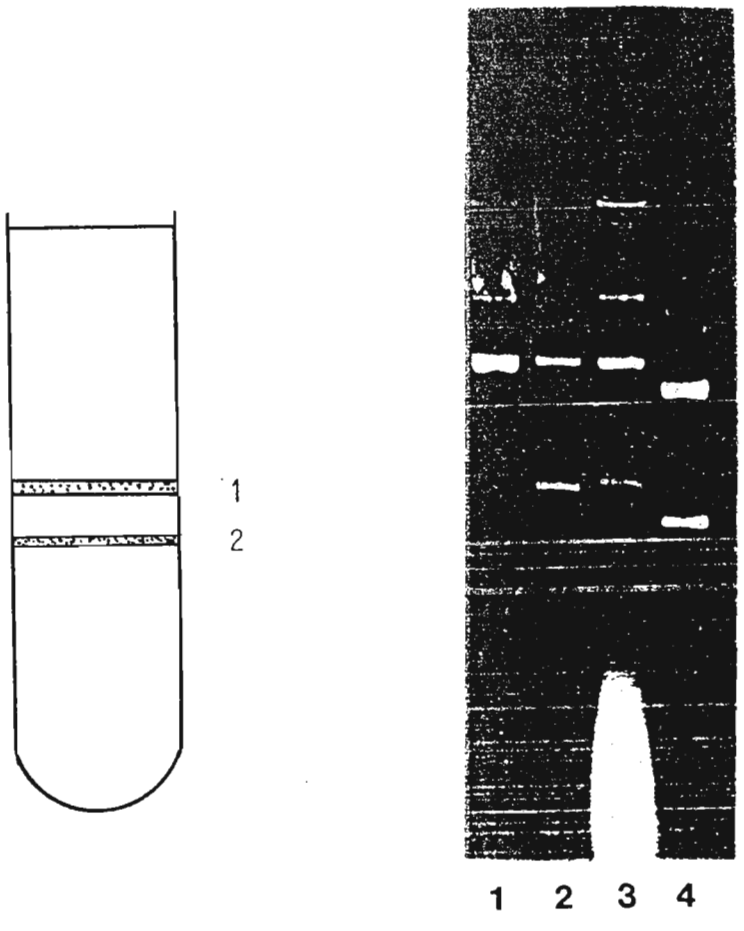
Thin layer chromatograms were placed in contact with Hyperfilm β max X - ray film for 48 - 72 hours. The X - ray film was developed in Phenisol X - ray film developer (Ilford), for 2 - 3 minutes, with agitation, immersed in stop solution for 30 seconds and fixed in rapid fixer for 3 minutes. Solutions were prepared according to manufacturers' instructions.

5.3 Results and Discussion

5.3.1 Preparation of pSV2cat DNA

The plasmid - containing *E. coli* were grown from an agar slope onto agar plates. Single colonies from the streaked plates were used to amplify the plasmid in suspension cultures by methods 1 and 2. The difference in the methods lay in the preparation of growth media and lysozyme digestion of the bacterial cell walls (Sections 5.2.1.3 and 5.2.1.5). Method 1 entailed boiling and rapid cooling, following lysozyme treatment under alkaline conditions. Bacterial DNA remains denatured under these conditions (Maniatis *et al.*, 1982) while closed circular plasmid DNA molecules regain their native configuration upon cooling. Method 2 made use of alkaline conditions and Triton X - 100 for bacterial cell lysis. A return to neutral pH results in renaturation of plasmid DNA while *E. coli* DNA remains denatured. Nevertheless, both preparation methods involved the differential sedimentation step for the removal of larger *E.coli* DNA strands, together with the lysed cell debris, to yield the crude plasmid preparation.

Plasmid DNA was purified by CsCl density gradient ultracentrifugation in the presence of ethidium bromide. Since the covalently closed circular plasmid DNA binds much less of the intercalating dye than the linear bacterial DNA, it bands at a higher density than the linear or nicked DNA (Radloff *et al.*, 1967). Thus, highly purified bands were obtained (Figure 5.2 a). The ethidium bromide



a b

Figure 5.2a : Cesium chloride density gradient ultracentrifugation of pSV2cat plasmid; 1 = nicked circular DNA; 2 = supercoiled circular DNA. Bands were visualized under UV illumination.

b : Agarose gel electrophoresis of pSV2cat plasmid DNA prepared by method 1. Lanes 1 and 2 contain top and bottom bands respectively, lanes 3 and 4 contain pSV2cat plasmid crude preparation and pBR322 DNA respectively.

was removed from the plasmid preparations by either water - saturated butanol (method 1) or isopropanol saturated with CsCl of density 1,574 g / ml (method 2).

The concentration of pSV2cat DNA was determined using the formula OD_{260} = 1 = 40 µg DNA (Humphreys *et al.*, 1975). Plasmid yield by method 1 was 2 mg / l of culture medium while the yield with method 2 was 5 mg / l culture medium. Higher yield by method 2 was probably due to the use of a highly enriched medium which allowed a denser growth of the plasmid - carrying bacteria. Another reason could be the gentler bacterial lysis procedure used in method 2, which would reduce losses of plasmid DNA through nicking or linearization. UV spectral analysis yielded an OD_{280} / OD_{260} ratio of 0,7 , indicating slight contamination with protein.

5.3.2 Electrophoretic analysis of pSV2cat DNA

The purity of the top and bottom bands after ultracentrifugation was assessed by agarose gel electrophoresis (Figure 5.2 b). The crude preparation showed linear, nicked circular and circular supercoiled DNA. The top band (Figure 5.2 a) contained nicked circular DNA and was collected fairly pure (lane 1, Figure 5.2 b). The bottom band contained supercoiled DNA, but was contaminated with nicked circular DNA (lane 2). Contamination possibly occurred during collection of the plasmid bands after CsCl density gradient ultracentrifugation.

5.3.3 Restriction analysis of pSV2cat DNA

*Eco*R1 and *Pst*1 restriction of pSV2cat were carried out as described in Section 5.2.3. Restriction fragments were separated on 1,25% agarose mini gels (Figure 5.3). The size of each fragment was determined from a plot of log of marker fragment molecular weight versus its mobility in the electrophoretic field. The fragment sizes corresponded closely with published values (Gorman, 1986). Since separation of restricted fragments was done on mini gels, errors in determination of fragment lengths were expected.

5.3.4 Transfection of cells in culture with pSV2cat recombinant DNA by LDL receptor mediation

5.3.4.1 Chloramphenicol acetyltransferase (CAT) activity

Some bacterial strains of *Escherichia coli*, *Streptococcus aureus*, *Diplococcus pneumoniae and Agrobacterium tumefaciens* are resistant to the antibiotic chloramphenicol (Shaw, 1967; Shaw and Brodsky, 1968; Shaw, 1975). This resistance phenotype is due to the inactivation of the antibiotic by the enzyme chloramphenicol acetyltransferase (CAT) which is induced by episomal resistance factors (Shaw, 1967). The plasmid - borne CAT gene codes for the CAT enzyme which inactivates chloramphenicol by acetylating the 3'- hydroxy position (Shaw, 1975). The 1 - acetylated derivative is produced by intramolecular conversion of chloramphenicol 3 - acetate to chloramphenicol 1 - acetate. Subsequent enzymatic acetylation of chloramphenicol 1 - acetate yields the 1,3 - di -

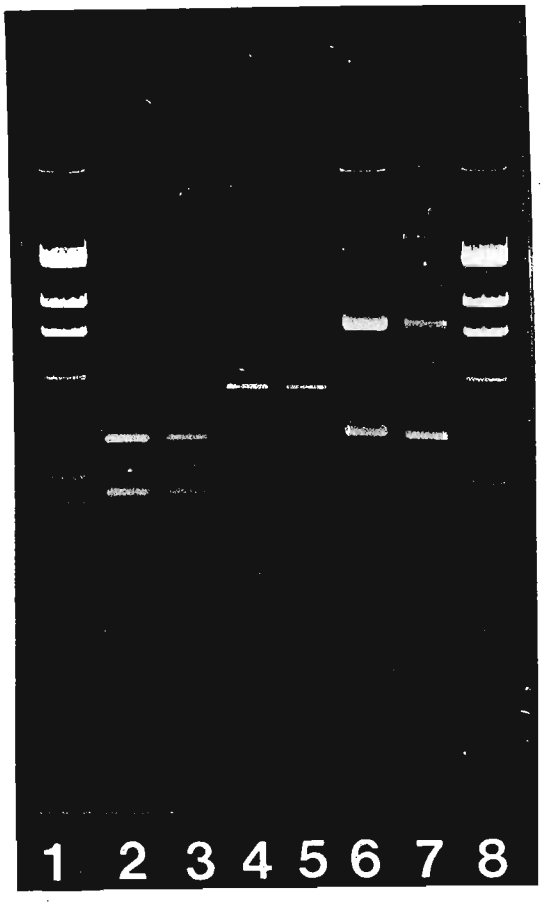


Figure 5.3 : *Eco*R1 and *Pst*1 digestion of pSV2cat DNA. Lanes 1 and 8 : DNA molecular weight marker; lanes 2 and 3 : *Eco*R1 digestion of pSV2cat DNA; lanes 4 and 5 : *Pst*1 digestion of pSV2cat DNA; lanes 6 and 7 : pSV2cat DNA. Components in lanes 2, 4 and 6 were from pSV2cat DNA prepared by method 1 (Section 5.2.1) and those in lanes 3, 5 and 7 were from the plasmid prepared by method 2 (Section 5.2.2).

acetylated chloramphenicol (Figure 5.4).

Use has been made of purified CAT enzyme to acetylate [^{14}C] chloramphenicol at 37°C, for 1 hour, in the presence of Acetylcoenzyme A (i.e., under ideal conditions). Chloramphenicol and its acetylated derivatives were extracted into ethylacetate and separated by thin layer chromatography as described in Section 5.2.5.2.2. Spots were located by UV illumination (Figure 5.5 a) and autoradiography (Figure 5.5 b). Quantitation of acetylated products in a liquid scintillation counter showed that 58,8% of the chloramphenicol had been converted to chloramphenicol 3-acetate, 7,6% to chloramphenicol 1-acetate and 28,5% to chloramphenicol 1,3-diacetate. Only 5,1% of the [^{14}C] chloramphenicol remained unreacted and was detectable by autoradiography (Figure 5.5b) and not by UV illumination. The [^{14}C] chloramphenicol standard appeared as a single UV absorbing spot but consistently showed contamination on the autoradiograms.

5.3.4.2 Control experiments

The prime aim of the experimental work in this chapter was to transfect cells in culture with the recombinant pSV2cat expression vector by LDL receptor mediation. In order to confirm that transfection could be carried out with some degree of success by this method, several control experiments had to be carried out.

