AN EVALUATION OF THE MECHANISMS BY WHICH THE HUMAN PLACENTA ACQUIRES CHOLESTEROL FOR STEROID AND MEMBRANE SYNTHESIS

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ABSTRACT

This thesis examines the mechanisms by which the human placenta acquires cholesterol for steroid and membrane synthesis. Lipoprotein metabolism in normal and abnormal pregnancies is discussed, and an evaluation of the classifications of lipoproteins presented. Methods of preparation and characterisation of lipoproteins are reviewed and the rationale for the chosen methodologies discussed.

Lipoproteins, isolated from human blood by rate zonal ultracentrifugation, were characterised by polyacrylamide and agarose gel electrophoresis and by double immunodiffusion.

Dually-perfused human placental lobules were used to study the acquisition of ^{125}I-lipoproteins and ^{14}C-cholesterol from the maternal circulation and to investigate ^{14}C-cholesterol metabolism into ^{14}C-progesterone. The results indicated that Very Low Density Lipoprotein (VLDL) has a higher transfer of activity than any of the other lipoprotein classes. This transfer could be inhibited by chloroquine suggesting that receptor-mediated endocytosis was the likely mechanism for VLDL uptake by the placenta.

To verify the presence of lipoprotein receptors, binding studies were performed on a microvillous membrane preparation from human placenta. Receptor numbers and affinity constants were obtained for the lipoprotein classes. High capacity, high affinity binding of VLDL was demonstrated where the maximal binding capacity was over four times greater, and the affinity constant was one order of magnitude larger than that of Low Density Lipoprotein (LDL). The affinity of High Density Lipoprotein (HDL) for the microvillous membrane receptor was approximately one hundred times lower than that for VLDL.

Scatchard analysis suggested a single population of receptor sites for which each lipoprotein class competes with differing affinities. The results for VLDL binding indicated the presence of 2.1×10^{11} receptors per mg of membrane protein and an affinity constant of 3.5×10^{-10} M. Self- and cross-inhibition studies showed that VLDL preferentially binds to the receptors as it can displace ^{125}I-LDL more readily than LDL itself.

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ABBREVIATIONS

A

ATP	Adenosine Tri-Phosphate

C

C	Centigrade
Ca	Calcium
cL	Centilitre(s)
cm	Centimetre(s)
Co	Cobalt
cpm	Counts per Minute

D

D	Density
DC	Direct Current
dL	Decilitre(s)
dpm	Disintegrations per Minute

E

EDTA	Disodium Ethylenediamine Tetra-acetic Acid
EGF	Epidermal Growth Factor

F

Fg	French Gauge

G

G	Gravity
g	gramme(s)

H

h	Hour
Hb	Haemoglobin
HCl	Hydrochloric Acid
HDL	High Density Lipoprotein
Hg	Mercury

I

i.d.	Internal Diameter
IDL	Intermediate Density Lipoprotein
Ig	Immunoglobulin
IU	International Unit(s)
IVS	Intervillous Space

K

K	Potassium

L

L	Litre(s)
LDL	Low Density Lipoprotein
LPDS	Lipoprotein Deficient Serum

M and μ

M	Molar (Moles per Litre)
M	Mass
mA	Milliamp(s)
mCi	Millicurie(s)
Mg	Magnesium
mg	Milligramme(s)
min	Minute
ml	Millilitre(s)
mM	Millimolar (Millimoles per Litre)
mm	Millimetre(s)
Mn	Manganese
μg	Microgramme(s)
μL	Microlitre(s)
μM	Micromolar (Micromoles per Litre)
Mol. Wt.	Molecular Weight

N

n	Number of Experiments
Na	Sodium
NaBr	Sodium Bromide
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
ng	Nanogramme(s)

O

o.d.	Outside Diameter

P

PCA	Perchloric Acid
pg	Picogramme(s)
PMSF	Phenylmethylsulphonylfluoride
POP	Dimethylphenyloxazolylphenyloxazololphenyl
PTA	Phosphotungstic acid

R

rpm	Revolutions per Minute

S

SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SE	Standard Error of the Mean
Sf	Svedberg Units

T

TEMED	N,N,N',N'-tetramethylethylenediamine
TCA	Trichloroacetic acid

U

u/ml	Units per Millilitre

V

V	Volume
VHDL	Very High Density Lipoprotein
VLDL	Very Low Density Lipoprotein

W

w/v	Weight by Volume

THE PROBLEM

It is known that the feto-placental unit requires a large amount of cholesterol for steroid and membrane synthesis. First-trimester abortions and intra-uterine growth retardation are associated with decreased steroid hormone production. This may be due to an inability of the placenta to acquire sufficient cholesterol from the mother, or a derangement in endogenous production of cholesterol or steroids.

This thesis examines the acquisition of cholesterol from the maternal circulation by the human placenta.

CHAPTER ONE

CHOLESTEROL AND LIPOPROTEIN METABOLISM

1.1. INTRODUCTION

Both the fetus and the placenta require fatty acids and cholesterol for the synthesis of complex lipids such as phospholipids, glycolipids, sphyngolipids, cholesteryl esters and triacylglycerol. These lipids are vital cellular components used for membrane biosynthesis, signal transmission, the synthesis of pulmonary surfactant and energy storage. From the eighth week of gestation the human placenta becomes the dominant source of steroid hormone production such that by late pregnancy approximately 250 mg of progesterone are produced per day (Contractor and Pearlman, 1960). In multiple pregnancies up to 800 mg of progesterone per day may be produced (Simpson and MacDonald, 1981).

It has been shown by Battaglia and Meschia (1978) and Jones and Rolph (1985) that the amount of fatty acids derived from the maternal circulation varies from species to species. Hummel et al (1975, 1983) showed that in the rat, between days 15 and 19, 10% of fetal fatty acids were derived from the maternal circulation, but at 21 days, 50% of the fatty acids were supplied by the maternal circulation.

In the human, it is known that fatty acids injected into the maternal circulation prior to delivery can be found in the fetal vein. If Intralipid (a soyabean-derived

emulsion) is infused, significant amounts of the same artificial fatty acids are found in the fetal circulation, but in a different composition from that of the native Intralipid. This makes it likely that there are specific transport mechanisms for selected fatty acids within the placenta. Elphick et al (1978) have suggested that the triglycerides may be hydrolysed to free fatty acids and then re-esterified within the placenta prior to release to the fetus.

1.2. LIPOPROTEINS

Fats are insoluble in water, and are therefore carried in the blood in the form of lipoproteins. These consist of a core of cholesteryl esters and triglycerides, surrounded by a phospholipid coat. Contained within the phospholipid coat are proteins called apolipoproteins. The human plasma lipoproteins are divided into four major classes according to their hydrated density: chylomicrons, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL). The structure and classification of lipoproteins will be discussed more fully in Chapter Two.

1.3. LIPOPROTEIN FORMATION

Chylomicrons are synthesised by the intestine to transport dietary triacylglycerols and cholesterol from the site of absorption in the intestinal epithelium to various tissues,

principally the liver, via lymphatic systems (as chyle) and blood. The hydrolysis of chylomicrons by lipoprotein lipase, which is found on the endothelial surfaces of the blood vessels, produces a lipoprotein relatively deficient in triacylglycerols and rich in cholesterol, which is known as chylomicron remnants. These remnants are normally removed from the plasma compartment by the liver. The liver in turn synthesises VLDL and LDL which redistribute the fatty acids around the body.

The hydrolysis of triacylglycerols generates free fatty acids which are used by cells as a form of energy source. This depletion of triglycerides and cholesterol from the VLDL produces the next lipoprotein in the series, LDL. The LDLs are probably the major cholesterol-transporting lipoproteins, and LDL receptors have been found on many tissues including the steroid producing cells of the ovary and the human placenta. Macrophages found associated with the endothelium of blood vessels have a high number of these receptors, and it is thought that LDL is the lipoprotein primarily responsible for atherogenesis formation.

High Density Lipoproteins are a heterogenous class of lipoproteins. Heiss et al (1980) have shown that there is a negative correlation between HDL levels and cardiovascular disease. Mahley (1982) suggested that HDL is the mechanism by which cholesterol is picked up from the

tissues and transported to the liver for excretion. The role of HDL in placental metabolism has not yet been elucidated.

1.4. THE PLACENTAL ACQUISITION OF FATTY ACIDS

There are two mechanisms by which the placenta may acquire fatty acids from the maternal circulation.

(i) Triacylglycerols may be removed from lipoproteins such as chylomicra, VLDL and LDL by lipoprotein lipase which is known to exist on the surface of the human placenta (for review see Coleman 1989). This provides a high local concentration of free fatty acids for direct diffusion into the placental cells.

(ii) Fatty acids and cholesterol may both be obtained by receptor-mediated endocytosis of the entire ipoprotein molecule. It is known that placental tissue has a significant number of LDL receptors present on the microvillous surface (Alsat et al, 1985). Winkel et al (1981) have shown the conversion of LDL cholesterol to progesterone by cultured human trophoblast cells. The pathway of biosynthesis of the steroids from cholesterol is summarised in Figure 1.1.

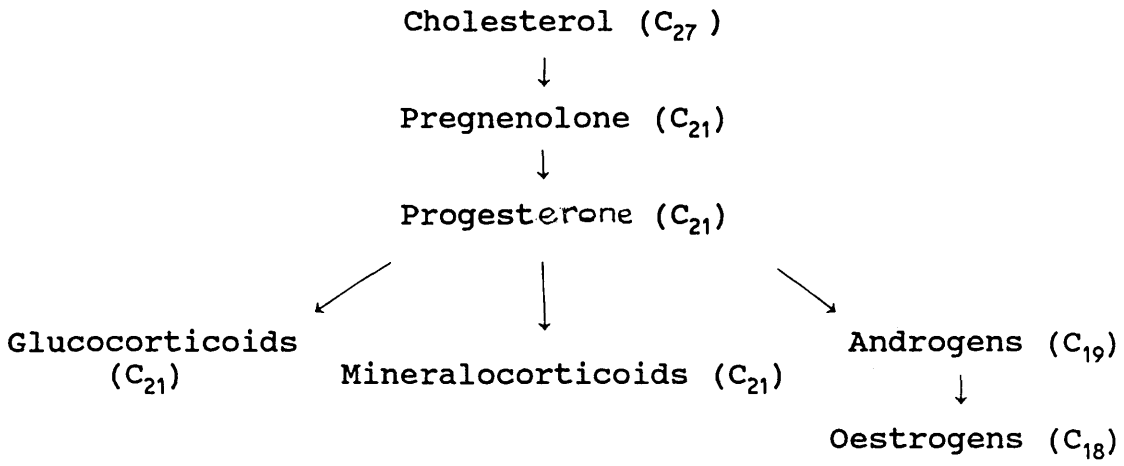


Figure 1.1. Synthetic Pathways of Steroid Hormones

1.5. RECEPTOR-MEDIATED ENDOCYTOSIS

Goldstein and Brown (1974) formulated the concept of receptor-mediated endocytosis to explain the observation that regulation of cellular cholesterol metabolism depended on the binding of LDL to the cell surface.

Anderson et al (1976) verified this theory by demonstrating that receptors for LDL were clustered in coated pits following the binding of ligand to these receptors and that the coated pits were subsequently pinched off from the surface to form vesicles within the cell. The existence of these pits had been known since 1964 (Roth and Porter) but their association with LDL and other ligands had not been appreciated. Subsequently Pearse (1975) showed that the coating was, in fact, a single protein, clathrin, and found

that the coated vesicles contained a number of different macromolecules.

The concepts of receptor-mediated endocytosis became much more significant with the finding that a genetic defect in receptor production caused a decrease in LDL uptake and internalisation, causing familial hypercholesterolaemia (Fredrickson et al, 1972). These patients have a very high incidence of myocardial infarction and atheroma formation.

Over the last decade it has been discovered that receptor-mediated endocytosis is the mechanism by which many macromolecules, including viruses, are internalised (Goldstein et al 1978, Pastan and Willingham 1981, Bretscher and Pearse 1984). More than twenty five specific receptors have been observed to participate in receptor-mediated endocytosis. These include the iron transport protein transferrin; the vitamin B_{12} transport protein, transcobalamin II; alpha-2-macroglobulin and various growth factors such as epidermal growth factor; platelet derived growth factor; insulin and luteinising hormone. The entry of these macromolecules into the cell may be governed by their intracellular metabolism. It is probable that the viruses and toxins which use this mechanism do so by opportunistic binding to the receptors.

The process of receptor-mediated endocytosis is initiated when receptors on the cell surface bind the macromolecules

and slide laterally into the clathrin-coated pits. When sufficient molecules are within the pit area, the pits invaginate to form endocytotic vesicles. After shedding the clathrin, the vesicles aggregate to form endosomes whose contents then become acidified by ATP driven proton pumps. In this acidic environment, the ligand and receptor become separated and the endosome either becomes attached to lysosomes for degradation, or open out releasing the ligand into the intracellular environment and the receptors return to the cell membrane for recycling.

There are several pathways by which receptor-mediated endocytosis occurs. They all share one common feature in that the receptors move to the coated pits which subsequently become coated vesicles. However, there are some differences in the mechanisms which instigate movement to the coated pits as well as variations in the routes which the receptors and ligands follow after entering the cells. These differences allow a classification of receptor-mediated endocytosis.

Firstly, there is a continuous movement of receptors to the coated pits and thence into the cell whether ligand is attached or not, and secondly, the receptors wait on the surface until bound to a ligand, whereupon they are captured by a coated pit. It appears that LDL falls into the former category.

The mechanism by which receptors move to the coated pits may be simple diffusion or it may involve a more directed type of propulsion (Bretscher 1984). The rate of diffusion of receptors on the cell surface is probably sufficiently fast to explain this movement (Goldstein et al 1981) but there is considerable evidence that this diffusion occurs in the direction of the coated pits.

1.5.1. Routes of endocytosis

The evidence suggests that all endocytotic vesicles enter the cell in the same way, but after that there are four principal routes which the vesicle may follow.

(i) <u>The receptor recycles and ligand metabolised</u>

This pathway is the classic mechanism described for the endocytosis of LDL, insulin and alpha-2-macroglobulin. The receptors and ligand part company within the endosome due to the acidic environment. The receptors then either bud off into a separate sub-vesicle of the endosome and are thereby transferred to the cell surface again or, more commonly, the endosome fuses with the Golgi apparatus allowing separation of ligands and receptors into different compartments. This releases the receptors which are then transported to the surface and the ligand is then targetted to the lysosomes. After their return to the surface, the LDL receptors are said to remain clustered ready to form coated pits. However, the work of Paavola et al (1985)

suggests that at least in granulosa cells cultured from the rat ovary, the LDL binding sites are not typically pre-clustered before interaction with ligand as they are in fibroblasts. This is an ideal mechanism for those ligands which are needed in considerable quantity as it does not require the constant formation of new receptors.

It does appear that there is a difference in the mechanism of recycling between different cell types. Paavola et al (1985) found some delay in the internalisation of the LDL and the receptor expression depended on the state of luteinisation of the cell, so that the receptor numbers and perhaps their mechanism of action, may be altered by exogenous hormones. This mechanism may be important in the control of endocytosis by the placenta.

(ii) Receptor recycles, ligand recycles

Octave et al (1983) first propounded this mechanism for the transferrin receptor. The transferrin/receptor complex did not appear to dissociate within the endosome under the normal acidic environment. However, it seems that iron is stripped from transferrin at the acidic pH (approximately pH 5.5) within the endosome. The apo-transferrin/receptor complex thus returns to the surface intact having deposited its load of iron within the cell. Apo-transferrin (transferrin without its iron) dissociates from the receptor at the neutral pH of the extracellular fluid. Therefore, when the complex reaches the surface, the apo-

transferrin dissociates from the receptor and is carried off in the bloodstream ready to bind to two more iron atoms. The receptor is now free to bind to another iron-containing transferrin molecule.

Studies by Unanue (1984) and Pernis (1985) suggest that this system of recycling may be of importance within the immune system as the mechanism of processing antigen prior to presenting it to effector cells. It is known that macrophages and certain B lymphocytes have very active receptor-mediated endocytotis systems.

(iii) Receptor degraded, ligand degraded

Epidermal growth factor (EGF) was the first macromolecule described which appeared to be processed in this way (Carpenter and Cohen 1979). After the ligand/receptor complex has been acidified within the endosome, both components seem to be degraded by lysosomal activity. How this system is effected is unclear as EGF dissociates from its receptor at acidic pHs. The same authors suggest that there are two classes of EGF receptors, 80% of which are internalised and degraded, and 20% follow kinetics suggestive of recycling.

(iv) Receptor transported, ligand transported

It has been suggested that this is the mechanism for the transport of immunoglobulins (IgA, IgM) across epithelial surfaces such as mammary epithelia for excretion into milk

and across liver cells for excretion into the bile (Solari and Kraehenbuhl 1984, Mostov et al 1984).

It is apparent that cells have different mechanisms for utilising their receptors. Indeed, some cells may well use several different mechanisms for the same receptor. This implies that these mechanisms must be regulated so as to allow a single cell to process the same receptor by different routes at different times according to its needs and that different cells may process the same receptor by different routes.

To explain this concept it is reasonable to suppose that each receptor contains different functional domaines. It must contain a binding domaine that is specific for a given set of ligands, and regions that allow it to interact with other molecules so that it can be transported to various sites within the cell. This enables the receptor to move from one compartment to another either being acted upon at each stage or not. This implies that there must be a sequential system of signals (Goldstein et al 1985).

1.6. LIPID METABOLISM IN PREGNANCY

It is known that in human pregnancy, there is a two- to threefold increase in plasma triglycerides, and slightly lesser increases in cholesterol and phospholipid (Knopp and Warth (1973). The differential compositions of the

lipoprotein classes are now well known (Gofman et al 1954; Warth et al 1975; Potter and Nestel 1979; Farhaeus et al 1985; Knopp et al 1986). From Figure 1.2. it can be seen that the concentration of all the lipoprotein fractions increase during pregnancy. The VLDL cholesterol and triglyceride increase approximately 2.5-fold over the pre-pregnancy levels, and LDL cholesterol increases 1.6-fold. The HDL cholesterol increases maximally to mid-gestation by 1.4-fold, and then declines to almost pre-pregnancy levels by term. These rises in lipoprotein levels would support the hypothesis that the placenta acquires its fatty acids from lipoproteins, probably by receptor-mediated endocytosis.

All these changes appear to be mediated by hormones, and can be mimicked by the high dose oestrogen combined contraceptive pill (Knopp et al 1982) (see Figure 1.3.). However, Applebaum-Bowden et al (1989) (see Figure 1.4.) found that postmenopausal women prescribed oestrogen for hormone replacement therapy had a 26% **reduction** in their LDL cholesterol and an increase of 72% in their HDL triglyceride levels. This is the opposite of what one would expect if the lipoprotein changes in pregnancy were mediated by oestrogen alone.

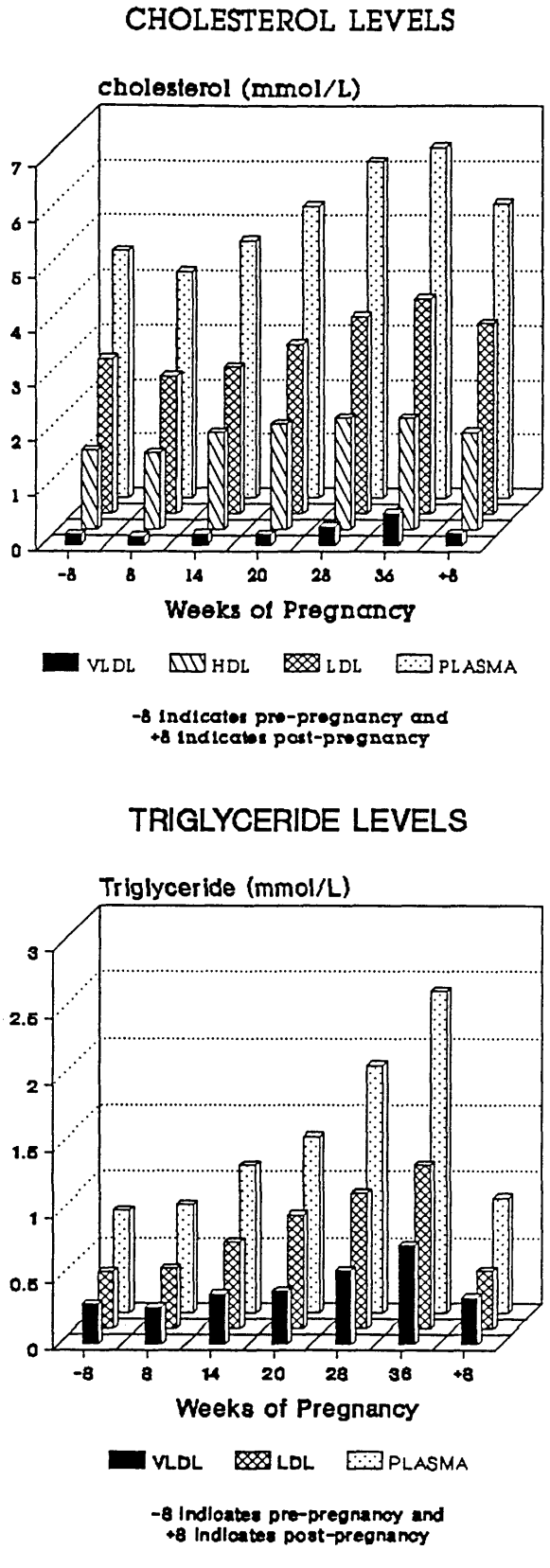


Figure 1.2. Cholesterol and Triglyceride Levels in Pregnancy

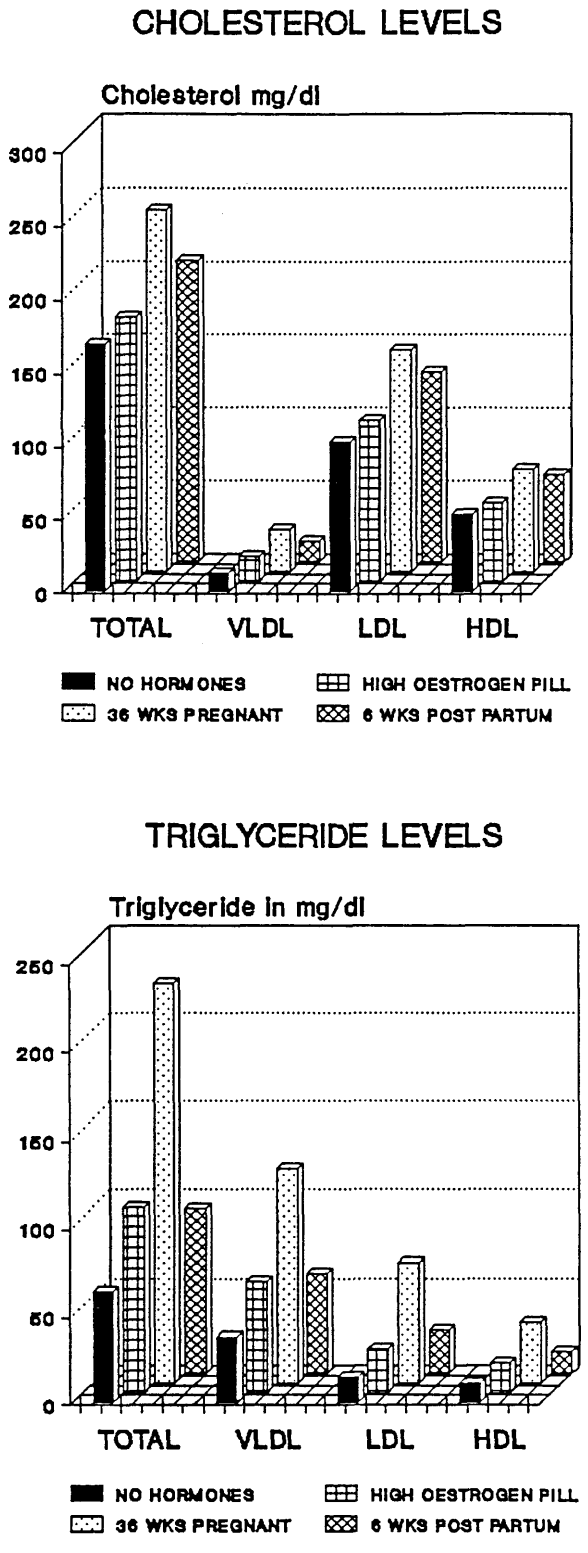


Figure 1.3. **Cholesterol and Triglyceride Levels in Pregnancy and with a High Oestrogen Contraceptive Pill (Knopp et al, 1982)**

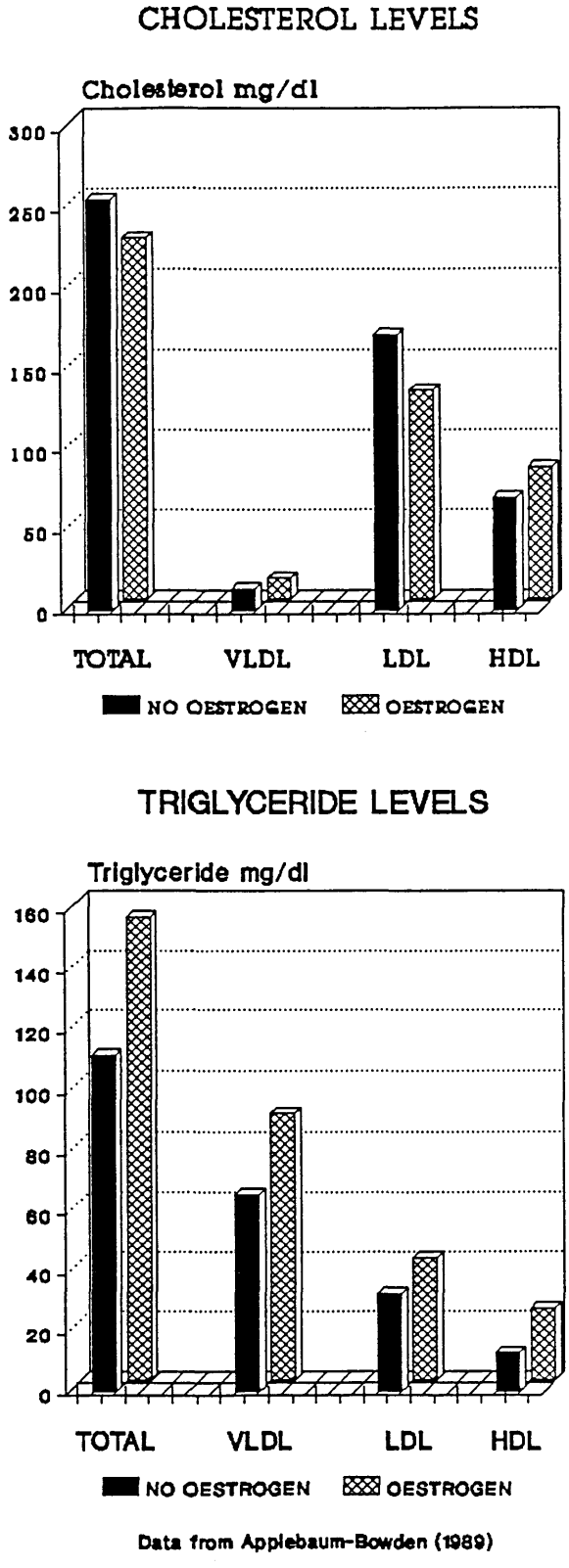
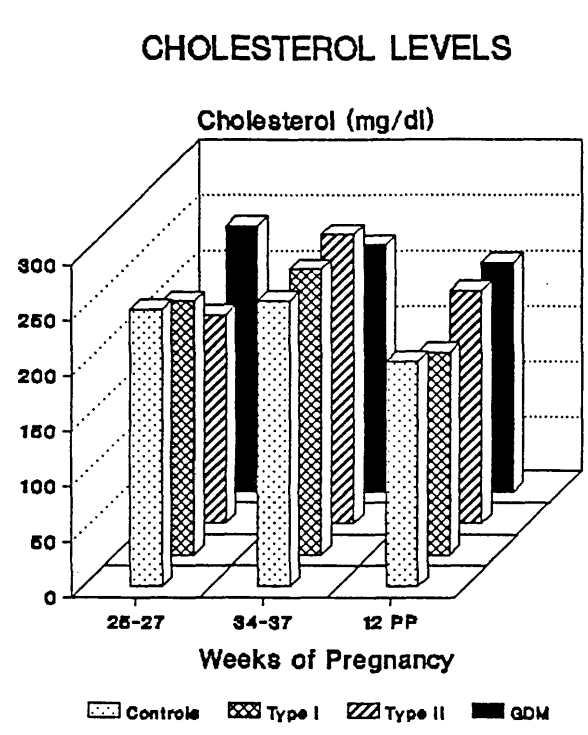


Figure 1.4. **Cholesterol and Triglyceride Levels with Hormone Replacement Therapy (Appelbaum-Bowden, 1989)**

1.7. LIPOPROTEIN METABOLISM IN DIABETES MELLITUS AND IN DIABETIC PREGNANCY

The most common form of human diabetes is the non-insulin dependent diabetes, also known as maturity-onset, or type II, diabetes and is commonly associated with obesity. Rigg et al (1980) have pointed out that during pregnancy there is a marked difference between the juvenile-onset type diabetes and the maturity-onset type diabetes. During pregnancy the increase in insulin requirements for juvenile, or type I, diabetics is approximately 38%, while it can be 100% in mature-onset, or type II diabetics. Knopp et al (1981) have suggested that the maturity onset diabetes is very similar to gestational diabetes, and is a different disease from the insulin-dependent juvenile-onset type.

Howard (1987) has shown that there is an abnormal composition of lipoproteins in maturity-onset diabetes. This abnormality is particularly marked in the VLDL portion of lipoproteins, such that in diabetic ketoacidosis, extreme elevations of VLDL have been recognised (Bierman et al 1966; Bagdade et al 1967).



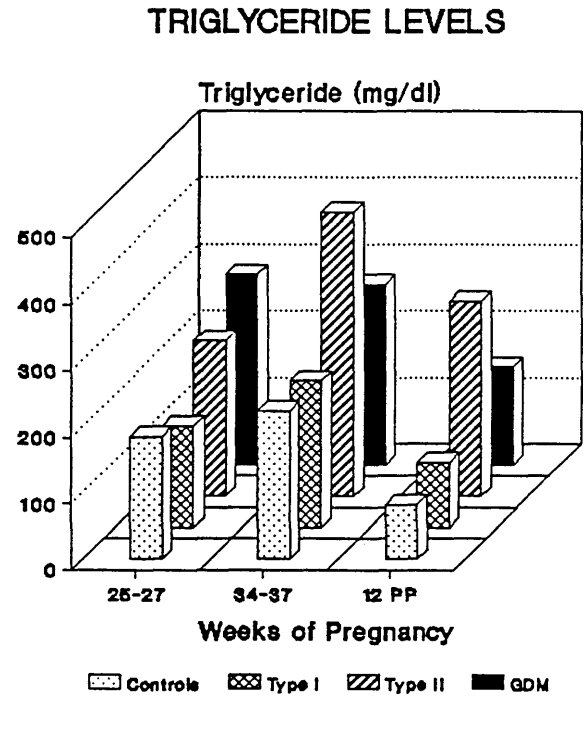


Figure 1.5. Cholesterol and Triglyceride Levels in Normal and Diabetic Pregnancy (Knopp et al, 1981)

It is interesting to note that in the maturity-onset type diabetes there is an abnormality of the VLDL fraction, such that the triglyceride content is much greater than normal, although the cholesterol content remains approximately the same (see Figure 1.5.). This may be due to the effect of a reduction in the efficiency of the lipoprotein lipase which is dependent on insulin (Nikkila 1984).

This hyperlipidaemia is related to a certain extent to the accuracy of the diabetic control; that is, the better the control, the less the hyperlipidaemia, although in some gestational diabetics, despite good control, this hyperlipidaemia, particularly in the VLDL group, still occurs (Howard 1987). In juvenile-onset, or insulin-dependent diabetes, the lipoprotein profile is essentially similar to that found in normal pregnant women, but does show variations if the diabetes becomes poorly controlled, for example due to infection.

CHAPTER TWO

CLASSIFICATION, SEPARATION AND CHARACTERIZATION OF PLASMA LIPOPROTEINS

2.1. STRUCTURE OF LIPOPROTEINS

Lipoproteins are the means by which the body transports lipids in a water based medium. This is achieved by wrapping the hydrophobic lipids up in an hydrophilic envelope, enabling a stable dispersion of the lipids to be formed in water. The hydrophobic core of lipoproteins consists of cholesteryl esters and triglycerides enclosed by an hydrophilic envelope of phospholipid, unesterified cholesterol and specific proteins. These proteins are called apo-lipoproteins and confer specificity and stability to the particle.

The exact biophysical mechanism by which lipoproteins are formed is not clear, but mixtures of lipids can readily be made into opaque emulsions which are similar to breast milk, and can be turned into clear microemulsions (Bowcott and Schulman 1943).

2.2. CLASSIFICATION OF LIPOPROTEINS

Attempts have been made to classify lipoproteins according to their physical and chemical characteristics, but none has been entirely satisfactory. The lipid and protein content of lipoproteins vary considerably and, in addition, lipoproteins form a continuous spectrum within any single given physical characteristic. For example, the density of

lipoproteins range from chylomicra (density <0.94 g/ml), to Very High Density Lipoprotein (density >1.25 g/ml). The division into different classes is therefore arbitrary.

2.2.1. Hydrated density

This classification, described by Havel et al (1955) and perfected by Lindgren (1975) in the Donner Laboratory, fractionates lipoproteins by suspending them in a solvent of known density and ultracentrifuging the mixture. The lipoproteins of lesser density float to the top and can be removed. If the density of the remaining mixture is again raised and ultracentrifuged, the lipoproteins falling between the two densities can be removed. This produces a flexible classification as the division of the densities can be as small as desired. The generally accepted density divisions are shown in Table 2.1.