Since LDL or ECDI-LDL was complexed to pSV2cat DNA (supercoiled,

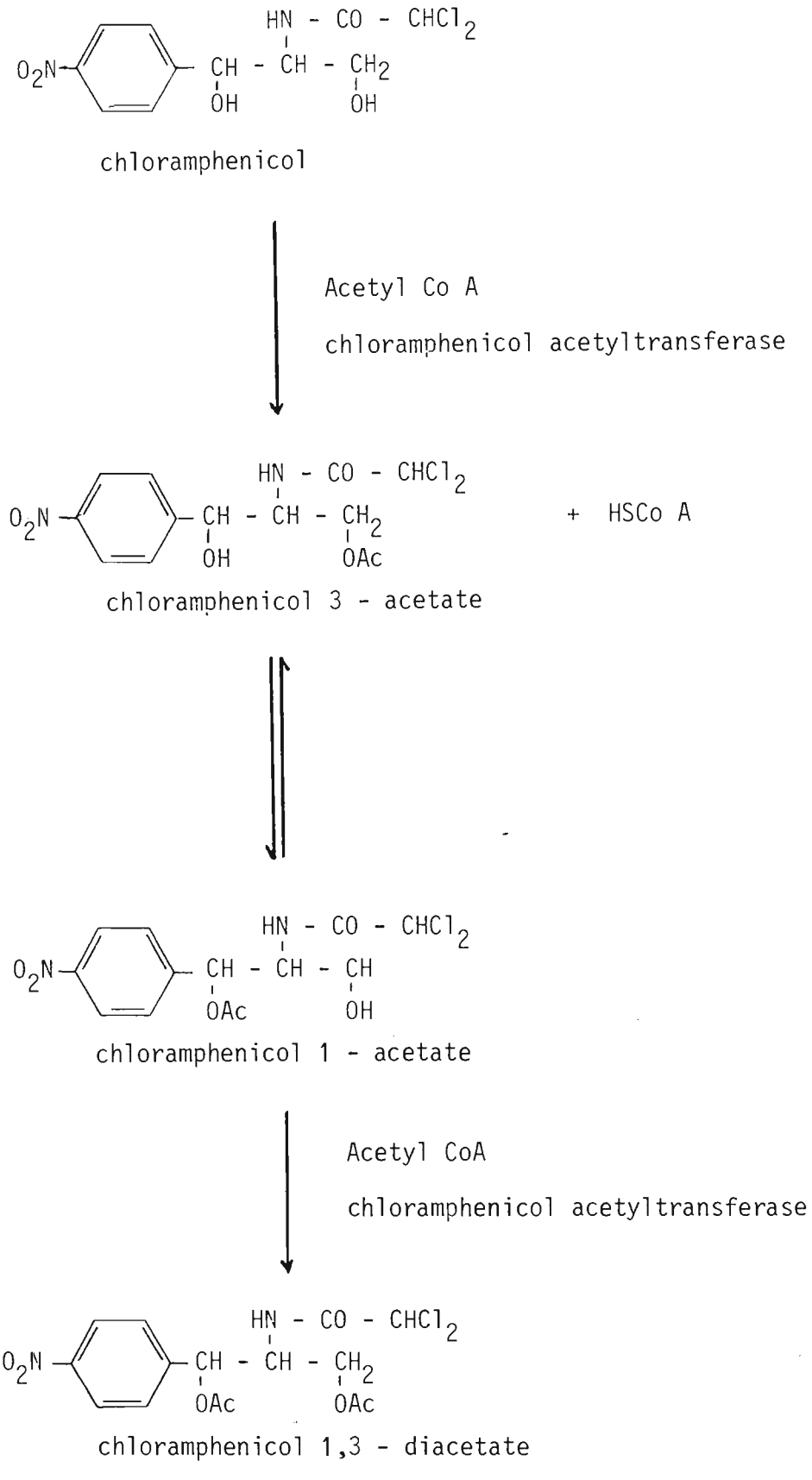


Figure 5.4 : The enzymatic acetylation of chloramphenicol with chloramphenicol acetyltransferase.

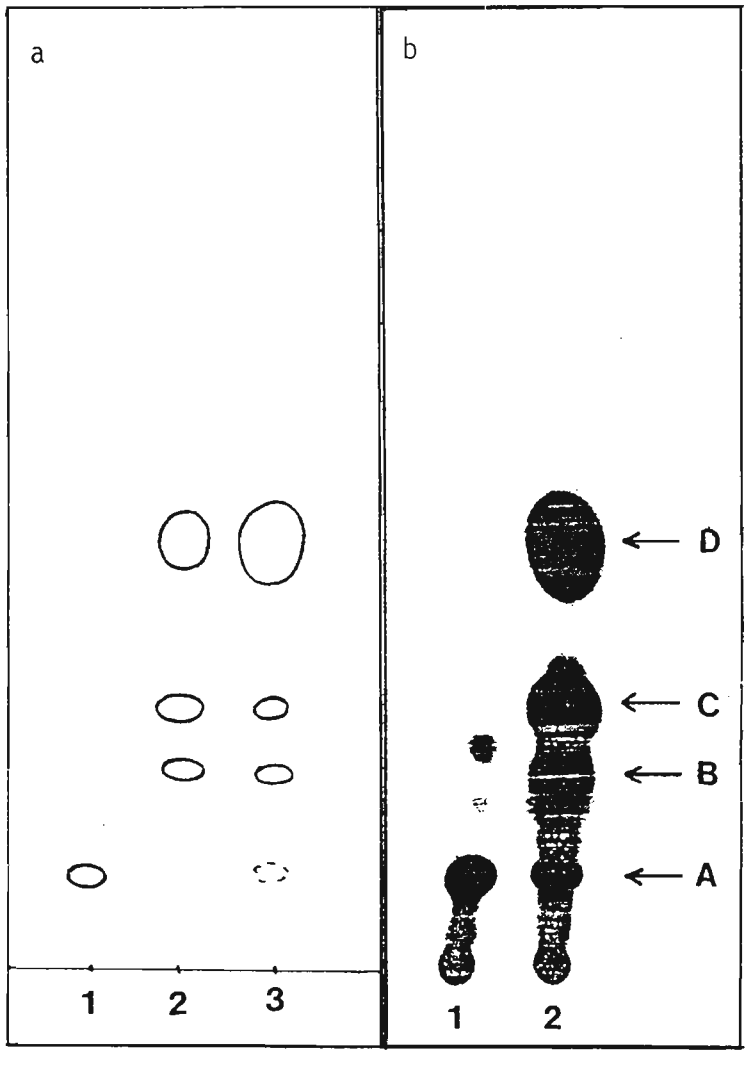


Figure 5.5a : Thin layer chromatography of [^{14}C] chloramphenicol (1), enzymatically acetylated products of [^{14}C] chloram - phenicol (2) and chemically prepared derivatives of chloramphenicol (3). Spots were located by UV illimination.

b : Autoradiogram of thin layer chromatogram (a).

A = chloramphenicol, B = chloramphenicol 1 - acetate, C = chloramphenicol 3 - acetate, D = chloramphenicol 1,3 - diacetate.

Section 5.3.1) for the transfections, control experiments were carried out by applying (i) LDL only, (ii) ECDI - LDL only, or (iii) pSV2cat alone, to monolayers in control flasks. Cells in control flasks were incubated at 37°C for 48 hours and harvested. The cell-free extracts were assayed for chloramphenicol acetyl - transferase (CAT) activity as described in Section 5.2.5.2.2. Results showed no CAT activity in any of the controls (Figure 5.6). A single spot obtained on the autoradiograms, between the monoacetylated derivatives, B and C, was also found in the chloramphenicol standard and was probably a contaminant. The acetylated derivatives are indicated by arrows (Figure 5.6) and constitute products of the purified enzyme (enzyme control). Enzyme controls were used with most CAT assays of transfected cell extracts, as reference for locating the acetylated chloram - phenicol products. The results in Figure 5.6 are those obtained with human lung fibroblasts and are representative of controls in all cell lines used for transfection with LDL - pSV2cat or ECDI - LDL - pSV2cat complexes.

5.3.4.3 Transfection of eukaryotic cells by LDL receptor - mediation

Transfection by LDL receptor - mediation was attempted on human skin fibroblasts, HeLa cells, CV - 1 cells, human fetal fibroblasts and human lung fibroblasts grown in culture. Cells were incubated for up to 48 hours with LDL - pSV2cat complex or ECDI - LDL - pSV2cat complex (Section 5.2.5.2). The cells were harvested and lysed (Section 5.2.5.3). The cell extracts were then assayed for CAT

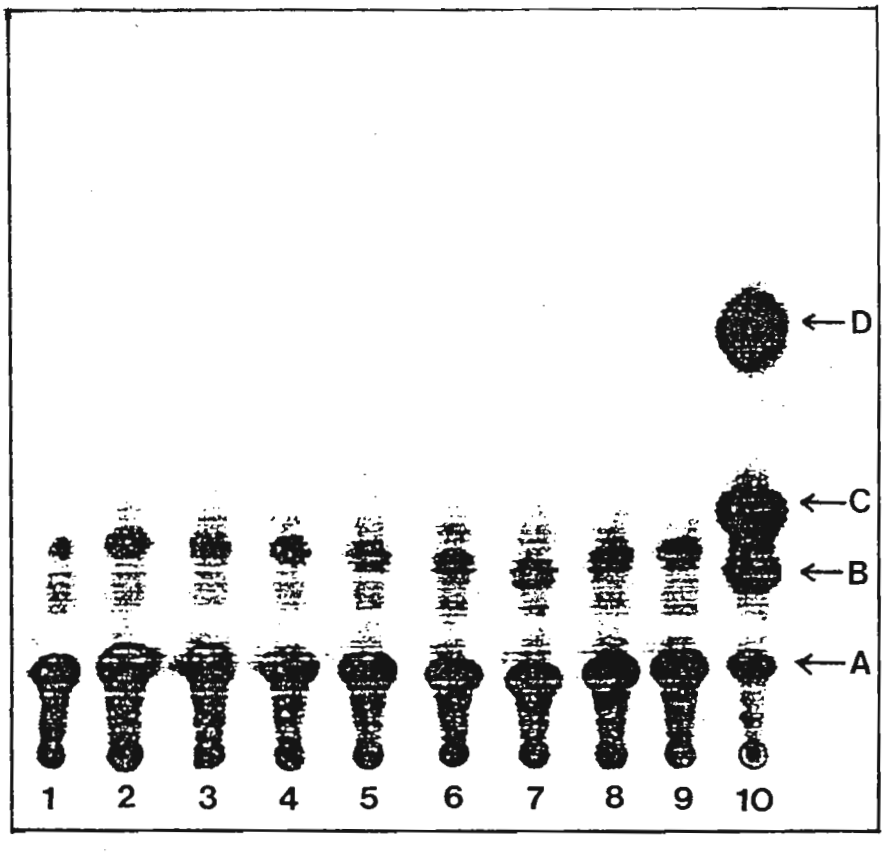


Figure 5.6 : Autoradiogram of typical control experiments carried out on all cell lines used for transfection by LDL receptor - mediated endocytosis. Results presented here are chloramphenicol acetyltransferase enzyme assays carried out on cell extracts of human lung fibroblasts to which either LDL (2 and 3), ECDI - LDL (4 and 5) or pSV2cat DNA (6, 7, 6 and 9 - containing 2,5 µg, 5 µg, 10 µg and 15 µg DNA respectively) were added and incubated at 37°C for 48 hours. [^{14}C] chloramphenicol standard = 1; 10 represents [^{14}C] chloramphenicol subjected to acetylation by purified chloramphenicol acetyltransferase enzyme. A,B,C and D are defined in the legend of Figure 5.5b.

activity (Section 5.2.5.2.2). [^{14}C] Chloramphenicol was used for the assays,(all assays were for one hour) and acetylated products were separated by thin layer chromatography, located by autoradiography (Section 5.2.5.2.3) and quantitated by liquid scintillation counting. With each cell type used to carry out transfection by LDL receptor - mediated endocytosis (Section 5.2.5.2) the calcium phosphate - DNA coprecipitation method of transfection was also carried out (Section 5.2.5.1). Untransfected cell extracts were used as controls for the $Ca_3(PO_4)_2$ - DNA transfected methods.

Normal skin fibroblasts could not be transfected efficiently by LDL receptor mediation. Both LDL - pSV2cat and ECDI - LDL - pSV2cat transfected cells exhibited low efficiencies of transfection in these cells (Figure 5.7). Up to 10 µg of plasmid DNA was used in the studies. The efficiency of transfection by LDL receptor - mediation was assessed by assaying for CAT activity for exactly one hour and quantitating the conversion of [^{14}C] chloramphenicol to acetylated products. About 0,1% of the chloramphenicol was converted to acetylated products in the skin fibroblasts transfected with both LDL - pSV2cat and ECDI - LDL - pSV2cat complexes and were barely detecteble by autoradiography.

HeLa cells also showed a very low efficiency of transfection by LDL receptor - mediated endocytosis (Figure 5.8). CAT assays yielded up to 0,4% acetylation. CV - 1 cells showed lower efficiencies of trans - fection (Figure 5.9) than HeLa cells. Up to 10 µg of DNA was used in the experiments.