Most researchers use a standard temperature of 20°C. for altering the density, and allowance must be made for this change in density if the centrifugation is performed at any other temperature.

When great precision is required, allowance must be made for the redistribution of salts that occurs during ultracentrifugation, as this produces a small density gradient within the centrifuge tube.

Lipoprotein	Density g/ml
Chylomicrons	<0.94
Very Low Density Lipoprotein (VLDL)	0.94 - 1.006
Intermediate Density Lipoprotein (IDL)	1.006 - 1.02
Low Density Lipoprotein (LDL)	1.02 - 1.063
High Density Lipoprotein (HDL)	1.063 - 1.20
HDL1 (HDLc)	1.06 - 1.07
HDL2	1.063 - 1.125
HDL3	1.125 - 1.20
Very High Density Lipoprotein (VHDL)	1.20 - 1.25

Table 2.1. Classification of Lipoproteins according to density

2.2.2 Floatation Rate

Lipoproteins float upwards at differing rates in the analytical ultracentrifuge; the rates being measured in Svedberg units (Sf). This again is a flexible classification as the lipoproteins under discussion can be described accurately by their floatation rate at a specific temperature and solvent density. Four categories have been defined, which are only broadly equivalent to the classes defined by ultracentrifugation (Table 2.2.).

Sf - >400	Floatation rate of >400 Svedbergs in a sodium chloride solution of density of **1.063** g/ml. These approximate to the chylomicrons.
Sf - 20-400	Floatation rate of 20-400 Svedbergs in a sodium chloride solution of density **1.063** g/ml. This approximates to VLDL.
Sf - 0-20	Floatation rate of 0-20 Svedbergs in a sodium chloride solution of density **1.063** g/ml. This is sometimes divided into Sf 12-20 (approximating to IDL) and Sf 0-12 (approximating to LDL).
F - 1.20	Floatation rate of 0-9 Svedbergs in a sodium chloride/sodium bromide solution of density **1.20** g/ml. This approximates to HDL, and is usually divided into F - 1.20, 3.5-9.0 (HDL2), and F - 1.20, 0-3.5 (HDL3).

(**F** denotes a different analytical density).

Table 2.2. Classification of Lipoproteins According to Floatation Rate

2.2.3 Particle Size

Stone et al (1970, 1971) proposed a classification based on the size of the lipoprotein particles. The size was estimated by a combination of their ability to scatter light, and their passage through filters of specific pore size. However, it is difficult to obtain filters with accurately defined pore size, particularly for the smaller particles. Particle size can also be estimated by the electron microscope, but this only appears to be suitable for very fresh samples. It may also suffer from artefacts of fixation.

2.2.4 Electrophoretic Mobility

Electrophoresis is a simple technique to perform, particularly with the commercially prepared agar/agarose sheets. Table 2.3. shows the classification of lipoproteins according to their electrophoretic mobility.

Chylomicrons	Particles which are too large to migrate out of the well.
Alpha-lipoproteins	Particles with a mobility similar to alpha 1 globulins, hence their name.
Pre-beta lipoproteins	Particles with a mobility somewhat greater than the beta lipoproteins (sometimes known as alpha 2 lipoproteins from alpha 2 globulins).
Beta lipoproteins	Particles which migrate at the same rate as beta globulins.

Two minor groups of lipoproteins have been described:-

Lipoprotein x	found in patients with obstructive jaundice, and migrates to the cathode at a pH of 8.0.
Lp(a)	known as "sinking prebeta lipoprotein", a normal component of plasma.

Table 2.3. Classification of Lipoproteins According to Electrophoretic Mobility

2.2.5 Classification According to Apo-lipoproteins

Alaupovic et al (1979, 1983) postulated a system of lipoprotein nomenclature based on the apolipoproteins.

2.2.5.1. The definition of apo-lipoproteins

A considerable number of proteins can be separated from lipoproteins. These are usually loosely attached by non-specific bonds although some, for example albumin, may be firmly attached. The significance of these is not yet clear. It is generally accepted that apolipoproteins are present on the particle for a specific purpose or a variety of purposes; to confer stability to the particle, act as an enzyme or coenzyme, or act as the ligand for receptor-mediated endocytosis of the particle.

2.2.5.2. Polymorphism

Apo-lipoproteins were given letters of the alphabet at the time of their isolation. However, some lipoprotein preparations thought to be homogenous were found later to be mixtures of different lipoproteins, for example apo-lipoprotein A, which was subsequently divided into AI, AII and AIII (which is also known as apo-lipoprotein D). Apo-lipoprotein G, AIV, apo-lipoprotein H (β_2 glycoprotein - 1), apo-lipoprotein (a) (which may be a polymorphic form of apo-lipoprotein B) may meet the criteria for inclusion

as apo-lipoproteins. Polymorphic forms are denoted by Arabic numerals, for example, apo-lipoprotein E-1, E-2 and apo-lipoprotein C-III-1.

In Alaupovic's system (1979, 1983), an apo-lipoprotein must be capable of being the only protein in the lipoprotein, thereby producing lipoprotein families. Some particles, however, carry more than one apo-lipoprotein. Osborne and Brewer (1977) circumvented this problem by suggesting that those lipoproteins with a single apo-lipoprotein should be termed a primary lipoprotein, and those with two or more, a secondary lipoprotein. Alaupovic et al (1983) called these lipoproteins associated complexes of families. It is debatable, however, whether those with polymorphic forms should be primary or secondary, or should have a separate classification.

The classification is also complicated by the fact that apo-lipoproteins appear to be added and removed from the particle during its time in the intra-vascular compartment.

2.3. SEPARATION OF PLASMA LIPOPROTEINS

To separate lipoproteins in useful quantities for experimentation, there are three main methods; ultracentrifugation, precipitation and chromatography.

2.3.1. Isolation by Sequential Ultracentrifugation

Lipoprotein classes are fractionated according to their hydrated density by having serial alterations in the solvent density. This method was propounded by Havel et al (1955). The principle of this method is to adjust the solvent density of the plasma to the lower limit of the fraction that it is proposed to isolate, and then centrifuge the preparation at approximately 100,000 G for 12-18 hours. The lipoproteins of a lesser density than the solvent will float to the top of the tube, while the other lipoproteins and plasma proteins will be centrifuged towards the bottom. If the lipoproteins at the top of the tube are then removed, the density of the infranate can be raised to a new level, and the ultracentrifugation repeated.

By continuing in this manner, the sample can be fractionated into different densities of the desired distribution. Due to the overlap of the lipoproteins at the junction between the supranatant and the infranatant, it is customary to purify the preparation by repeated ultracentrifugations within the desired density groups. The researcher must be aware that repeated manipulation may degrade the lipoproteins.

The time and speed of ultracentrifugation depends on the rotor head that is used. It is better to use a longer,

slower ultracentrifugation as the separation of the lipoproteins can sometimes be improved, as time is allowed for the lipoproteins at the bottom of the tube to float to the top.

2.3.2 Isolation by Density Gradient Ultracentrifugation

Lipoproteins can be separated into their various density classes by either a continuous or discontinuous density gradient ultracentrifugation.

Density gradient ultracentrifugation can be performed by using a density gradient in a long swinging bucket ultracentrifuge rotor, or by rate zonal ultracentrifugation.

2.3.2.1. Single Tube Density Gradient Ultracentrifugation

The density gradient is formed from sodium bromide solution which is pumped into the tube with a mechanical density gradient former to produce a continuous, or discontinuous, gradient. The sample is then applied to the top of the tube, and the preparation centrifuged. The bucket is then removed (with care in order not to disturb the density gradient), and the sample aspirated.

2.3.2.2. Rate Zonal Ultracentrifugation

Rate zonal floatation of lipoproteins in a density gradient ultracentrifugation has been described in detail by Wilcox and Heimberg (1970) and Patsch et al (1974 and 1980). It is possible to separate all the lipoprotein classes in one ultracentrifugation. However, better resolution is achieved when two ultracentrifugations with different density gradients are employed; the first yielding VLDL, IDL and LDL; and the second, with the higher density gradient, yielding HDL and its sub-divisions.

2.3.3. Precipitation Techniques

The techniques available for the fractionation of lipoproteins using precipitation do not have a classification system of their own, and the fractions that are precipitated do not coincide particularly well with any of the recognised methods of classification. Once the lipoproteins have been precipitated and redissolved, they have to be fully characterised by using one of the standard techniques; either electrophoresis or ultracentrifugation.

However, the precipitated fractions approximate to the alpha and beta lipoproteins produced by electrophoresis and, for convenience, are described by these names, although they do not precisely correspond.

2.3.3.1. Low Temperature Ethanol Fractionation

This technique, developed by Cohn et al (1950), uses precisely controlled changes in pH, dielectric constant, ionic strength, temperature and protein concentration. Acetate and carbonate buffers are used to alter the pH and ionic strength. Ethanol is used to control the dielectric constant, and the temperature is maintained at -10°C. The fractions produced bear his name.

2.3.3.2. Precipitation with Sodium Phosphotungstate

Burstein (1970) developed a technique using sodium phosphotungstate, an inorganic precipitant of high molecular weight. In the presence of magnesium ions, sodium phosphotungstate will precipitate all the lipoproteins including HDL.

2.3.3.3. Precipitation with Heparin and Dextran Sulphate

The most commonly used method was devised by Burstein and Scholnick (1973). Sulphated polysaccharides will react with plasma proteins at physiological pH to produce soluble complexes. Lipoproteins are precipitated when the concentration of the polysaccharide is low, and the pH is maintained between 7.5 and 8.6. Using a mixture of

sulphated polysaccharide, and one of the divalent cations Ca^{2+}, Mg^{2+}, Mn^{2+} or Co^{2+} the various main classes of lipoproteins can be separated out.

2.3.4. Chromatography

Chromatography is a useful technique for purifying and separating lipoproteins but, like precipitation techniques, the products have to be characterised by ultracentrifugation or electrophoresis.

2.3.4.1. Gel Filtration

The lipoprotein sample is passed through gel columns of various pore size. However, the resolution of this technique is poor, and it produces a large elution volume requiring concentration.

2.3.4.2. Affinity chromatography

The advent of monoclonal antibodies and specific antibodies to the various apo-lipoproteins makes immuno-adsorption chromatography a useful technique for separating lipoproteins from large volumes of fluid. However, it is the apo-lipoprotein that is recognised, and this may limit its usefulness. Immobilised heparin and lectin have been used for affinity chromatography, although the resolution is unsatisfactory.

2.3.5. Discussion

2.3.5.1. Ultracentrifugation

Sequential ultracentrifugation is the standard method for separating lipoprotein fractions, and forms the basis for the classification of VLDL, IDL, LDL and HDL. The only suitable rotor head for sequential ultracentrifugation in the laboratory where this research was undertaken was an MSE 6 x 14 swinging bucket which limited the volume of samples to 78 ml. By contrast, 120 ml samples could be processed by rate zonal ultracentrifugation. In addition, sequential ultracentrifugation takes 72 hours to fractionate lipoproteins, compared with 140 minutes by rate zonal ultracentrifugation.

These disadvantages are shared by single tube density gradient ultracentrifugation, where even smaller sample volumes are used. Two further disadvantages occur with single tube density gradient ultracentrifugation. Firstly, the lipoprotein sample forms a continuous spectrum, which do not form discrete bands. Thus it is difficult to obtain a pure sample, however good the removal of the density gradient. Secondly, the centrifuge tubes must be filled and unloaded while the rotor is at rest; under these conditions diffusion may distort both the gradient and the lipoprotein position within the gradient.

The technique of rate zonal floatation in the ultracentrifuge overcomes all these disadvantages. Large or small volumes may be used, and the density gradient is loaded into the rotor which is rotating at a sufficient speed to maintain the density gradient. Pure representative samples of each lipoprotein fraction can be obtained.

Using sequential ultracentrifugation initially, pure samples of the lipoprotein fractions were prepared, and these formed the standard against which all subsequent samples, prepared by rate zonal ultracentrifugation, were compared.

2.3.5.2. Precipitation techniques

The disadvantage of these techniques is that they do not correspond precisely with the classification of lipoproteins, and therefore a second method of isolation is required. This, in principle, is a sound practice. However, the more the lipoprotein molecules are manipulated, the more likely they are to become denatured. These techniques, therefore, were not used in this study.

2.3.5.3. Chromatography

Gel chromatography has poor resolution of lipoproteins, and a large elution volume. Immuno-adsorption chromatography,

with specific antibodies, is useful when processing large sample volumes, for example, perfusates, and radio-active lipoproteins, when the use of a titanium rate zonal rotor head is precluded. Rabbit anti-LDL was produced by injecting LDL, purified by sequential ultracentrifugation, into rabbits to use for immuno-adsorption. This technique was found subsequently to be unnecessary for this study, but the rabbit anti-LDL was used for the characterisation of the lipoproteins using Ouchterlony plates.

In summary, the methods used to prepare lipoprotein fractions were:

a) small volumes of lipoprotein were separated using sequential ultracentrifugation as a standard;

b) larger volumes of lipoproteins were separated using rate zonal ultracentrifugation and compared with those obtained by sequential ultracentrifugation;

c) ^{125}I-lipoproteins were purified on Sepharose columns prior to use.

2.4. CHARACTERISATION OF LIPOPROTEIN FRACTIONS

Lipoprotein preparations are a mixture of particles that differ with respect to size, density, electric charge and chemical composition. As such, no two samples will be identical, and each preparation has to be characterised by several properties to ensure comparability.

2.4.1. Electrophoresis

The basis of electrophoretic identification of lipoproteins is that they have an isoelectric point at approximately pH 5.5, above which they are negatively charged. This enables the lipoproteins to be moved by an electric current. The classification of lipoproteins according to their electrophoretic mobility has been described in Section 2.2.4. The five principal electrophoretic techniques are described.

2.4.1.1. Paper Electrophoresis

Strips of paper are placed horizontally over two electrodes, with the ends in an electrolyte buffer solution of pH 8.6. Albumin can be added to the buffer to improve the band separations. The plasma sample is applied to the cathode end of the paper, and a DC current passed across the electrodes. The best results are obtained with a constant voltage power supply, although a constant current

supply can be used. The papers are dried in an oven, and the lipoproteins are then developed by staining with either Oil Red O or Sudan Black.

2.4.1.2. Cellulose Acetate Electrophoresis

The technique is a similar system to the paper electrophoresis. The bands run quicker and have a tendency to be sharper, but care must be taken to select the appropriate acetate as some sheets stain non-specifically with Oil Red O or Sudan Black.

2.4.1.3. Agarose Gel Electrophoresis

This method, devised by Noble (1968), is faster than paper electrophoresis and has good resolution. The staining qualities of agarose plates are superior to acetate. However, it is difficult to prepare agar plates of a uniform thickness and consistency, and poor quality plates produce an uneven running of the samples. This method also requires the fresh preparation of gels. Commercial, ready to use gels with a long shelf life are available. These give good results but are more expensive.

2.4.1.4. Polyacrylamide Gel Electrophoresis

This is a sophisticated method for producing a sieve of various pore sizes for the separation of particles of

different molecular weights. The intact lipoprotein molecule is very large, requiring the use of polyacrylamide gradient gels. In addition, the top of the gradient must have a very low concentration of polyacrylamide in order to produce a large pore size, thus making the top of the gel extremely fragile. The gel is removed from its container after the electrophoresis run, and the lipoproteins stained either with Sudan Black or Oil Red O.

The addition of sodium dodecyl sulphate (SDS) removes the apo-lipoproteins from the lipoproteins, and the identification of the molecular weights of the apo-lipoproteins is performed on a discontinuous gel. This technique identifies the lipoproteins by their apo-lipoprotein moeity. The VLDL fraction contains apo-lipoprotein B and E, and the LDL fraction contains apo-lipoprotein B alone. Cooling apparatus is required to prevent the system overheating.

Maguire et al (1989) have described a technique for using SDS-glycerol polyacrylamide slab gels capable of resolving apo-lipoproteins with molecular weights from 8,000 to greater than 550,000 on a single gel.

2.4.1.5. Immunoelectrophoresis

Immunoelectrophoresis uses specific, preferably monoclonal, antibodies to the moiety to be isolated and identified.

The procedure is essentially similar to the electrophoresis of lipoproteins in agarose gel. After electrophoresis, however, specific antibody is placed in a trough between each pair of wells, and diffusion of the antibody, which takes approximately three days, is allowed to occur.

2.4.1.6. Ouchterlony Immunodiffusion Plates

This technique utilises the precipitation lines formed when antigen meets antibody. Antigen is placed in the centre well of an agar plate and antibodies in serial dilutions are placed in wells around it. Precipitation lines occur - their density being dependent on the concentration of antibody in each well (Ouchterlony, 1970).

2.4.2. Discussion

The characterisation of lipoprotein samples had to achieve two objectives. Firstly, to ensure that there was no contamination of the lipoprotein samples with unwanted proteins, particularly albumin, and secondly, to ensure that there was consistency between the lipoprotein samples.

Paper electrophoresis was found to take a long time to perform, and gave inconsistent results. Acetate sheets which did not pick up Sudan Black and Oil Red O stains were difficult to obtain. The techniques of paper and cellulose acetate electrophoresis were therefore abandoned.

In order to perform agarose gel electrophoresis, the worker initially prepared his own agarose gel plates, but found several disadvantages. It was difficult to achieve uniform consistency in the agarose gel over the entire plate, leading to an irregular running pattern, and the gels had to be prepared on the day of use. These disadvantages were overcome by using commercially-prepared agarose plates, which had a long shelf life, were easy to use, and gave consistent results. Although the agarose gel electrophoresis utilises a different classification from the ultracentrifugation preparation, it was adequate to show a consistency between lipoprotein samples.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis gave consistent and reproducible results for determining the molecular weights of the apo-lipoproteins, and verifying the consistency of the apo-lipoprotein content of the lipoprotein samples.

When this research was undertaken, few specific antibodies were available, thus limiting the usefulness of immunoelectrophoresis. Immunodiffusion in the form of a simple Ouchterlony plate was useful in confirming the absence of albumin and the presence of apo-lipoprotein B in the lipoprotein samples.

In summary, the methods used for the characterisation of the lipoprotein samples were as follows:

a) A representative sample of the lipoprotein class was prepared by rate zonal ultracentrifugation.

b) Agarose gel electrophoresis confirmed consistency between samples.

c) SDS-polyacrylamide gel electrophoresis of the apo-lipoproteins in the lipoprotein samples confirmed the sole presence of apo-lipoproteins B and E in the VLDL fraction, apo-lipoprotein B in the LDL fraction, no apo-lipoprotein E in the HDL fraction, and confirmed the absence of any other contaminating proteins;

d) Ouchterlony immunodiffusion confirmed immunologically the presence of apo-lipoprotein B in VLDL and LDL, and the absence of albumin in any of the lipoprotein samples.

The details of the methods employed are described fully in the next chapter.

CHAPTER THREE

MATERIALS AND METHODS FOR PREPARATION AND CHARACTERISATION OF LIPOPROTEINS

3.1. INTRODUCTION

This chapter addresses the materials and methodology required for the isolation and characterisation of VLDL, IDL, LDL and HDL. Chapter Two outlines the methods available for the separation of the lipoprotein fractions, and discusses in general terms the reasons for the choice of techniques which were adopted for this study.

Lipoproteins are particularly susceptible to degradation from enzymes within the plasma, oxidation from ambient oxygen, microbiological degradation and excessive manipulation. To minimise these effects, samples should be processed as rapidly as possible at a temperature between 0 and 4°C.

To reduce the effects of oxidation, disodium ethylenediamine tetraacetic acid (EDTA), to a concentration of 1.0 mM, was added to the plasma and all solutions used during the preparation and manipulation of the lipoprotein samples. The EDTA also acts as an anti-coagulant by chelating calcium ions so plasma can be separated from the erythrocytes by centrifugation, thus avoiding the time taken for clot formation and retraction. Solutions should be degassed before use, and the lipoprotein, if stored, should be kept between 0-4°C overlaid with nitrogen, in a stoppered flask. It is advisable to use the lipoproteins within three weeks of taking the sample.

Aseptic precautions should be adopted, and the sample should be filtered through a 22 micron milipore filter as bacteria, particularly the Pseudomonas group, will still grow at 4°C. If the lipoprotein is not to be used in a biological system, sodium azide or thiomersyl can also be added at 2.0 mM and 0.2 mM concentrations respectively.

The components of the lipoprotein are held together by covalent forces which are relatively easily disrupted. Manipulation should be kept to a minimum compatible with the purity of the preparation and the characterisation of the lipoprotein which will be dictated by the requirements of the experiment to be performed.

3.2. BLOOD SAMPLES FOR LIPOPROTEIN ISOLATION

250 ml of blood was taken by venepuncture using a 16G Butterfly needle, three-way tap and a 60 ml syringe, from normal, healthy volunteers who had fasted overnight. After withdrawing 50 ml, the three-way tap was turned and the blood from the syringe was ejected into a conical flask containing 92.5 mg EDTA, to produce a 1 mM final concentration. The remaining 200 ml was withdrawn in the same way.

The blood was immediately centrifuged at 4°C. for 15 minutes at 2700 rpm in an MSE Mistral 4L Centrifuge. This speed was selected as sufficient to sediment the red cells while

avoiding haemolysis. The plasma was removed with a pipette and centrifuged at 15000 rpm for 30 minutes at 4°C. in an MSE High Speed 18 Centrifuge. The opalescent layer at the top of the tube, containing the bulk of the chylomicrons, was removed with a pipette and discarded. The plasma was raised to the appropriate density (see Section 3.3.) prior to ultracentrifugation.

3.3. ALTERATION OF PLASMA DENSITY

The density of plasma needs to be altered for both sequential and rate zonal ultracentrifugation. When calculating density alterations, the density of plasma is assumed to be 1.006 g/ml, and approximately 6% of the sample volume is occupied by plasma proteins. Therefore, the partial solvent volume is 0.94 times the actual volume.

3.3.1. Dialysis

Dialysis of the sample against a solvent of the required density is the easiest and gentlest method. Analytical grade sodium bromide or sodium chloride is used to make up the solutions of the required density. However, its disadvantage is that it takes at least 24 hours, during which time the lipoprotein may undergo oxidation, or bacterial and enzymatic degradation. This method was therefore used infrequently in this study.

3.3.2. The Addition of A Solution of Required Density

A solution of known density in the appropriate volume is added to the sample to raise the density to the specified value by using the equation:

$$DV = d_1 v_1 + d_2 v_2$$

where D d_1 and d_2 are the densities of the final mixture, the plasma and the diluent respectively. V v_1 and v_2 are the corresponding volumes.

The sample is diluted with at least one volume of solvent, which gives a considerable dilution. As the volume of the appropriate rotor head in the Laboratory was 112 ml, this did not allow more than 45 ml of plasma to be processed in a single ultracentrifugation. This method was adequate for the initial preparative work in this study.

A more precise method was put forward by Lindgren (1975) which takes into account the non-linearity of the relationship between the concentration and density of a salt solution. The precision required in this study did not require the use of this more sophisticated formula.

3.3.3. Addition of Solid Salt

Solid analytical grade sodium bromide is added to the sample in a precisely calculated amount to raise the density to the required value. The simplest method to raise the density using solid salt was proposed by Radding and Steinberg (1960), who devised the formula:

$$M = \frac{v(d_2 - d_1)}{1 - V'd_2}$$

where M is the weight of salt to be added to a volume v to change its density from d_1 to d_2 at a stated temperature. V' is the partial specific volume of the salt at that relevant temperature and concentration. The values of V' of the sodium chloride, sodium bromide, potassium bromide at temperatures between 5 and 25°C. can be obtained from the relevant tables, reproduced in the Appendix, which are derived by Mills et al (1984) from data of Baxter and Wallace (1916).

This method was used most frequently as it allowed the largest plasma volume to be used. The quantity of analytical grade sodium bromide was calculated using the above formula, and weighed. The sample of plasma was added, and stirred gently at 4°C. until the salt dissolved.

3.4. ULTRACENTRIFUGATION

3.4.1. Sequential Ultracentrifugation

Havel et al (1955) described the method used in this study to separate the lipoprotein fractions. However, chylomicrons and VLDL have a density less than that of plasma and sequential separation of these two classes is therefore unsatisfactory, and are best separated by rate zonal floatation (see Section 3.4.2.).

The initial centrifugation at 15,000 rpm in the MSE High Speed 18 removed most of the chylomicron fraction (Section 3.2.). The sample was ultracentrifuged in polycarbonate centrifuge tubes at 28,000 rpm for 3 hours at 10°C. The fraction at the top of the tube contained chylomicron remnants and VLDL.

The density of the plasma sample was assumed to be 1.006 g/ml, and this was raised by the addition of solid analytical grade sodium bromide (Sigma Pharmaceuticals, Poole, Dorset, UK) using the formula stated in Section 3.3.3. to a density of 1.02 g/ml. The mixture was ultracentrifuged in a 6 x 14 swinging bucket rotor head at 28,000 rpm for 16 hours at 5°C. The supernatant was removed by tube slicing (see Section 3.4.3.) which yielded IDL, density 1.006 - 1.02 g/ml.

The remaining plasma was assumed to have a density of 1.02 g/ml and solid sodium bromide was added to produce a density of 1.063 g/ml. Re-ultracentrifugation of the sample yielded LDL, again removed by tube slicing. To produce the HDL fraction, the sample density was raised to 1.20 g/ml, and ultracentrifuged at 28,000 rpm for 48 hours at 10°C.

3.4.2. Rate Zonal Ultracentrifugation

Wilcox and Heimberg (1970) and Patsch et al (1974 and 1980) described methods for rate zonal floatation of lipoproteins in B14, B15 and TI-14 titanium rotors. In this study, their technique was modified to suit an MSE titanium B14 rotor.

3.4.2.1. Sample Preparation

A blood sample was taken and prepared as described in Section 3.2. and the density raised to 1.30 g/ml (see Section 3.3.3.).

3.4.2.2. Setting up the Density Gradient

Solutions:

Light Solution

2 litres distilled water with 350 μM EDTA adjusted to pH 7.4 with NaOH.

Heavy Solution

2 litres sodium bromide solution of density 1.30 (measured with an hydrometer) with 350 μM EDTA adjusted to pH 7.4 with NaOH.

There are two methods used for setting up density gradients:

i) A cam of variable shape which dictates the proportions of the light and heavy solutions pumped into the rotor.

ii) Different volumes of the light and heavy solutions are used and run in under gravity.

The latter method was used, which required two glass cylinders (LS and HS) joined together at the bottom with tubing and a tap (T). A stirrer (S) was inserted into the first cylinder, just above the outlet to a peristaltic pump (P), which was connected to the inlet port of the rate zonal rotor (see Figure 3.1.).

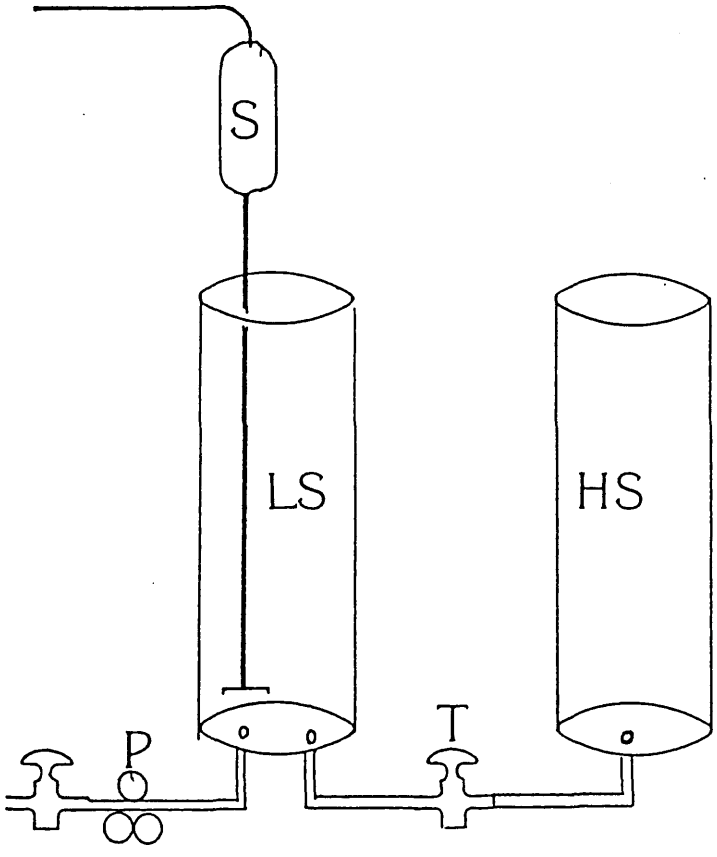


Figure 3.1. Diagram of Density Gradient Former

The technique is best illustrated by using an example. If the serum volume is 120 ml, and a 30 ml cushion (NaBr, density 1.30 g/ml, to flush the sample into the rotor) is required at the end of loading, then the gradient volume will be the volume of the rotor minus this volume, i.e. 670 -150 ml = 520 ml.

To determine the differing volumes of light and heavy solutions is a matter of trial and error. The proportion of heavy to light solution will be the ratio of their densities, 1:1.3, thus the volume of the light solution will need to be multiplied by 1/1.3 = 0.7692.

An appropriate sounding volume, for example 300 ml, of light solution is then multiplied by this figure to yield 230 ml of heavy solution which adds up to a total volume of 530 ml, therefore the volume has been overestimated. Thus trying a volume of 295 ml of light solution gives a volume of 226 ml of heavy solution which adds up to 521 ml of gradient.

3.4.2.3. Method

An MSE titanium B14 zonal rotor with a volume of 670 ml was used, and its characteristics have been described in detail by Anderson et al (1967).

295 ml of light solution was put into the cylinder with the stirrer, and 226 ml of heavy solution was put into the other cylinder. The tap between the two was opened, and the pump started simultaneously. In this way, the appropriate proportions of light and heavy solutions were run into the rotor. The density gradient was pumped in from the rim of the rotor towards the centre (rim feed), thus the densest part of the gradient was at the periphery of the rotor. The sample was pumped into the densest portion of the gradient, and therefore its density had to be raised to this level, that is, 1.30 g/ml.

In these experiments, the gradient was loaded at a loading speed of 3500 rpm. By trial and error, Patsch et al (1974, 1980) had determined that the appropriate centrifugation time was 140 minutes at a speed of 42000 rpm at 10°C. These conditions gave optimum separation of the VLDL, IDL and LDL from the HDL and protein which remained mixed at the periphery. A density gradient between 1.0 and 1.4 g/ml with an ultracentrifugation at 42000 rpm at 10°C. for 24 hours gave optimal separation of the HDL from the plasma proteins.

The zonal rotor was unloaded at 3500 rpm by pumping sodium bromide solution of density 1.30 g/ml into the periphery, forcing the gradient out through the centre port. The gradient was collected in 10 ml aliquots and read in a spectrophotometer at 280 nm to determine the protein

content. The density was measured using a 5 ml density flask and the results plotted on a graph of which Figure 3.3. is an example. Figure 3.2. shows a density gradient using sodium bromide without the addition of plasma to validate the technique.