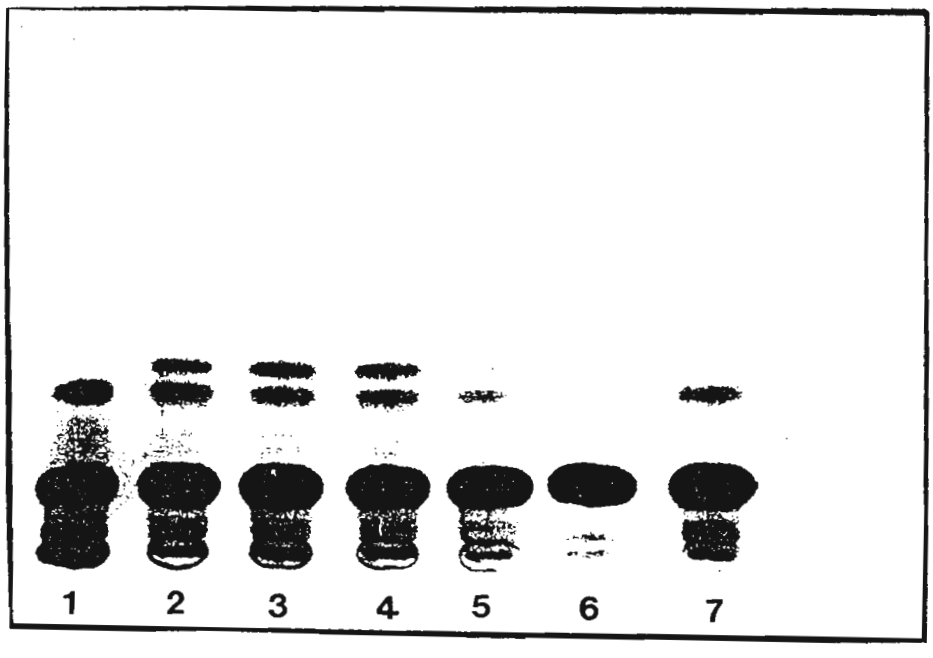


Figure 5.7 : Autoradiogram of cell extracts from normal skin fibroblasts transfected by $Ca_3(PO_4)_2$ - pSV2cat and lipoprotein - pSV2cat techniques. 1 : [^{14}C] chloram - phenicol standard; 2, 3 and 4 : calcium phosphate - DNA transfections using 2,5 , 5 and 7,5 µg DNA respectively; 5, 6 : transfection with LDL - pSV2cat and ECDI - LDL - pSV2cat complexes respectively; 7 : pSV2cat DNA control.

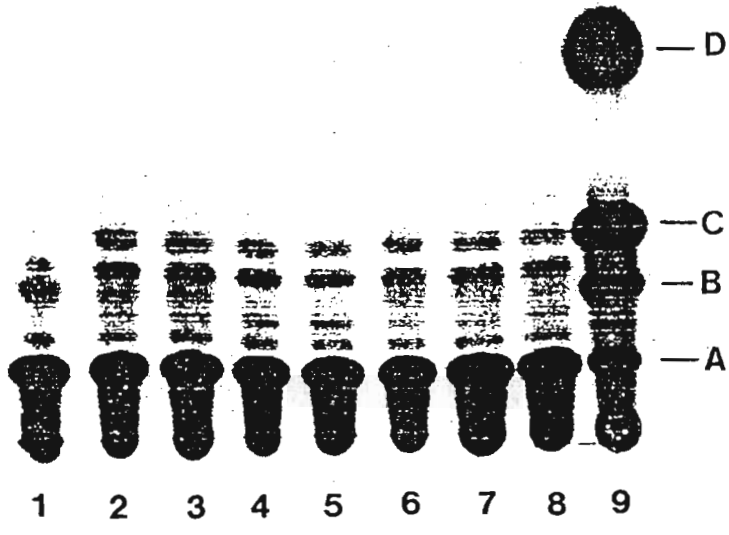


Figure 5.8 : HeLa cells transfected by LDL receptor - mediated endocytosis and calcium - precipitated DNA technique. 1 : [^{14}C] chloramphenicol standard; 2, 3 : calcium phosphate - pSV2cat - transfected cell extracts (2,5 µg and 5 µg DNA respectively); 4, 5 : LDL - pSV2cat - transfected cell extracts; 6, 7 : ECDI - LDL - pSV2cat transfected cell extracts; 8 : pSV2cat DNA control; 9 : enzyme control.

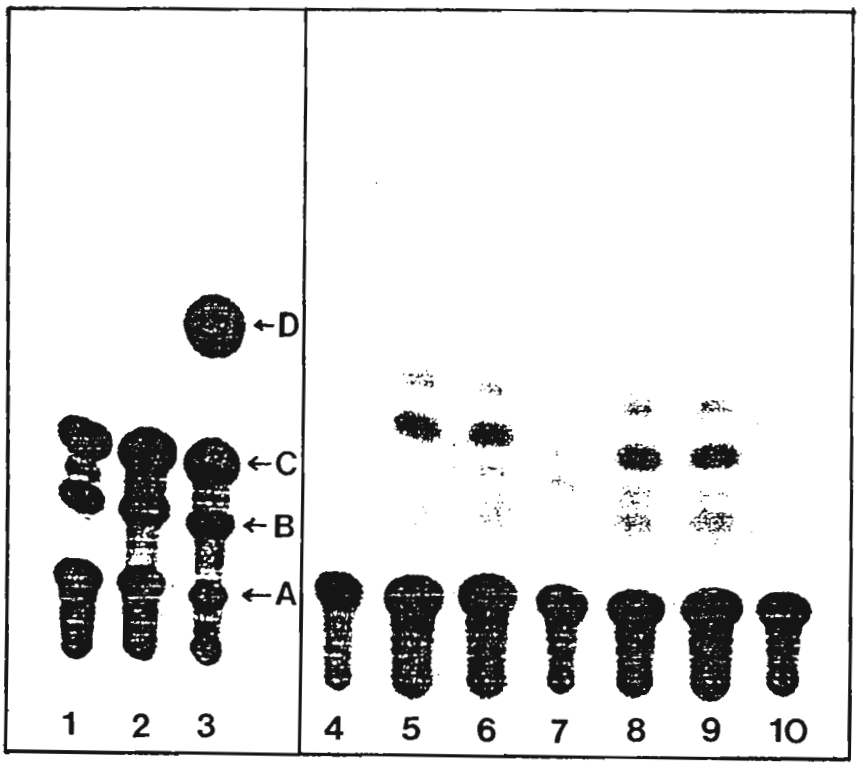


Figure 5.9 : Autoradiograms of CV - 1 cell extracts assayed for CAT activity following transfection with calcium phosphate - pSV2cat (1 and 2), LDL - pSV2cat (5 and 6), and ECDI - LDL - pSV2cat complexes (8 and 9).Controls were as follows : 3 : enzyme control; 4 : LDL; 7 : ECDI - LDL; 10 : pSV2cat DNA control.

Fetal fibroblasts showed slightly higher transfection efficiencies than the above cell lines. The CAT assays yielded up to 1,05% acetylation with cell extracts from LDL - pSV2cat transfected cells and 1,2% in ECDI - LDL - pSV2cat transfected cells. The amount of DNA in each case varied between 2,5 and 25 µg (Figure 5.10). The lipoprotein was kept constant at 100 µg (protein content).

The highest efficiency of transfection by LDL receptor - mediated endocytosis occurred in human lung fibroblasts in culture. Using LDL - pSV2cat complex in a ratio of 100 µg LDL to 25 µg DNA per flask, a time study for the transfection was carried out over a period of 48 hours (Figure 5.11). Cell extracts assayed for CAT activity showed that maximum acetylation (4,4%) occurred in cells harvested at 32 hours post - transfection. Nevertheless, activity was detectable 1,5 hours post - transfection.

When ECDI - LDL : DNA were used in a ratio of 100 µg : 25 µg, precipitation of the complexes occurred upon incubation at room temperature, prior to application to monolayers. This resulted in lower efficiencies of transfection of the lung fibroblasts. Lower concentrations of ECDI - LDL or pSV2cat or higher dilution were employed to overcome this problem. This resulted in higher CAT activities in the ECDI - LDL - pSV2cat transfected cells (Figure 5.12).

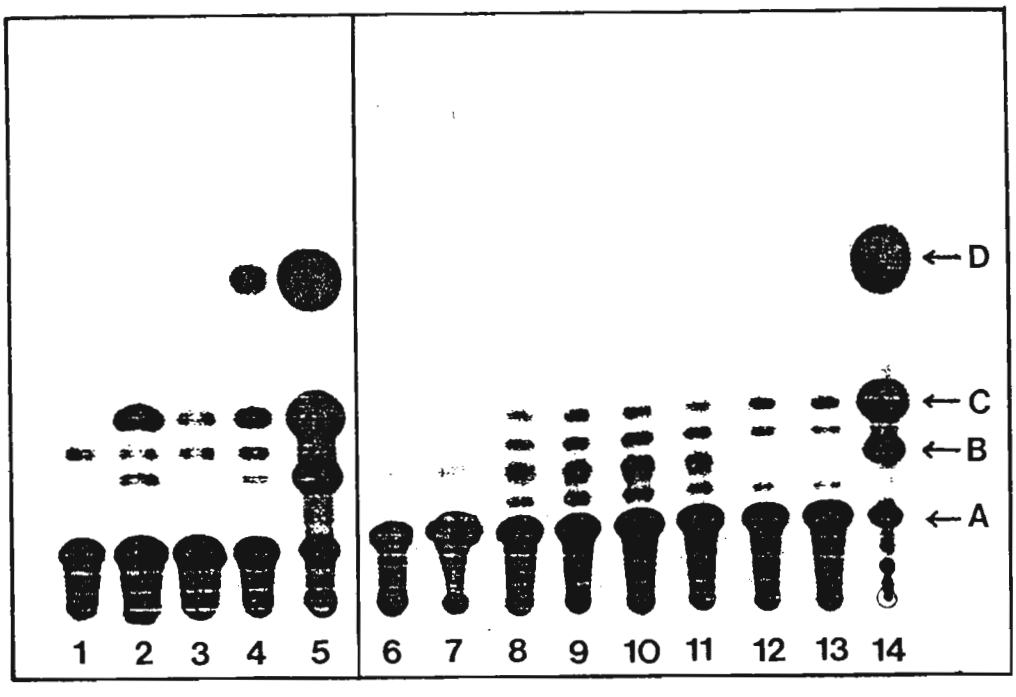


Figure 5.10 : CAT activities expressed in fetal fibroblasts.
1 : untransfected control; 2 - 4 : calcium phosphate - pSV2cat transfected cell extracts; 5 and 14 : enzyme controls; 6 : chloramphenicol; 7 : pSV2cat DNA control; 8, 9 and 10 : LDL - pSV2cat - transfected cell extracts (5, 15 and 25 µg DNA respectively); 11, 12 and 13 : ECDI - LDL - pSV2cat - transfected cell extracts (5, 15 and 25 µg DNA respectively).