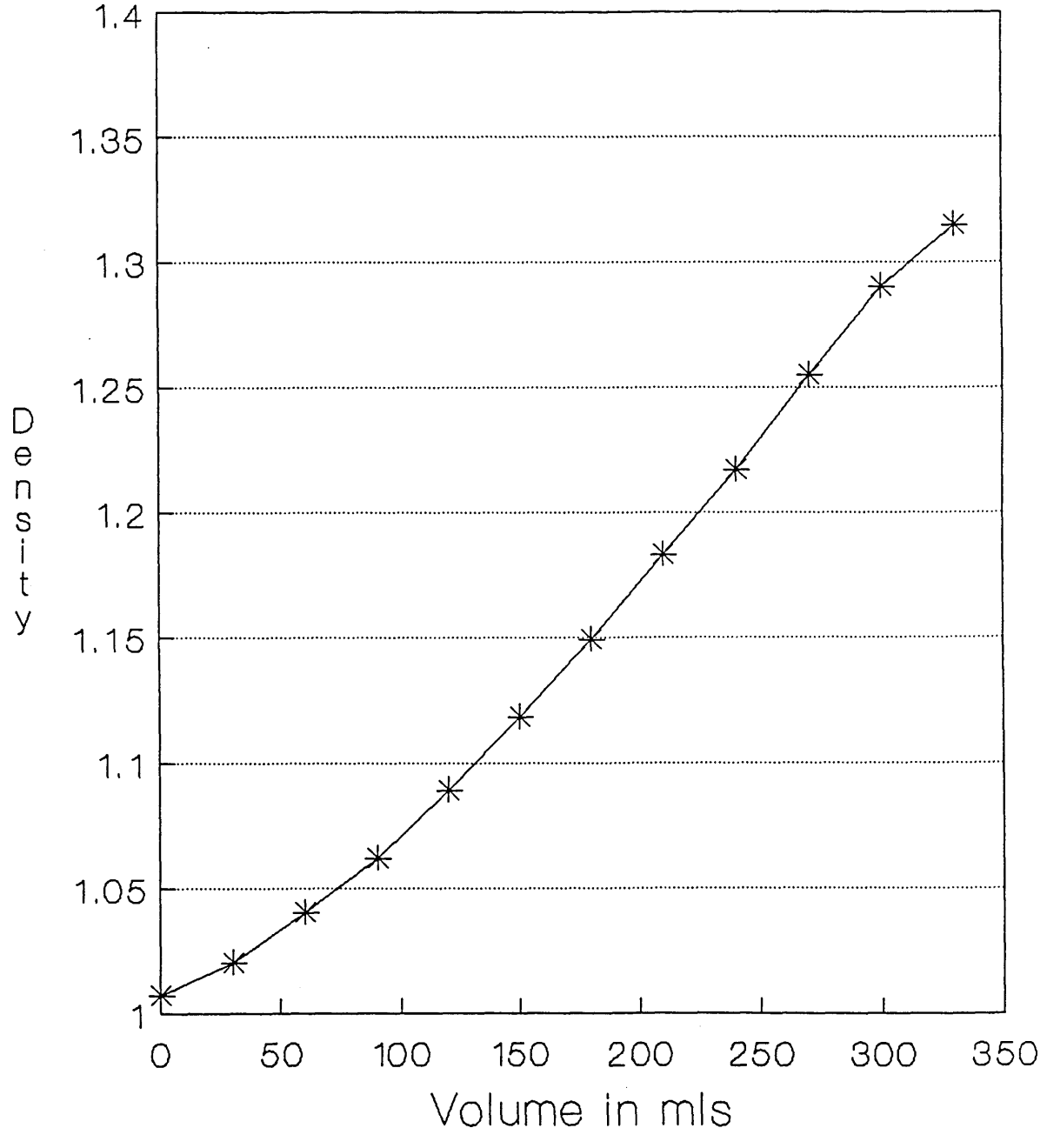


Figure 3.2. Graph of Sodium Bromide Density Gradient without the Addition of Plasma

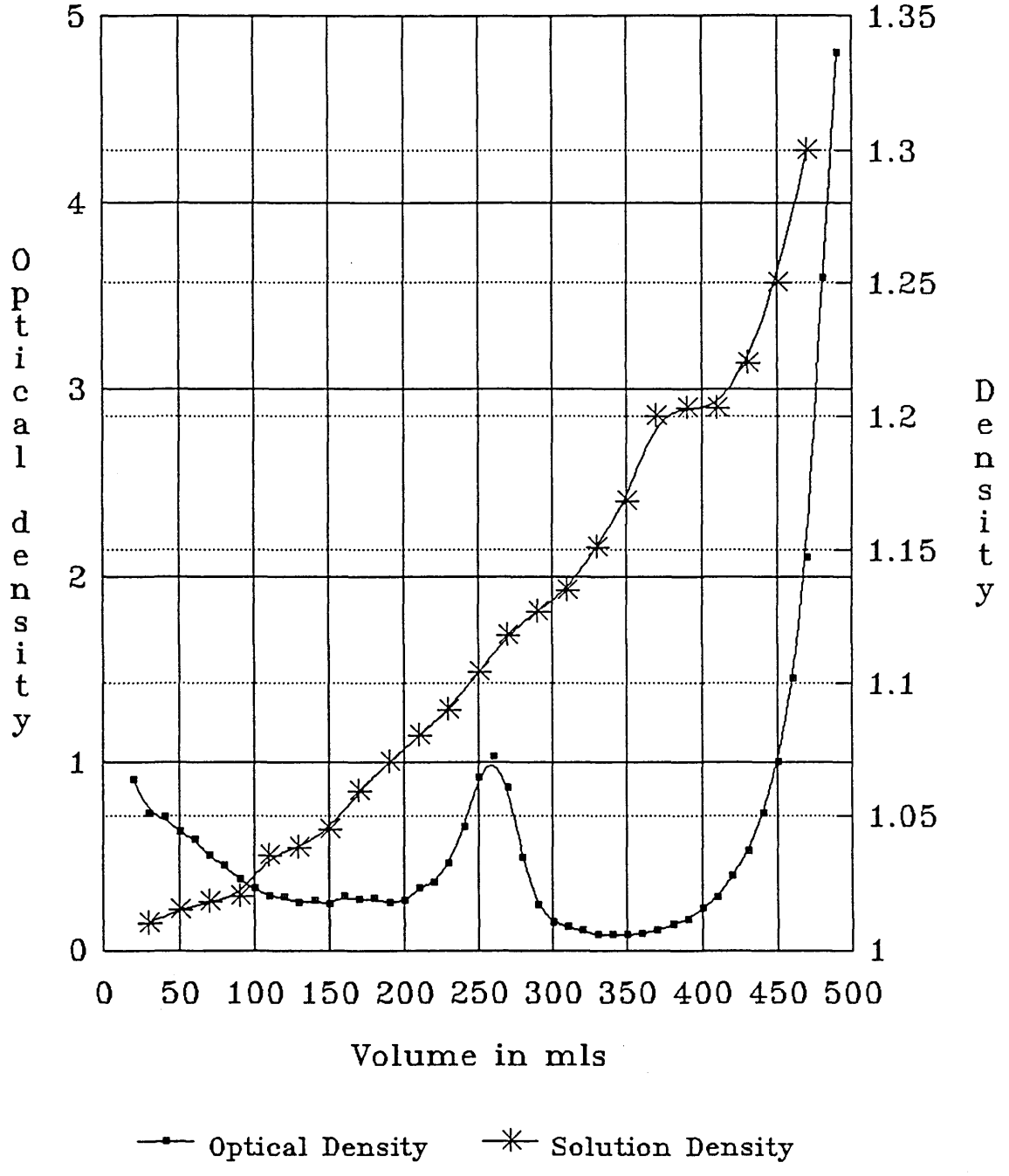


Figure 3.3. **Graph of Sodium Bromide Density Gradient and Optical Density of Zonal Ultracentrifugation with 50 ml Human Plasma**

A representative sample of each lipoprotein class was taken, and passed through a 22 micron filter. The samples were concentrated by placing them in large bore Visking tubing and covering them with solid polyethylene glycol (mol. wt. 20,000).

The samples were re-ultracentrifuged, without making any density alterations, in a six by fourteen swinging bucket rotor for 16 - 20 hours at 28000 rpm at 10°C. The lipoprotein was collected by tube slicing (see Section 3.4.3.).

3.4.3. Tube Slicing

Supernatants can be harvested from ultracentrifuged tubes in three ways: aspirating from the top of the tube; tube slicing and puncturing the bottom of the tube; and aspirating the infranate. The most efficient method of removing the supernatant was found to be by tube slicing.

The tube slicing equipment consisted of two aluminium alloy plates which could be screwed together with thumb screws. A flat, triangular stainless steel blade was fixed to a square alloy handle 8" long with a circular palm pad on the end. The aluminium plates had been ground to allow the blade and its square handle to slide horizontally between the two plates. A hole was bored at one end of the plates to accommodate two silicone rubber washers and the

centrifuge tube. The apparatus was firmly mounted in a horizontal position on the bench.

The blade and washers were lightly coated in a thin film of silicone lubricant prior to use. The centrifuge tubes were removed from the buckets, and the interface marked with a black pen. The centrifuge tube was carefully pushed through the washers until the mark reached the level of the blade. The blade was firmly and rapidly sliced through the tube, and the supernatant aspirated with a pipette. This procedure was repeated with the other tubes and the lipoprotein containing fractions were pooled.

3.5. LIPOPROTEIN CHARACTERISATION

The classification of lipoproteins according to their electrophoretic mobility has been described in Section 2.2.4. and the selected techniques are now described.

3.5.1. Agarose Gel Electrophoresis

The method proposed by Noble (1968) was used in this study. Noble found that better results were obtained by adding a small amount of agar to the agarose gel.

3.5.1.2. Solutions

1. 0.05 Molar Barbital buffer pH 8.6

diethyl barbituric acid 2.76 g; sodium barbiturate 15.4 g; made up to 1500 ml with distilled water.

2. Fat Red 7B dye

Fat Red 7B powder 112 mg (Sigma), dissolved in 500 ml absolute methanol, stirred overnight and matured for three days at room temperature.

3. Working dye

Slowly add 2 ml of distilled water to 10 ml of stock Fat Red dye, stirring constantly.

4. Bromophenol blue

Bromophenol blue 25 mg made up to 10 ml with 0.1 M phosphate buffer pH 7.4

5. Bovine albumin solution

Bovine albumin 2.5 g (Sigma), dissolved in 10 ml normal saline (0.9% w/v) and pH adjusted to 8.6 with 1 M Tris buffer.

Agarose, agar and lipid-free bovine albumin fraction V were all obtained from Sigma.

3.5.1.3. Method

0.2 g of agarose was added to 40 ml of 0.05 M barbital buffer and brought to a gentle boil in a lightly stoppered small flask. 0.06 g of agar was added to 10 ml of 0.05 M barbital buffer and heated with care in a flask while stirring constantly. When both these solutions were clear and colourless, they were allowed to cool in a water bath to 45-50°C. The 10 ml of agar solution was added to the agarose and stirred. To this mixture, 1 ml of bovine albumin solution was added, and stirred constantly.

The gel was carefully pipetted onto clean, warmed glass plates (10 x 10 cm), and left to cool. The lipoprotein samples were streaked onto the agarose plate one third of the way from the cathode electrode, and a spot of bromophenol blue solution applied at the same level. The plates were placed on a cooling plate at 25°C. in the electrophoresis tank. Wicks of double thickness filter paper, which had been immersed in the barbital buffer, were then laid on each end. A constant current of 25 mA was applied until the bromophenol marker had run approximately 8 cms. The apparatus was then switched off.

The Fat Red 7B dye was poured onto the plate and allowed to stand for three minutes. The plate was washed in methanol, but it did not clear sufficiently to see the bands. The plates were therefore left in the dye overnight, and

cleared with methanol. This produced bands which were irregular across the plate. The conclusion drawn was that this was due to the poor quality of the plate. Repeated attempts to obtain better quality plates were unsuccessful.

Corning agarose electrophoresis plates were therefore purchased and gave good results. These worked very well and stained easily with the Fat Red 7B in three minutes and gave clear bands.

The use of the Corning agarose plates, required a different barbital buffer, as follows:

> Barbital Buffer 0.05 M, pH 8.6
> Sodium barbital 5.83 g; barbital 1.2 g; sodium chloride 0.33 g; EDTA 0.23 g; made up to 2 litres with distilled water.

Corning plates have wells in which to place the samples and, using an LKB 2103 power supply in constant voltage mode, 90 volts DC was applied for 45 minutes. The plates were dried in a drying oven and stained with Fat Red 7B as described above.

3.5.2. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

3.5.2.1. Theory of Polyacrylamide Gel Formation

The sieving effect of the sodium dodecyl sulphate (SDS) polyacrylamide gel is the critical factor in these separations. The density of the gel network, that is the pore size, is varied according to the size of the protein sub-units being studied. Acrylamide gel is formed from acrylamide monomer which is then cross-linked by using N,N'-methylene bisacrylamide. Other cross-linking agents, for example N,N'-diallyltartardiamide or N,N'-bisacrylylcystamine, can be used which confer different properties on the gels.

The pore size, that is the density of cross-linking, can be varied in two ways:-

1. The concentration of both acrylamide monomer and N,N'-methylene bisacrylamide can be increased, which causes the pore size to decrease. This is usually expressed as %T, i.e. the total acrylamide and cross-linker in the gel. This may be expressed as:-

$$\%T = \frac{\text{g of acrylamide + g of cross-linker x 100}}{\text{100 ml of solution}}$$

2. If the amount of cross-linker is changed, the pore size also changes. This is expressed as %C, the proportion of cross-linker to the total acrylamide and cross-linker concentration in the gel. This may be expressed as:-

$$\%C = \frac{\text{g of cross-linker} \times 100}{\text{g of (acrylamide + cross-linker)}}$$

It is important to use degassed solutions as oxygen interferes with the uniform polymerisation of the acrylamide.

Polyacrylamide gels can be cast in various sizes of rods, for one-dimensional electrophoresis, or slabs, for two-dimensional electrophoresis. Discontinuous gels can be cast and are capable of separating the markedly differing high molecular weight apo-lipoprotein B and the other, much lower molecular weight, apo-lipoproteins and albumin.
The technique described by Weber and Osborn (1969) was used.

3.5.2.2. Solutions

IT IS IMPORTANT THAT THE ACRYLAMIDE MONOMER AND THE N,N'-BIS ACRYLAMIDE ARE HANDLED WITH GREAT CARE AS THEY ARE BOTH POTENT NEUROTOXINS.

1. Acrylamide solution

Acrylamide monomer 22.2 g; N,N'-bisacrylamide 0.6 g; made up to 100 ml with distilled water. Degassed and stoppered.

2. SDS/Phosphate buffer 0.2 M, pH 7.2

Sodium dodecyl sulphate 2 g, made up to 1 litre with distilled water. Degassed and stoppered.

3. Ammonium persulphate solution

Ammonium persulphate 150 mg made up to 100 ml with distilled water.

4. Bromophenol blue

Bromophenol blue 25 mg made up to 10 ml with 0.1 M phosphate buffer.

5. Coomassie blue stain

Coomassie blue R 250 (Sigma) 1.25 g;
50% ethanol in distilled water 454 ml; glacial acetic acid 46 ml.

6. TEMED

N,N,N',N'-tetramethylethylenediamine solution (Sigma)

7. Destaining solution

Glacial acetic acid 30 ml; methanol 20 ml; distilled water 350 ml.

3.5.2.3. Gel Preparation

Gel rods of 5 mm in diameter were made by using precision bore glass tubing, of this internal diameter, cut to fit the LKB electrophoresis tank. The glass was cleaned thoroughly by soaking the tubes in chromic acid, rinsed with distilled water, and finally rinsed in a 0.1% solution of Triton X-100. The rods were drained and dried in an oven.

When cooled, a double thickness of Parafilm was placed over one end of the glass rods, which were placed in a vertical rack. The verticality of the rack was checked with a bubble level.

A dual density discontinuous gel column was used with T = 7.5% at the bottom of the tube, and T = 3.75% at the top. To make up a T = 7.5% gel the following proportions were used:-

Gel buffer 20 ml

Acrylamide solution 13.5 ml

Distilled water 4.5 ml

Ammonium persulphate solution 2 ml

TEMED 0.06 ml

This solution was pipetted into the glass tubes to half fill them and, to prevent meniscus formation, distilled water was gently layered on the top using a 25 guage needle and a 1 ml syringe. The mixture started to set within 20 minutes of the addition of the ammonium persulphate catalyst.

The gels were allowed to set completely at room temperature for two hours under a fluorescent light source to provide ultra violet light, facilitating the polymerisation of the acrylamide. The second gel, T = 3.75%, was made using the following proportions:-

Gel buffer 20 ml

Acrylamide solution 6.75 ml

Distilled water 11.25 ml

Ammonium persulphate solution 2 ml

TEMED 0.06 ml

The distilled water was carefully removed from the top of the gel column by inverting the tube and draining onto filter paper. The tubes were placed in a vertical rack and

the 3.75% gel was carefully layered on to the top of the first gel. Distilled water was again layered onto the top of the second gel column to avoid the meniscus formation.

When the 3.75% gel had set after two hours at room temperature and under ultra violet light, the columns were transferred to a refrigerator at 4°C. until used.

3.5.2.4. Sample Preparation

The lipoprotein samples were dialysed overnight against a 0.1 M phosphate buffered saline pH 7.4, with 0.1 M EDTA at 4°C. 0.02 ml of 15% SDS in phosphate buffer was added to 0.2 ml of the lipoprotein samples to give a 1.5% solution, to maintain the SDS:protein proportion at approximately 3:1 by weight. 1 μL of bromophenol blue solution was added to the sample mixture.

3.5.2.5. Electrophoresis

The LKB apparatus was set up according to the manufacturer's instructions, diluting the gel buffer 1:1 with distilled water as the electrode buffer. The samples were layered onto the top of the gel column, and the power switched on. The gels were run until the bromophenol blue was approximately three-quarters of the way down the tube. The power was switched off and the tubes removed from the tank.

The fragile gels were removed from their glass tubes by gently injecting distilled water down the side of the column with a 27 gauge needle and a 1 ml syringe. The gels were laid in a dish and stained by leaving them in the Coomassie blue dye overnight. The gels were cleared by washing them in distilled water and destained with the destaining solution. A tube with a known standard mixture of proteins of known molecular weight was run on each occasion to act as a standard to measure the molecular weights of the apo-lipoproteins.

3.5.3. Immunological Characterisation

The purity of the lipoprotein samples was ascertained by using two Ouchterlony plates; one with anti-apo-lipoprotein B, and the other with anti-human albumin (Sigma).

The Ouchterlony plate was made by dissolving 1% agar (Pifco Laboratories, Detroit, Michigan, USA) in 0.85% sodium chloride containing 0.1% w/v sodium azide. The gel was melted in a hot water bath and poured into Petri dishes and allowed to cool and solidify. Wells were then cut according to the requirements.

The antigen, in this case the lipoprotein, was placed in the centre well and neat antibody, and serial dilutions of half, quarter, one-eighth and one-sixteenth strength antibody were placed in separate wells around it.

Lines of precipitation, known as lines of identity, formed when antigen/antibody complexes precipitated. This technique verified the presence of apo-protein B and the absence of albumin confirming the purity of the samples as determined by SDS-polyacrylamide gel electrophoresis.

3.6. IODINATION OF LIPOPROTEINS

This procedure was based on McFarlane's iodine monochloride method with the modifications suggested by Bilheimer et al (1972). The method described produces radio-actively labelled ^{125}I-lipoprotein with the following characteristics:

i) a specific radio-activity of 200-600 cpm per nanogram of protein;

ii) less then 5% of the radio-activity is extractable into a 2:1 chloroform:methanol mixture;

iii) greater than 95% of the radio-activity is precipitable by 10% trichloracetic acid.

3.6.1. Solutions

1. Glycine/sodium hydroxide buffer
   1 M, pH 10

2. Iodine monochloride stock solution 33 mM (see below for method)

3. Buffer A
   Sodium chloride 150 mM; EDTA 0.24 mM; adjusted to pH 7 with sodium hydroxide

4. Low density lipoprotein at protein concentration of 20-40 mg/ml in buffer A

5. Sodium iodide (^{125}I) Carrier-free 2 mCi in 0.1 ml of 0.1 M sodium hydroxide (The Radiochemical Centre, Amersham, England)

6. Sephadex G-25 Column 10 ml equilibriated with Buffer A

7. 2.64 mM working solution of iodine monochloride
   Iodine monochloride stock solution 1 ml plus sodium chloride 2 M 12.5 ml

3.6.2. Method for Preparing Iodine Monochloride Stock Solution

150 mg sodium iodide was dissolved in 8 ml of 6 M hydrochloric acid in a conical flask. 99 mg anhydrous sodium iodate was dissolved in 2 ml of distilled water. The iodate solution was forcibly injected into the iodide/HCl solution with a syringe and needle to avoid precipitation of iodine and the mixture was made up to 40 ml with distilled water. 5 ml carbon tetrachloride was added to this and shaken well, and the upper organic phase was discarded. If a faint red colour appeared in the carbon tetrachloride, indicating the presence of free iodine, the carbon tetrachloride washing was repeated. Any residual carbon tetrachloride was evaporated by passing humidified air through the solution for one hour. The final volume was made up to 45 ml with distilled water. This produced the stock solution which could be stored in the dark at 4°C. for up to two months.

3.6.3. Method for Iodinating LDL

All steps were performed at 0 - 4°C. in a fume cupboard designated for radio-active use. Surgical gloves, mask and a plastic apron were worn.

A conical tipped test tube was used for the reaction. 0.1 ml of 1 M glycine-sodium hydroxide buffer pH 10, and 3.2 mg

of LDL protein in 0.2 ml of buffer A was placed in this tube. 0.2 ml of 1 M glycine-sodium hydroxide buffer pH 10 was added to 2 mCi of sodium ^{125}I in 0.1 ml of 0.1 M sodium hydroxide.

This mixture was added to the LDL solution and Parafilm stretched over the top. Using a 1 ml syringe, 0.42 ml of iodine monochloride working solution was injected forcefully into the vial while it was being vortexed. The duration of vortexing should not exceed two seconds. The mixture was incubated in ice for five minutes and 0.28 ml of buffer A was added to the iodination mixture.

The whole mixture was applied to a Sephadex G-25 column and the eluate collected in 1 ml aliquots while 3.5 ml of buffer A was applied to the top of the column. The aliquots with maximal radio-activity were combined and dialysed against three changes of 3 litres each of buffer A until the dialysate had less than 3000 cpm/ml (usually 24 hours at 4°C.).

After dialysis, the ^{125}I-LDL solution was centrifuged for 30 minutes at 4°C. at 10,000 rpm. The supernatant was sterilised by passage through a Milipore filter and stored under nitrogen at 4°C., and used within two weeks.

CHAPTER FOUR

PLACENTAL PERFUSION AND THIN LAYER CHROMATOGRAPHY

4.1. INTRODUCTION

The perfusion of placentae of animals, either in situ or as isolated preparations, is a well estabished approach to the investigation of placental function. However, the problems associated with human placental perfusion have resulted in many human placental function studies being performed on inappropriate or damaged placental preparations. (For review, see Miller et al, 1976). This led Krantz et al (1962) and Panigel (1971) to develop methods for the in vitro perfusion of the whole, and Schneider et al (1972) of a selected lobule, of the placenta. These workers employed a variety of perfusates, ranging from outdated bank, or pooled fetal blood, to various physiological salt solutions.

Panigel (1971) made a detailed comparison of the effects on human placental ultrastructure of fetal side perfusion with Hanks and Earle's salt solutions diluted with human plasma, and dual perfusion with diluted heparinised adult human blood. This showed that the first manifestation of syncitiotrophoblast injury, and later in the other cell types, was severe swelling and rupture of the cytoplasmic membrane systems, swelling and disruption of mitochondria, intracellular oedema and loss of microvilli. This damage was much more severe in the perfusions with salt solutions than with diluted adult blood perfusates. These problems

of placental viability were highlighted by Penfold et al in 1981.

Contractor et al (1984) compared the open circuit perfusion of a placental lobule with Earle's solution, with a closed circuit perfusion of a lobule using a perfusate of fresh autologous fetal blood diluted with Earle's solution. They used light and electron microscopy to examine the placental tissue from the closed circuit perfusion. They showed that there was little change over a period of two hours, while after three hours the tissue showed some oedema and microvillous damage, but no sign of cell swelling and little mitrochondrial damage. They concluded that the viability of the perfused human placental lobule depends on the type of perfusate used, and that the use of a blood enriched perfusate is of considerable value in the maintenance of the viability of the perfusion preparation.

Eaton et al (1985) used creatinine as a measure of the efficiency and viability of the perfusion preparation by demonstrating that the transfer of inert creatinine showed a linear rise throughout the perfusion. The creatinine rise also acted as an index of the overlap of the two circulations, and the available exchange area and therefore could be used as a within-experiment control.

Two methods are commonly used for placental perfusion. The first uses an open circuit which has a large reservoir of perfusate running through both the maternal and fetal side, producing a single pass system (Schneider et al, 1972). The second uses a closed circuit on both the maternal and fetal sides of the placenta, producing a continuously circulating perfusate (Contractor et al (1984). Closed circuit perfusion is the only suitable method if autologous maternal and fetal blood are used as their limited availability results in a small volume of perfusate.

For this study, a microperfusion system, with a volume of 10 ml, was developed initially for perfusing a small area of placenta with gold-labelled ligand for electron microscopy studies. Paavola et al (1985) have recently used gold-labelled LDL to trace the intracellular metabolism of LDL. Gold-labelling of lipoproteins was not found necessary in this study, and the microperfusion system was therefore used to produce a high concentration of ^{14}C-cholesterol in a small area of placenta.

4.2. PLACENTAL PERFUSION

A perfusion cabinet (see Figure 4.2.), capable of maintaining a temperature of 37°C., was humidified and used to house the perfusion apparatus.

4.2.1. Perfusion Circuit

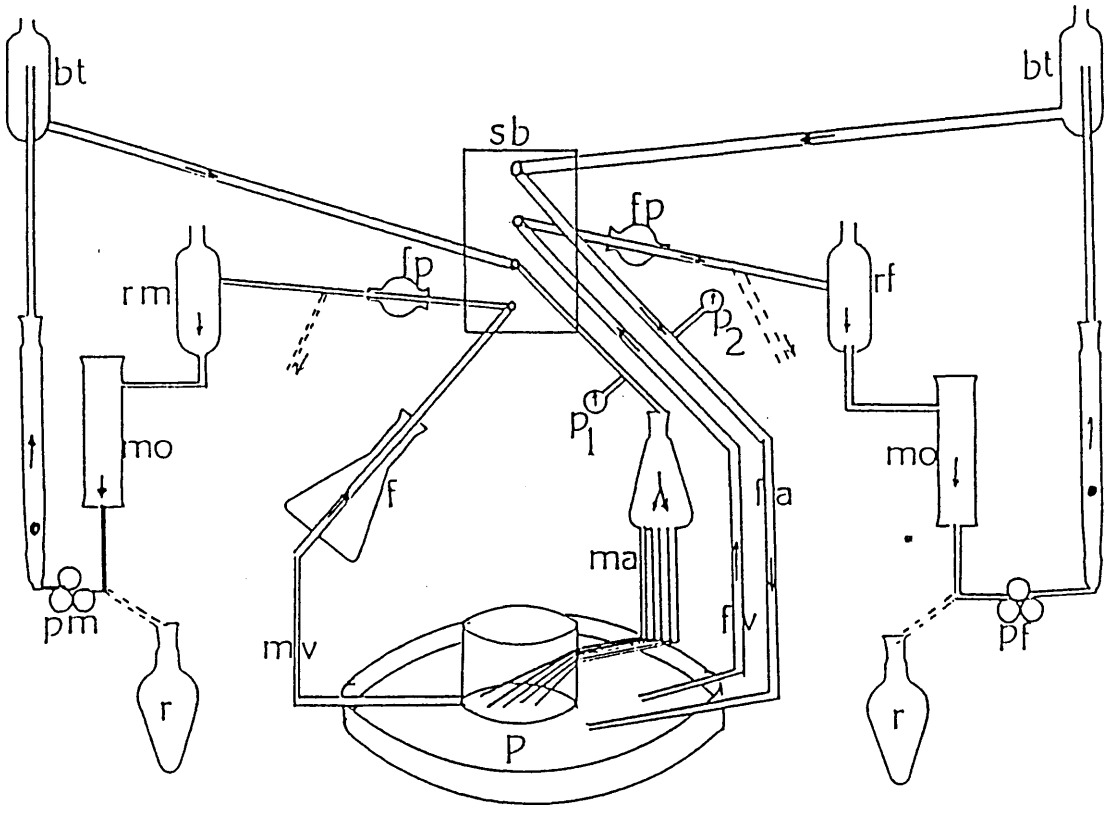


Figure 4.1. Diagram of Placental Perfusion Apparatus

The perfused placental lobule **(p)** is fixed in a perspex chamber (see text for details). The fetal perfusate is pumped by a peristaltic pump **(pf)** through a flow meter **(fm)** into a bubble trap **(bt)** and then to the sampling block **(sb)** before entering an artery on the chorionic plate **(fa)**. The perfusion pressure is monitored at this point **(p_2)**. The venous drainage **(fv)** passes to the sampling block and then to the fetal reservoir **(rf)**. An electromagnetic flow probe **(fp)** monitors the venous outflow rate. In a closed circuit system the reservoir feeds a membrane oxygenator **(mo)** which completes the circuit. In the open circuit experiments fluid is drawn from another reservoir **(r)** and runs to waste distal to the flow probe. On the maternal side a similar circuit is present. The arterial inflow **(ma)** is from a manifold feeding five cannulae which penetrate the intervillous space through the basal plate. Perfusion pressure is recorded proximal to this point **(p_1)**. The venous drainage from the chamber **(mv)** has an in-line filter **(f)**. The open circuit arrangements (broken lines) are as for the fetal side.

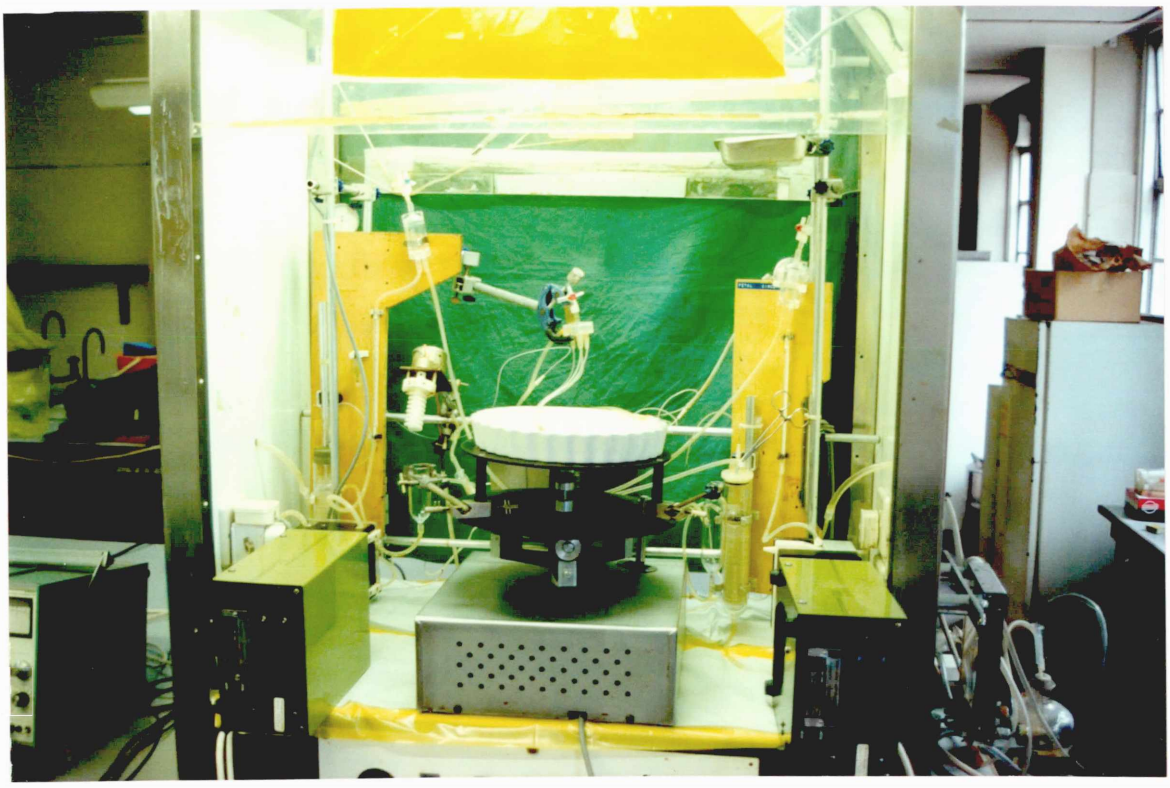


Figure 4.2. The Perfusion Cabinet

A perspex annulus was permanently covered with a silicone rubber sheet. Both the sheet and the ring had a segment removed so that following the inversion of the placenta, maternal side uppermost, the fetal cannulae were not occluded by the weight of the placenta.

The maternal and fetal circuits are essentially similar. The maternal venous fluid returns from the intervillous space (IVS) of the placenta through multiple venous openings in the decidual plate. Mixing of the fluids in the space between the placenta and the venous drainage was effected by the rocking motion of the stand. The gentle

eccentric rotational motion avoided stagnation of the perfusing fluids without displacement of the soft perfusion catheters. This venous pool then drained through a blood filter (f) (Travenol, Norfolk, UK) into a small reservoir (rm) which was constantly stirred to ensure adequate mixing. The fetal venous drainage was effected via a polythene 8Fg umbilical catheter (C.R. Bard International Ltd, Essex, UK) into a reservoir (rf).

Each line then passed into a membrane oxygenator (mo) which was an adaptation of the type used for small organ perfusion by Folkman, Cole and Zimmerman (1966). It consisted of 10 m of silicone rubber tubing (i.d. 0.25 mm, o.d. 0.5 mm) wrapped in a single layer around an inner glass core 3 cm in diameter and inserted in a close fitting outer glass sleeve. The oxygenating gas mixture passed up through the rubber tubing and perfusion fluid passed downwards in a thin layer over the outside of the tubing. Back pressure from the pumps was essential to provide surface stirring and to achieve adequate oxygenation. This oxygenator had the advantage of having a small, 10 ml, priming volume; it was also easily cleaned and the coil membrane could be replaced when necessary.

Watson-Marlowe peristaltic pumps (pm and pf) were used for the perfusion of both circulations. The perfusate passed through electromagnetic flow meters (fp) (Devices Instruments Ltd, Welwyn Garden City, UK). Tycos pressure

gauges (p_1, p_2) (Short and Mason, London, UK) were connected into the circuit by fine bore pressure tubing to monitor the perfusion pressures. Both arterial lines then passed through bubble traps (bt). The fetal arterial supply was through a 6 Fg infant feeding catheter (Warne Surgical Products, Andover, Hants, UK) the holes of which had been enlarged. The maternal supply was through five 5 Fg polythene infant feeding catheters attached to a specially made perspex manifold (ma).

The volume of perfusion fluid was 80-100 ml in the fetal circuit and 130-150 ml in the maternal circuit. Silicone rubber tubing (i.d. 3.5 mm, o.d. 5.5 mm), silicone rubber sheet and oxygenator tubing were obtained from Adpol Ltd, Sussex, UK.

4.2.2. Perfusion Fluids

The perfusion fluid consisted of TC199 tissue culture medium with 20 ml/L Dextran 40 solution (50 g Dextran of av. mol. wt. 40, sodium chloride 4.5 g, water for injection to 500 ml) and 5000 IU/L of heparin. The fetal blood drained from the umbilical cord and from the perfused portion of the placenta was added to the perfusate to produce an haematocrit of 10% - 20%. The maternal perfusate contained maternal blood drained from the placenta and the kidney dish, in which the placenta had been placed after delivery. This was filtered prior to

addition to the circuit. If there was inadequate maternal blood to perfuse the maternal circulation, fetal red cells were added to the maternal circuit. By using autologous red cells, it was hoped to avoid any possible antigen/antibody reactions and avoided the necessity of using cross-matched adult blood. The other advantage of using fetal red cells is that they have a greater affinity for oxygen than adult haemoglobin, thereby compensating for the low haematocrit.