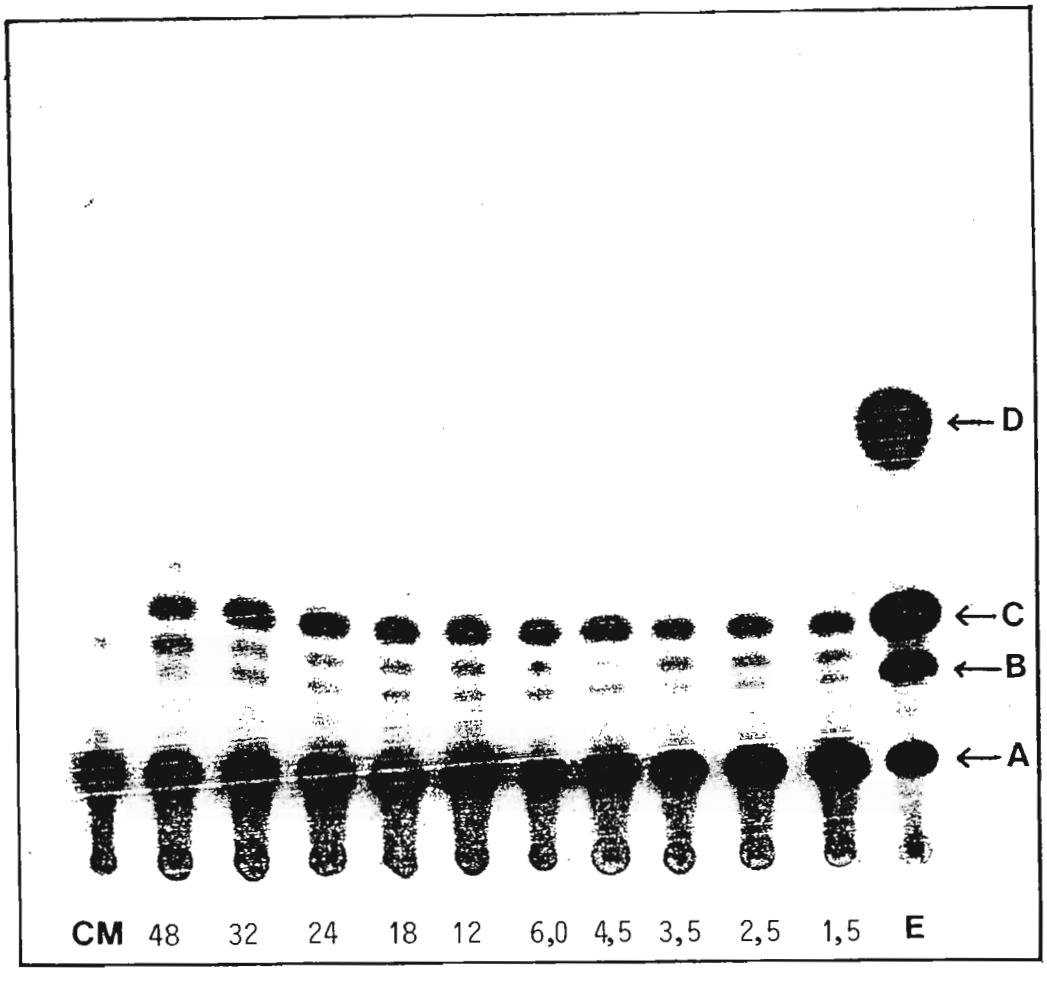


Figure 5.11 : Transfection of human lung fibroblasts by LDL receptor-mediated endocytosis. The cells were transfected with LDL - pSV2cat complexes and harvested between 1,5 and 48 hours, as indicated. CM = chloramphenicol; E = enzyme control. The ratio of LDL to DNA per flask was 100 µg to 25 µg. A - D are defined in the legend of Figure 5.5b.

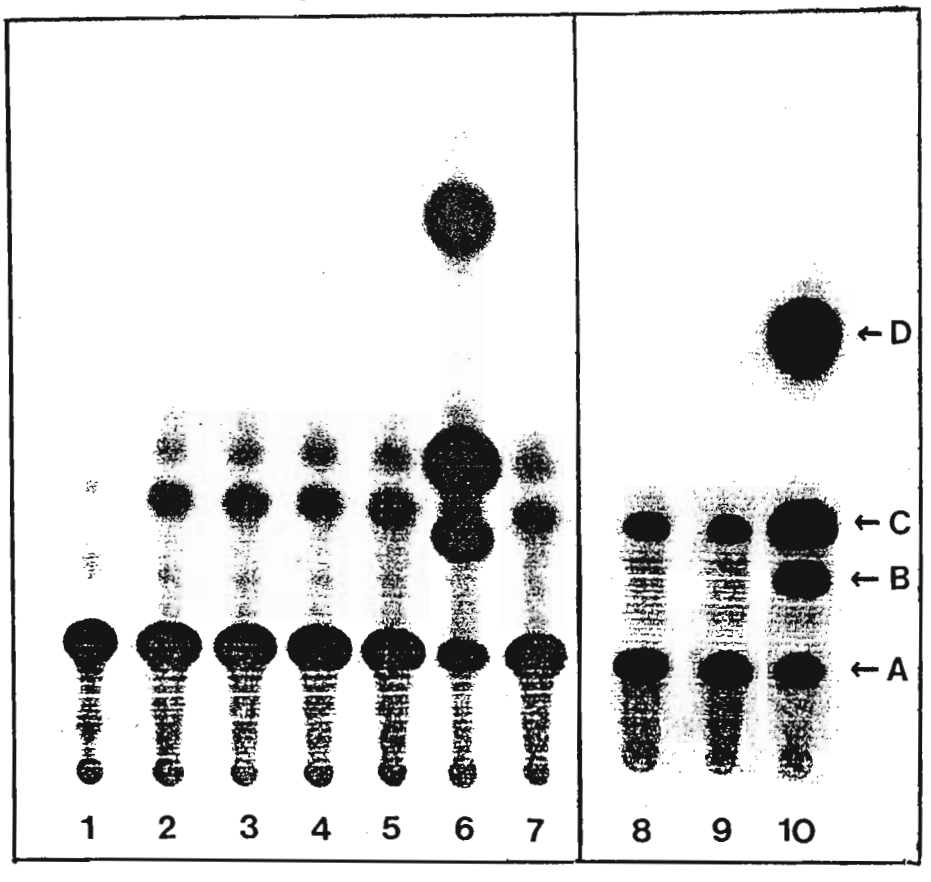


Figure 5.12 : CAT activity in human lung fibroblasts in culture, transfected by LDL receptor - mediation. 1 : chloram - phenicol; 2, 3, 4, 5 : ECDI - LDL transfected extracts (10 µg, 2,5 µg, 5 µg and 10 µg, DNA respectively); 7 : ECDI - LDL - pSV2cat transfected cell extracts (complex prepared at high dilution with 25 µg DNA); 8, 9 : calcium phosphate - pSV2cat - transfected cell extracts; 6, 10 : enzyme controls. ECDI - LDL was decreased to 50 µg in 3, 4 and 5, and maintained at 100 µg in 2 and 7. A - D are defined in the legend of Figure 5.5b.

5.4 Concluding remarks

The recombinant expression vector pSV2cat was amplified in *E. coli* HB101 by two methods (Maniatis *et al.*, 1982; Gorman, 1986). The second method gave a higher yield of plasmid because a richer medium was used for bacterial growth and a gentler method was used for bacterial cell lysis.

The purified plasmid vector was complexed to either native LDL or ECDI - LDL. The LDL - pSV2cat and ECDI - LDL - pSV2cat complexes were subsequently applied to cells in culture to carry out transfec - tions by LDL receptor - mediated endocytosis. Of all the cell lines used, human skin fibroblasts and CV - 1 cells showed the lowest efficiency of transfection and were barely detectable by autoradiography. Fetal fibroblasts and HeLa cell extracts of the low density lipoprotein - pSV2cat transfected cells assayed for CAT activity showed a greater amount of acetylated (chloramphenicol) products. The highest amount of acetylation occurred in human lung fibroblasts transfected with LDL - pSV2cat and ECDI - LDL-pSV2cat complexes. In all experiments, 10 μg ct DNA was added to all 25 cm^2 flasks prior to transfections, to guard against entry of pSV2cat DNA by processes other than LDL receptor - mediated endocytosis.

Since DNA controls showed no acetylated products, the lipoprotein - DNA complexes were assumed to bind to LDL receptors on the plasma membrane and internalize by LDL receptor - mediated endocytosis.

Lipoprotein - DNA complexes were shown to be successfully internalized by LDL receptor - mediated endocytosis in Chapter 3. Degradation products of both LDL and DNA were indicative of internalization by LDL receptor mediation and subsequent lysosomal degradation. The internalization of LDL - pSV2cat and ECDI - LDL - pSV2cat complexes by LDL receptor mediation is also subject to a similar fate. However, some DNA probably escapes lysosomal degradation and is subsequently expressed. Since the pSV2cat recombinant contains a eukaryotic SV 40 promoter (Gorman *et al.*, 1982), it can induce eukaryotic cellular mechanisms to express the foreign gene, albeit transiently. Thus successful transfection of pSV2cat could be assayed by the expression of the CAT enzyme. However, when compared with the calcium phosphate - DNA coprecipitation method, the efficiency of transfection was low in the cell lines used. Human lung fibroblasts showed the highest efficiency of transfection from all cell lines used to study transfections by LDL receptor - mediated endocytosis. Differences in efficiencies of transfection thus could be a function of cell selectivity. Nevertheless, the pSV2cat DNA was able to escape lysosomal degradation to some extent and induce expression in normal human cell lines.

The CAT gene has been utilized also in expression vectors containing the α - gonadotropin gene (Darnell and Boime, 1985). Transfection of the α - gonadotropin gene was thus monitored in placental and tumour cells by the expression of the linked CAT gene. In the placental cells transfection efficiency was 10 - fold higher than in tumour cells.

However, in these and other cell lines transcription was enhanced or induced by either cAMP or butyrate, and the method of transfection was the calcium phosphate - DNA technique (Darnell and Boime, 1985; Gorman *et al.*, 1983; Gorman, 1986). In addition to using the SV 40 tandem repeat sequences as eukaryotic enhancers of transcription, other plasmid constructs expressing the CAT gene have made use of the Malony murine sarcoma virus (MSV) enhancers (Laimins *et al.*, 1982), Rous sarcoma virus (RSV) long terminal repeats (Burke and Mogg, 1985; Gorman *et al.*,1982), Adenovirus type 12 (Ad 12) promoters (Kruckzek and Doerfler, 1983), or HSV promoters (McLaughlan *et al.*,1985) for expression of CAT activity in eukaryotic cells. Transfection by the above plasmid constructs has been carried out in CV - 1 monkey kidney cells, HeLa cells, placental cells, $LMTK^-$ mouse cells, NIH 3T3 mouse cells, CHO cells and MEFC mouse cells, and expression has been detected by assays for CAT activity.

McLauchlan and coworkers (1985) have used plasmid constructs with the CAT gene linked to an HSV terminator fragment to study the effects of deletions on CAT activity, in HeLa cells and to identify consensus sequences in the eukaryotic mRNA. The authors have been able to show that the -YGTGTTYY- (Y = pyrimidine) sequence is essential for efficient formation of mRNA 3' - termini.

The $pRSVcat^{amb38}$ vector has been used to quantitate amber expression in various mammalian cells (Burke and Mogg, 1985). Suppressor tRNA does not occur naturally in mammalian cells and its successful introduction into mammalian cells was monitored by assaying for

REFERENCES

ARKIGG, A., GARDNER, S. D. and GREENAWAY, P. J. (1981), J. Gen. Virol., 55, 247.

ALADJEM, F. (1966), Nature, 209, 1003.

ALAUPOVIC, P. (1981), Advances in Physiological Sciences, 35, 3.

ALAUPOVIC, P., LEE, D. M. and Mc CONATHY, W. J. (1972), Biochim. Biophys. Acta, 260, 689.

ALBERS, J. J. and CHEUNG, M. C. (1979), In Report of the high density lipoprotein methodology workshop. Ed. K. Lippel. NIH publ., no. 82 - 1661, pp. 252 - 265. U. S. Dept. of Human Services, Bethesda.

ALDEN, C. J. and KIM, S - H. (1979), in Stereodynamics of Molecular Systems. Ed. R. H. Sarma , pp. 331 - 350. Pergamon press, New York.

ALWINE, J. C., KEMP, D. J. and STARK, G. R. (1977), Proc. Natl. Acad. Sci. U.S.A., 74, 5350.

ANDERSON, R. G. W., BROWN, M. S. and GOLDSTEIN, J. L.(1981), J. Cell. Biol., 88, 441.

ANDERSON, M. L. M. and YOUNG, B. D. (1985), in Nucleic Acid Hybridization, a practical approach. Ed. B. D. Hames and S. J. Higgins , pp. 73 - 111. IRL Press, Oxford.

ANDERSON, W. F., KILLOS, L., SANDERS - HAIGH, L.,KRETSCHMER, P. J. and DIACUMAKOS, E. G. (1980), Proc. Natl. Acad. Sci. U.S.A., 77, 5399.

ARIATTI, M., HAWTREY, R., KHAN, Z. and HAWTREY, A. O. (1986), IRCS Med. Sci., 14, 382.

REFERENCES

ARKIGG, A., GARDNER, S. D. and GREENAWAY, P. J. (1981), J. Gen. Virol., 55, 247.

ALADJEM, F. (1966), Nature, 209, 1003.

ALAUPOVIC, P. (1981), Advances in Physiological Sciences, 35, 3.

ALAUPOVIC, P., LEE, D. M. and Mc CONATHY, W. J. (1972), Biochim. Biophys. Acta, 260, 689.

ALBERS, J. J. and CHEUNG, M. C. (1979), In Report of the high density lipoprotein methodology workshop. Ed. K. Lippel. NIH publ., no. 82 - 1661, pp. 252 - 265. U. S. Dept. of Human Services, Bethesda.

ALDEN, C. J. and KIM, S-H. (1979), in Stereodynamics of Molecular Systems. Ed. R. H. Sarma , pp. 331 - 350. Pergamon press, New York.

ALWINE, J. C., KEMP, D. J. and STARK, G. R. (1977), Proc. Natl. Acad. Sci. U.S.A., 74, 5350.

ANDERSON, R. G. W., BROWN, M. S. and GOLDSTEIN, J. L.(1981), J. Cell. Biol., 88, 441.

ANDERSON, M. L. M. and YOUNG, B. D. (1985), in Nucleic Acid Hybridization, a practical approach. Ed. B. D. Hames and S. J. Higgins , pp. 73 - 111. IRL Press, Oxford.

ANDERSON, W. F., KILLOS, L., SANDERS - HAIGH, L.,KRETSCHMER, P. J. and DIACUMAKOS, E. G. (1980), Proc. Natl. Acad. Sci. U.S.A., 77, 5399.

ARIATTI, M., HAWTREY, R., KHAN, Z. and HAWTREY, A. O. (1986), IRCS Med. Sci., 14, 382.

ARRAND, J. E. (1985), in Nucleic Acid Hybridization, a practical approach. Ed. B.D. Hames and S.J. Higgins, pp. 17 - 45. IRL Press, Oxford.

ASSMANN, G. and JABS, H-U. (1985), Biochem. Soc. Trans., 13, 19.

AULINSKAS, T. H., van der WESTHUYZEN, D. and COETZEE, G. A. (1983), Artherosclerosis, 47, 159.

BACHORIK, P. S. (1979), in Report of the high density lipoprotein Methodology Workshop. Ed. K. Lippel. NIH Publication no. 82 - 1661, pp. 164 - 166. U.S. Dept. of Human Services, Bethesda.

BARRETT, A. J. (1984), Biochem. Soc. Trans., 12, 899.

BARTLETT, G. R. (1959), J. Biol. Chem., 234, 466.

BASU, S. K., GOLDSTEIN, J. L., ANDERSON, R. G. W. and BROWN, M. S. (1976), Proc. Natl. Acad. Sci. U.S.A., 73, 3178.

BEATTIE, D. S., CLEJAN, L. and BOSCH, C. G. (1984), J. Biol. Chem., 259, 10526.

BEISIEGEL, U., SCHNEIDER, W. J. S., BROWN, M. S. and GOLDSTEIN, J. L. (1982), J. Biol. Chem., 257, 13150.

BEISIEGEL, U., SCHNEIDER, W. J., GOLDSTEIN, J. L., ANDERSON, R. G. W. and BROWN, M. S. (1981), J. Biol. Chem., 256, 11923.

BELL, F. P. (1976), in Low Density Lipoproteins. Ed. C.E. Day and R.S. Levy, pp. 111 - 133. Plenum Press, New York.

BENOIST, C. and CHAMBON, P. (1981), Nature, 290, 304.

BERMAN H. M. and NEIDLE, S. (1979), in Stereodynamics of Molecular Systems. Ed. R.H. Sarma, pp. 376 - 382. Pergamon Press, N.Y.

BERNFELD, P. (1958), in The Lipoproteins: Methods and Clinical Significance. Ed. F. Homburger and P. Bernfeld, pp. 24 - 36. S. Karger, Basle.

BERNFELD, P., DONAHUE, V. M. and BERKOWITZ, M. E.(1957), J. Biol. Chem., 22, 51.

BESTERMANN, J. M. and LOW, R. B. (1983), Biochem. J., 210, 1.

BIERMAN, E. L., STEIN, O. and YECHEZKIEL, S. (1974), Circ. Res., 35, 136.

BILHEIMER, D. W., EISENBERG, S. and LEVY, R. I. (1972), Biochimica et Biophysica Acta, 260, 212.

BLIGHT, E. G. and DYER, W. J. (1959), Can. J. Biochem. Physio., 37, 911.

BOLIVAR, F., RODRIGUEZ, R. L., GREENE, P. J., BETLACH, M. C., HEYNEKER, H. L., BOYER, H. W., CROSA, J. H. and FALKOW, S. (1977), Gene, 2, 95.

BRADLEY, W. A., HWANG, S-L. C., KARLIN, J. B., LIN A. H. Y., PRASAD, S. C., GOTTO Jr., A. M. and GIANTURCO, S. H. (1984), J. Biol. Chem., 259, 14728.

BRAGDON, J. H., HAVEL, R. J. and BOYLE, E. (1956), J. Lab. Clin. Med., 48, 36.

BRISETTE, L. and NOËL, S-P. (1986), J. Biol. Chem., 261, 6847.

BROWN, M. S., ANDERSON, R. G. W. and GOLDSTEIN, J. L. (1983), Cell, 32, 663.

BROWN, M. S., DANA, S. E. and GOLDSTEIN, J. L. (1974), J. Biol. Chem., 249, 789.

BROWN, M. S., DANA, S. E. and GOLDSTEIN, J. L. (1973), Proc. Natl. Acad. Sci., U.S.A., 70, 2162.

BROWN M. S., DANA, S. E. and GOLDSTEIN, J. L. (1975a), J. Biol. Chem., 250, 4025.

BROWN, M. S., DANA, S. E. and GOLDSTEIN, J. L. (1975b), Proc. Natl. Acad. Sci., 72, 2925.

BROWN, M. S., FAUST, J. R. and GOLDSTEIN, J. L. (1975c), J. Clin. Invest., 55, 783.

BROWN, M. S. and GOLDSTEIN, J. L. (1974a), Proc. Natl. Acad. Sci., USA 71, 788.

BROWN, M. S. and GOLDSTEIN, J. L. (1974b), J. Biol. Chem., 249, 7306.

BROWN, M. S. and GOLDSTEIN, J. L. (1975), Cell, 6, 307.

BROWN, M. S. and GOLDSTEIN, J. L. (1976a), Science, 191, 150.

BROWN, M. S. and GOLDSTEIN, J. L. (1976b), Cell, 9 ,663.

BROWN, M. S. and GOLDSTEIN, J. L (1979), Proc. Natl. Acad. Sci., 76, 3330.

BROWN, M. S. and GOLDSTEIN, J. L. (1984), Scientific Amer., 251, 52.

BROWN, M. S., SOBHANI, M. K., BRUNSCHEDE, G. Y. and GOLDSTEIN, J. L. (1976), J. Biol. Chem., 251, 3277.

BROWN, D. D. and STERN, R. (1974), Ann. Rev. Biochem., 43, 667.

BURKE, J. F. and MOGG, A. E. (1985), Nucl. Acids Res., 13, 1317.

BURNSTEIN, M. and SCHOLNICK, H. R. (1974), Nouv. Rev. Franc. d´Hématol., 14, 131.

BURNSTEIN, M., SCHOLNICK, H. R. and MORFIN, R. (1970), J. Lipid Res., 11, 583.

CAPECCHI, M. R. (1980), Cell, 22, 479.

CARDIN, A. D., WITT, K. R., BARNHART, C. L. and JACKSON, R. L. (1982), Biochemistry, 21, 4503.

CARDIN, A. D., WITT, K. R., CHAO, J., MARGOLIUS, H. S., DONALDSON, V. H. and JACKSON, R. L. (1984), J. Biol. Chem., 259, 8522.

CARROLL, R. M. and RUDEL, L. L. (1983), J. Lipid Res., 24, 200.

CAWLEY, L. P. and MINARD, B. (1969), Lab. Synopsis (Behring), 2, 56.

CELIS, J. E. (1984), Biochem. J., 223, 281.

CELIS, J. E., KALTOFT, K. and BRAVO, R. (1980), in Introduction of Macromolecules into viable mammalian cells. Ed. Baserga, R.,Croce, C. and Rovera, G., pp. 99 - 123. Alan R. Liss, New York.

CHAIT, A., BIERMAN, E. L. and ALBERS, J. J. (1979), Diabetes, 28, 914.

CHAN, L., WEI, C-F., LI, W-H., YANG, C-Y., RATNER, P., POWNALL, H., GOTTO, A. M. Jr. and SMITH, L.C. (1985), J. Biol. Chem., 260, 2629.

CHAPMAN, D. (1973), in Form and Function of Phospholipids. Ed. Ansell, G.B., Dawson, R.M.C. and Hawthorne, J.N., pp. 117 - 142. Elsevier Scientific Publishing Co., Amsterdam.

CHEN, H. W., HEINIGER, H. and KANDUTSCH, A. A. (1975), Proc. Natl. Acad. Sci., U.S.A., 72, 1950.

CHEN, P. S., TORIBARA, T. Y. Jr. and WARNER, H. (1956), Anal. Chem., 28, 1756.

CHU, G. and SHARPE, P. A. (1981), Gene, 13, 197.

CHUNG, B. H., WILKINSON, T., GEER, J. C. and SEGREST, J. P. (1980), J. Lipid Res., 21, 284.

COHEN, S., CHANG, A., BOYER, H. and HELLIG, R. (1973), Proc. Natl. Acad. Sci., U.S.A., 70, 3240.

COLBERE - GARAPIN, F., HORODNICEANU, F., KOURILSKY, P. and GARAPIN, A - C. (1981), J. Mol. Biol., 150, 1.

COLEMAN, R. (1973), in Form and Function of Phospholipids. Ed. Ansell, G. B., Dawson, R.M.C. and Hawthorne, J.N., pp. 345 - 375. Elsevier Scientific Publishing Co., Amsterdam.

CORNWELL, D. G. and KRUGER F. A.. (1961), J. Lipid Res., 2, 110.

CRYER, A. (1985), Biochem. Soc. Trans., 13, 27.

CUATRECASAS, P. (1974), Ann. Rev. Biochem., 43, 169.

DAILEY, H. A. and STRITTMATTER, P. (1979), J. Biol. Chem., 254, 5388.

DANA, S. F., BROWN, M. S. and GOLDSTEIN. J. L. (1977), Biochem. Biophys. Res. Comm., 74, 1369.

DARNELL, R. B. and BOIME, I. (1985), Mol. Cell Biol., 5, 3157.

DAVIS, R. A. and MALONE - MC NEAL, M. (1985), Biochem. J., 227, 29.

DAWSON, G., KRUSKI, A. W. and SCANU, A. M. (1976), J. Lipid Res., 17, 125.

DEAN, R. T. (1984), Biochem. Soc. Trans., 12, 911.

DEAN, R. T., JESSUP, W. and ROBERTS, C. R. (1984), Biochem. J., 217, 27.

DECKELBAUM, R. J., SHIPLEY, G.G. and SMALL, D. M. (1977), J. Biol. Chem., 252, 744.

DEMPSEY, M. E. (1974), Ann. Rev. Biochem., 43, 967.

De PAMPHILIS, M. L. and WASSARMAN, P.M. (1980), Ann. Rev. Biochem., 49, 627.

DEVOS, R., VAN EMMELO, J., CONTRERAS, R. and FIERS, W. (1979), J. Mol. Biol., 128, 595.