4.2.3. Method

The circuit was primed with perfusion fluid, ensuring that all air bubbles were caught by bubble traps and expelled. The pumps were then set to run slowly to ensure that the perfusate was oxygenated. The arterial lines were placed in the corresponding reservoirs to form a closed circuit.

A freshly delivered human placenta was immediately transferred to the laboratory in the labour ward. The maternal surface was examined, and an intact lobule was selected. If the maternal surface was badly lacerated or the fetal vessels torn, the placenta was discarded.

The placenta was placed maternal-side down in a ceramic flan dish as this was found to be the correct size for the majority of placentae. The dish was placed within the perfusion cabinet on the rocking table. The fetal venous

drainage cannula was inserted into the umbilical vein and fetal blood was drained into the fetal circuit reservoir (Figure 4.3.). The fetal arterial line was inserted closer to the perfused lobule by stripping the amnion from the chorial plate (Figure 4.4.), and making a small arteriotomy in the fetal surface of the arteriole. A catheter was inserted and tied into position with a suture passed beneath the arteriole (Figure 4.5.). The proximal end of the arteriole was occluded by ligation, and this tie was further used to secure the fetal arterial catheter onto the chorial plate in line with the arteriole (Figures 4.6 and 4.7.). The fetal venous cannula was pushed into the corresponding vein and secured in position by transfixion of the cord and double ligation (Figure 4.8.). Thus the fetal circulation of the selected lobule was established.

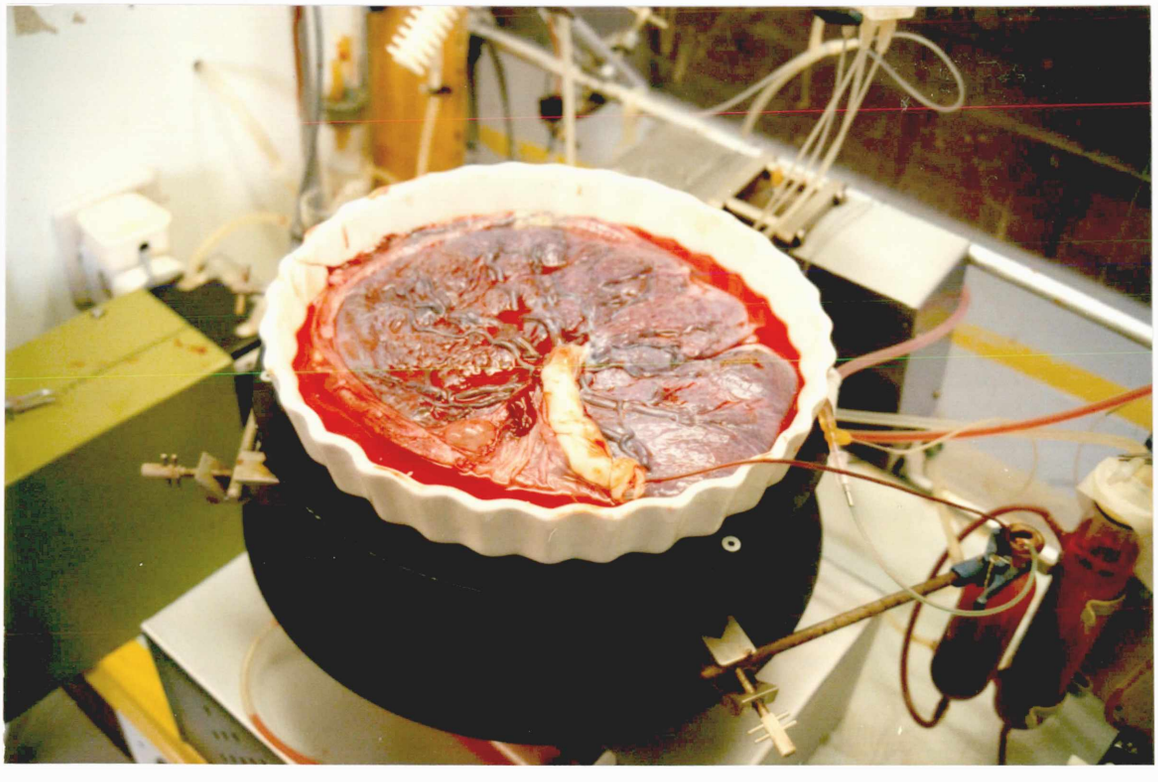


Figure 4.3. Fetal Venous Drainage

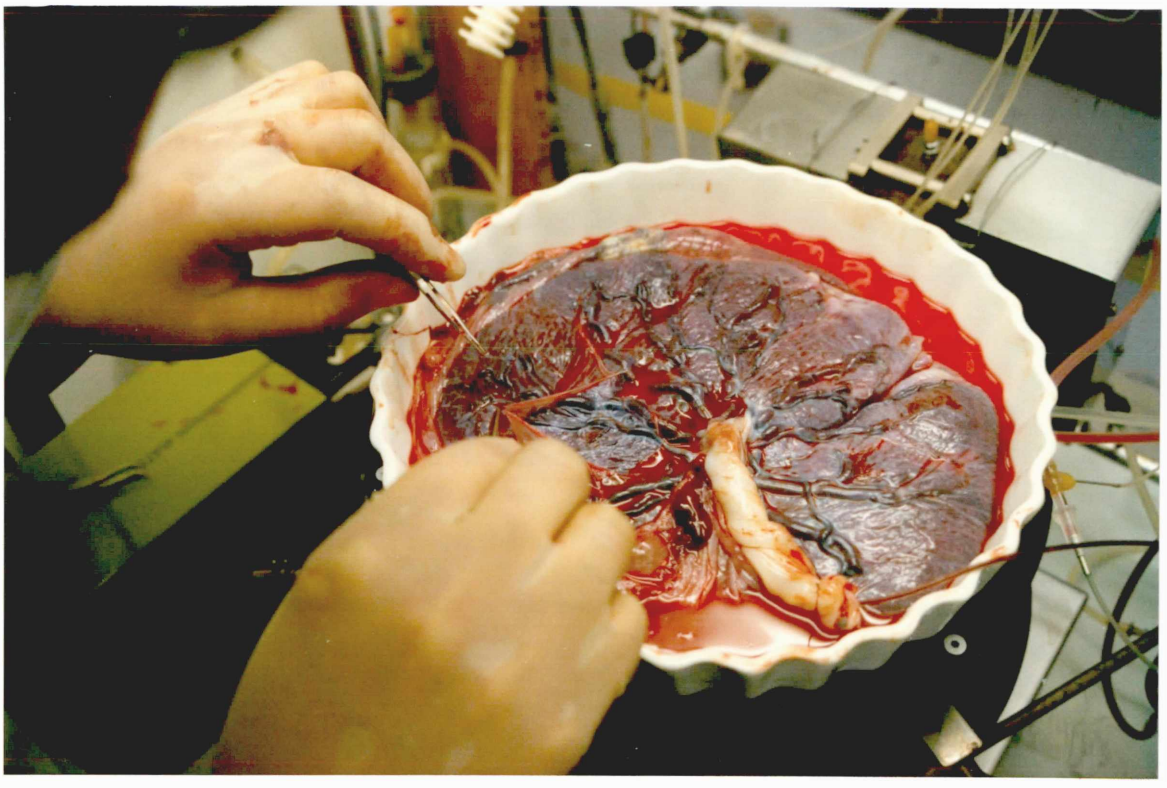


Figure 4.4. Stripping the Chorion

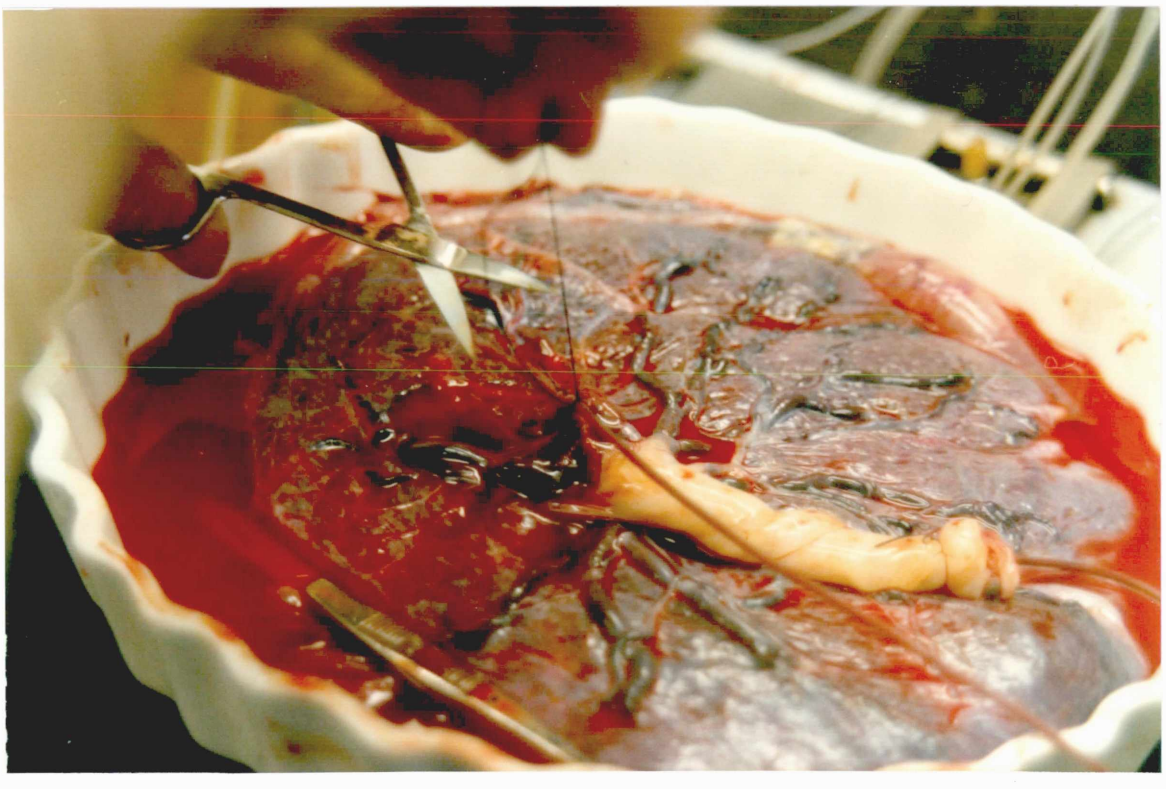


Figure 4.5. Suturing the Catheter into the Arteriole

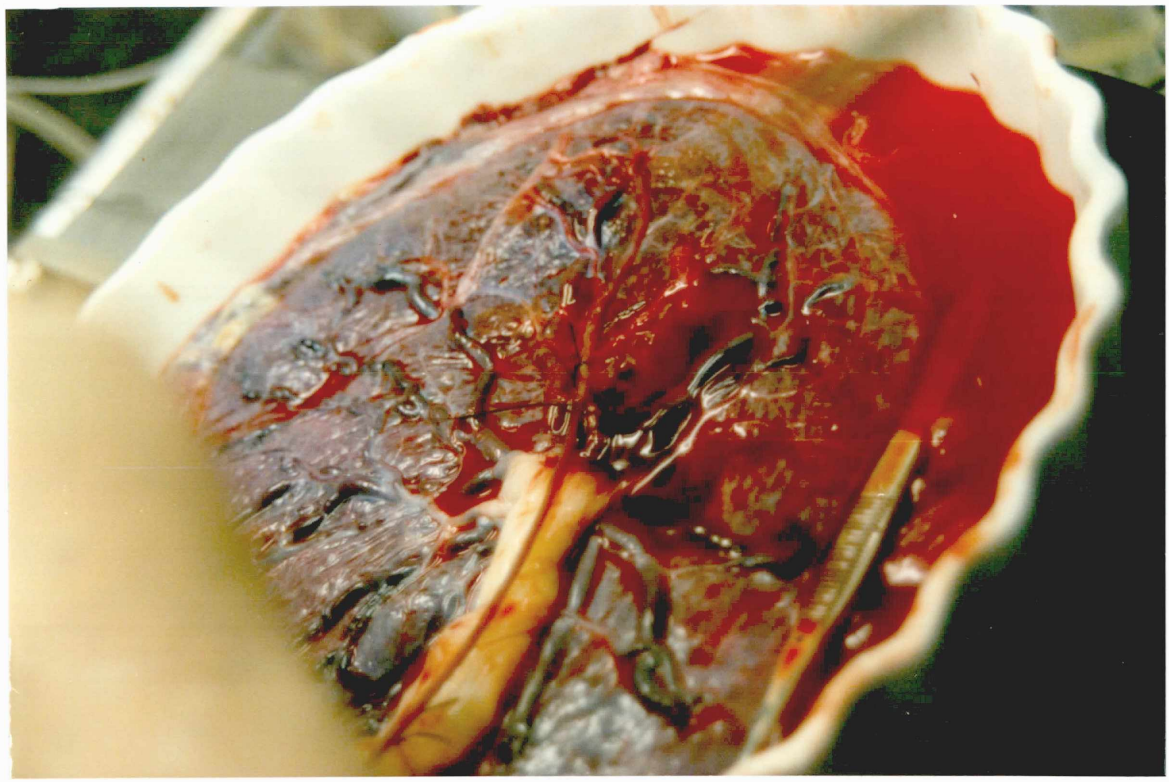


Figure 4.6. **Suturing the Proximal Arteriole**

Figure 4.7. **Suturing the Cannula in line with the Arteriole**

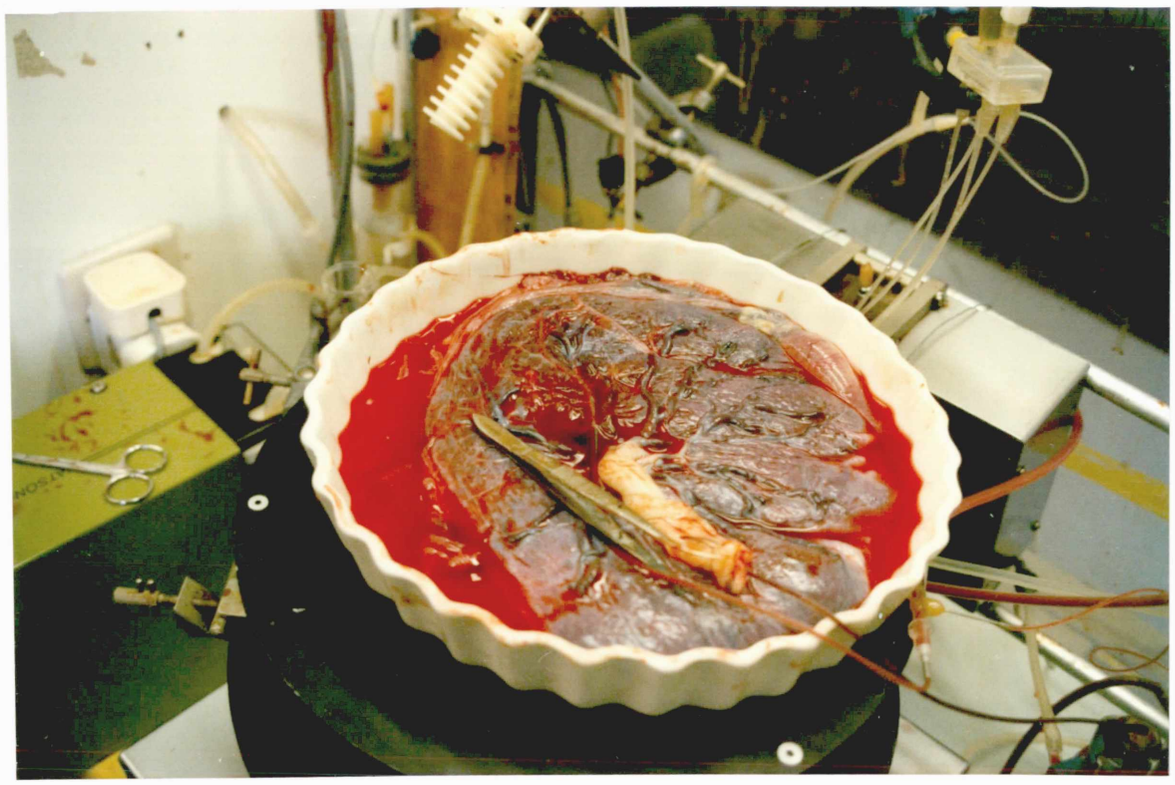


Figure 4.8. Inserting the Venous Cannula to Drain the Chosen Fetal Site

The area to be perfused on the maternal side was identified by the slight initial blanching due to the fetal perfusion. The intervillous space was cannulated by inserting five polyvinyl catheters through the basal plate (Figure 4.9.).

The perfused lobule was isolated from the rest of the placenta by placing an upper circular chamber onto the placental surface. The chamber was secured by passing long screws through the placental tissue, and screwing the top and base plates together (Figure 4.10.). The maternal arterial cannulae passed through the wall of the chamber and the venous effluent drained by gravity through two

openings close to the placental surface. Venous drainage was assisted by tilting the preparation towards the drainage holes, and by the rocking movement of the perfusion table.

When the dual perfusion had been established, the fetal oxygenator was switched off to mimic as closely as possible the physiological situation of the fetal circulation acquiring its oxygen from the maternal side. The fetal circulation was established within 5-10 minutes of delivery of the placenta, thereby avoiding undue hypoxia.

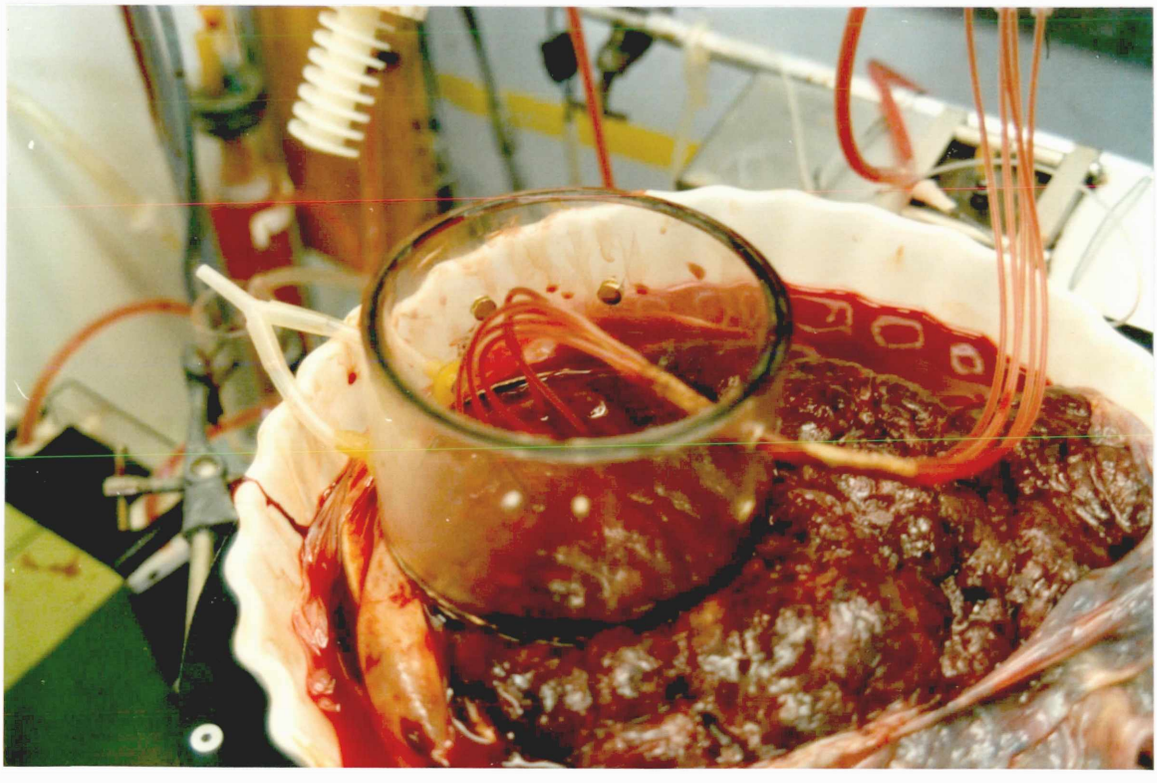


Figure 4.9. Inserting Maternal Cannualae through the Basal Plate

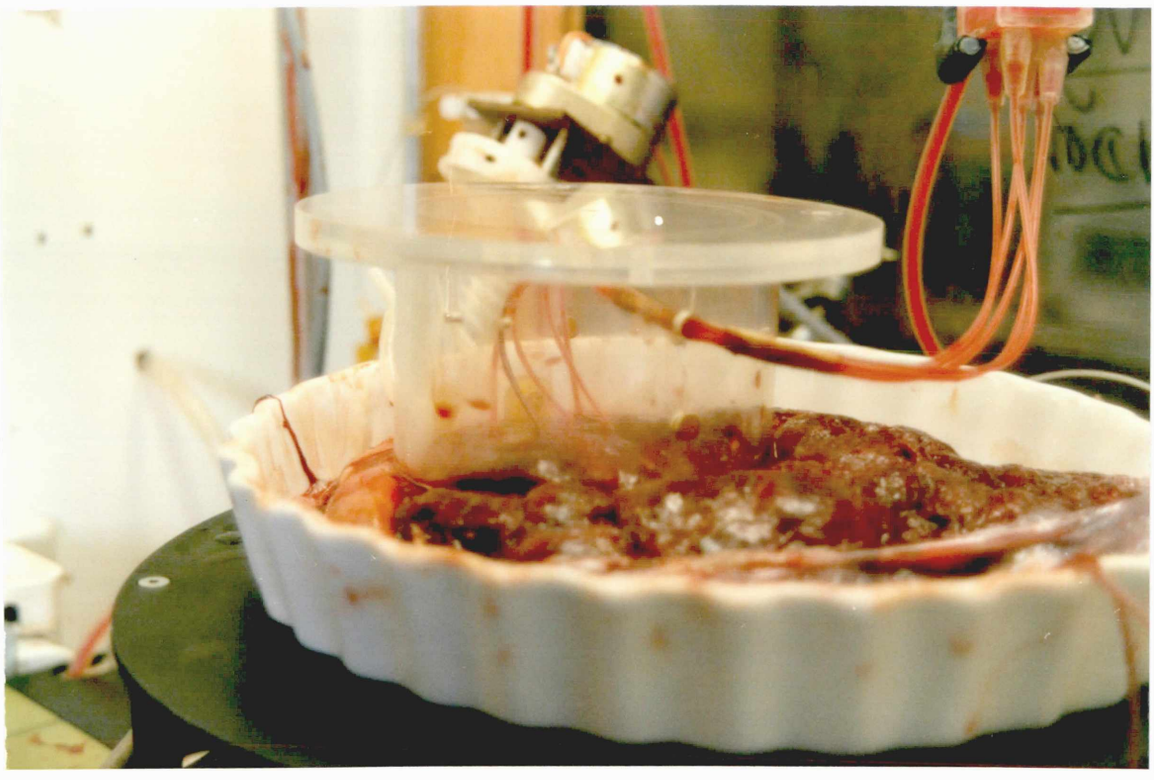


Figure 4.10. Isolating the Placental Lobule

The perfusion rate was 5-7 ml/minute with a perfusion pressure of 40-90 mm Hg on the fetal side, and 20-24 ml/minute, with a perfusion pressure of 20-40 mm Hg, on the maternal side. The pH and perfusate gasses were monitored by taking regular samples, and analysed with a Corning blood gas analyser. The pH was adjusted if necessary by the addition of sodium bicarbonate, and the blood gases maintained at their correct level by adjusting the amount of carbon dioxide and oxygen supplied to the maternal oxygenator. The status of the perfusion was monitored by electro-magnetic flow meters, pressure gauges and the stability of the perfusion volumes. If the perfusion

pressure rose, or the perfusate volume on the fetal side decreased, the experiment was terminated.

To ensure that no damage to the placenta occurred, and that the perfusion overlap of the fetal and maternal circuits was sufficient, 30 mg of creatinine dissolved in a small volume of TC199 solution was added to the maternal circuit as an inert diffusion marker (Eaton et al 1985). This internal control ensured that only experiments with transfers within defined limits were included.

Once the perfusion had been satisfactorily established, a 2 ml background sample was removed from both the maternal and fetal reservoirs. The labelled lipoprotein was added to the maternal distribution head at time zero, and 2 ml samples were removed from the maternal reservoir at 30 minute intervals and the fetal reservoir at 15 minute intervals for the two hour perfusion period. The perfusion volumes were maintained by replacing the sample removed with an equal volume of stock perfusate.

1 ml of the samples was counted and 0.5 ml was added to 1 ml of perchloric acid (PCA), left overnight at 4°C., centrifuged and the supernatant removed. Both the supernatant and resuspended pellet were counted to give the PCA precipitable activity. The remaining 0.5 ml was centrifuged to sediment the erythrocytes, and the

supernatants removed and stored briefly at 4°C., or frozen at -20°C., until assayed for creatinine.

4.2.4. Creatinine Estimation

Creatinine concentrations were determined using a commercial assay kit (Sigma) on a Zeiss PMQ II spectrophotometer at a wavelength of 500 nm. This procedure did not require an initial deproteinisation step, and corrected for any non-creatinine chromogen in the sample. The creatinine concentrations obtained during the experiments were corrected for the endogenous creatinine concentrations determined from the background samples. Values were then converted to give the amount of creatinine transferred and expressed as percentages of the added dose of creatinine.

4.3. MICRO-PERFUSION TECHNIQUE

A micro-perfusion technique was devised by the worker in order to achieve a high density of ligand in a small area of placenta. This was only feasible on the maternal side as the fetal vasculature could not be cannulated to drain and supply this small area. Thus the technique was only suitable for demonstrating placental acquisition of substrate from the maternal circulation and its intracellular distribution (e.g. by gold-labelling and

electron microscopy). It was not suitable for materno-fetal transfer studies.

A perfusion was established as described in Section 4.2. with the addition of an extra, smaller, occlusive column (a 30 ml syringe cut to different column heights) on the maternal side. One 5 Fg infant feeding catheter was inserted through the basal plate within the column and a second 6 Fg infant feeding catheter, with extra holes, was curled round the inside of the column and lay on the placental tissue to provide the venous drainage. An extension to the fetal peristaltic pump provided the circulation, although it was necessary to insert a 30 cm vertical tube to moderate the pressure fluctuations and act as a bubble trap.

A micro-oxygenator was made by wrapping the oxygenator tubing round a steel rod and placing this within the circuit reservoir. The micro-circuit volume was 15-20 ml.

4.4. ^{125}I-LIPOPROTEIN PERFUSIONS

4.4.1. Introduction

The placental perfusion system was used to determine whether ^{125}I-labelled lipoproteins were acquired by the placenta used in the in vivo model.

4.4.2. Protocol

Perfusion fluids were modified according to the experiment performed. To avoid any competitive inhibition of radio-labelled lipoprotein uptake, the perfusion fluid on the maternal side was composed of washed erythrocytes suspended in an iso-osmotic solution of Earle's medium, sodium bicarbonate, heparin 2,500 IU/L, Dextran 8 g/L (17,900 mol. wt.) and AB rhesus negative lipoprotein deficient plasma (see section 4.5.2.). The amount of heparin used should not have affected the lipoprotein binding to the receptors.

Perfusions were performed in <u>triplicate</u> with the following ^{125}I-labelled lipoproteins:

i) ^{125}I-VLDL;
ii) ^{125}I-IDL;
iii) ^{125}I-LDL;
iv) ^{125}I-HDL.

In order to exclude the possibility of any interference in lipoprotein binding by Dextran, two further perfusions with ^{125}I-LDL were performed using an iso-osmotic solution of Gelofusin instead of Dextran, with sodium bicarbonate, washed maternal red cells and Earle's solution. Chloroquine, an acidotrophic agent which has been shown to block the receptor-mediated endocytosis of transferrin in this perfusion system (Contractor and Eaton, 1986) was

added to the maternal circuit in separate perfusions to ascertain if the mechanism of lipoprotein transfer was via receptor-mediated endocytosis.

4.5. ^{14}C-CHOLESTEROL PERFUSION

4.5.1. Introduction

^{14}C-cholesterol was incorporated into LDL and VLDL as described in Section 4.5.2. To determine whether ^{14}C-cholesterol LDL was taken up by the placenta, and the ^{14}C-cholesterol converted to steroids, three perfusions were performed in three different ways. It was intended to wash the perfusion with a solution containing heparin at a concentration sufficiently high to cause dissociation of the endogenous LDL from their receptors, and maximise the absorption of ^{14}C-cholesterol LDL.

The steroids were extracted from the perfusates and placentae, and separated by thin-layer chromatography on silica gel plates (see Section 4.6.).

4.5.2. Incorporation of ^{14}C-Cholesterol Into Lipoproteins

The method described by Roberts et al (1985) was used. 250 ml of blood was taken from a normal human volunteer as described in Section 3.2. and the LDL was obtained from the

plasma by rate zonal ultracentrifugation. The LDL sample was filtered through a 22 micrometer filter into sterile siliconised containers and the air above the sample displaced with nitrogen before stoppering. The protein content of an aliquot was estimated using Lowry's technique (see Appendix).

Lipoprotein deficient serum was prepared by centrifuging 10 ml of plasma raised to a density of 1.215 g/ml with solid sodium bromide and centrifuged at 29,000 rpm at 15 °C. for 36 hours. The lipoproteins were removed by tube slicing and the serum remnant was deemed lipoprotein free.

The lipoprotein samples and the lipoprotein deficient serum were dialysed against degassed phosphate buffered saline with 1 mM EDTA at pH 7.4 for 48 hours. Nitrogen was blown into the flasks prior to sealing them.

A sample of ^{14}C-cholesterol was obtained from Amersham Laboratories which contained 50 μCi of ^{14}C-cholesterol dissolved in toluene. The toluene was evaporated from 0.352 mg of ^{14}C-cholesterol with a gentle, filtered stream of nitrogen. As soon as the solid formed, it was redissolved in 0.3 ml of acetone. The ^{14}C-cholesterol in acetone was added drop by drop to the lipoprotein deficient serum while being continuously and gently stirred in a sterile container. 3 mg of LDL protein was added to the lipoprotein deficient serum, and the sealed tube was placed

on a rocker and incubated for 30 minutes at 4°C. This serum was raised to a density of 1.063 with solid sodium bromide and centrifuged at 28,000 rpm at 4°C. for 20 hours.

The lipoprotein was removed by tube slicing and placed in sterile siliconised containers under nitrogen. The infranate was stored in the same way. 100 μL aliquots of both the infranate and the lipoprotein were counted to determine the percentage of ^{14}C-cholesterol incorporation into the lipoprotein. This technique was also used for incorporating cholesterol into VLDL.

4.5.3. Protocol

a) The ^{14}C-cholesterol LDL was divided into three aliquots of 0.3 ml each.

b) One aliquot was used in a micro-circuit perfusion with 3 ml of lipoprotein deficient serum (LPDS).

c) One aliquot was used in a micro-circuit perfusion with no LPDS.

d) The remaining aliquot was used to perfuse a whole placental lobule (see Section 4.2.3.).

e) Another whole placental lobule was also perfused with ^{14}C-cholesterol, not incorporated into LDL, plus LPDS.

4.5.3.1. Protocol of Micro-perfusion Technique

a) The fetal side perfusion was established.

b) The micro-circuit perfusion on the maternal side was established after a normal maternal-side perfusion had been set up.

c) The maternal side was washed for 5 minutes with 5 mg/ml heparin in 8 g/L Dextran Earle's solution.

d) The maternal side was washed for a further 25 minutes with 8 g/L Dextran Earle's solution on open circuit to remove the heparin.

e) Heparin levels in the wash solution were measured to ensure that all the heparin was washed out.

f) A closed circuit perfusion was established.

g) Base line samples were taken.

h) 0.3 ml of ^{14}C-cholesterol LDL (plus 3 ml LPDS in appropriate perfusion) was added to the micro-circuit.

i) The lobule was perfused for one hour.

j) All perfusates, micro-, fetal and maternal, were collected, divided into two, centrifuged and separated. All samples were frozen at -20°C.

k) All remaining maternal blood was squeezed out of the placental micro-perfusion area.

The lobule was exised and washed thoroughly in phosphate buffered saline at 0°C., blotted and frozen at -20°C.

4.5.4. Test Devised for Heparin Estimation

To determine whether the heparin used to remove LDL from the placental receptors had been washed out of the placental perfusion, a rapid method of estimating heparin levels was devised. The test was based on the standard thrombin time test.

4.5.4.1. Solutions

1. 8 g/L Dextran Earle's solution (normal perfusate)

2. Normal plasma
   10 ml of blood from a normal volunteer was collected into 1 ml of 0.11 M sodium citrate. This was centrifuged and the plasma separated. 0.5 ml aliquots of plasma were frozen at -20°C.

3. Thrombin stock

   Distilled water 1 ml and normal saline 3 ml, were added to a vial of thrombin and gently mixed, to give a concentration of 50 u/ml. This was divided into 0.5 ml aliquots and frozen at -20°C.

4. Calcium chloride 0.025 M

5. Working thrombin 5 u/ml

   Thrombin stock 0.5 ml

   Calcium chloride 5 ml

6. Working thrombin 7 u/ml

   Thrombin stock 0.5 ml

   Calcium chloride 3.5 ml

7. Working thrombin 10 u/ml

   Thrombin stock 0.5 ml

   Calcium chloride 2.5 ml

4.5.4.2. Method

100 µL of 8 g/L Dextran Earle's solution was added to 100 µL of freshly thawed plasma and warmed to 37°C. in a water bath. 100 µL of 5 u/ml working thrombin was added and a stopwatch started. The clock was stopped when a clot formed. Differing concentrations of working thrombin were

required depending on the concentration of heparin in the perfusate. A graph of heparin concentation against time was established by experimentation from which the heparin concentration in the perfusate could be calculated.