DIACUMAKOS, E. G. (1980), in Introduction of Macromolecules into Viable Mammalian cells. Ed. Baserga, R., Croce, C. and Rovera, G., pp. 85 - 98. Alan Liss Inc., New York.

DICKSON, R., ABELSON, J., BARNES, W. and REZNIKOFF, W. (1975), Science, 187, 27.

DOBROTA, M. and HINTON, R. H. (1984), Biochem. Soc. Trans., 12, 1021.

DONOVAN, J. J., SIMON, M. I., DRAPER, R. K. and MONTAL, M. (1981), Proc. Natl. Acad. Sci., U.S.A., 78, 172.

DRIKAMER, K. (1981), J. Biol. Chem., 256, 5827.

DRIKAMER, K. and MAMON, J. F. (1982), J. Biol. Chem., 257, 15156.

DUBENSKY, T. W., CAMPBELL, B. A. and VILLARREAL, L. P. (1984), Proc. Natl. Acad. Sci., U.S.A., 81 , 7529.

DUGIACZYK, A., BOYER, H. W. and GOODMAN, H. M. (1975), J. Mol. Biol., 96, 171.

DUNCAN, R., KOPECEK. J. and LLOYD, J. B. (1984), Biochem. Soc. Trans., 12, 913.

DUNN, W. A., HUBBARD, A. L. and ARONSON, N. N. Jr. (1980), J. Biol. Chem., 255, 5971.

EDELSTEIN, C. (1979), in Report of the High density Lipoprotein Methodology Workshop. Ed. K. Lippel. NIH pulication, no. 82 - 1661, pp. 325 - 327. U.S. Dept. of Human Services, Bethesda.

EFSTRADIADIS, A., KAFATOS, F. C., MAXAM, A. M. and MANIATIS, T. (1976), Cell, 7, 278.

ENQUIST, L. W., VAN DE WOUDE, G. F., WAGNER, M., SMILEY, J. R. and SUMMERS, W. C. (1979), Gene, 7, 335.

FAUST, J. R., GOLDSTEIN, J. L. and BROWN, M. S. (1977), J. Biol. Chem., 252, 4861.

FEINBERG, A. P. and VOGELSTEIN, B. (1983), Anal. Biochem., 132, 6.

FELLIN, R., AGNOSTINI, B., ROST, W. and SEIDEL, D. (1974), Clin. Chim. Acta., 54, 325.

FIERS, W., CONTRERAS, R., DUERINCK, F., HAEGEMAN, G., ISERENTANT, D., MERREGAERT, J., MINJOU, W., MOLEMANS, F., RAEYMAEKERS, A., BERGHE, V., VOLCKAERT, G. and YSEBAERT, B. (1976), Nature, 260, 500.

FINEAN, J. B. (1973), in Form and Function of Phospholipids. Ed. Ansell, G.B., Dawson, R.M.C. and Hawthorne, J.N., pp. 171 - 203. Elsevier Publishing Co., Amsterdam.

FISKE, C. H. and Subbarow, Y. (1925), J. Biol. Chem., 66, 375.

FLESS, G. M., ROLIH, C. A. and SCANU, A. M. (1984), J. Biol. Chem., 259, 11470.

FONG, B. S., RODRIGUES, P. O. and ANGEL, A. (1984), J. Biol. Chem., 259, 10168.

FOREMAN, J. R., KARLIN, J. B., EDELSTEIN, C., JUHN, D. J., RUBENSTEIN, A. H. and SCANU, A. M. (1977), J. Lipid Res., 18, 759.

FRAENKEL - CONRAT, H. (1957), Methods in Enzymology, 4, 247.

FREDRICKSON, D. S., LEVY, R. I. and LEES, R. S. (1967a), New England J. Med., 276, 34.

FREDRICKSON, D. S., LEVY, R. I. and LEES, R. S. (1967b), New England J. Med., 276, 94.

FREDRICKSON, D. S., LEVY, R. I. and LEES, R. S. (1967c), New England J. Med., 276, 148.

FREDRICKSON, D. S., LEVY, R. I. and LEES, R. S. (1967d), New England J. Med., 276, 215.

FREDRICKSON, D. S., LEVY, R. I. and LEES, R. S. (1967e), New England J. Med., 276, 273.

FRESHNEY, R. I. (Ed.). (1986), Animal Cell Culture - A Practical Approach. IRL Press, Oxford.

FRIEFELDER, D. (1982), Physical Biochemistry : Application to Biochemistry and Molecular Biology. W. H. Freeman and Co., San Franscicso.

GALTON, D. J., STOCKS, J. and REES, A. (1985), Biochem. Soc. Trans., 13, 31.

GASSER, C. S. and SCHIMKE, R. T. (1986), J. Biol. Chem., 261, 6938.

GEFTER, M. L. (1975), Ann. Rev. Biochem., 44, 45.

GEREN, L. M., O'BRIEN, P., STONEHUERNER, J. and MILLET, F. (1984), J. Biol. Chem., 259, 2155.

GHOSH, S. (1976), in Low density Lipoproteins. Ed. Day, C.E. and Levy, R.S., pp. 371 - 419. Plenum Press, New York.

GIDEZ, L. I., MILLER, G. J., BURNSTEIN, M. and EDER, H. A. (1979), in Report of the High Density Lipoprotein Methodology Workshop, ed. Lippel, K. NIH Publication, no. 82 - 1661, p 328. U.S. Dept. of Health and Human Services, Bethesda.

GLASS, E. (1982), Gene Function.: E. coli and its Heritable Elements. Croom Helm Ltd., London.

GLOMSET, J. (1969), Meth. Enzymol., 15, 543.

GODFREY, W., DOE. B. and WOFSY. L. (1983), Proc. Natl. Acad. Sci., U.S.A., 80, 2267.

GOEDDEL, D.V., HEYNEKER, H. L., HOZUMI, T., ARENTZEN, R., ITAKURA, K., YANSURA, D. G., ROSS, M. J., MIOZZARI, G., CREA, R. and SEEBURG, P. H. (1979), Nature, 281, 544.

GOFMAN, J. W. (1958), in The Lipoproteins : Methods and Clinical Significance. Ed. Homburger, F. and Bernfeld, P., pp. 47 - 70. S. Karger, Basle.

GOLDSTEIN, J. L., BASU, S. K., BRUNSCHEDE, G. Y. and BROWN, M. S. (1976), Cell, 7, 85.

GOLDSTEIN, J. L. and BROWN, M. S. (1974), J. Biol. Chem., 249, 5153.

GOLDSTEIN, J. L. and BROWN, M. S. (1977), Ann. Rev. Biochem., 46, 897.

GOLDSTEIN, J. L., DANA, S. E., FAUST, J. R., BEAUDET, A. L. and BROWN, M. S. (1975), J. Biol. Chem., 250, 8487.

GOLDSTEIN, J. L., HELGESON, J. A. S. and BROWN, M. S. (1979a), Ann. Rev. Biochem., 254, 5403.

GOLDSTEIN, J. L., HO, Y. K., BASU, S. K. and BROWN, M. S. (1979b), Proc. Natl. Acad. Sci., U.S.A, 76, 333.

GOLDFARB, D. S., RODRIGUEZ, R. L. and DOI, R. H. (1982), Proc. Natl. Acad. Sci., U.S.A., 79, 5886.

GORDHAN, H., ARIATTI, M. and HAWTREY, A. O. (1986), IRCS Med. Sci., 14, 1118.

GORMAN, C. (1986), in DNA cloning, a practical approach, vol II. Ed. Glover, D.M., pp. 143 - 190. IRL Press, Oxford.

GORMAN, C. M., HOWARD, B. H. and REEVES, R. (1983), Nucl. Acids Res., 11, 7631.

GORMAN, C. M., MERLINO, G. T., WILLINGHAM, M. C., PASTAN, I. and HOWARD, B. H. (1982a), Proc. Natl. Acad. Sci., U.S.A, 79, 6777.

GORMAN, C. M., MOFFAT, L. F. and HOWARD, B. H. (1982b), Mol. Cell Biol., 2, 1044.

GOTTO, A. M., LEVY, R. I., LINGRED, F. T. and FREDERICKSON, D. S. (1969), Biochim. Biophys. Acta, 176, 667.

GRAESSMANN, A., GRAESSMANN, M. and MUELLER, C. (1980), Methods Enzymol., 65, 816.

GRAESSMANN, M. and GRAESSMANN, A. (1976), Proc. Natl. Acad. Sci., 73, 366.

GRAESSMANN, M. and GRAESSMANN, A. (1983), Methods Enzymol., 101. 484.

GRAHAM, F. L., BACHETTI, S., Mc KINNON, R., STANNERS, C., CORDELL, B. and GOODMAN, H. M. (1980), in Introduction of Macromolecules into Viable Mammalian Cells. Ed. Baserga, R., Croce, C. and Rovera, G., pp. 3 - 25. Alan R. Liss Inc., New York.

GRAHAM, F. L. and van der EB, A. J. (1973), Virology, 52, 456.

GRUSS, P., DHAR, R. and KHOURY, G. (1981), Proc. Natl. Acad. Sci., U.S.A., 78, 943.

GUSTAFSON, A. (1965), J. Lipid Res., 6, 512.

HABERLAND, M. E., OLCH, C. L. and FOLGELMAN, A. M. (1984), J. Biol. Chem., 259, 11305.

HANIG, M. (1958), in The Lipoproteins : Methods and Clinical Significance. Ed. Homburger, F. and Bernfeld, P., pp. 71- 74. S. Karger, Basle.

HARDY, E. (1981), Bacterial Plasmids. Thomas Nelson and Sons, Hong Kong.

HARDY, K. G. (1986), in DNA Cloning : Vol. II. Ed. Glover, D.M., pp. 1 - 17. IRL Press, Oxford.

HARIKUMAR, P. and REEVES, J. P. (1984), Biochem. Soc. Trans., 12, 906.

HARLEY, E. H., OLLIVER, C. L., RHODES - HARRISON, L., MEW, R. T., LECATSAS, G. and NAUDE, W. duT. (1982), S.A.J. Sci., 78, 112.

HATCH, F. T. and LEES, R.S. (1968), Adv. Lipid Res., 6, 1.

HAVEL, R. J., EDER, H. A. and BRAGDON, J. H. (1955), J. Clin. Invest., 34, 1345.

HAWTREY, R., ARIATTI, M. and HAWTREY A. O. (1986), IRCS Med. Sci., 14, 380.

HELENIUS, A. and SIMONS, K. (1971), Biochemistry, 10, 2542.

HINKLE, D. C. and CHAMBERLIN, M. J. (1972), J. Mol. Biol., 70, 157.

HINTON, D. M., BAEZ, J. A. and GUMPORT, R. I. (1978), Biochem., 17, 5091.

HINTON, D. M., BRENNAN, C. A. and GUMPORT, R. I. (1982), Nucleic Acids Res., 10, 1877.

HINTON, R. H., KOWALSKI, A. J. and MALLINSON, A. (1973), Clin. Chim. Acta., 44, 267.

HOLLEY, R. W. (1966), Sci. Amer., 214, 30.

HOMBERGER, F. and BERNFELD, P. (Ed.) (1958), The Lipoproteins : Methods and Clinical Significance. Buchdruckerei Ludin A.G., Basle.

HUCKETT, B., GORDHAN, H., HAWTREY, R., MOODLEY, N, ARIATTI, M. and HAWTREY, A. O. (1986), Biochem. Pharmacol., 35, 1249.

HUI, D. Y., INNERARITY, T. L., MILNE, R. W., MARCEL, Y. L. and MAHLEY, R. W. (1984), J. Biol. Chem., 259, 15060.

HUMPHREYS, G. O., WILLSHAW, G. A. and ANDERSON, E. S. (1975), Biochim. Biophys. Acta., 383, 457.

HUTCHISON, N. J., LANGER-SAFER, P. R., WARD, D. C. and HAMKALO, B. A. (1982), J. Cell Biol., 95, 609.

ITAYA, K. and UI, M. (1966), Clin. Chim. Acta., 14, 361.

JANSEN, H. and HÜLSMANN, W. C. (1985), Biochem. Soc. Trans., 13, 24.