4.5.4.3. Conclusion

Using the heparin assay, the concentration of heparin required to remove lipoprotein from the receptors, could not be removed by washing. Even after a 90 minute wash heparin was detectable at 12,000 IU/L, and this aspect of the experiment was abandoned. The experiments outlined in Section 4.5.3. were therefore performed without the heparin wash.

4.6. EXTRACTION OF PROGESTERONE AND PRECURSORS FROM PLACENTA AND PERFUSATE BY THIN-LAYER CHROMATOGRAPHY

4.6.1. Introduction

The human placenta at term produces approximately 250 mg of progesterone per day (Contractor and Pearlman 1960). The ^{14}C-cholesterol perfusions were designed to determine which of the mechanisms of cholesterol acquisition were used by the human placenta. To determine whether the ^{14}C-cholesterol had been metabolised the steroids, pregnenolone and progesterone, were separated and counted to identify the ^{14}C incorporation.

The placental tissue and perfusates were extracted with a mixture of chloro form and ethanol, concentrated by evaporation, and the steroids separated by thin-layer chromatography on silica gel plates. The steroid bands were removed from the silica plates and counted.

4.6.2. Solutions

1. Extraction solution

Chloroform	200.0 ml
Ethanol (new)	200.0 ml

2. Solvent

Chloroform	300.0 ml
Methanol	13.5 ml
Water	1.125 ml

3. Silica gel

Silica gel G60	25.0 g
Distilled water	65.0 ml

4. Controls

Cholesterol	1.0 mg
Progesterone	1.0 mg
Pregnenolone	1.0 mg
Methanol	1.0 ml

5. 1% PPO/0.05% POPOP Toluene

2,5-diphenyloxazole 20.0 g

1,4-bis-2-(-phenyloxazolyl)benzene

1.0 g, made up to 2 litres with Toluene

6. **Vanillin reagent**

Vanillin 1 g

Aqueous orthophosphoric acid 50% w/v 100 ml

4.6.3. Equipment

Vacuum pump capable of being connected to a rotating flask connected to a double Leibig condenser

Silica gel plate spreader

Screw driven sample streaker

Homogeniser

4.6.4. Extraction of Tissue and Perfusate

The placental tissue was weighed and 150 ml of extraction solution was added, and the tissue homogenised. The homogenate was filtered with a No. 41 Whatman filter paper with light vacuum filtration. The tissue and filter paper were rehomogenised and extracted with a further 100 ml extraction fluid. This process was repeated once more.

The extracts were combined and concentrated by warming gently to 40°C. in a water bath while rotating and evacuating. The residuum, consisting of a waxy deposit, was dissolved with a minimum of chloroform, usually 1.5-2.0 ml, and 100 μL counted.

The perfusate was extracted by shaking perfusate and extraction solution together, separating and repeating the process twice more. The extracts were combined and concentrated as above.

4.6.5. Preparation of Silica Gel Plates

Four 20 x 20 cm glass plates were cleaned in chromic acid, thoroughly rinsed in distilled water, and dried in an oven. The plates were placed on the table of the plate spreader and the table was levelled with a bubble level. The silica gel solution was freshly prepared and poured into the bucket of the plate spreader. The bucket was then moved in a steady, controlled manner over the plates to spread the gel. The plates were allowed to dry and then placed in an oven at 105°C. for 30 minutes to activate the gel.

4.6.6. Chromatography

A glass chromatography tank was lined with filter paper and was equilibriated with 100 ml of solvent 4-6 hours at room

temperature. The lid was lightly greased with silicone grease to ensure an airtight seal.

0.5 ml of concentrated chloroform extract was aspirated into a sample applicator syringe and the sample was streaked approximately 2 cm from the edge along one end of the plate. 200 μL of the standard solution of steroids was applied to one side of the plate to provide markers. The plate was placed in a vertical rack in the chromatography tank and allowed to run until the solvent had reached a line drawn 10 cm from the origin. The plate was removed and dried.

The plate was lightly sprayed with vanillin solution in a fume cupboard, and developed by placing it in an oven at 120°C. until the lines were clearly defined.

4.6.7. Counting Chromatography Plates

The standard markers indicated the position of the three steroids, cholesterol, progesterone and pregnenolone. A fine line was drawn across the plate at the level to be counted. A cotton wool plug, to act as a filter, was lightly inserted into the end of a glass pipette which had been drawn to give a slightly flattened tip approximately 1.0 mm across. A gentle vacuum was applied to the end of the pipette, which was pushed gently across the silica gel plate, thereby aspirating the silica gel into the pipette.

When all the silica had been collected, the tip of the pipette was placed in a counting vial, and the vacuum switched off. The silica gel was gently tapped out by rubbing the slightly serrated finger grips of Mayo dissecting forceps across the pipette.

The progesterone, pregnenolone and cholesterol bands were collected in this way. A blank was also taken from the top of the plate. To each vial was added 1 ml methanol and 0.9 ml Soluene followed by the scintillation solution, and counted in a Hewlett Packard 4000 Series Counter using internal quench standards.

CHAPTER FIVE

RESULTS OF PLACENTAL PERFUSIONS

5.1. RESULTS OF PLACENTAL LOBULE PERFUSIONS

5.1.1. Introduction

Three VLDL, three IDL, four LDL and three HDL placental lobule perfusions were performed as described in Sections 4.2. and 4.4. using washed autologous red cells and an iso-osmotic TC199 perfusate. The lipoproteins were iodinated as described in Section 3.6. The VLDL perfusions have been described fully to illustrate the method used.

5.2. RESULTS OF VLDL PERFUSIONS

5.2.1. Preparation of Mix Standards

To assess the activity of the added ^{125}I-VLDL solution, 100 μL was added to 2 ml of TC199 solution and four 100 μL aliquots (Mix) were counted to give a mean total count. The mean total count and the volume of the Mix added to the perfusate allowed calculation of the total dose of ^{125}I-lipoprotein which was added to the maternal perfusate. Four 100 μL aliquots of the ^{125}I-VLDL Mix were each added to 0.4 ml 1% bovine serum albumin solution and 1 ml of 15% TCA was added to precipitate all the protein. The tubes were centrifuged and the supernatant and precipitate separated, counted and expressed as a percentage of the total count. This indicated the amount of ^{125}I bound to macromolecules as the free iodine and low molecular weight breakdown products would not be precipitated.

Mix Standards	% precipitable activity (expressed as % total counts)			
VLDL 1	90.6	91.0	90.7	91.1
VLDL 2	89.7	89.7	89.6	89.3
VLDL 3	89.1	89.1	89.4	89.5

Mean 89.7 ± 1.3 (SD, n = 12)

Table 5.1. ^{125}I-VLDL Mix Standards

5.2.2. Maternal Side Perfusate Results

1 ml aliquots of the maternal perfusate were removed at 30, 60, 90 and 120 minutes. The volume removed was replaced each time with Stock Perfusate and the residual counts were corrected for the counts removed.

% dose remaining in maternal circuit

Time in Minutes	30	60	90	120
VLDL 1	89.9	89.6	85.0	83.3
VLDL 2	87.0	85.0	81.6	79.6
VLDL 3	90.9	84.2	66.0	65.0
Mean	89.3	86.3	77.0	76.0
± SD	2.0	2.9	10.7	9.7
± SE	1.2	1.7	6.2	5.6
n	3	3	3	3

Table 5.2. Percentage ^{125}I-VLDL Remaining in Maternal Circuit

To determine the stability of the ^{125}I-VLDL in the maternal circuit 0.5 ml of perfusate was added to 1 ml of 15% TCA in the same way as in the Mix Standards (see Section 5.1.2.).

% precipitable activity

(expressed as a % total counts)

Time in minutes	**30**	**60**	**90**	**120**
VLDL 1	85.0	86.6	87.4	87.4
VLDL 2	87.9	87.7	87.6	87.5
VLDL 3	85.5	85.3	85.7	86.6
Mean	86.1	86.5	86.9	87.2
± SD	1.6	1.2	1.0	0.5
± SE	0.9	0.7	0.6	0.3
n	3	3	3	3

Table 5.3. **Percentage of Precipitable Activity in the Maternal Circuit**

5.2.3. Fetal Side Perfusate Results

Samples of the fetal side perfusate were taken every 15 minutes, and the volume and count adjusted as described in Section 5.1.3. The samples were treated in the same way as the maternal perfusate to give the percentage of ^{125}I transferred.

% dose transferred (total activity)

Time in Minutes	15	30	45	60	75	90	105	120
VLDL 1	0.4	1.5	2.0	2.3	2.8	3.2	3.3	3.7
VLDL 2	0.1	0.3	0.6	0.7	1.1	1.2	1.3	1.6
VLDL 3	0.2	0.6	0.9	1.1	1.3	1.5	1.8	1.9
Mean	0.2	0.8	1.2	1.4	1.7	2.0	2.1	2.4
± SD	0.2	0.6	0.7	0.8	0.9	1.1	1.0	1.1
± SE	0.1	0.4	0.4	0.5	0.5	0.6	0.6	0.7
n	3	3	3	3	3	3	3	3

Table 5.4. Percentage of ^{125}I Transferred to the Fetal Perfusate

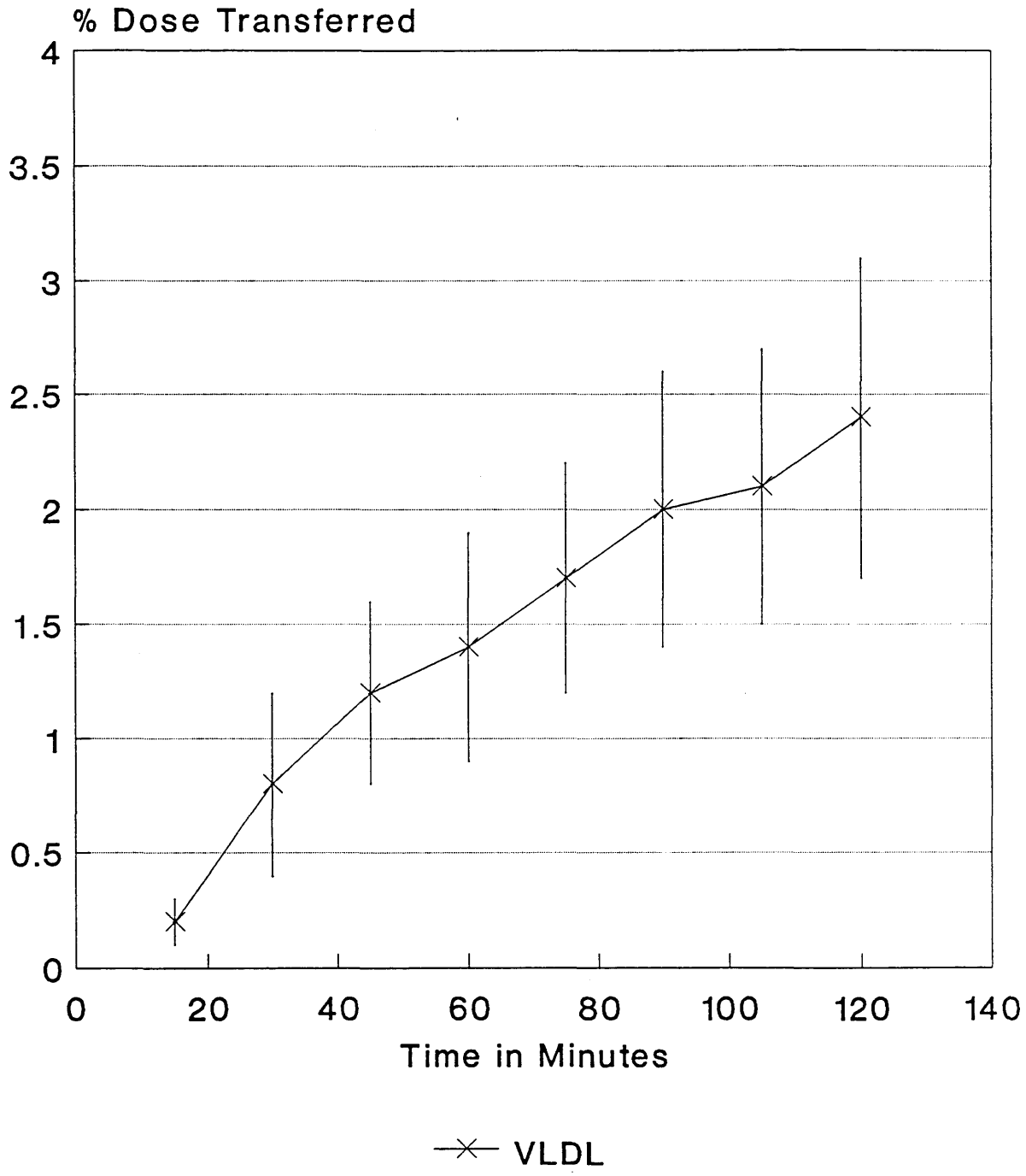


Figure 5.1. VLDL Transfer to Fetal Circulation

TCA was added to 0.5 ml aliquots of the fetal perfusate and centrifuged. The percentage of precipitable activity indicates the quantity of ^{125}I attached to macromolecules, presumably VLDL.

% precipitable activity

(expressed as % total)

Time in Minutes	**15**	**30**	**45**	**60**	**75**	**90**	**105**	**120**
VLDL 1	55.3	55.8	55.8	50.9	55.6	52.2	50.1	49.1
VLDL 2	28.8	26.8	27.1	24.9	26.0	24.5	25.9	26.7
VLDL 3	58.8	52.8	48.3	47.6	45.5	46.3	44.7	38.7
Mean	47.6	45.1	43.7	41.1	42.4	41.0	40.2	38.2
± SD	16.4	15.9	14.9	14.2	15.0	14.6	12.7	11.2
± SE	9.5	9.2	8.6	8.2	8.7	8.4	7.3	6.5
n	3	3	3	3	3	3	3	3

Table 5.5. Percentage of Precipitable Activity in the Fetal Perfusate

5.2.4. Placental Lobule Results

The perfused placental lobule was excised, squeezed to remove perfusate, blotted thoroughly, homogenised with phosphate buffered saline and 100 μL aliquots counted. The results are expressed as the percentage of the added dose which remains within the tissue. The TCA/PTA precipitable activity indicates the amount of ^{125}I bound to macromolecules.

VLDL 1	Tissue contained 9.0% of dose
VLDL 2	Tissue contained 7.1% of dose
VLDL 3	Tissue contained 4.2% of dose
Mean	**6.8 ± 2.4% (SD, n = 3)**

Table 5.6. Percentage of ^{125}I Dose Remaining Within the Placental Lobule

VLDL 1	68.1, 68.2, 68.4, 67.9
VLDL 2	77.1, 73.4, 74.9, 73.2
VLDL 3	45.9, 48.3, 49.1, 44.9, 53.5
	Mean 62.5 ± 12.1% (SD, n = 13)

Table 5.7. Percentage of Total TCA/PTA Precipitable Activity in the Placental Lobule Homogenate

5.3. RESULTS OF IDL PERFUSIONS

5.3.1. ^{125}I-IDL Mix Standards

Mix standards	% precipitable activity (expressed as % total)
IDL 1	88.8, 89.1, 89.2, 89.0
IDL 2	96.2, 96.2, 96.3, 96.3
IDL 3	95.7, 95.9, 95.8, 95.8
	Mean 93.7 ± 3.5% (SD, n = 12)

Table 5.8. ^{125}I-IDL Mix Standards

5.3.2. Maternal Side Perfusate Results

	% dose remaining in maternal circuit			
Time in Minutes	**30**	**60**	**90**	**120**
IDL 1	76.34	74.5	73.3	69.6
IDL 2	99.6	94.1	83.8	81.9
IDL 3	111.6	110.1	73.0	89.4
Mean	95.8	92.9	76.9	80.3
± SD	17.9	17.8	6.0	10.0
± SE	10.4	10.3	3.5	5.8
n	3	3	3	3

Table 5.9. Percentage of ^{125}I-IDL Remaining in Maternal Circuit

% precipitable activity

(expressed as % total counts)

Time in Minutes	**30**	**60**	**90**	**120**
IDL 1	91.3	91.3	91.3	91.3
IDL 2	93.8	93.8	94.1	94.0
IDL 3	91.7	91.7	92.7	91.6
Mean	92.3	92.3	92.7	92.3
± SD	1.3	1.3	1.4	1.5
± SE	0.8	0.8	0.8	0.9
n	3	3	3	3

Table 5.10. Percentage of Precipitable Activity in the Maternal Circuit

5.3.3. Fetal Side Perfusate Results

% dose transferred (total activity)

Time in Minutes	15	30	45	60	75	90	105	120
IDL 1	0.10	0.27	0.35	0.51	0.56	0.69	0.81	0.91
IDL 2	0.01	0.07	0.14	0.24	0.34	0.45	0.57	0.67
IDL 3	0.12	0.37	0.78	0.68	1.09	1.17	1.47	1.82
Mean	0.08	0.24	0.42	0.48	0.66	0.77	0.95	1.13
± SD	0.06	0.15	0.33	0.22	0.39	0.37	0.47	0.61
± SE	0.03	0.09	0.19	0.13	0.22	0.21	0.27	0.35
n	3	3	3	3	3	3	3	3

Table 5.11. Percentage of ^{125}I Transferred to the Fetal Perfusate

% precipitable activity

(expressed as % total)

Time in Minutes	15	30	45	60	75	90	105	120
IDL 1	61.9	57.0	51.8	53.6	52.7	53.5	53.1	52.0
IDL 2	51.0	50.6	57.4	53.2	56.2	56.9	56.2	57.2
IDL 3	54.7	54.9	56.8	53.6	56.0	53.5	53.8	53.3
Mean	55.9	54.2	55.3	53.5	55.0	54.6	54.4	54.2
± SD	5.5	3.3	3.1	0.2	2.0	2.0	1.6	2.7
± SE	3.2	1.9	1.8	0.1	1.1	1.1	0.9	1.6
n	3	3	3	3	3	3	3	3

Table 5.12. Percentage of Precipitable Activity in the Fetal Perfusate

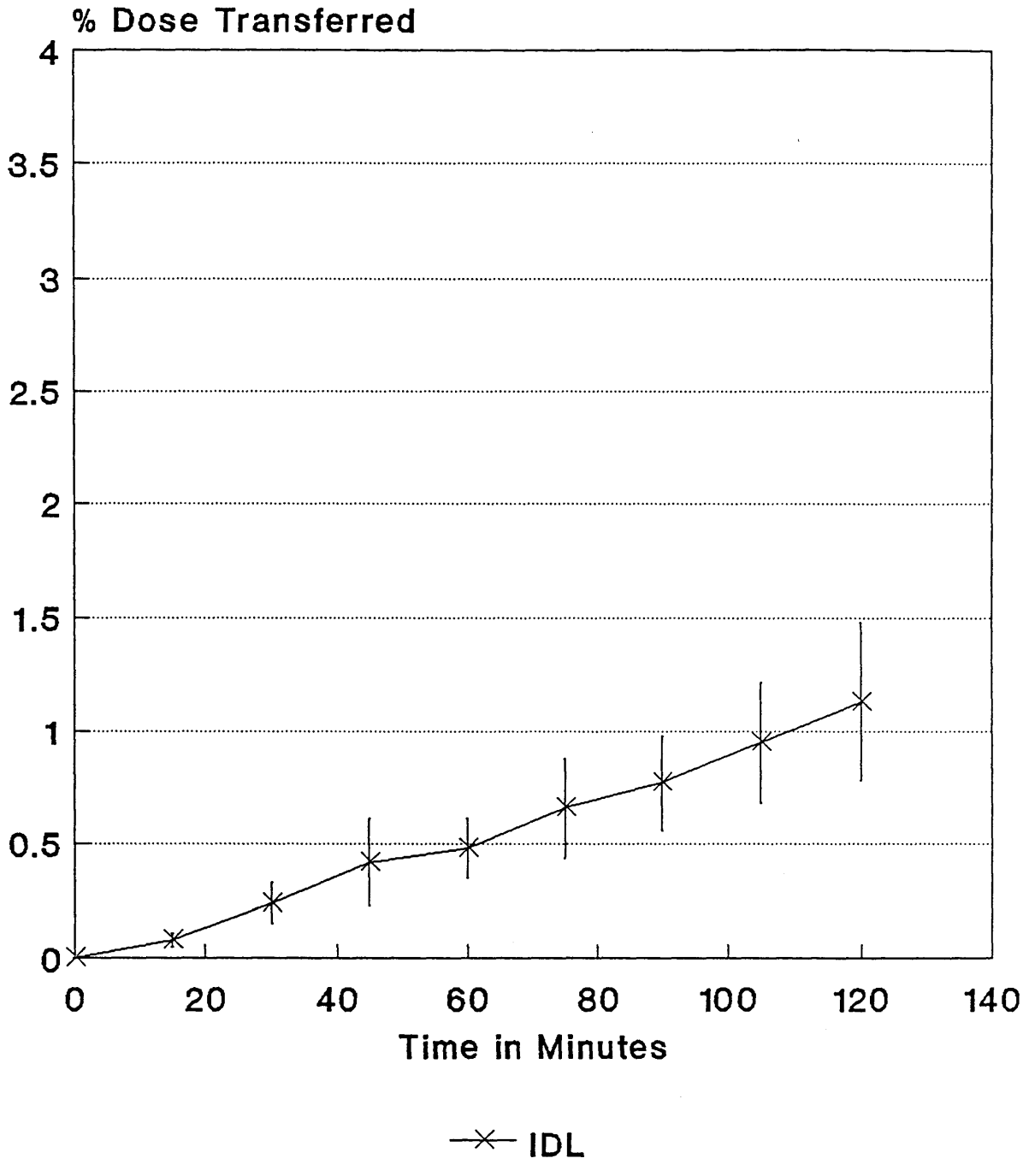


Figure 5.2. IDL Transfer to Fetal Circulation

5.3.4. Placental Lobule Results

IDL 1 Tissue contained 4.6% of dose

IDL 2 Tissue contained 13.3% of dose

IDL 3 Tissue contained 4.0% of dose

Mean 7.3% ± 5.2 (SD, n = 3)

Table 5.13. Percentage of ^{125}I Dose Remaining Within the Placental Lobule

IDL 1 73.4, 73.7, 73.8, 75.2

IDL 2 77.2, 76.1, 77.7, 77.6

IDL 3 69.1, 67.9, 71.4, 67.4

Mean 73.4% ± 3.7 (SD, n = 12)

Table 5.14. Percentage of Total TCA/PTA Precipitable Activity in the Placental Lobule Homogenate

5.4. RESULTS OF LDL PERFUSIONS

5.4.1. ^{125}I-LDL Mix Standards

Mix standards	% precipitable activity (expressed as % total counts)
LDL 1	97.0, 97.7, 96.6, 98.9
LDL 2	94.6, 94.6, 94.7, 94.6
LDL 3	90.6, 89.9, 89.8, 90.0
	Mean 94.5 ± 2.8% (SD, n = 16)

Table 5.15. ^{125}I-LDL Mix Standards

5.4.2. Maternal Side Perfusate Results

	% dose remaining in maternal circuit			
Time in Minutes	**30**	**60**	**90**	**120**
LDL 1	89.8	92.7	91.5	89.4
LDL 2	102.2	101.8	98.3	96.6
LDL 3	94.8	95.0	92.8	92.9
LDL 6	102.0	89.6	80.3	78.2
Mean	97.2	94.8	90.7	89.3
± SD	6.0	5.2	7.5	7.9
± SE	3.0	2.6	3.8	4.0
n	4	4	4	4

Table 5.16. Percentage of ^{125}I-LDL Remaining in Maternal Circuit

% precipitable activity

(expressed as % total)

Time in Minutes	**30**	**60**	**90**	**120**
LDL 1	95.5	95.6	95.4	95.5
LDL 2	96.6	96.6	96.6	96.4
LDL 3	95.1	95.5	95.5	95.8
LDL 6	98.3	98.2	98.2	98.2
Mean	96.4	96.5	96.4	96.5
± SD	1.4	1.3	1.3	1.2
± SE	0.7	0.6	0.7	0.6
n	4	4	4	4

Table 5.17. Percentage of Precipitable Activity in the Maternal Circuit

5.4.3. Fetal Side Perfusate Results

% dose transferred (total activity)

Time in Minutes	15	30	45	60	75	90	105	120
LDL 1	0.12	0.25	0.38	0.47	0.57	0.64	0.78	0.77
LDL 2	0.09	0.34	0.48	0.66	0.82	0.94	1.15	1.21
LDL 3	0.24	0.62	0.85	1.04	1.24	1.36	1.50	1.63
LDL 6	0.00	0.01	0.04	0.04	0.05	0.06	0.07	0.08
Mean	0.11	0.31	0.44	0.55	0.67	0.75	0.88	0.90
± SD	0.10	0.25	0.33	0.42	0.50	0.55	0.61	0.65
± SE	0.05	0.13	0.17	0.21	0.25	0.27	0.31	0.33
n	4	4	4	4	4	4	4	4

Table 5.18. Percentage of ^{125}I Transferred to the Fetal Perfusate

% precipitable activity

(expressed as % total)

Time in Minutes	15	30	45	60	75	90	105	120
LDL 1	66.8	69.7	69.3	70.5	65.7	62.9	62.1	59.5
LDL 2	67.5	65.1	62.6	62.8	61.2	59.9	57.0	52.1
LDL 3	53.5	55.7	52.3	53.5	47.9	50.3	47.6	43.7
LDL 6	86.1	61.1	47.9	44.2	39.5	32.9	28.9	23.6
Mean	68.5	62.9	58.0	57.8	53.6	51.5	48.9	44.7
± SD	13.4	5.9	9.7	11.4	12.0	13.5	14.6	15.5
± SE	6.7	3.0	4.9	5.7	6.0	6.8	7.3	7.7
n	4	4	4	4	4	4	4	4

Table 5.19. Percentage of Precipitable Activity in the Fetal Perfusate

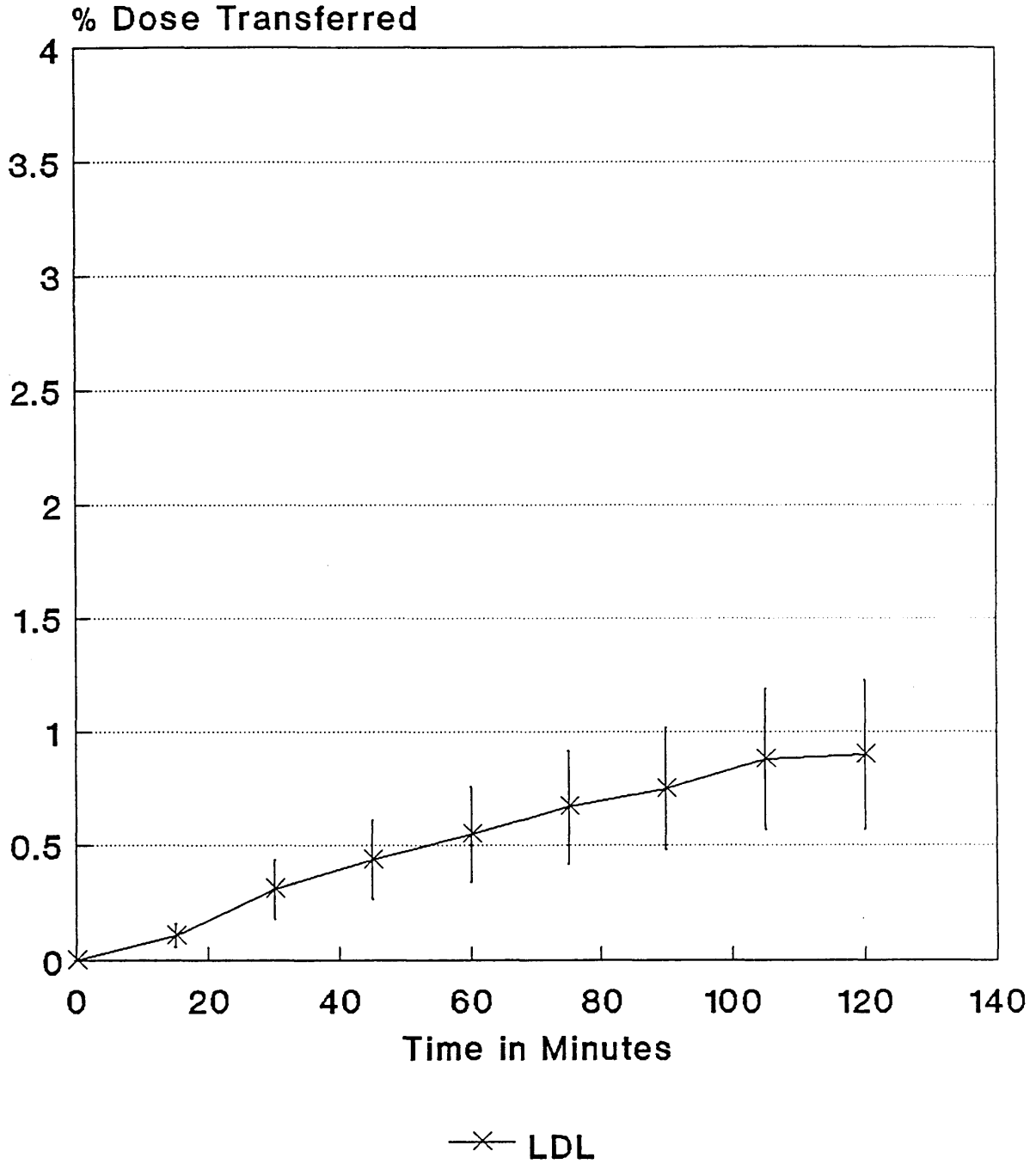


Figure 5.3. LDL Transfer to Fetal Circulation

5.4.4. Placental Lobule Results

LDL 1	Tissue contained 2.4% of dose
LDL 2	Tissue contained 1.9% of dose
LDL 3	Tissue contained 2.6% of dose
LDL 6	Tissue contained 3.6% of dose

Mean 2.6 ± 0.7% (SD, n = 4)

Table 5.20. Percentage of ^{125}I Dose Remaining Within the Placental Lobule

LDL 1	66.5, 68.7, 69.0, 68.3
LDL 2	72.0, 71.6, 70.1, 69.8
LDL 3	71.6, 71.5, 71.1, 72.0
LDL 6	91.5, 91.2, 91.6, 91.7

Mean 75.5 ± 9.7% (SD, n = 16)

Table 5.21. Percentage of Total TCA/PTA Precipitable Activity in the Placental Lobule Homogenate

5.5. RESULTS OF HDL PERFUSIONS

5.5.1. ^{125}I-HDL Mix Standards

Mix standards	% precipitable activity (expressed as % of total counts)
HDL 1	99.5, 99.5, 99.5, 99.5
HDL 2	99.8, 99.8, 99.8, 99.8
HDL 3	99.4, 99.4, 99.4, 99.3
	Mean 99.6 ± 0.2% (SD, n = 12)

Table 5.22. ^{125}I-HDL Mix Standards

5.4.2. Maternal Side Perfusate Results

	% dose remaining in maternal circuit			
Time in Minutes	**30**	**60**	**90**	**120**
HDL 1	100.87	93.79	90.17	88.98
HDL 2	80.19	78.78	78.59	78.22
HDL 3	91.71	88.41	85.55	86.12
Mean	90.9	87.0	84.8	84.4
± SD	10.4	7.7	5.8	5.6
± SE	6.0	4.4	3.4	3.2
n	3	3	3	3

Table 5.23. Percentage of ^{125}I-HDL Remaining in Maternal Circuit

% precipitable activity

(expressed as % total)

Time in Minutes	**30**	**60**	**90**	**120**
HDL 1	99.1	99.0	99.0	99.0
HDL 2	99.5	99.4	99.5	99.4
HDL 3	99.2	99.2	99.3	99.1
Mean	99.3	99.2	99.3	99.2
± SD	0.2	0.3	0.3	0.2
± SE	0.1	0.1	0.1	0.1
n	3	3	3	3

Table 5.24. Percentage of Precipitable Activity in the Maternal Circuit

5.5.3. Fetal Side Perfusate Results

% dose transferred (total activity)

Time in Minutes	15	30	45	60	75	90	105	120
HDL 1	0.05	0.06	0.07	0.11	0.13	0.16	0.18	0.19
HDL 2	0.00	0.01	0.03	0.04	0.05	0.06	0.07	0.09
HDL 3	0.01	0.04	0.06	0.07	0.08	0.08	0.10	0.12
Mean	0.02	0.04	0.06	0.07	0.09	0.10	0.12	0.13
± SD	0.03	0.03	0.03	0.04	0.04	0.05	0.06	0.05
± SE	0.02	0.01	0.02	0.02	0.02	0.03	0.03	0.03
n	3	3	3	3	3	3	3	3

Table 5.25. Percentage of ^{125}I Transferred to the Fetal Perfusate

% precipitable activity

(expressed as % of total)

Time in Minutes	15	30	45	60	75	90	105	120
HDL 1	46.4	44.4	45.5	43.7	44.7	44.2	41.8	43.4
HDL 2	71.1	59.4	56.5	55.5	51.2	52.0	49.6	48.3
HDL 3	49.1	58.6	61.4	57.6	60.0	55.4	55.1	58.5
Mean	55.5	54.1	54.5	52.3	52.0	50.5	48.8	50.1
± SD	13.5	8.4	8.1	7.5	7.7	5.7	6.7	7.7
± SE	7.8	4.9	4.7	4.3	4.4	3.3	3.9	4.4
n	3	3	3	3	3	3	3	3

Table 5.26. Percentage of Precipitable Activity in the Fetal Perfusate

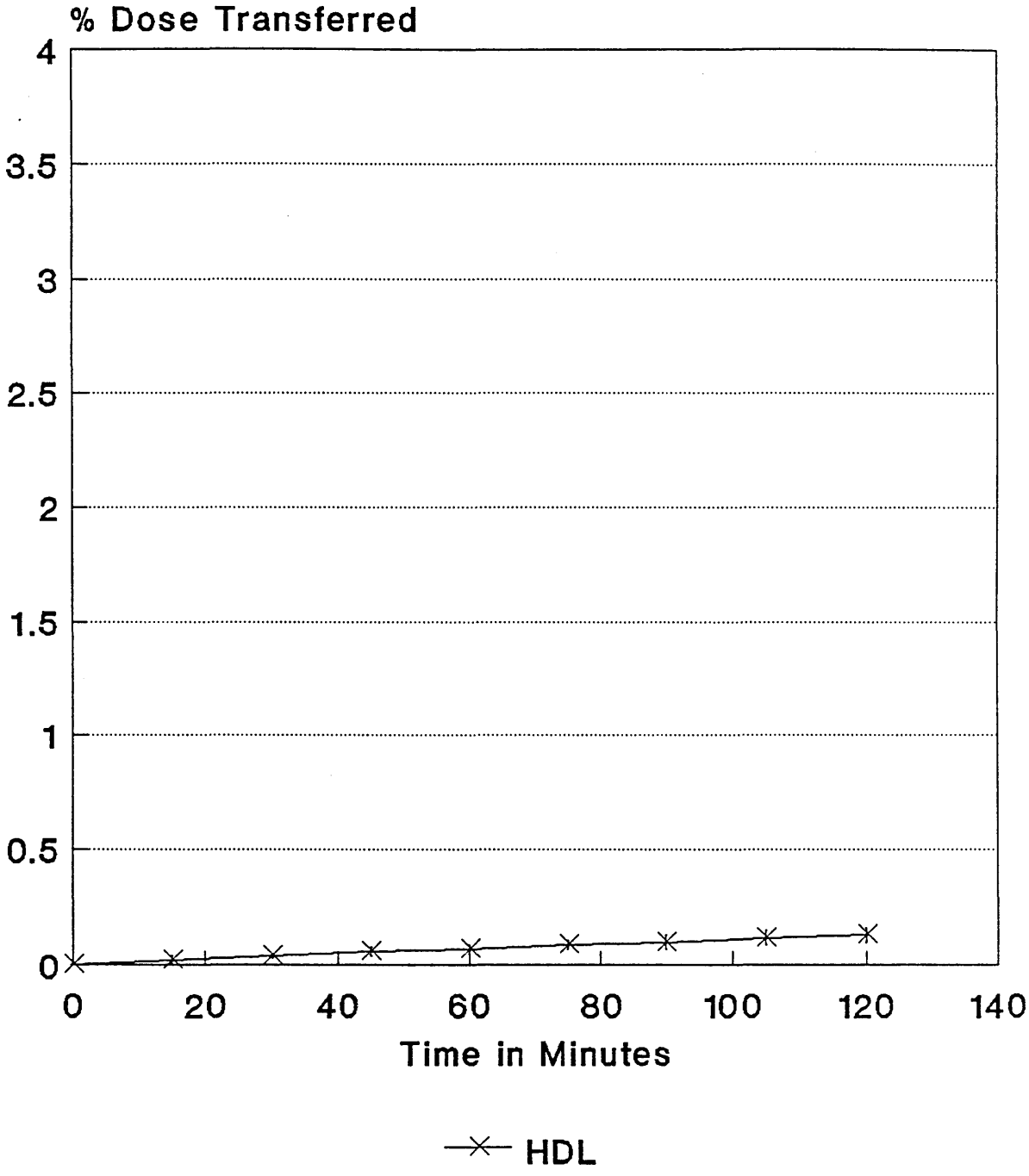


Figure 5.4. HDL Transfer to Fetal Circulation

5.5.4. Placental Lobule Results

HDL 1	Tissue contained 7.0% of dose
HDL 2	Tissue contained 3.1% of dose
HDL 3	Tissue contained 4.4% of dose

Mean 4.8 ± 2.0 (SD, n = 3)

Table 5.27. Percentage of ^{125}I Dose Remaining Within the Placental Lobule

HDL 1	93.6, 93.6, 94.1, 93.6
HDL 2	93.1, 93.5, 93.4, 93.1
HDL 3	93.6, 93.8, 93.5, 93.4

Mean 93.5 ± 0.3% (SD, n = 12)

Table 5.28. Percentage of Total TCA/PTA Precipitable Activity in the Placental Lobule Homogenate

5.6. RESULTS OF ^{125}I-VLDL PERFUSIONS WITH 10 mM CHLOROQUINE

Chloroquine is known to block receptor-mediated endocytosis (see Section 4.4.). To confirm that receptor-mediated endocytosis is the mechanism by which the placenta acquires VLDL, chloroquine was added to the maternal perfusate to a final concentration of 10 mM in three further perfusions with ^{125}I-VLDL as described in Section 5.2. The results are shown in Figure 5.5.

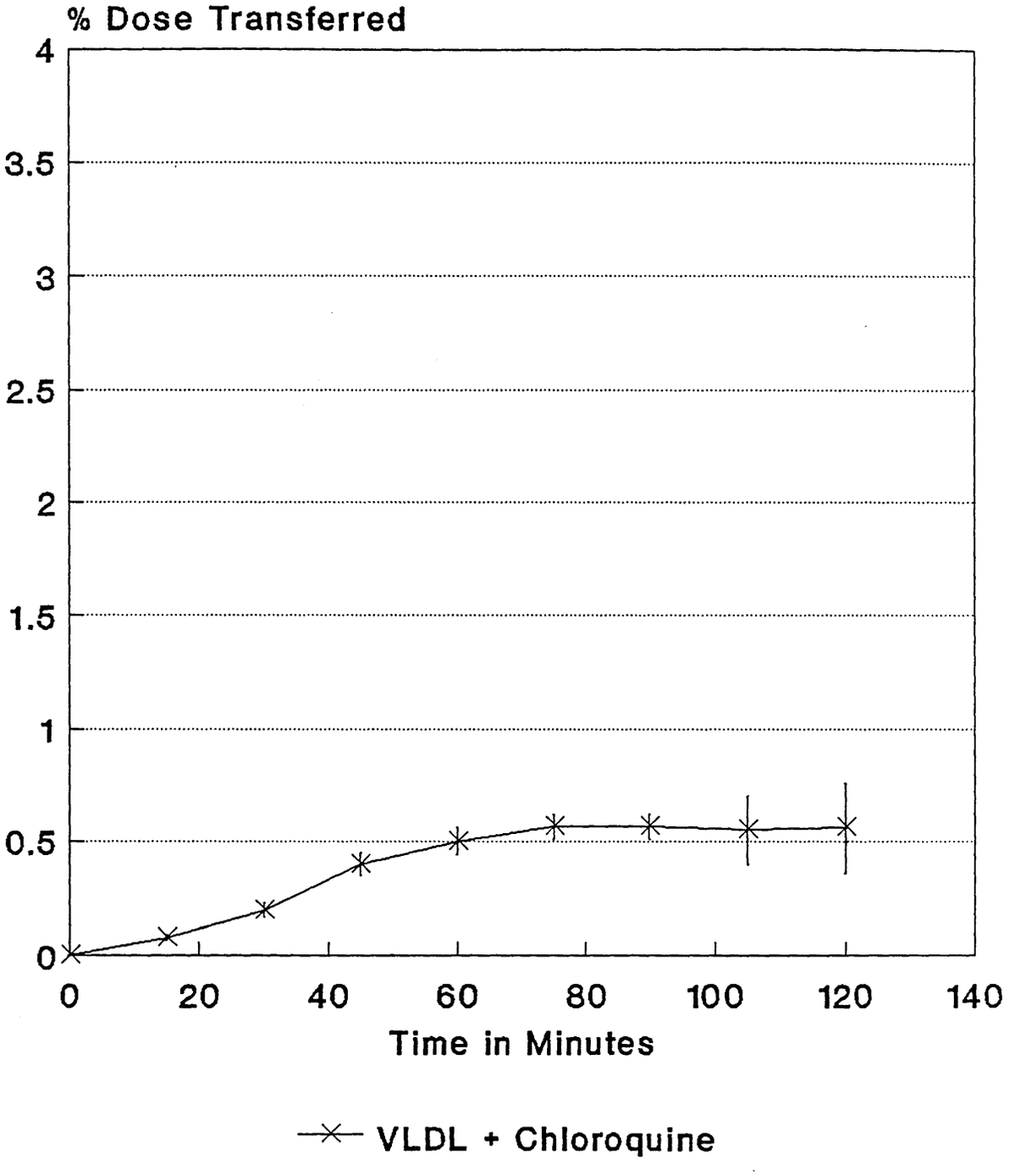


Figure 5.5. ^{125}I-VLDL Transfer to the Fetal Circulation with the Addition of 10 mM of Chloroquine

5.7. RESULTS OF ^{14}C-CHOLESTEROL PERFUSIONS AND THIN-LAYER CHROMATOGRAPHY

The micro-perfusions were performed as outlined in Section 4.5.3. The tissues were homogenised and the perfusate and tissues extracted and subjected to thin layer chromatography. The cholesterol, pregnenolone and progesterone bands were counted and showed minimal ^{14}C-cholesterol and **NO** ^{14}C-pregnenolone or ^{14}C-progesterone in the tissues.

5.8. DISCUSSION OF PLACENTAL PERFUSION RESULTS

Figure 5.6. shows the combined results of the maternal to fetal transfer experiments with the different lipoprotein classes. This indicates that VLDL has a much higher transfer of ^{125}I-activity than any of the other lipoprotein classes. The constant precipitable activity on the maternal side of between 80-90% indicates that the lipoprotein molecules appear to remain intact during the placental lobule perfusion. In the fetal perfusate the precipitable activity is 20-50% of the total fetal activity. This would suggest that the majority of the ^{125}I within the fetal circuit is either free or is attached to small molecules indicating that there is probably not a major transfer of intact lipoprotein across the placenta. The tissue counts would support this view, particularly as

the VLDL count is significantly higher than the other lipoprotein classes.

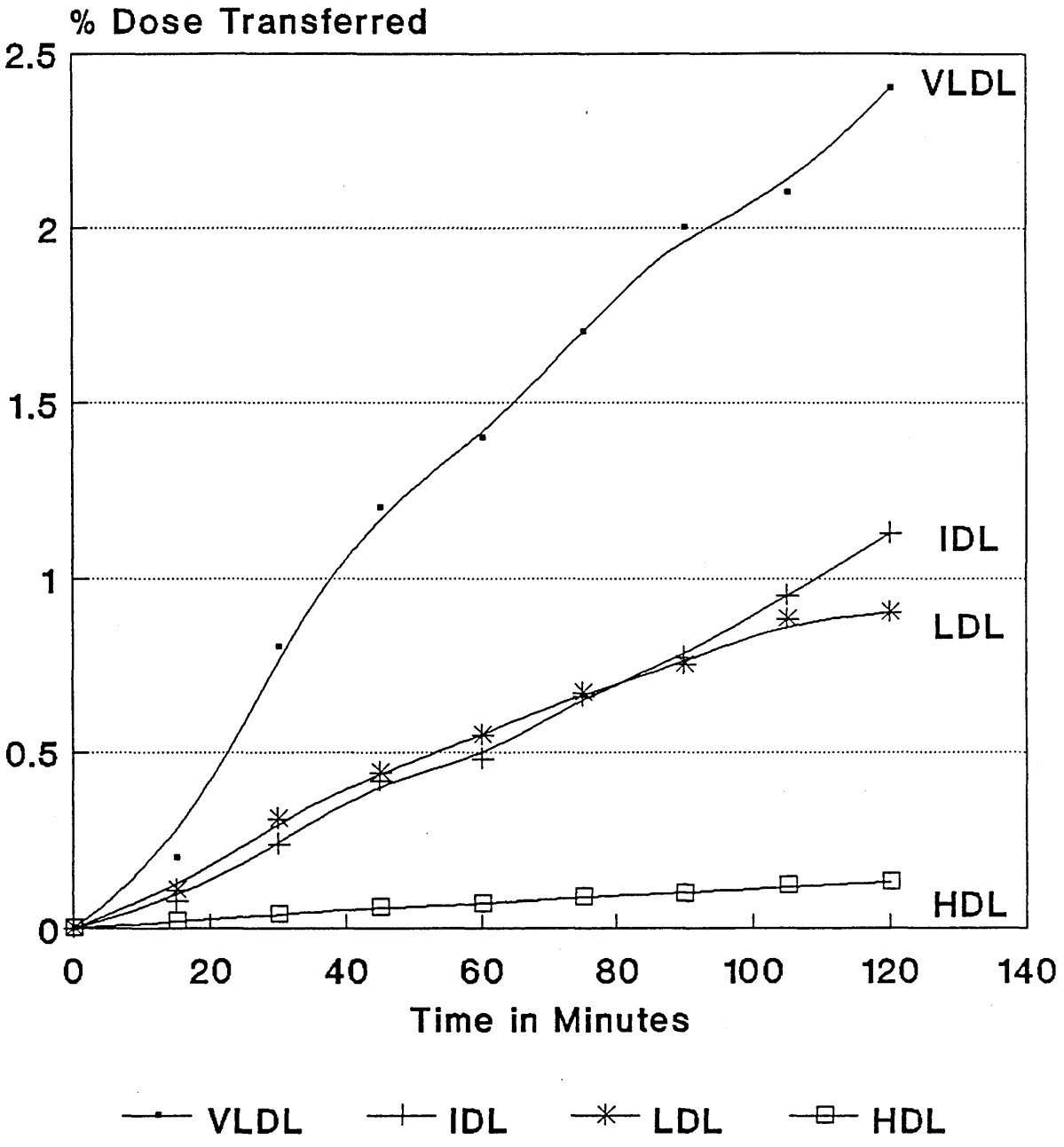


Figure 5.6. Maternal to Fetal Transfer of all Lipoprotein Classes

Figure 5.7. shows the counts within the tissues. IDL1 is the mean of the three IDL experiments. However, one experiment showed a particularly high residual count, probably due to inadequate squeezing and blotting of the placental lobule, and IDL2 is the mean of the two remaining experiments. This is probably a more accurate reflection of the true tissue accumulation of ^{125}I-IDL.

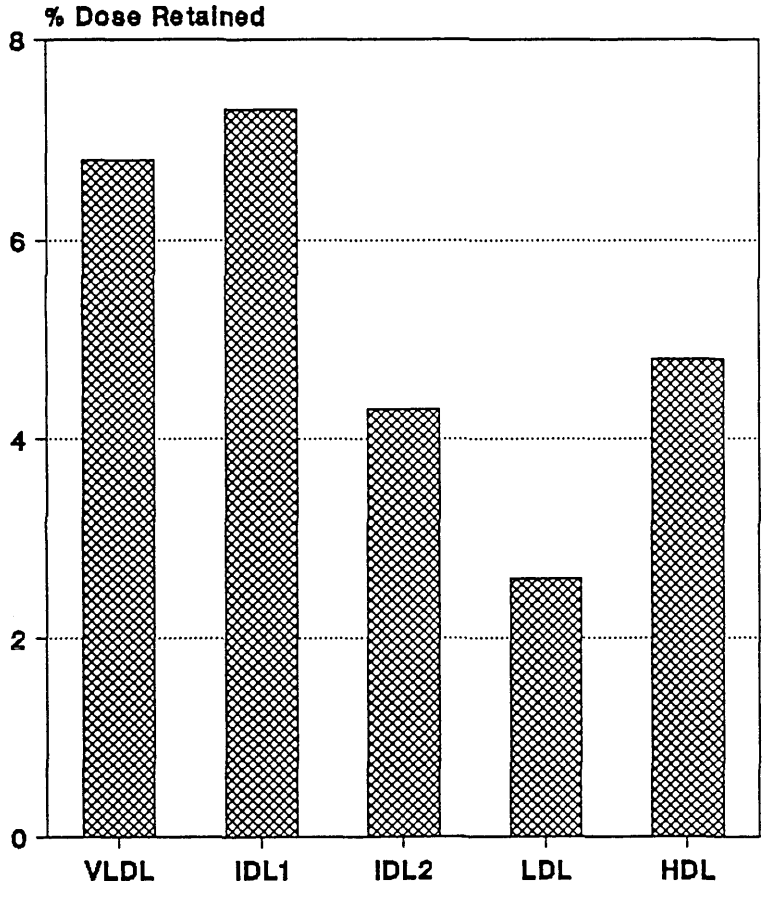
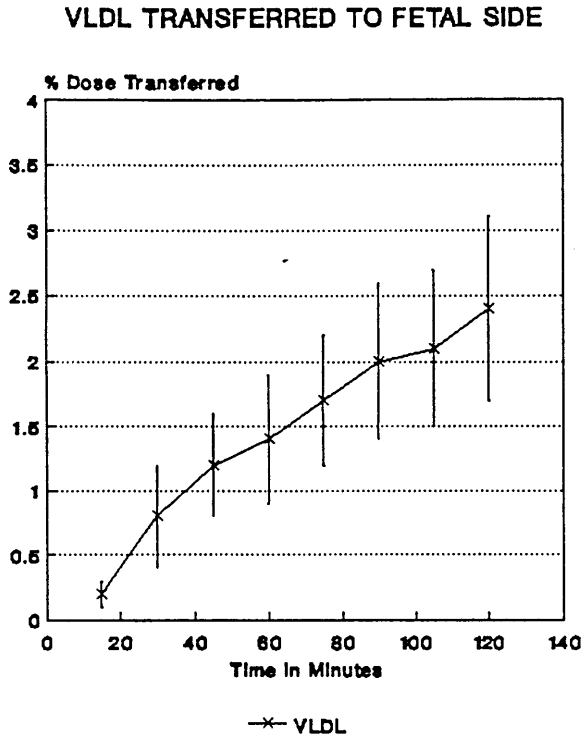


Figure 5.7. Tissue Accumulation of ^{125}I-Labelled Lipoproteins

Figure 5.8. compares the transfer of ^{125}I-VLDL to the fetal circulation with and without chloroquine. It clearly shows that the uptake of VLDL by the placental lobule is inhibited by the addition of chloroquine, suggesting that receptor-mediated endocytosis is the mechanism by which VLDL is acquired by the placenta.

The ^{14}C-cholesterol LDL perfusion experiments confirm that the placenta does not appear to use LDL as its principal source of cholesterol, nor does it seem to acquire cholesterol from the serum in a free form. Having used lipoprotein-deficient plasma and washed fetal red cells for the perfusate, it is unlikely that endogenous cholesterol or lipoproteins could be competing for absorption.

The results from the perfusion of ^{125}I-labelled lipoproteins and ^{14}C-cholesterol LDL perfusions would suggest that the main mechanism for cholesterol absorption is by the receptor-mediated endocytosis of VLDL. In order to verify this theory membrane binding studies on a microvillous membrane preparation of the human placenta were performed. These included competetive binding between LDL and VLDL.



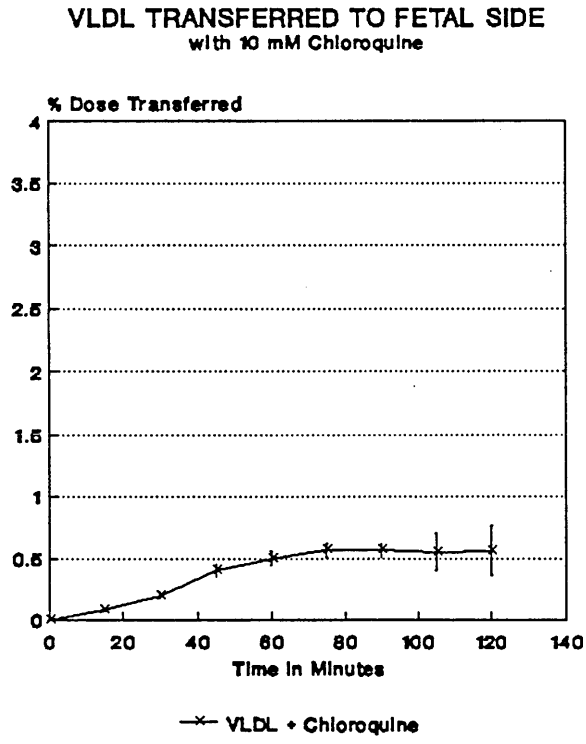


Figure 5.8. Transfer of ^{125}I-VLDL to the Fetal Circulation With and Without the Addition of Chloroquine

CHAPTER SIX

MEMBRANE BINDING

6.1. INTRODUCTION

A potent stimulus for research into receptors was the realisation that breast tumours may be hormone-dependent, and receptor status affected the method of treatment. The initial mathematical analysis of results of receptor binding kinetics is based on the early work of Scatchard (1949) who investigated ion binding to proteins, and was extended by Braunsberg (1984). The estimation of receptor characteristics involves the separation of binding data into high affinity-low capacity (receptor) and low affinity-high capacity (non-receptor or non-specific binding) components since a correction needs to be made for the non-specific binding of ligands to non-receptor sites. In addition to the calculation of receptor numbers, the estimation of association, or dissociation, constants provide measures of affinity.

If the ratio of the quantities of bound ligand over free ligand is plotted against the bound ligand, a straight line graph should result if the ligand is binding to a single class of receptors. The x intercept of the linear regression analysis gives an indication of the quantity of ligand bound and an indication of the receptor numbers.

The different mechanisms of receptor-mediated endocytosis have been outlined in Chapter One. Whatever the mechanism of receptor movement or the fate of the endocytotic

vesicle, it is the receptor numbers, and their affinity for the ligand, which, in part, determine the quantity of ligand internalised. Thus, an estimate of receptor numbers is a useful indicator of the activity of that particular tissue. Affinity and competitive binding assays give an indication of the relative affinities of different ligands for the receptor.

Membrane binding studies, using a microvillous membrane preparation from the trophoblast (not the amnion and chorion) give an in vitro estimation of placental receptor activity. In vivo studies are ethically difficult to perform, particularly in late pregnancy, unless non-radio active labelling techniques using stable isotopes and a mass spectrometer are used.

There are two principal techniques used for membrane binding studies; firstly ultracentrifugation, a technique devised by Basu et al (1978), and secondly the use of milipore filters devised by Rudling and Peterson (1985). Lipoproteins have a tendency to adhere to inert substances such as glass beads which can, under some circumstances, mimic the saturation kinetics exhibited by receptors (Dana et al, 1977). Alsat et al (1982) published a method for the binding of LDL and HDL to a microvillous membrane preparation based on the technique devised by Basu. For this study a modification of Alsat's technique was used

involving a microcentrifuge centrifugation instead of ultracentrifugation.

6.2. SYNCYTIOTROPHOBLAST MICROVILLOUS MEMBRANE PREPARATION

6.2.1. Introduction

Crude homogenates of the placenta have been used for estimation of various enzyme kinetics, but there is now a much greater awareness of the different metabolic activities of the different tissues within the placenta. Smith and Brush (1978) developed a technique for producing a microvillous membrane enriched preparation of the placenta. This technique was modified by Contractor, Das and Oakey (1982) who used the enzymes alkaline phosphatase and 5'-nucleotidase to identify the apical membrane facing the maternal plasma and $Na^+ + K^+$ - activated ATPase and adenylate cyclase to identify the basal fetal side. The technique used in this study was that developed by Contractor et al (1982). To determine normal receptor numbers the placentae used were from normal, term (37-41 weeks) pregnancies with apparently normal babies who had exhibited no evidence of fetal distress during labour.

6.2.2. Solutions

1. Phosphate buffered saline 10 mM pH 7.2
   Sodium phosphate 10 mM; sodium chloride 0.15 M.

2. Tris/HCl buffer pH 7.3 +
   Phenylmethylsulphonylfluoride (PMSF) 1 mM

3. Sucrose 25% solution in distilled water

4. Sucrose 37% solution in distilled water

6.2.3. Method

A freshly-delivered placenta was placed on ice and the basal plate cut off. Apparently healthy portions of villous tissue were excised and placed in ice-cold phosphate buffered saline. Care was taken to avoid the large placental vessels. The tissue was gently rinsed to remove the blood, placed on a glass plate, which was lying on a bed of ice, and blotted with tissue paper. The tissue was then teased out with a pair of dissecting forceps and a small spatula to remove the fibrous tissue, which was discarded. The remaining microvillous-rich tissue was placed in a fresh container of ice-cold phosphate buffered saline. Phosphate buffered saline was added until the

total volume was approximately ten times the volume of the tissue.

The mixture was gently stirred at 4°C for 30 minutes to allow the microvilli to separate, and was then centrifuged in an MSE 4L at 800 G for ten minutes, also at 4°C. to remove excess tissue and red blood cells. The cloudy supernatant was centrifuged for one minute at 1000 G at 4°C. to remove any miscellaneous particles. This supernatant was then centrifuged in an MSE 18 at 50,000 G for one hour at 4°C producing a crude membrane pellet which was resuspended in 10 mM Tris/HCl buffer.

A discontinuous sucrose gradient was produced by gently pouring 37% sucrose solution into a polycarbonate centrifuge tube and, with a slow pump and screw table so that the tip of the layering cannula was always in the meniscus, 25% sucrose solution was carefully layered above it. The resuspended membrane pellet was carefully layered on to the top of the sucrose gradient which was then centrifuged at 50,000 G for sixteen hours at 4°C.

After centrifugation, three bands could be seen in the gradient, with the microvillous rich portion at the interface of the density gradients. The supernatant was gently removed, again with a slow pump and screw table, and discarded. The interface band was collected and

diluted 1:4 with Tris/HCl buffer, and the infranate discarded.

The microvillous rich suspension was centrifuged at 100,000 G for one hour at 4°C. The supernatant was removed and discarded, and the pellet resuspended in the Tris/HCl buffer. This centrifugation wash was repeated twice.

The final pellet was resuspended in Tris/HCl buffer to which two drops of azide (0.02%) had been added. This usually gave a final volume of between 3 and 4 ml of a milky solution, which was stored under nitrogen at 4°C. and used within four weeks.

6.3. MEMBRANE BINDING TECHNIQUE

6.3.1. Solutions

1. Incubation buffer pH 7.4
   Sodium chloride 100 mM; calcium chloride 1 mM; Tris-HCl 10 mM; delipidated bovine albumin 2%

2. Membrane solution 250 μg/100 μL
   equivalent to 2.5 mg/ml.
   Stock membrane solution had a Lowry protein estimation performed to determine the protein level and the concentration was then

adjusted with incubation buffer to give a protein content of 2.5 mg/ml.

3. Lipoprotein (unlabelled) 500 μg/80 μL
   Lipoprotein stock solution had a Lowry protein estimation performed and the concentration adjusted with incubation buffer to give 500 μg/80 μL of unlabelled lipoprotein.

4. Lipoprotein (iodinated) 1 μg/100 μL
   0.5 ml stock solution was put through a 2.5 ml G25 Sephadex column prior to use to remove any free iodine and the material eluted in the void volume was subjected to a Lowry protein estimation. The concentration was adjusted with incubation buffer to give 1 μg/100 μL ^{125}I-lipoprotein. Four 100 μL aliquots were counted on each occasion as standards.

6.3.2. Validation of Membrane Binding Technique

6.3.2.1. Centrifugation of Membranes

To determine whether the microcentrifuge centrifugation for 15 minutes was adequate to precipitate the membranes, a sample of membranes was iodinated as described in Section

3.6., and the concentration adjusted with incubation solution to give 250 mg/100 μL. A 100 μL aliquot was suspended in 250 μL incubation solution and centrifuged in the microcentrifuge. The supernatant was removed, the pellet was washed and re-microcentrifuged for 15 minutes, and the supernatant wash was added to the original supernatant. The combined supernatants were ultracentrifuged at 100,000 G for 15 minutes. The supernatant was again removed, put into a counting tube, and the pellet, if any, was resuspended and counted. The microcentrifuge tube containing the membranes, the ultracentrifuged supernatant and the ultracentrifuge tube containing any possible membranes were all counted.

The results of this experiment showed that the membrane preparation used was pelleted in the microcentrifuge. This was confirmed by duplicating the first membrane binding experiment using the ultracentrifuge technique in parallel with the microcentrifuge technique (see Section 7.1.3.).

6.3.2.2. To Determine the Number of Washes Required

The number of washes required to remove the free lipoproteins was determined by washing a membrane pellet three times and counting the supernatant each time. The conclusion drawn was that one wash was adequate (see Figure 7.2.).

6.3.3. To Determine the Optimum Binding Time

250 μg of membranes were incubated with 1 μg of ^{125}I-LDL for various lengths of time. The system was designed to have a constant incubation volume of 360 μL, and the incubations were performed in duplicate.

6.3.3.1. Method

Tubes A

100 μL membrane solution (250 μg)

160 μL incubation buffer

At time zero

100 μL iodinated LDL (1 μg) was added

Tubes B

100 μL membrane solution (250 μg)

80 μL incubation buffer

At time zero

100 μL iodinated LDL (1 μg) and

80 μL unlabelled LDL (500 μg) were added

Blanks

1. 260 μL incubation buffer
   100 μL iodinated LDL (1 μg)
   No membranes

2. 180 μL incubation buffer
   80 μL unlabelled LDL (500 μg)
   100 μL iodinated LDL (1 μg)

3. 260 μL incubation buffer
   100 μL membranes
   No LDL

Tubes A and B were incubated for 20,40,60,80 and 120 minutes on ice at between 0 and 4°C. The microcentrifuge tubes were centrifuged in an MSE Microcentaur for 15 minutes. The supernatant was aspirated and placed in a counting tube. 360 μL of incubation buffer was pipetted into the microcentrifuge tubes, and recentrifuged for 15 minutes. The supernatant from this wash was added to the original supernatant. The blanks were treated in exactly the same way. The microcentrifuge tubes were placed in counting tubes. The supernatant and pellets were counted in a Hewlett Packard Auto Gamma 5650 Counter.

6.4. CONCENTRATION BINDING

6.4.1. Introduction

In these experiments the concentration of the labelled lipoprotein varied, and the binding time was constant at one hour. The incubation volumes were kept constant at 360 μL and the binding was performed in the presence and

absence of an excess of unlabelled lipoprotein to determine the specific binding.

After analysing the results of the first two LDL binding experiments, it was found that there was a significant variation between the binding of different placentae. All subsequent experiments were therefore performed on the pooled membranes of a minimum of six placentae to achieve a consistent membrane preparation. The experiments were performed in the presence of an excess of the same lipoprotein to obtain the binding constants, and in the presence of different lipoproteins, that is competitive binding, to assess their relative affinities for the receptor.

6.4.2. Solutions

1. Incubation buffer pH 7.4
   Sodium chloride 100 mM; calcium chloride 1 mM; Tris-HCl 10 mM; delipidated bovine albumin 2%
2. Membrane solution 250 μg/100 μL
   = 2.5 mg/ml
   Stock membrane solution had a Lowry protein estimation performed to determine the protein level and the concentration was then adjusted with incubation buffer to give a protein content of 2.5 mg/ml.

3. Lipoprotein (unlabelled) 500 μg/80 μL

Lipoprotein stock solution had a Lowry protein estimation performed and the concentration adjusted with incubation buffer to give 500 μg/80 μL of unlabelled lipoprotein.

4. Lipoprotein (iodinated) 1 μg/100 μL

0.5 ml stock solution was put through a 2.5 ml G25 Sephadex column and a Lowry protein estimation performed. The concentration was adjusted with incubation buffer to give 1 μg/100 μL ^{125}I-lipoprotein. Four 100 μL aliquots were counted on each occasion.

6.4.3. Method

Tubes A

100 μL membrane solution

230, 200, 170, 140, 110, 80 μL

of incubation buffer

At time zero

30, 60, 90, 120, 150, 180 μL

^{125}I- lipoprotein were added.

Constant volume 360 μL was maintained.

Tubes B

100 μL membrane solution

150, 120, 90, 60, 30, 0 μL of

incubation buffer

At time zero

80 μL of unlabelled lipoprotein and

30, 60, 90, 120, 150, 180 μL of

^{125}I-lipoprotein were added.

Constant volume 360 μL was maintained.

The microcentrifuge tubes were centrifuged and the pellets washed and counted as described in Section 6.3.2.1.

6.4.4. LDL Binding Experiments No. 1 and 2

The membranes from one placenta were prepared as described in Section 6.2. A concentration binding experiment was performed using ^{125}I-LDL and an excess of unlabelled LDL to determine the specific binding. The results of this experiment have been given in detail in Section 7.2.1.

A second LDL binding experiment using a microvillous membrane preparation from a different placenta was performed giving significantly different results (see Section 7.2.2.). and a pooled membrane preparation was subsequently used for all experiments (see Section 6.4.1.).

6.4.5. Membrane Binding Studies with VLDL, LDL and HDL using a Pooled Microvillous Membrane Preparation

6.4.5.1. VLDL Binding Studies

For these studies a pooled microvillous membrane preparation with the concentration adjusted to 125 μg/100 μL was used with ^{125}I-labelled VLDL and an excess of unlabelled VLDL. The experiment was performed three times as outlined in Section 6.4. and the results are shown in Section 7.3.1.

6.4.5.2. LDL Binding Studies

The LDL binding studies were performed with the same pooled microvillous membrane preparation as used in the VLDL studies. ^{125}I-LDL was competed with an excess of unlabelled LDL and repeated four times. The results are shown in Section 7.3.2.

6.4.5.3. HDL Binding Studies

Again using the same pooled microvillous membrane preparation at a concentration of 125 μg/100 μL, the HDL self-inhibiting binding was determined with three experiments. The results are shown in Section 7.3.3.

6.4.6. Self- and Cross-Inhibition of VLDL and LDL Binding

The specificity of the binding of VLDL and LDL to the placental microvillous membrane preparation was examined by determining the effect of increasing concentrations of unlabelled VLDL on the binding of ^{125}I-VLDL and ^{125}I-LDL. This was repeated using an increasing concentration of unlabelled LDL and determining the binding against ^{125}I-VLDL and ^{125}I-LDL. Each of these experiments was repeated three times and the results are shown in Section 7.3.4.