JAY, E., SETH, A. K., ROMMENS, J., SOOD, A. and JAY, G. (1982), Nucleic Acids Res., 10, 6319.

JOHNSON, A. D., PABO, C. O. and SAHER, R. T. (1980), Methods Enzymol., 65, 839.

KABARA, J. J. (1962), Methods Biochem. Anal., 10, 263.

KANE, J. P. (1973), Anal. Biochem., 53, 350.

KARLSSON, S., HUMPHRIES, R. K., GLUZMAN, Y. and NIEHUIS, A. W. (1985), Proc. Natl. Acad. Sci., U.S.A., 82, 158.

KASUGA, M., ZICK, Y., BLITH, D. L., KARLSSON, F. A., HARING, H. U. and KAHN, C. R. (1982), J. Biol. Chem., 257, 9891.

KELLY, T. J. Jr. and SMITH, H. O. (1970), J. Mol. Biol., 51, 393.

KESSLER, C., NEUMAIER, P. S. and WOLF, W. (1985), Gene, 33, 1.

KHORANA, H. G. (1961), Some Recent Developments in the Chemistry of Phosphate esters of Biological interest. John Wiley and Sons, Inc, New York.

KHORANA, H. G. (1978), Bioorganic Chemistry, 7, 351.

KINGSLEY, D. M. and KRIEGER, M. (1984), Proc. Natl. Acad. Sci., 81, 5454.

KIRSTEN, E. S. and WATSON, J. A. (1974), J. Biol. Chem., 249, 6104.

KOLATA, G. (1984), Science, 223, 1376.

KORN, E. D. (1955), J. Biol. Chem., 215, 1.

KOSTNER, G. M. and LAGGNER, P. (1979), in Report of the High Density Lipoprotein Methodology Workshop. Ed. Lippel, K. NIH Publication, pp. 343 - 355. U.S. Dept. of Human Services, Bethesda.

KOVANEN, P. T., BROWN, M. S. and GOLDSTEIN, J. L. (1979), J. Biol. Chem., 254, 11367.

KRIEGER, M., BROWN, M. S., FAUST, J. R. and GOLDSTEIN, J. L. (1978a), J. Biol. Chem., 253, 4093.

KRIEGER, M., GOLDSTEIN, J. L. and BROWN, M. S. (1978b), Proc. Natl. Acad. Sci., U.S.A., 75, 5052.

KREIGER, M., SMITH, L.C., ANDERSON, R. G. W., GOLDSTEIN, J. L., KAO, Y. J., POWNALL, H. J., GOTTO, A. M. Jr. and BROWN, M. S. (1979), J. Supramol. Structure, 10, 467.

KRUCZEK, I. and DOERFLER, W. (1983), Proc. Natl. Acad. Sci., 80, 7586.

LAGGNER, P. (1976), in Low Density Lipoproteins. Ed. Day, C.E. and Levy, R.S. Plenum Press, New York, pp 49 - 69.

LAIMINS, L. A., KHOURY, G., GORMAN, C., HOWARD, B. and GRUSS, P. (1982), Proc. Natl. Acad. Sci., U.S.A., 79, 6453.

LAMBETH, J. D., GEREN, L. M. and MILLETT, F. (1984), J. Biol. Chem., 259, 10025.

LEE, A. G. (1977), Biochim. Biophys. Acta., 472, 285.

LEE, D. M. (1976), in Low Density Lipoproteins. Ed. Day, C.E. and Levy, R.S., pp. 3 - 47. Plenum Press, New York.

LEE, D. M. and ALAUPOVIC, P. (1974a), Biochem. J., 137, 155.

LEE, D. M. and ALAUPOVIC, P. (1974b), Atherosclerosis, 19, 501.

LEES, R. S. and HATCH, F. T. (1963), J. Lab. Clin. Med., 61, 518.

LEHNINGER, A. L. (1975), Biochemistry: the Molecular Basis of Cell Structure and Function, pp. 290 - 291. Worth Publishers Inc., New York.

Le PECQ, J-B. (1971), Methods of Biochem. Analysis, pp. 41 - 86. Ed. Glick, D. John Wiley and Sons Inc., New York.

LICHTENSTEIN, C. and DRAPER, J. (1986), in DNA Cloning, Vol II, ed. Glover, D.M., pp 67 - 119. IRL Press, Oxford.

LINDGREN, F. T., JENSEN, L. C. and HATCH, F. T. (1972), in Blood Lipids and Lipoproteins : Quantitation, Composition and Metabolism, ed. Nelson, G.J., pp. 181 - 274. John Wiley and Sons, New York.

LITTLEFIELD, J. (1974), Science, 145, 709.

LOBBAN, P. and KAISER, A. D. (1973), J. Mol. Biol., 79, 453.

LOWY, I., PELLICER, A., JACKSON, J. F., SIM, G-K, SILVERSTEIN, S. and AXEL, R. (1980), Cell, 22, 817.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, L. A. and RANDALL,R. J. (1951), J. Biol. Chem., 193, 265.

LOYTER, A., VAINSTAIN, A., GRAESSMANN, M. and GRAESSMANN,A. (1983), Exp. Cell Res., 143, 415.

LOYTER, A., ZAKAI, N. and KULKA, R. G. (1975), J. Cell Biol., 66, 292.

MACKETT, M., SMITH, G. L. and MOSS, B. (1986), in DNA Cloning: Vol. II, ed. Glover, D.M., pp. 191 - 211. IRL Press, Oxford.

MAHLEY, R. W. and WEISGRABER, K. H. (1979), in Report of the High Density Lipoprotein Methodology Workshop, ed. Lippel, K., NIH Publication no. 82 - 1661, pp. 356 - 367. U.S. Dept. of Human Services, Bethesda.

MAIZELS, N. M. (1973), Proc. Natl. Acad. Sci., 70, 3585.

MANIATIS, T., FRITSCH, E. F. and SAMBROOK, J. (1982), in Molecular Cloning : A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring, New York.

MANIATIS, T., PTASHNE, M., BARRELL, B. G., and DONELSON, J. (1974), Nature, 250, 394.

MANN, G. V. (1958), in The Lipoproteins : Methods and Clinical Significance, ed. Homburger, F. and Bernfeld, P., pp. 7 - 13. S. Karger, Basle.

MANN, G. V. (1961), Clin. Chem., 7, 275.

MANUELIDIS, L., LANGER - SAFER, P. R. and WARD, D. C. (1982), J. Cell Biol., 95, 619.

MARGOLIS, S. and CAPUZZI, D. (1972), in Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism, ed. Nelson, G.J., pp. 825-880. John Wiley and Sons Inc., New York.

MARKWELL, M. A. K., HAAS, S. M., TOLBERT, N. E. and BIEBER, L. L. (1981), Methods Enzymol., 72, 296.

MASON, P. J. and WILLIAMS, J. G. (1985), in Nucleic Acid Hybridization, a Practical Approach. Ed. Hames, B.D. and Higgins, S.J., pp. 113 - 117. IRL Press, Oxford.

MAXAM, A. M. and GILBERT, W. (1977), Proc. Natl. Acad. Sci., 74, 560.

MAZZONE, T. and CHAIT, A. (1982), Arteriosclerosis, 2, 487.

Mc FARLANE, A. S. (1958), Nature, 158, 53.

Mc LAUCHLAN, J., GAFFNEY, D., WHITTON, J. L. and CLEMENTS, J. B. (1985), Nucl. Acids Res., 13, 1347.

MERTZ, J. E. and DAVIS, R. W. (1972), Proc. Natl. Acad. Sci.,U.S.A., 69, 3370.

MILLER, A. D., ECKNER, R. J., JOLLY, D. J., FRIEDMANN, T. and VERMA, I. M. (1984), Science, 223, 630.

MILLET, F., de JONG, C., PAULSON, L. and CAPALDI, R. A. (1983), Biochemistry, 22, 546.

MILLET, F., DARLEY-USMAR, V. and CAPALDI, R. A. (1982), Biochemistry, 21, 3857.

MOKRASCH, L. C. (1975), Methods Enzymol., 35, 334.

MONAHAN, J. J., Mc REYNOLDS, L. A. and O' MALLEY, B. W. (1976), J. Biol. Chem. , 251, 7355.

MORRISETT, J. D., JACKSON, R. L. and GOTTO, A. M. Jr. (1975), Ann. Rev. Biochem., 44, 183.

MUNYON, W., KRAISELBURD, E., DAVIS, D. and MANN, J. (1971), J. Virol., 7, 813.

MULLIGAN, R. and BERG, P. (1981), Proc. Natl. Acad. Sci., U.S.A., 78, 2072.

NAKANISHI, S., ADHYA, S., GOTTESMAN, M. and PASTAN, I. (1973), J. Biol. Chem., 248, 5937.

NEUFELD, E. F, LIM, T. W. and SHAPIRO, L. J. (1975), Ann. Rev. Biochem., 44, 357.

NOBLE, R. P. (1968), J. Lipid Res., 9, 693.

NYSTRÖM, E. and SJÖVALL, J. (1975), Methods Enzymol., 35, 378.

OMARY, M. B. and TROWBRIDGE, I. S. (1981), J. Biol. Chem., 256, 12888.

ONCLEY, J. L. (1958), in The Lipoproteins : Methods and Clinical Significance. Ed. Homburger, F. and Bernfeld, P., pp. 14 - 23. S. Karger, Basle.

ONCLEY, J. L., WALTON, K. W. and CORNWELL, D. G. (1957), J. Amer. Chem. Soc., 79, 4666.

OWEN, J. S., CHAVES, M. E. C. and CHITRANUKROH, A. (1985), Biochem. Soc. Trans., 13 , 20.

PAPADOPOULOS, N. M. (1978), Clin. Chem., 24, 227.

PAPAHADJOPOULOS, D. (1973), in Form and Function of Phospholipids, ed. Ansell, G.B., Dawson, R.M.C. and Hawthorne, J.N., pp. 143 - 169. Elsevier Scientific Publishing Co., Amsterdam.

PEARSE, B. M. F. (1975), J. Mol. Biol. , 97, 93.

PELLICER, A., ROBINS, B., WOLD, R., SWEET, J., JACKSON, I., LOWY, J. M., ROBERTS, G. K., SIMS, S., SILVERSTEIN, S. and AXEL, R. (1980), Science, 209, 1414.

PELLICER, A., WIGLER, M., AXEL, R. and SILVERSTEIN, S. (1978), Cell, 14, 133.

PEREBITYUK, A. N. and BORONIN, A. M. (1984), Mol. Biol.(Moscow), 18, 136.

PERUCHO, M., HANAHAN, D. and WIGLER, M. (1980), Cell, 22, 309.

PETERMANN, M. L., BARCLAY, M., ESCHER, G.and KAUFMAN, R. (1958), in The Lipoproteins : Methods and Clinical Significance, ed. Homburger, F. and Bernfeld, P.,pp. 75 - 86.
S. Karger, Basle.

PETERSON, G. L. (1977), Analytical Biochem., 83, 346.

PHILIPPSEN, P., KRAMER, R. A. and DAVIS, R. W. (1978), J. Mol. Biol., 123, 371.

PITAS, R. E., INNERARITY, T. L. and MAHLEY, R. W. (1983), Arteriosclerosis, 3, 2.

POTTER, H. and DRESSLER, D. (1979), DNA Recombination : *In vivo* and *in vitro* studies. Cold Spring Harbor Symp. Quant. Biol., 43, 969.

POTTER, H., WEIR, L. and LEDER, P. (1984), Proc. Natl. Acad, Sci., U.S.A., 81, 7161.

RADLOFF, R., BAUER, W. and VINOGRAD, J. (1967), Proc. Natl. Acad. Sci., U.S.A., 57, 1514.

REDGRAVE, T. G., ROBERTS, D. C. K. and WEST, C. E. (1975), Anal. Biochem., 65, 42.

REIJNGOUD, D-J. and TAGER, J. M. (1977), Biochim. Biophys. Acta., 472, 419.