CHAPTER SEVEN

RESULTS OF MEMBRANE BINDING EXPERIMENTS

7.1. TECHNIQUE VALIDATION

7.1.1. Membrane Centrifugation Experiment

This assessed the ability of the microcentrifuge to precipitate the microvillous membranes in a 15 minute centrifugation. The method has been described in Section 6.3.2.1., and the results have been plotted on a log scale graph (see Figure 7.1.).

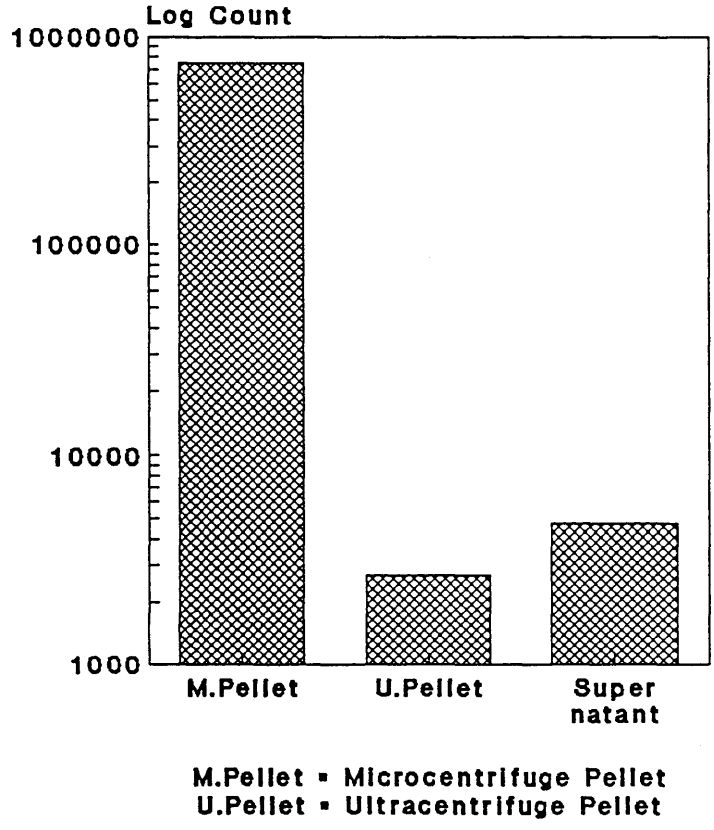


Figure 7.1. **Membrane Centrifugation: Comparison between Microcentrifugation and Ultracentrifugation**

7.1.2. Membrane Wash Experiment

This experiment assessed the number of washes required to remove the unbound ^{125}I-lipoprotein. 60μL and 100μL ^{125}I-HDL were incubated with 250μg of membranes for one hour, microcentrifuged and washed three times (see Section 6.3.2.2.). Figure 7.2. illustrates the results with and without the supernatant.

Experiment 1 with 60 μL of HDL

Supernatant dpm	431320.7 ± 1837.2
Pellet dpm	5982.5 ± 237.4
Wash 1	6411.9 ± 214
Wash 2	842.6 ± 102
Wash 3	578.3 ± 67

Experiment 2 with 210 μL of HDL

Supernatant dpm	1513149.5 ± 8907
Pellet dpm	13830.9 ± 49.3
Wash 1	18846.2 ± 547.6
Wash 2	2054.1 ± 6.5
Wash 3	1298.4 ± 4.2

Table 7.1. Membrane Wash Experiments

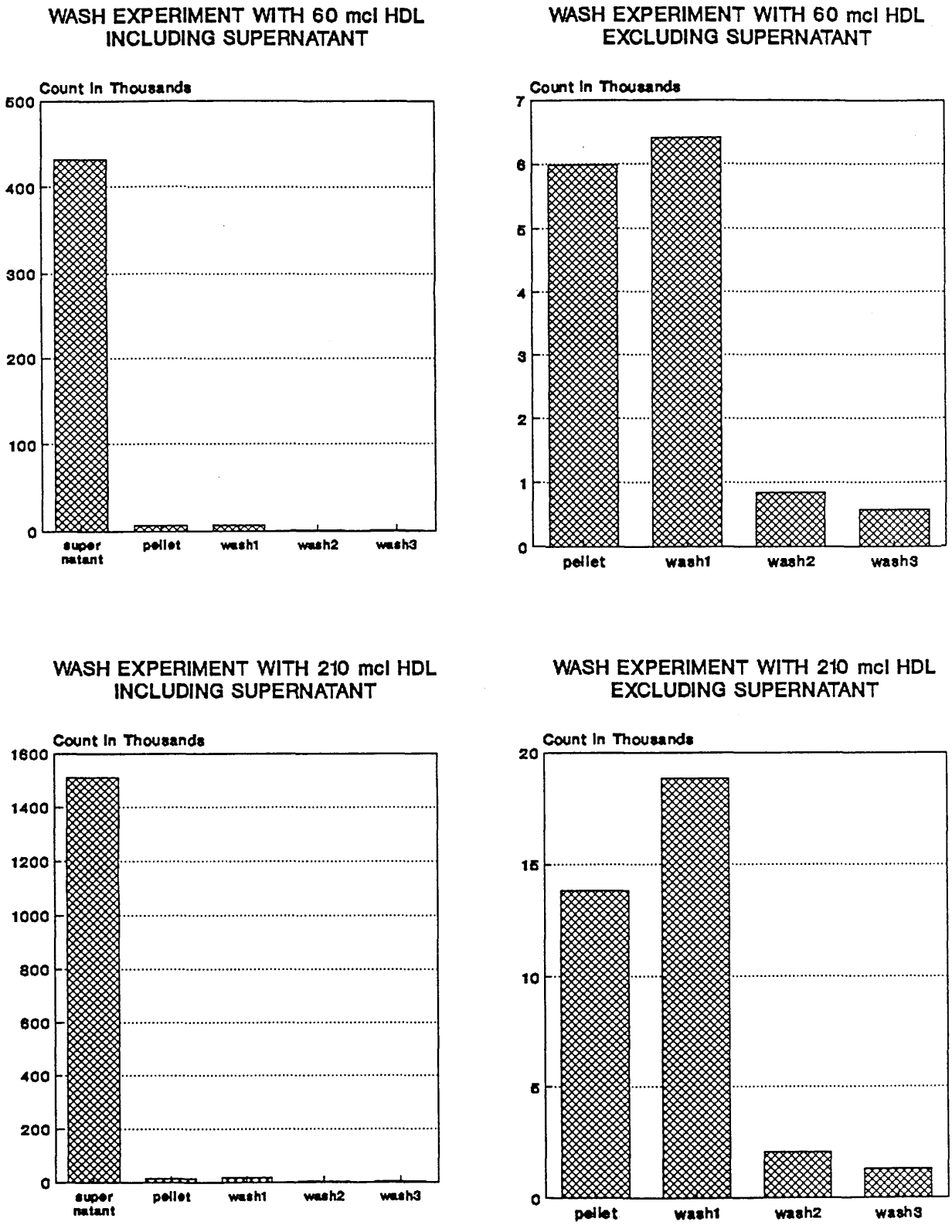


Figure 7.2. Membrane Wash Experiment

7.1.3. Time Course Experiment with Microcentrifuge and Ultracentrifuge Techniques in Duplicate

To determine the time required to complete the lipoprotein binding to the membranes, a time course binding experiment was performed (see Section 6.3.2.). This experiment was duplicated using the ultracentrifugation technique to validate the microcentrifuge technique, and the results are shown in Figure 7.3.

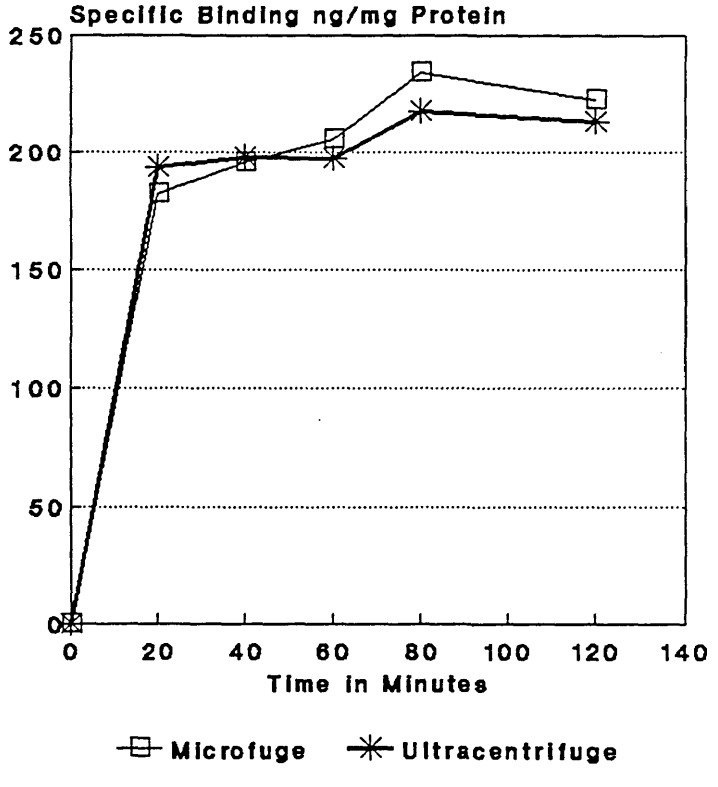


Figure 7.3. Time Course Binding of LDL: Comparison between Microfuge and Ultracentrifuge

7.1.4. Conclusion of Technique Validation

The results show that all the membranes were precipitated in the microcentrifuge in 15 minutes and that one wash was adequate. The residual counts in the washes were almost certainly due to the unbinding of ^{125}I-lipoprotein from the membranes. The technique was validated by the duplicated time binding experiment.

7.2. LDL MEMBRANE BINDING EXPERIMENT

The method for this experiment has been described in Section 6.4. and uses a microvillous membrane preparation from a single placenta. An excess of unlabelled LDL was used to determine the specific binding of LDL. A computer programme was written to analyse the data (see Appendix), but have been tabled fully to illustrate the method.

7.2.1. LDL Binding No.1

Standards

^{125}I-LDL Standard (100 μL containing 1 μg ^{125}I-LDL)

cpm - blank	% efficiency	dpm
408130.9	75.91	537651.09
414690.1	76.54	541795.27
405802.3	75.71	535995.65
405336.1	76.15	532286.4

Mean 536932.2 ± 3942.4

Table 7.2. Standards for LDL Binding No. 1

Non-specific binding (pellet dpm corrected for non-specific tube binding obtained in presence of 500 μg unlabelled LDL)

^{125}I-LDL volume μL	^{125}I-LDL quantity ng	Corrected dpm	Mean of duplicates
30	300	15271.7	
		11885.5	13578.6
60	600	33647.4	
		26837.0	30242.2
90	900	34161.7	
		35285.0	34723.8
120	1200	51719.3	
		42014.2	46866.8
150	1500	49775.8	
		54471.4	52023.6
180	1800	66592.3	
		75351.7	70972.0

Table 7.3. Non-Specific Binding of LDL Binding No. 1

^{125}I-LDL Added ng	^{125}I-LDL Bound ng
300	25.3
600	56.3
900	64.7
1200	87.3
1500	96.9
1800	132.2

Table 7.4. Non-Specific ^{125}I-LDL Bound of LDL Binding No. 1

Total binding (pellet dpm corrected for non-specific binding to tube obtained in absence of unlabelled LDL)

^{125}I-LDL volume μL	^{125}I-LDL quantity ng	Corrected dpm	Mean of duplicates
30	300	42218.5	
		37200.4	39709.5
60	600	70750.9	
		70594.9	70672.9
90	900	91704.8	
		87242.2	89473.5
120	1200	104382.6	
		109298.3	106840.5
150	1500	122350.3	
		125001.4	123675.9
180	1800	139339.4	
		136855.9	138097.7

Table 7.5. Total Binding of LDL Binding No. 1

^{125}I-LDL Added ng	^{125}I-LDL Bound ng
300	74.0
600	131.6
900	166.6
1200	199.0
1500	230.3
1800	257.2

Table 7.6. Total ^{125}I-LDL Bound of LDL Binding No. 1

Specific binding (total binding - non-specific binding)

^{125}I-LDL Added	Counts
300	39709.5 - 13578.6 = 26130.9
600	70672.9 - 30242.2 = 40430.7
900	89473.5 - 34723.8 = 54749.7
1200	106840.5 - 46866.8 = 59973.7
1500	123675.9 - 52023.6 = 71652.3
1800	138097.7 - 70972.0 = 67125

Table 7.7. Specific Binding of ^{125}I-LDL

^{125}I-LDL Added ng	^{125}I-LDL Bound ng	^{125}I-LDL Bound/mg Membranes	Bound / Free
300	48.7	194.8	0.1623
600	75.3	301.2	0.1255
900	102.0	408.0	0.1133
1200	110.7	442.8	0.0922
1500	133.4	533.6	0.0889
1800	125.0	500.0	0.0694

Table 7.8. Results of ^{125}I-LDL Binding Experiment No. 1

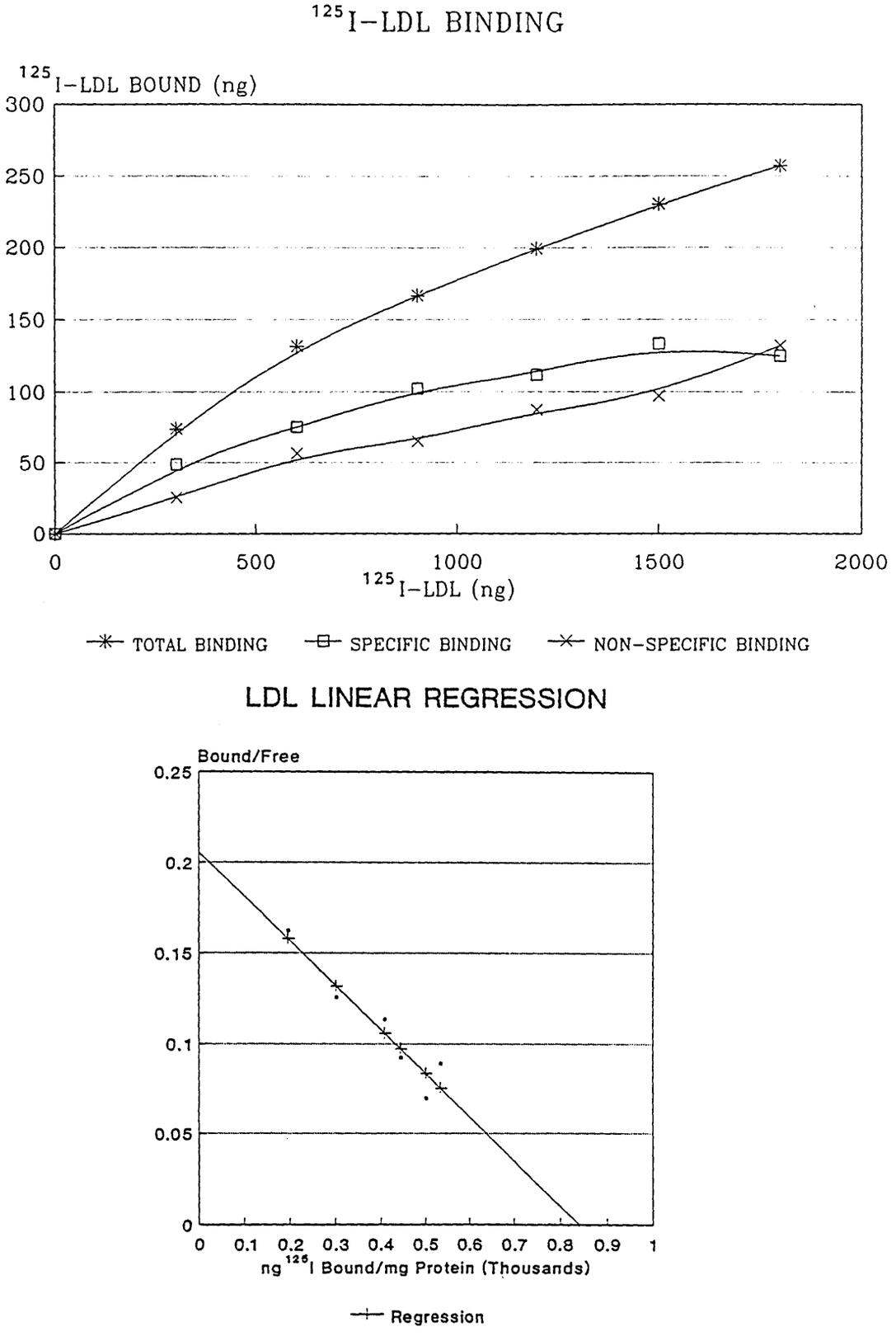


Figure 7.4. **^{125}I-LDL Binding Experiment Showing Total, Specific and Non-Specific Binding with the Linear Regression of the Bound/Free versus ^{125}I-LDL Bound**

Figure 7.4. shows the specific binding of ^{125}I-LDL to membranes from a single placenta and the linear regression analysis of the bound/free compared with ^{125}I-LDL bound/mg protein.

7.2.1.1. Calculation of Scatchard Parameters

$y = mx + c$

$y = - 0.000244\ x + 0.205419$

$r = 0.9498$

When $x = 0$, then $y = c$

Therefore $y = 0.205419$

When $y = 0$, then $x = \frac{-c}{m}$

$$x = \frac{0.205419}{0.000244}$$

$x = 841.881$

Therefore Intercept on y axis = 0.2054
Intercept on x axis = 841.9

Therefore amount of ^{125}I-LDL bound = 841.9 ng protein per mg of membrane protein

Assuming LDL is 21% protein by weight, there are 4009.0 ng LDL per mg of membrane protein.

Assuming a molecular weight for LDL of 2.75×10^6

1 mole	=	2.75×10^6g
1 g	=	$\frac{1}{2.76 \times 10^6}$ moles
1 g	=	3.63636×10^{-7} moles
1 ng	=	3.63636×10^{-16} moles
4.009.0 ng	=	1.4578×10^{-12} moles

Now 1 mole contains 6.022×10^{23} molecules

Therefore 1.4578×10^{-12} moles contain
$(6.022 \times 10^{23}) \times (1.4578 \times 10^{-12})$ molecules

$$= 8.78 \times 10^{11} \text{ molecules}$$

Assuming there is one molecule per receptor:

Number of receptors = 8.78×10^{11} receptors per mg of membrane protein.

The binding constant is calculated from the slope of the line of the Scatchard plot.

The amount of lipoprotein bound per mg of membrane protein in 360 μL of incubation medium in converted to give moles^{-1}.

The dissociation constant (K_d, units of M) is the reciprocal of the slope.

The slope is found by dividing the intercept of the y axis by the intercept of the x axis.

From the previous calculation:

1.44578×10^{-12} moles of LDL are bound in 360 μL of incubation medium

$$\text{Moles of LDL bound/L} = \frac{1.4578 \times 10^{-12}}{360} \times 10^{6}$$

$$= 4.0494 \times 10^{-9} \text{ moles/L}$$

$$\text{Therefore slope} = Ka = \frac{0.2054}{4.0494 \times 10^{-9}}$$

$$Ka = 5.1 \times 10^{7} M^{-1}$$

$$K_d = 1.97 \times 10^{-8} M$$

7.2.2. LDL Binding No. 2

The LDL binding experiment was repeated using the microvillous membrane preparation from a different placenta. The results are shown in the table below.

^{125}I-LDL Added ng	^{125}I-LDL Bound ng	^{125}I-LDL Bound/mg Membranes	Bound / Free
150	1.97	15.74	0.014
300	2.79	22.32	0.010
450	4.26	34.11	0.010
600	9.2	73.59	0.017
750	10.55	84.37	0.015
900	11.16	89.25	0.013
1050	15.23	121.83	0.016

Table 7.9. Results of ^{125}I-LDL Binding Experiment No. 2

7.2.3. Discussion

It is evident from the two experiments that there is 100-fold difference in the amount of ^{125}I-bound to the membranes. It was deduced that the placenta from which the membranes for LDL Binding No. 1 was in an active phase of cholesterol absorption and had a higher than average number of receptors which exhibited a greater than normal affinity, whereas the second placenta was in a quiescent phase. This variation lends some support to the theory of receptor number and receptor affinity control described in Section 1.5..

7.3. RESULTS OF MEMBRANE BINDING STUDIES WITH VLDL, LDL AND HDL USING A POOLED MICROVILLOUS MEMBRANE PREPARATION

7.3.1. VLDL Binding Experiments

Three experiments were performed using ^{125}I-VLDL competing with unlabelled VLDL. The results are shown in Figure 7.5. For these experiments the computer programme written to analyse the results (see Appendix) was modified to feed the data directly into a statistics programme to produce the required graphs.

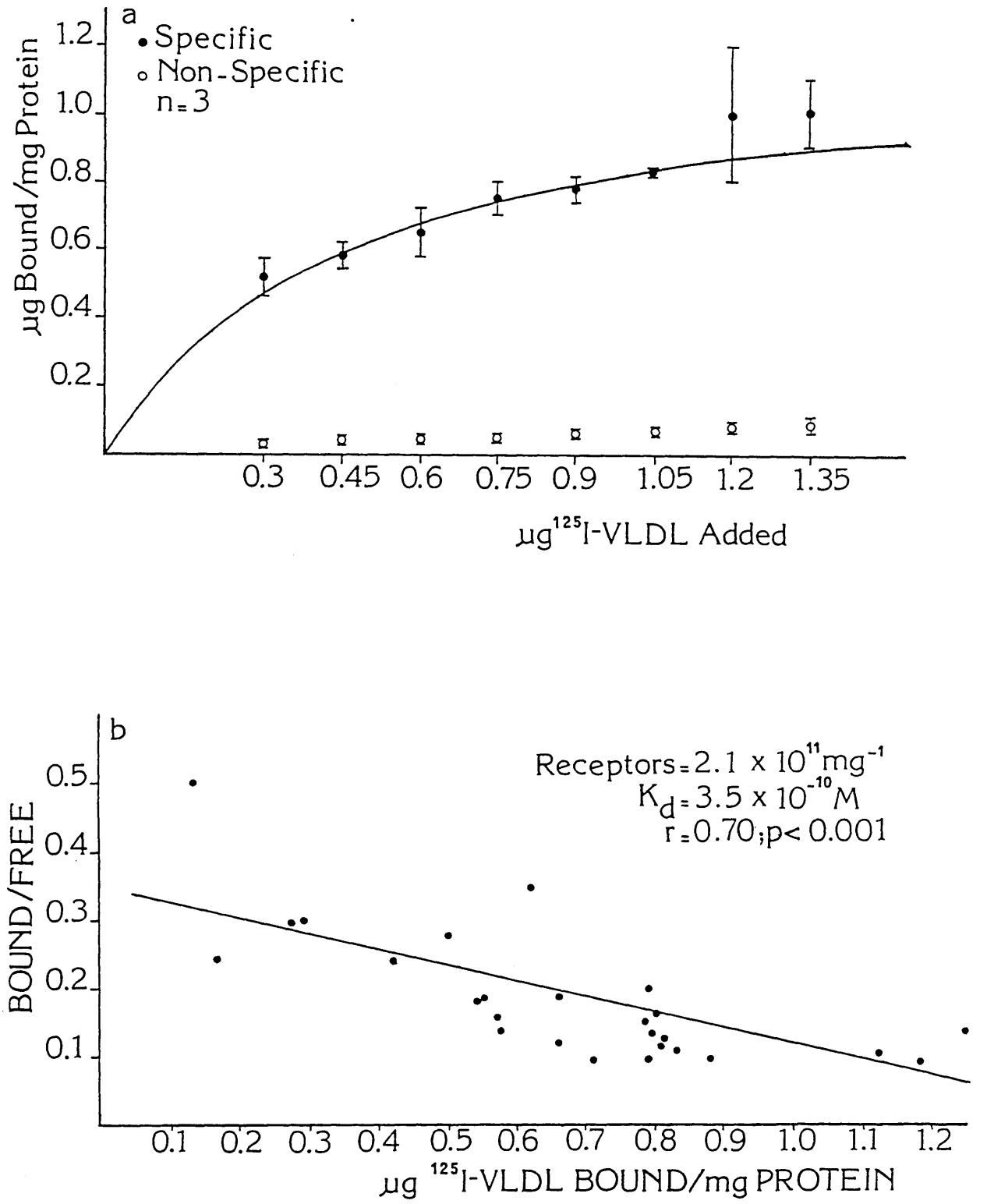


Figure 7.5. VLDL Binding

7.3.2. LDL Binding Experiments

Four experiments were performed using ^{125}I-LDL competing with unlabelled LDL and the results are shown in Figure 7.6.

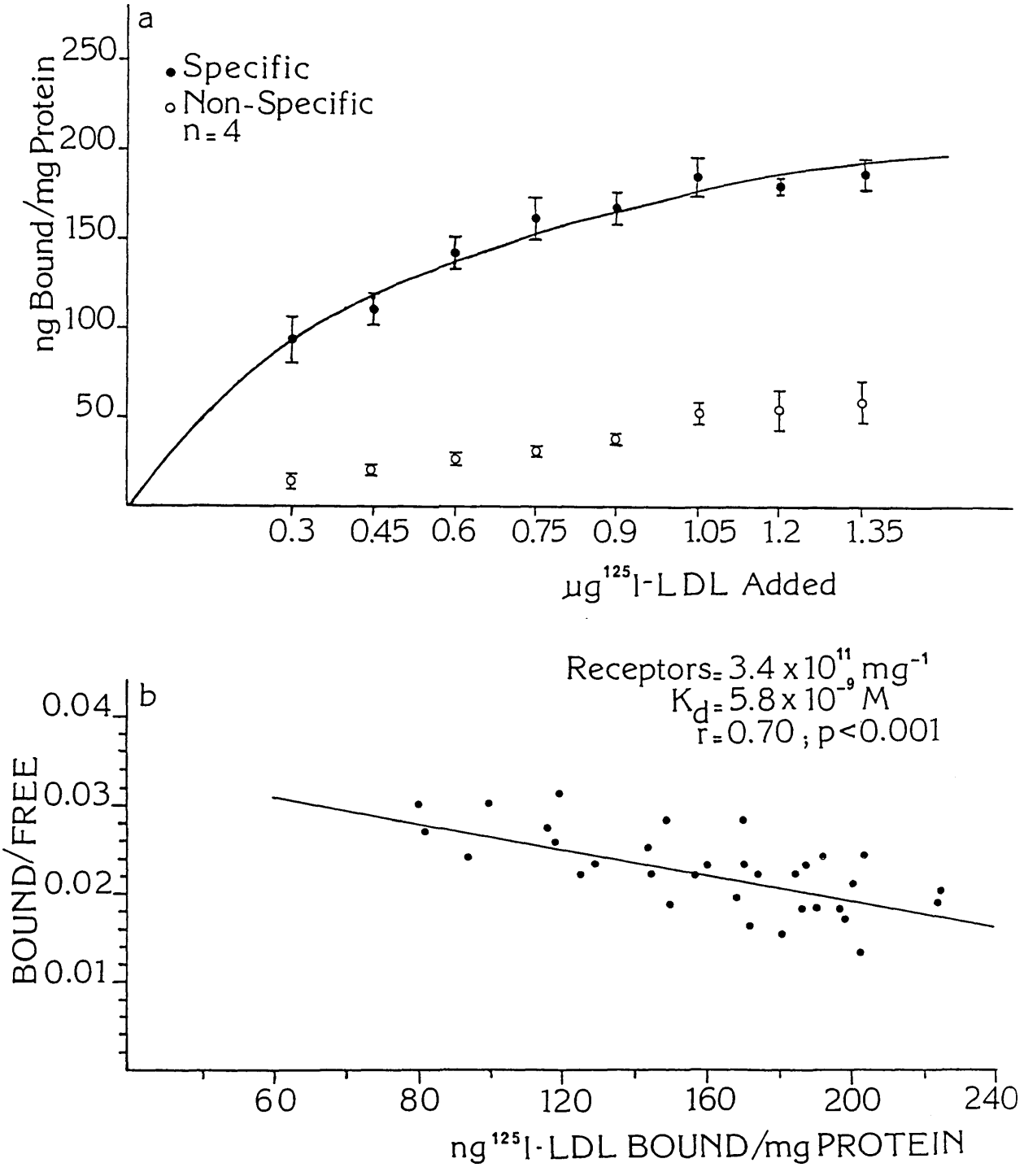


Figure 7.6. LDL Binding

7.3.3. HDL Binding Experiments

The HDL binding experiments were performed three times and the results are shown in Figure 7.7.

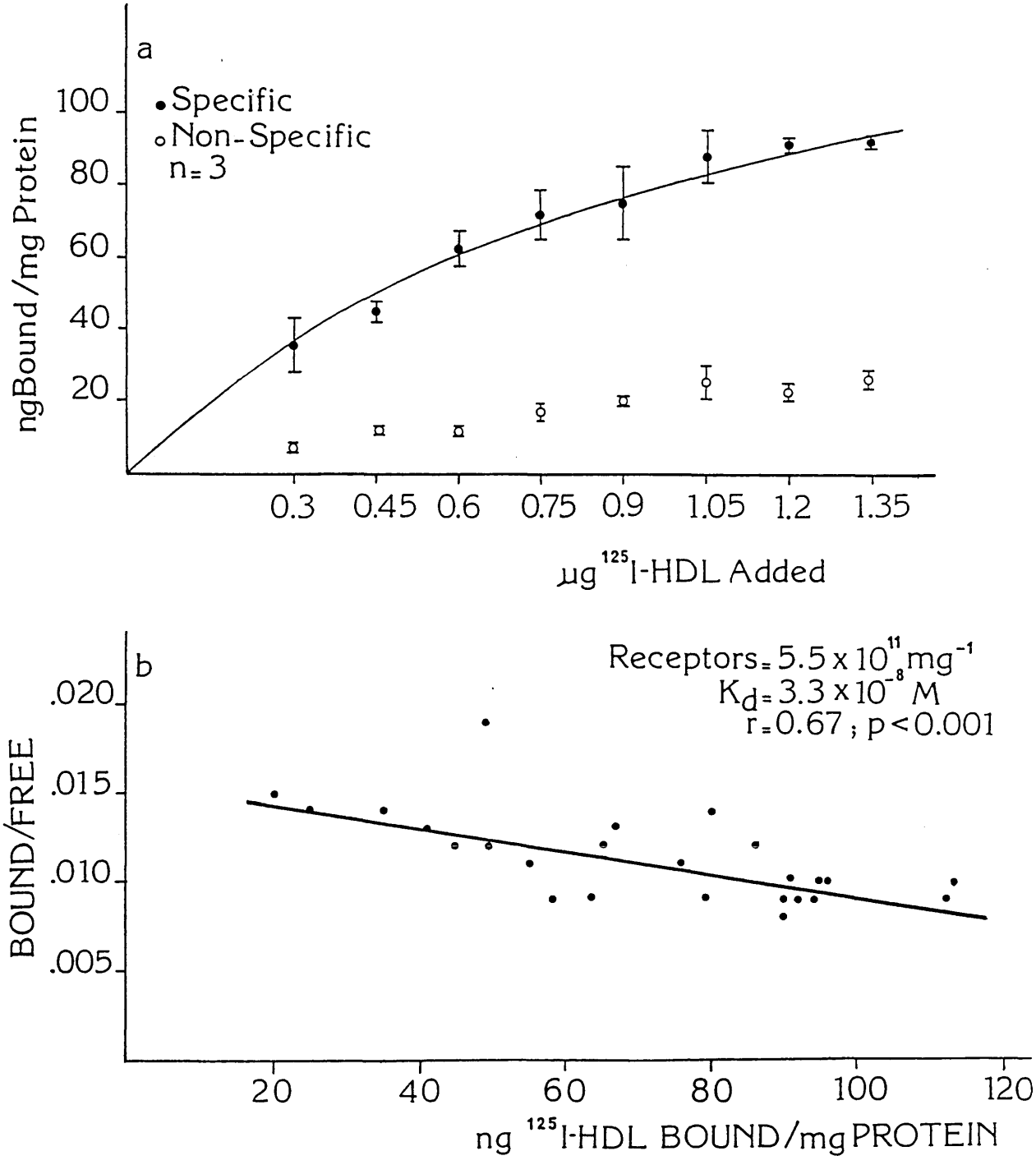


Figure 7.7. HDL Binding

7.3.4. Self- and Cross-Inhibition of VLDL and LDL Binding

The methodology of this experiment is explained in Section 6.4.6.

7.3.4.1. ^{125}I-VLDL Competing with Unlabelled VLDL and LDL

Increasing amounts of unlabelled VLDL and then, in a separate experiment, increasing amounts of unlabelled LDL were competed against ^{125}I-labelled VLDL. Figure 7.8. shows the results of experiments which were each repeated three times.