RIGBY, P. W. J., DIECKMANN, M., RHODES, C. and BERG, P. (1977), J. Mol. Biol., 113, 237.

ROBERTS, D. C. K., MILLER, N. E., PRICE, S. G. L., CROOK, D, CORTESE, C., LaVILLE, A., MASANA, L. and LEWIS, B. (1985), Biochem. J., 226, 319.

ROBERTS, R. J. (1983), Nucl. Acids Res., 11, 135.

ROENSCH, L. F. and BLOHM, T. R. (1976), in Low Density Lipoproteins, ed. Day, C.E. and Levy, R.S., pp. 93 - 110. Plenum Press, New York.

RONINSON, I. B. (1983), Nucl. Acids Res., 11, 5413.

RUDEL, L. L. and MORRIS, M. D. (1973), J. Lipid Res., 14, 364.

RUDEL, L. L, LEE, J. A., MORRIS, M. D. and FELTS, J. M. (1974), Biochem. J., 139, 89.

RUSSELL, D. W., YAMAMOTO, T., SCHNEIDER, W. J., SLAUGHTER, C. J., BROWN, M. S. and GOLDSTEIN, J. L. (1983), Proc. Natl. Acad. Sci., U.S.A., 80, 7501.

SALSER, W. A. (1974), Ann. Rev. Biochem., 43, 923.

SANGER, F., NICKLEN, S. and COULSON, A. R. (1977), Proc. Natl. Acad. Sci., U.S.A., 74, 5463.

SANGER, F. and COULSON, A. R. (1978), FEBS Letters, 87, 107.

SCAGNOS, G. and RUDDLE, F. H. (1981), Gene, 14, 1.

SCHAFFNER, W. (1980), Proc. Natl. Acad. Sci., U.S.A., 77, 2163.

SCHLEGEL, R. A. and MERCER, W. E. (1980), in Introduction of Macromolecules into Viable Mammalian Cells, ed. Baserga, R, Croce, C. and Rovera, G., pp. 145 - 155. Alan R. Liss, New York.

SCHNEIDER, D. L. (1981), J. Biol. Chem., 256, 3858.

SCHNEIDER, J. A., JONAS, A. J., SMITH, M. L. and GREENE, A.A. (1984), Biochem. Soc. Trans., 12, 908.

SCHNEIDER, W. J., BASU, S. K., McPHAUL, M. J., GOLDSTEIN, J. L. and BROWN, M. S. (1979), Proc. Natl. Acad, Sci., U.S.A., 76, 5577.

SCHNEIDER, W. J., BEISIEGEL, U., GOLDSTEIN, J. L. and BROWN, M. S. (1982), J. Biol. Chem., 257, 2664.

SCHROEPFER, G. J. Jr., SHERRIL, B. C., WANG, K-S., WILSON, W. K., KISIC, A. and CLARKSON, T. B. (1984), Proc. Natl. Acad. Sci., U.S.A., 81, 6861.

SEIDEL, D. (1979), in Report on the High Density Lipoprotein Methodology Workshop, ed. Lippel, K., pp. 84 - 96. NIH Publication no. 82 - 1661. U.S. Dept. Human Services, Bethesda.

SEIF, I., KHOURY, G. and DHAR, R. (1979), Cell, 18, 963.

SHARP, P. A., SUGDEN, B. and SAMBROOK, J. (1973), Biochemistry, 12, 3055.

SHAW, W. V. (1967), J. Biol. Chem., 242, 687.

SHAW, W. V. (1975), Methods Enzymol., 43, 737.

SHAW, W. V. and BRODSKY, R. F. (1968), J. Bacteriol., 95, 28.

SHAW, J. E., HUANG, E-S. and PAGANO, J. S. (1975), J. Virol., 16, 132.

SHORE, B. and SHORE. V. (1969), Biochemistry, 8, 4510.

SHORE, V. G. and SHORE, B. (1972), in Blood Lipids and Lipoproteins : Quantitation, Composition and Metabolism, ed. Nelson, G. J., pp. 789 - 824. John Wiley and Sons Inc., New York.

SILVERSTEIN, S. C., STEINMAN, R. M. and COHN, Z. A. (1977), Ann. Rev. Biochem., 46, 669.

SINSHEIMER, R. L. (1977), Ann. Rev. Biochem., 46, 415.

SKIPSKI, V. P. (1972), in Blood Lipids and Lipoproteins : Quantitation, Composition and Metabolism, ed. Nelson, G.J., pp. 471 - 583. John Wiley and Sons Inc., New York.

SKIPSKI, V. P. and BARCLAY, M. (1969), Methods in Enzymology, 14, 530.

SKIPSKI, V. P., PETERSON, R. F. and BARCLAY, M. (1964), Biochem. J., 90, 374.

SMITH, H. O. and WILCOX, K. W. (1970), J. Mol. Biol., 51, 379.

SOUTHERN, E. M. (1975), J. Mol. Biol., 98, 503.

SPEAKE, B. K., PARKIN, S. M. and ROBINSON, D. S. (1985), Biochem. Soc. Trans., 13, 29.

SRINIVASAN, S. R., RADHAKRISHNAMURTHY, B. and BERENSON, G. S. (1979), in Report of the High Density Lipoprotein Methodology Workshop, ed Lippel, K., pp. 70 - 83. U.S. Dept. of Human Services, Bethesda.

STACEY, D. W. (1980), in Introduction of Macromolecules into Viable Mammalian Cells, ed. Baserga, R., Croce, C. and Rovera, G., pp. 100 - 125. Alan R. Liss Inc., New York.

St. CLAIR, R. W., LEIGHT, M. A. and BARAKAT, H. A. (1986), Arteriosclerosis, 6, 170.

STEER, C. J., KEMPNER, E. S. and ASHWELL, G. (1981), J. Biol. Chem., 256, 5851.

STRAIN, A. J. and WYLLIE, A. H. (1984), Biochem. J., 218, 475.

STRAUBINGER, R. M., HONG, K., FRIEND, D. S. and PAPAHADJOPOULOS, D. (1983), Cell, 32, 1069.

SUTCLIFFE, J. G. (1978), Nucleic Acids Res., 5, 2721.

SZYBALSKI, W., SZYBALSKA, E. H. and RAGNI, G. (1962), National Cancer Institute Monograph, 7, 75 - 89.

TAGER, J. M., JONSSON, L. V. M., AERTS, J. M. F., ELFERINK, R.P.J., SCHRAM, A. W., ERIKSON, A. H. and BARRANGER, J. A. (1984), Biochem. Soc. Trans., 12, 902.

TAKAYAMA, M., ITOH, S., NAGASAKI, T. and TANIMIZU, I. (1977), Clin. Chim. Acta, 79, 93.

TEICHMAN, R. J., TAKEI, G. H. and CUMMINS, J. M. (1974), J. Chromatography, 88, 425.

TIMKOVICH, R. (1977), Anal. Biochem., 79, 135.

UNGEWICKELL, E. (1984), Biochem. Soc. Trans., 12, 978.

UNGEWICKELL, E. and BRANDON, D. (1981), Nature, 239, 420.

USHIRO, H. and COHEN, S. (1980), J. Biol. Chem., 255, 8363.

VAHERI, A. and PAGANO, J. S. (1965), Virology, 27, 435.

VAINSTEIN, A., RAZIN, A., GRAESSMANN, A. and LOYTER, A. (1983), Methods Enzymol., 101, 492.

Van BERKEL, T. J. C., KRUIJT, J. K., SPANJER, H. H., NAGELKERKE, J. F., HARKES, L. and KEMPEN, H. J. M. (1985), J. Biol. Chem., 260, 2694.

Van der WESTHUYZEN, D. R., COETZEE, G. A., DEMASIUS, I. P. C., HARLEY, E. H., GEVERS, W., BAKER, S. G. and SEFTEL, H. C. (1984), Arteriosclerosis, 4, 238.

VOGEL, T., WEISGRABER, K. H., ZEEVI, M. I., BEN-ARTZI, H., LEVANON, A. Z., RALL, S. C. J. Jr., INNERARITY, T. L., HUI, D. Y., TAYLOR, J. M., KANNER, D., YAVIN, Z., AMIT, B., AVID, H., GORECKI, M.and MAHLEY, R. W. (1985), Proc. Natl. Acad. Sci., U.S.A., 82, 8696.

WARNICK, G. R. and ALBERS, J. J. (1979), in Report of the High Density Lipoprotein Methodology Workshop, ed. Lippel, K., pp. 53 - 68. NIH publication No. 82 - 1661, U.S. Dept. Health and Human Services, Bethesda.

WATSON, J. D., TOOZE, J. and KURTZ, D. T. (1983), Recombinant DNA : A Short Course. W.H. Freeman and Co., New York.

WIBERG, F. C., SUNNERHAGAN, P., KALTOFT, K., ZEUTHEN, J. and BJURSELL, G. (1983), Nucl. Acids Res., 11, 7287.

WEILAND, H. and SEIDEL, D. (1973), Clin. Chem., 19, 1139.

WIGLER, M., SILVERSTEIN, S., LEE, L-S., PELLICER, A., CHENG, Y-C. and AXEL, R. (1977), Cell, 11, 223.

WIKLUND, O., DYER, C. A., TSAO, B. P.and CURTISS, L.K. (1985), J. Biol. Chem., 260, 10956.

WILCOX, G., MEURIS, P., BASS, R. and ENGLESBERG, E. (1974), J. Biol. Chem., 249, 2946.

WILSON, K. and GOULDING, K. H. (Ed.). (1986), A Biologists' Guide to Principles and Techniques of Practical Biochemistry. Pp. 153 - 197.

WINTER, G. and BROWNLEE, G. G. (1978), Nucl. Acids Res., 5, 3129.

WITTER, R. F. and WHITNER, Y. S. (1972), in Blood Lipids and Lipoproteins : Quantitation, Composition and Metabolism, ed. Nelson, G.J., pp. 75 - 111. John Wiley and Sons Inc., New York.

YAMAMOTO, K. R. and ALBERTS, B. M. (1976), Ann. Rev. Biochem., 45, 721.

YOUNG, B. D. and ANDERSON, M. L. M. (1985), Quantitative Analysis of Solution Hybridization in Nucleic Acids Hybridization, a Practical Approach, ed. Hames, B.D. and Higgins, S.J., pp. 47 - 71. IRL Press, New York.

YOUNG, S. G., WITZTUM, J. L., CASAL, D. C., CURTISS, L. K. and BERNSTEIN, S. (1986), Arteriosclerosis, 6, 178.

ZAK, B. (1957), Amer. J. Clin. Pathology, 27, 583.

ZECHNER, R., DIEPLINGER, H., ROSCHER, A. and KOSTNER, G. M. (1984), Biochem. J., 224, 569.

ZILVERSMIT, D. B., HUGHES, L. B. and BALMER, J. (1975), Biochim. Biophys. Acta, 409, 393.

ZILVERSMIT, D. B. and JOHNSON, L. W. (1975), Methods Enzymol., 35, 262.

ZLATKIS, A. and ZAK, B. (1969), Anal. Biochem., 29, 143.

ZUBAY, G. (1980), Methods Enzymol., 65, 856.

APPENDIX

All lipid standards were purchased from Sigma Chemical Co., St. Louis, U.S.A.

Restriction endonucleases and phospholipase C and D were purchased from Boehringer Mannheim, Germany.

Sephadex G25 was obtained from Pharmacia fine chemicals, Upsala, Sweden.

Human lung fibroblasts were obtained from Highveld Biologicals.

All radiochemicals were purchased from the Radiochemical Center, Amersham, England.

The plasmid pSV2cat was a gift from Professor E. Harley, Dept, of Chemical pathology, University of Cape Town.

Fetal fibroblasts and CV - 1 cells were a gift from Professor E. Harley and Dr R. Rubenstein, Dept. of Chemical Pathology, University of Cape Town.

Normal skin fibroblasts were a gift from Professor I. Jaylal and Mrs R. Hanif, Dept. of Chemical Pathology, University of Natal.

All other chemicals used were purchased from E.Merck,Damstadt, Germany.