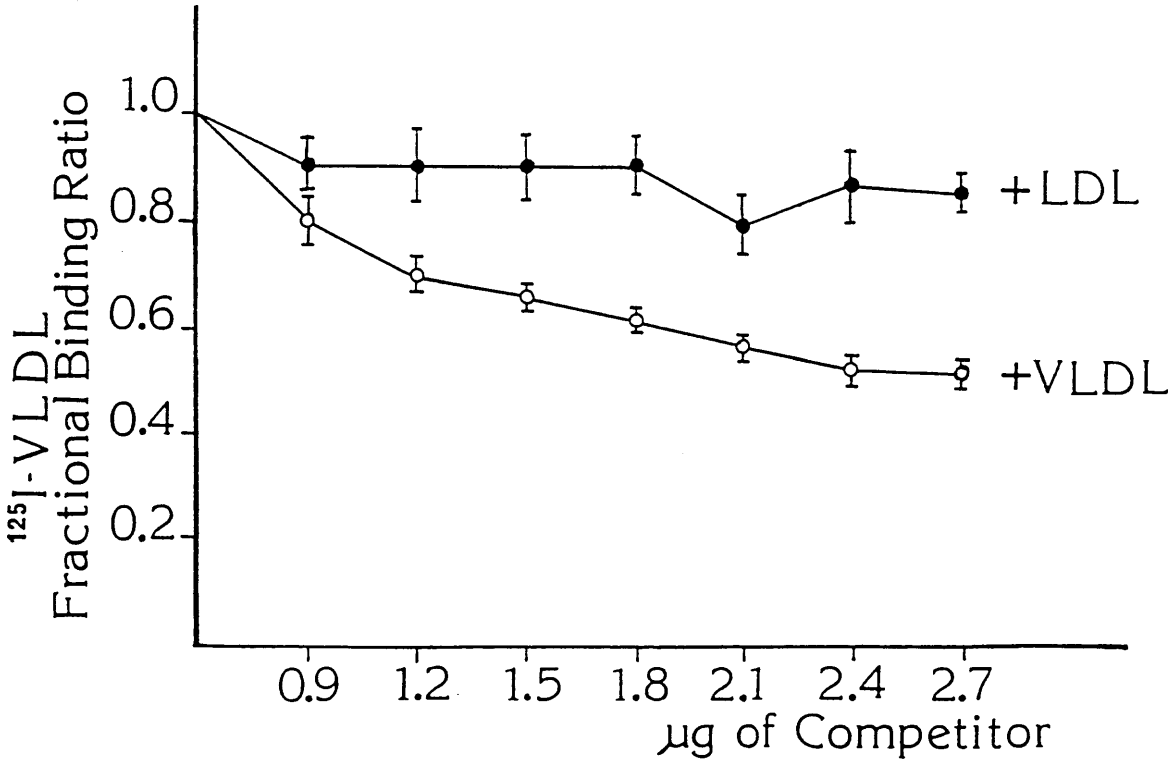


Figure 7.8. Self- and Cross-Inhibition of VLDL

7.3.4.2. ^{125}I-LDL Competing with Unlabelled VLDL and LDL

Increasing amounts of unlabelled VLDL and then, in a separate experiment, increasing amounts of unlabelled LDL were competed against ^{125}I-labelled LDL. These experiments were each repeated three times and the results are shown in Figure 7.9.

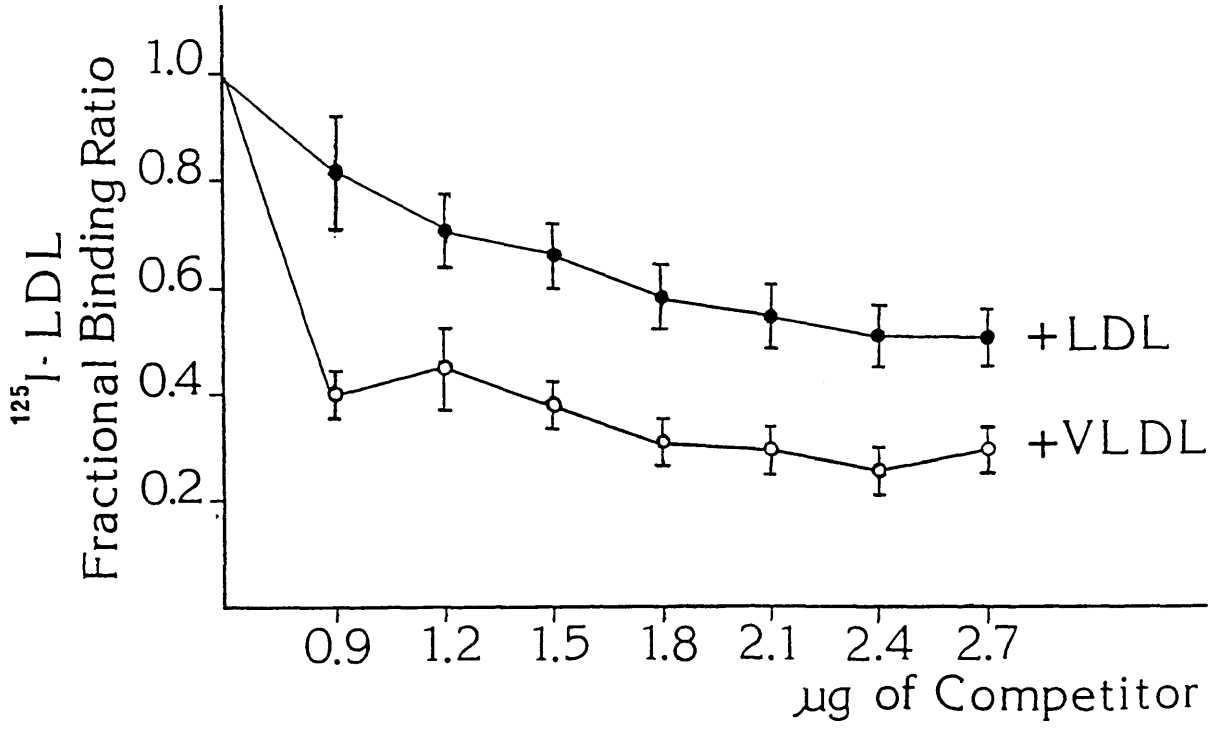


Figure 7.9. Self- and Cross-Inhibition of LDL

7.4. DISCUSSION OF THE RESULTS OF THE MEMBRANE BINDING EXPERIMENTS

The validation of the membrane binding technique used in this study showed that the membranes were all precipitated in the microcentrifuge and that only one wash was required to remove the free lipoprotein. The time course experiment showed that binding was complete by one hour and therefore the concentration experiments were performed with an incubation period of one hour at between 0-4°C. The marked difference in results between the first two LDL binding experiments indicated the necessity of using a pooled microvillous membrane preparation.

Figure 7.5. shows the specific and non-specific binding curves obtained by incubating increasing amounts of ^{125}I-VLDL with 125 μg of microvillous membrane proteins. The data is presented as the mean $\pm$ the standard error of three experiments in which each value was the mean of duplicates. A saturation curve weighted for the reciprocal of the standard errors of the mean has been fitted through the specific binding data. The calculated maximal binding capacity is 1215 $\pm$ 105 ng VLDL protein per mg of membrane protein.

The second part of Figure 7.5. shows a combined Scatchard analysis of the individual points in the three experiments. The linearity of the calculated regression line ($r = 0.70$,

$P < 0.001$) indicates a single class of binding sites. Assuming a mean protein content of 5% in VLDL and a relative molecular mass of 1×10^8 (Mills, Lane and Weech 1984) it can be calculated that there are approximately 2.1×10^{11} binding sites per mg of membrane protein and that the mean affinity constant for the binding is $2.9 \times 10^9 M^{-1}$.

The LDL binding experiments shown in Figure 7.6. were obtained in a similar manner to that described for VLDL except that the data is from four assays. The curve fitted through the specific binding data shows a maximal binding capacity of 270 $\pm$ 16 ng of LDL protein bound per mg of membrane protein which is 4.5-fold less than that of VLDL. The lower panel of the figure shows the Scatchard analysis, which is also linear ($r = 0.70$, $P < 0.001$), and using a relative molecular mass for LDL of 3×10^6 and a protein content of 21%, it can be calculated that there are 3.4×10^{11} receptors per mg of membrane protein with an affinity constant of $1.7 \times 10^8 M^{-1}$.

Results for the specific and non-specific binding for HDL are shown in Figure 7.7. The specific binding shows less tendency to saturate than was seen for LDL or VLDL. However, the fitted curve indicates a maximal binding of 178 $\pm$ 18 ng of HDL bound per mg of membrane protein. This is lower even than that found for LDL. The amount of HDL needed to achieve half-maximal saturation is nearly twice that for LDL. The lower panel shows the Scatchard analysis

of the HDL showing a linear result indicating again a single class of receptors ($r = 0.67$, $P < 0.001$). Using a mean protein content of 50% for HDL and a relative molecular mass of 3.5×10^5 it can be calculated that there are 5.5×10^{11} receptors per mg of membrane protein with an affinity constant of $3.0 \times 10^7 M^{-1}$.

The specificity of the binding of VLDL and LDL to the pooled placental microvillous membrane preparation was examined by increasing the concentrations of unlabelled VLDL and LDL as explained above. The results were expressed as the amount of binding in its presence as a percentage of that measured in the absence of any competitor. Figure 7.8. shows that the binding of ^{125}I-VLDL is strongly inhibited by increasing amounts of VLDL and at the highest concentration tested (2.7.μg of unlabelled competitor) the binding of the ^{125}I-VLDL was only 51% of the binding in the absence of the competitor. However, in the presence of a similar quantity of LDL it was reduced to only 85% of the control value.

In contrast, Figure 7.9. shows the effects of unlabelled VLDL and LDL on the binding of ^{125}I-LDL. It can be seen that while substantial self-inhibition was observed, VLDL was even more effective in blocking the binding of ^{125}I-LDL even at very low concentrations, causing an inhibition, at the highest concentration tested, of 31% of the control binding value.

CHAPTER EIGHT

CONCLUDING DISCUSSION

Particular interest has been paid recently to the role of lipoproteins in steroidogenesis since it has been shown that cholesterol is the major source of steroidogenic substrate in the adrenal gland, ovary and possibly also in the testis (Gwynne and Strauss 1982). The recent increase in evidence for the cellular uptake of large proteins by the mechanism of receptor-mediated endocytosis was initiated by Goldstein and Brown in 1974. Their work on the uptake of LDL by human fibroblasts has given rise to the concept of an LDL receptor which has been characterised in a large number of different cell types (Mahley and Innerarity 1983).

Subsequently the apo-lipoprotein moieties recognised by this receptor have been determined and since they occur in several lipoprotein classes, the receptor is more accurately known as the apo-lipoprotein B, E receptor. Most of the work on the placental apo-lipoprotein B, E receptor has involved its recognition of LDL as the main ligand (Alsat et al 1982, 1984, 1985, Cummings et al 1982, Winkel 1980). LDL is quantitatively the largest of the lipoproteins in the circulation and levels rise significantly during pregnancy.

The investigation of lipid metabolism in the fetus and the placenta is hampered by only being able to investigate certain aspects at any one time due to ethical and methodological difficulties. Workers have measured

maternal and cord plasma lipids in humans and animals; radio-labelled material has been infused into the mother and recovered in fetal blood or tissues; modified lipids have been infused into the mother and recovered in the fetus; placental slices and cultured placental cells have been used to study lipid utilisation, and various placental perfusion systems have been employed. All the methods used have provided important, but limited, data. Each method systematically excludes one or more aspects of the three interactive units; mother, placenta and fetus. Substrates may be metabolised by the placenta and therefore data on the transfer of lipids derived from perfusion methods alone may be difficult to interpret. Likewise, the in vivo techniques are difficult to interpret if fetal tissue simultaneously takes up or synthesises the substrate under investigation.

Early studies (Dancis et al 1973) suggested that placental transport might provide at maximum only 20% of the fatty acid that is stored as triacylglycerol in the human fetus. Jones (1976) suggested that the high rate of fatty acid synthesis by the fetal liver might make fatty acid transfer across the placenta unnecessary for fetal growth.

It has now been shown that in the rabbit, rat, primate and guinea pig lipid transport increases markedly during late gestation and it is believed that the placenta may transport as much as 50% of the fetal requirements for

fatty acids (Battaglia 1978, Munro 1983, Jones 1985, Coleman 1987, Thomas 1987). Ethical considerations have prevented direct data being acquired from human mothers and babies as the investigations require radio-active labelling and sacrificial techniques. However, in the human fetus, the fat stores increase from 1% to 15% of body weight during the third trimester of pregnancy (Coleman 1989). These fat stores are similar in composition to maternal diet and it is therefore reasonable to presume that fatty acid transport is essential for fetal growth during the third trimester of pregnancy (Hull and Elphick 1979).

The early studies employing perfused placentae and cotyledons produced results that are difficult to interpret. The techniques used suffered from methodological problems that have since been understood and modified (see Section 4.1.).

The present study examined two aspects of placental function pertaining to lipid metabolism. The placental uptake of lipoproteins has been examined by perfusing human placentae with radio-labelled lipoproteins. The metabolism of cholesterol has been investigated by incorporating ^{14}C-cholesterol into LDL, which was used in the perfusion of the placenta. There was no significant uptake of LDL by the perfused human placenta, or conversion of ^{14}C-cholesterol in LDL to ^{14}C-progesterone by the placenta.

This led to the study of alternative lipoprotein ligands as a source of cholesterol for the placenta.

Placentae were subsequently perfused using ^{125}I-labelled VLDL, IDL and HDL. From this initial work it appeared that VLDL was the preferred substrate, but further studies employing membrane binding techniques to investigate the apo-lipoprotein B,E receptor were required. Competetive binding between all the lipoprotein classes had not previously been performed, and it was necessary to undertake these investigations to substantiate the hypothesis that VLDL was the principal substrate used by the placenta for the acquisition of cholesterol.

Recently HDL has been recognised as the acceptor molecule for the removal of cholesterol and its transfer to the liver for excretion. It has also been implicated as a means of delivering cholesterol to steroidogenic tissues for hormone synthesis (for review see Norum et al 1983). Previous studies with placental microvillous membranes have indicated only partial inhibition binding by HDL (Alsat 1984) probably due to small amounts of HDLc which contains apo-lipoprotein E, known to have a high affinity for the apo-lipoprotein B,E receptor (Mahley and Innerarity 1983, 1984). Cummings et al (1982), using a mixed membrane preparation prepared from a placental homogenate, found a substantial binding of ^{125}I-HDL suggesting the presence of specific receptors. Winkel et al (1981) observed a linear

uptake of ^{125}I-HDL up to a concentration of 1000 μg/dl, with negligible degradation, and concluded that the binding sites had a low affinity and a high capacity. These sites could not, however, be ascribed to the microvillous membrane as these authors used a culture of mixed placental cells rather than pure trophoblast. It is well known that there are HDL receptors on the endothelium of blood vessels and these cells may have contaminated the preparation.

A well-established role for the apo-lipoprotein B,E receptor in fibroblasts and macrophages involves its ability to bind beta-VLDL (found in hypertriglyceridaemic individuals) but not normal VLDL (Gianturco et al 1978). This is mediated via the apo-lipoprotein E moiety (for review see Sparks and Sparks 1985). However, recent evidence suggests that in fibroblasts normal VLDL does bind to these receptors as would be expected since normal VLDL contains both apo-lipoprotein B and E (Mahley et al 1983, Yamamoto et al 1985, Innerarity et al 1986).

The present study provides the first demonstration of a high capacity, high affinity binding of normolipidaemic VLDL to the human placental microvillous membrane. The maximal binding capacity was over four times greater than that observed for LDL and the affinity constant was one order of magnitude larger than that of LDL. It is assumed that the apo-lipoprotein E moiety in the VLDL is responsible for these findings. It is of interest that the

maximal binding capacity for LDL of 270 ng/mg of membrane protein is twice that reported by Alsat et al in 1982. Two possible causes for these findings are, firstly that the microvillous membrane preparation used here was highly enriched, and secondly Alsat et al used a single placental membrane preparation rather than the pooled preparation found to be necessary in this study. The affinity constant found for LDL is similar to other published values for placental microvillous membranes (Alsat et al 1982, Cummings et al 1982) and also to that found for LDL in other cell types (Mahley and Innerarity 1983).

The present study also demonstrates for the first time the poor affinity of HDL for the microvillous membrane receptors. The HDL preparation used was known to be uncontaminated with HDLc as samples containing apo-lipoprotein E on SDS-gel electrophoresis were discarded. The HDL binding experiments employed a microvillous membrane preparation with minimal contamination from other tissues and used an HDL preparation with no apo-lipoprotein E, and therefore should reflect the true HDL binding characteristics. The finding that the HDL affinity constant is approximately 100 times lower than that for VLDL is in agreement with its poor inhibitory effect on the uptake of LDL demonstrated by Alsat et al in 1982.

The similarity in the number of binding sites determined from the Scatchard analyses, together with their linearity,

suggest that there is a single population of receptor sites for which each lipoprotein class is competing with differing affinities. This is confirmed by the self- and cross-inhibition data which clearly show that VLDL will preferentially bind to the receptors as it can displace ^{125}I-LDL more readily than LDL.

Workers in the laboratory where the work for this thesis was undertaken have recently shown that if ^{14}C-cholesterol is incorporated into VLDL and then perfused in the human placental model as described in Chapter Four, ^{14}C-pregnenelone and ^{14}C-progesterone have been isolated from the placental homogenate (Eaton, B.M., personal communication).

VLDL plays a major role in the transport of cholesterol to the tissues and ultimately in cholesterol homeostasis. The cholesterol ester content of VLDL is 11-14% of its mass and free cholesterol accounts for a further 5.1-8.4%. Thus normal VLDL could be an important source of cholesterol for steroid synthesis. The lipid content of VLDL increases significantly during pregnancy (Farhaeus et al 1985) whereas the rise in LDL is not so significant. These changes which result in the amount of cholesterol carried by VLDL, approaching one-third of that carried by LDL, together with the high affinity of the microvillous membrane receptor for VLDL, strongly suggests that in contrast to other steroidogenic tissues VLDL is an

important source of cholesterol for the human placenta. This would be an efficient mechanism for the acquisition of cholesterol as the amount gained per receptor cycle from VLDL is much greater than the amount of cholesterol obtained per cycle from LDL.

The effects of diabetes on pregnancy-associated hypertriglyceridaemia have been studied by Hollingsworth and Grundy (1982), and Knopp et al (1986). These workers have shown that there is a marked increase in the VLDL triglyceride throughout pregnancy, particularly in the type II (mature onset) and gestational diabetics. Women with gestational diabetes had twice the level of triglyceride than the normal controls and doubled their concentration of triglyceride by the second trimester, maintaining this very high level for the remainder of the pregnancy. Joven et al (1989) showed that in diabetics there was an increase of lipoprotein remnants and that the LDL cholesterol and triglyceride were higher in the type II than the type I diabetes confirming the earlier work by Knopp et al (1982). Ishibashi et al (1989) showed a linear relationship between an increase in the blood glucose and Hb A_{1c} levels and an increase in the blood triglyceride levels.

Szabo and Szabo (1974) first postulated that free fatty acids may have a much greater role to play in causing the macrosomia in babies of diabetic mothers than previously had been thought. Shafrir and Barash (1987) showed that high levels

of triglycerides and free fatty acids in the maternal plasma of the rat promoted an increased passage of fats to the fetus. They suggested that the passage of lipids through the placenta is not direct, but served as an interim storage organ with its lipid content increasing in proportion to the maternal triglyceride and free fatty acid level. They found that VLDL triglycerides were taken up by the placenta and that the size of the free fatty acid pool corresponds to the extracellular fluid space.

It is feasible that the macrosomia occurring predominantly in the babies of gestational diabetics may be caused by the placenta acquiring two to three times the normal amount of triglyceride per molecule of cholesterol, (the probable rate limiting molecule in lipoprotein metabolism). This thesis supports Shafrir's view that the VLDL may be the source of the triglycerides rather than the free fatty acids as suggested by Szabo and Szabo (1974).

It is well known that macrosomia can occur during a pregnancy complicated by diabetes despite what appears to be perfect control of the blood glucose levels, as measured by serial blood glucoses throughout the day, glycosolated proteins and glycosolated haemoglobin. Pederson (1954, 1961) produced an hypothesis that the increased adipose tissue mass in neonates born to diabetic mothers is due to the accelerated triglyceride synthesis by the fetal fat cells. The substrate for this fat synthesis is the

abundant glucose that is transported across the placenta from the maternal to the fetal blood. This hypothesis gained wide acceptance because it grouped the biochemical and physiological observations into a logical sequence of events. Pederson postulated that chronic increase in fetal blood sugar would cause hyperplasia of the fetal pancreatic islets resulting in elevated plasma insulin levels. These two postulates were later proven (Driscoll 1960, Mintz 1972) giving the Pederson hypothesis very strong support.

From the work in this thesis, and from other workers, it would appear that Pederson's hypothesis may not be the only mechanism for the macrosomia found in babies of diabetic mothers.

Further study is needed to ascertain the causative factors of macrosomia. This would involve a prospective study of the Hb A_{1c} levels, blood glucose levels, triglyceride and cholesterol levels in VLDL, LDL and HDL throughout the pregnancy of normal women, type I diabetics, type II diabetics and gestational diabetics. Knopp et al (1985) investigated the relationship of infant birth size to maternal weight, maternal lipoproteins and apo-lipoproteins in normal pregnancies. They found that birth weight and/or birth weight ratio are weakly positively associated with maternal VLDL triglycerides.

There is a high incidence of congenital malformations in uncontrolled diabetics (Pederson 1977, Mills 1982). Currently the most common cause of perinatal death in the infants of diabetic mothers is congenital malformations (Freinkel 1980). The causes of these malformations have not been ascertained, but are thought to be multi-factorial in origin, part of which is due to the disordered lipoprotein metabolism that occurs in uncontrolled diabetics (Eriksson et al 1987). Further investigation of this aspect of lipoprotein metabolism should be considered.

Potter and Nestel (1979) showed that in hypertensive patients there was an increased level of plasma triglyceride and cholesterol and this was particularly marked in the VLDL fraction. Patients with essential hypertension, where there is no placental abnormality, have been observed to have higher birth weight babies (Hytten and Chamberlain, 1980). Further studies are required to examine the relationship between essential hypertension, VLDL triglyceride levels and high birth weight.

APPENDIX

A1.1. Tables of Partial Specific Volume

Partial specific volume (V) of potassium bromide in aqueous solutions of different density (d) and temperature

d	V5	V10	V15	V20	V25
1.0100	0.2688	0.2753	0.2811	0.2863	0.2909
1.0250	0.2712	0.2776	0.2834	0.2885	0.2930
1.0500	0.2751	0.2813	0.2869	0.2919	0.2962
1.0630	0.2769	0.2831	0.2886	0.2934	0.2977
1.0650	0.2773	0.2834	0.2889	0.2938	0.2980
1.0750	0.2787	0.2847	0.2901	0.2949	0.2991
1.1000	0.2820	0.2878	0.2930	0.2976	0.3016
1.1250	0.2851	0.2906	0.2956	0.2999	0.3038
1.1500	0.2880	0.2932	0.2979	0.3020	0.3057
1.2000	0.2933	0.2979	0.3020	0.3058	0.3091
1.2100	0.2944	0.2988	0.3028	0.3064	0.3097
1.2500	0.2980	0.3020	0.3056	0.3089	0.3119

Partial specific volume (V) of sodium bromide in aqueous solutions of different densities (d) and temperature

d	V5	V10	V15	V20	V25
1.0100	0.2074	0.2157	0.2235	0.2308	0.2376
1.0250	0.2110	0.2189	0.2263	0.2332	0.2396
1.0500	0.2165	0.2238	0.2306	0.2369	0.2427
1.0630	0.2192	0.2262	0.2327	0.2386	0.2442
1.0650	0.2195	0.2264	0.2329	0.2388	0.2443
1.0750	0.2214	0.2281	0.2344	0.2401	0.2454
1.1000	0.2257	0.2320	0.2377	0.2430	0.2477
1.1250	0.2297	0.2355	0.2407	0.2455	0.2497
1.1500	0.2332	0.2385	0.2434	0.2477	0.2516
1.2000	0.2392	0.2438	0.2478	0.2514	0.2544
1.2100	0.2403	0.2447	0.2487	0.2521	0.2551
1.2500	0.2443	0.2482	0.2516	0.2545	0.2569

Partial specific volume (V) of sodium chloride in aqueous solutions of different density (d) and temperature

d	V5	V10	V15	V20	V25
1.0100	0.2552	0.2697	0.2826	0.2939	0.3038
1.0250	0.2669	0.2804	0.2925	0.3032	0.3124
1.0500	0.2835	0.2956	0.3063	0.3159	0.3241
1.0630	0.2909	0.3022	0.3123	0.3213	0.3290
1.0650	0.2920	0.3032	0.3132	0.3220	0.3297
1.0750	0.2971	0.3077	0.3173	0.3257	0.3330
1.1000	0.3084	0.3178	0.3262	0.3337	0.3402
1.1250	0.3181	0.3264	0.3339	0.3406	0.3464
1.1500	0.3269	0.3344	0.3412	0.3473	0.3527
1.1900	0.3409	0.3477	0.3539	0.3596	0.3648

The values of v' of the sodium chloride, sodium bromide, potassium bromide at temperatures between 5-25°C. can be obtained from the relevant tables which are derived from data of Baxter and Wallace 1916 and are taken from Laboratory Techniques in Biochemistry and Molecular Biology - A Guidebook to Lipoprotein Technique (Mills, Lane and Weech).

A1.2. Lowry Method for Protein Estimation

Solutions

1. Copper tartrate/carbonate reagent
2. Ciotalteu reagent (BDH Pharmaceuticals)

Method

Copper tartrate/carbonate reagent was made by mixing 0.4 mls of 5% copper sulphate solution with 4.0 mls of 1% sodium tartrate. This solution was slowly added to 200 mls of 2% sodium carbonate with 0.1 Molar sodium hydroxide.

5 mls of copper tartrate/carbonate reagent was added to 1 ml of the protein solution. This was allowed to stand at room temperature for 15 minutes.

0.5 mls of Ciotalteu reagent was added to the solution and allowed to stand for a further 30 minutes at room temperature. The optical density was read with a spectrophotometer at 720 nm. The equivalent protein concentration was read from the prepared optical density curve.

A1.3. Preparation of Visking Tubing

Visking tubing is available in different diameters suitable for different volumes and quantities of dialysis and may be obtained from BDH Pharmaceuticals. The tubing is brown, but after preparation becomes clear.

The tubing is boiled for thirty minutes in 0.2 Molar sodium carbonate and rinsed thoroughly in distilled water, and boiled for a further one hour in 0.7 Molar acetate buffer pH 5.0 containing 0.5 mM EDTA. It was again washed thoroughly with distilled water and stored in a sealed container of 95% ethanol. The tubing is rinsed with distilled water prior to use.

A1.4. Computer Programme to Analyse Binding Experiments

The worker wrote the following programme in BBC BASIC for a BBC B Computer to analyse the results of the lipoprotein binding experiments. This analysis would normally require many duplicated procedures and is ideally suited for a computer programme. The raw data from the cpm counts and efficiency of the counts were entered into the computer programme and a table of nanogrammes added and nanogrammes bound and bound/free were printed out. This table could be fed directly into a statistics programme which produced the linear regression analysis.

```
 10 ENVELOPE 2,5,16,12,8,1,1,1,10,-10,0,-10,100,50
 20 SOUND 1,2,100,100
 30 #%=&20309
 40 REM SCATCHARD ANALYSIS
 50 REM R C S DE C 22.11.85
 60 CLS
 65 CLOSE#0
 70 DIM D P M (2)
 80 PRINT:PRINT
 90 PRINT CHR$(129);CHR$(157);CHR$(131);"    SCATCHARD
    ANALYSIS"
100 PRINT'':INPUT"TITLE OF EXPERIMENT  "Fname$
110 PRINT'':X=OPENOUT(Fname$)
120 PROCSTAN
130 PRINT':INPUT"HOW MANY REPLICATES  ",REP
140 PRINT#X,REP
150 DIM SDPM(REP):DIM PDPM(REP)
160 FIDFAC=0:PROCFIDDLE
170 PRINT':INPUT"HOW MANY SETS OF REPLICATES ARE THERE
    ";NUMSAMP
180 PRINT#X,NUMSAMP
190 DIM DICK(9,NUMSAMP)
200 FOR W=1 TO NUMSAMP
210 PRINT':PRINT CHR$(129);CHR$(157);CHR$(131);"VOLUME OF
    LIGAND  ";:INPUT LIG
220 PRINT#X,LIG
230 DICK(1,W)=LIG
240 DICK(2,W)=(ng/100)*LIG
250 PRINT"ng OF LIGAND = ";DICK(2,W)
260 PROCDPM
270 DICK(3,W)=A
280 DICK(4,W)=B
290 NEXT W
300 PRINT:PRINT
310 PRINT"TYPE IN DATA FOR NON-SPECIFIC BINDING"
320 PRINT
330 FOR W=1 TO NUMSAMP
340 PRINT"DATA FOR ";DICK(1,W);" UL SAMPLES"
350 PROCDPM
360 DICK(5,W)=A
370 DICK(6,W)=B
380 NEXT W
390 REM TABLE OF SPECIFIC BINDING AND BOUND/FREE
400 CLS
410 PRINT':PRINT CHR$(129);CHR$(157);CHR$(131);"TABLES OF
    SPECIFIC BINDING AND ":PRINT CHR$(129);CHR$(157);
    CHR$(131);"  BOUND/FREE FOR   ";Fname$
420 FOR W= 1 TO NUMSAMP
430 DICK(7,W)=(DICK(4,W)-DICK(6,W))/DPMNG
440 DICK(8,W)=(DICK(4,W)-DICK(6,W))/DICK(3,W)
445 DICK(9,W)=(DICK(7,W)*(1000/umem)
450 NEXT W
451 PRINT'':PRINT"SAMP. VOL.  M.CORR.SUP.DPM
    M.CORR.PELL.DPM"
453 FOR W=1 TO NUMSAMP
454 #%=&2010C
```

```
 455 PRINT DICK(1,W),DICK(3,W),DICK(4,W)
 458 NEXT W
 460 PRINT: PRINT
 461 PRINT'''"  SAMP.VOL.  XS.M.CORR.SUPP.DPM
     XS.M.CORR.PELL.DPM"
 463 FOR W=1 TO NUMSAMP
 465 PRINT DICK(1,W),DICK(5,W),DICK(6,W)
 468 NEXT W
 470 PRINT '''" ng ADDED"," ng BOUND"," ng BOUND/mg"," B/F"
 480 FOR W=1 TO NUMSAMP
 481 #%=&20309
 490 PRINT DICK(2,W),DICK(7,W),DICK(9,W),DICK(8,W)
 500 NEXT W
 510 CLOSE#X
1010 END
1020 DEF PROCDPM
1030 FOR Z=0 TO REP:SDPM(Z)=0:NEXT Z
1040 FOR D=1 TO REP
1050 INPUT"SUPERNATANT CPM  ",SCPM
1051 PRINT#X,SCPM
1070 INPUT"EFFICIENCY (%)  "EFFS
1071 PRINT#X,EFFS
1080 IF EFFS<0 GOTO 1070
1090 IF EFFS>100 GOTO 1070
1100 SDPM(D)=(SCPM*100)/EFFS
1120 NEXT D
1130 LET A=0
1140 FOR D=1 TO REP
1150 LET A=A+SDPM(D)
1160 NEXT D
1170 LET A=A/REP
1180 A=INT((A*10)+0.5)/10
1190 PRINT''
1200 PRINT CHR$(129);CHR$(157);CHR$(131);"MEAN
     SUPERNATANT DPM=  ";A
1210 FOR Z=0 TO REP:PDPM(Z)=0: NEXT Z
1220 FOR  D=1 TO REP
1230 INPUT"PELLET CPM  ",PCPM
1231 PRINT#X,PCPM
1240 INPUT"EFFICIENCY (%)  ",EFFS
1241 PRINT#X,EFFS
1250 IF EFFS <0 GOTO 1240
1260 IF EFFS >100 GOTO 1240
1270 PDPM(D)=(PCPM*100)/EFFS
1290 NEXT D
1300 LET B=0
1310 FOR D=1 TO REP
1320 LET B=B+PDPM(D)
1330 NEXT D
1340 LET B=B/REP
1350 B=INT((B*10)+0.5)/10
1360 PRINT''
1370 PRINT CHR$(129);CHR$(157);CHR$(131);"MEAN PELLET
     DPM=  ";B
1380 PRINT:PRINT"IS DATA CORRECT"
1390 PRINT:PRINT"YES OR NO"
```

```
1400 INPUT I$
1405 PRINT#X,I$
1410 IF LEFT$(I$,1)<>"Y"THEN PRINT"RE-ENTER DATA  "
     :GOTO 1030
1420 A=A+((B*FIDFAC)/100)
1430 PRINT:PRINT CHR$(129);CHR$(157);CHR$(131);"CORRECTED
     MEAN SUPERNATANT DPM=  ";A
1440 B=B-((B*FIDFAC)/100)
1450 PRINT:PRINT CHR$(129);CHR$(157);CHR$(131);"CORRECTED
     MEAN PELLET DPM=  ";B
1460 ENDPROC
2000 DEF PROCFIDDLE
2010 PRINT'':PRINT "% BOUND TO TUBE"
2030 PRINT:PRINT"ENTER BLANK COUNTS"
2050 PROCDPM
2130 FIDFAC=(B/A)*100
2140 PRINT''CHR$(131);CHR$(157);CHR$(132);"% BOUND TO
     TUBE",FIDFAC
2150 ENDPROC
2995 DEF PROCSTAN
3000 PRINT ''':PRINT"MIX STANDARDS"
3010 PRINT "INPUT STANDARD DATA"
3020 INPUT"NUMBER OF STANDARDS",NUMSTAN
3021 PRINT#X,NUMSTAN
3022 DIM STDPM(NUMSTAN)
3040 FOR I=1 TO NUMSTAN
3060 INPU"CPM  ",CPM
3061 PRINT#X,CPM
3070 INPUT"EFFICIENCY (%)  ",EFF
3071 PRINT#X,EFF
3080 IF EFF <0 THEN 3070
3090 IF EFF >100 THEN 3070
3100 STDPM(I)=(CPM*100)/EFF
3105 PRINT STDPM(I)
3120 NEXT I
3130 REM SUBROUTINE TO CHECK DATA
3140 PRINT"IS DATA CORRECT?"
3150 PRINT"YES OR NO"
3155 INPUT I$
3156 PRINT#X,I$
3160 IF LEFT$(I$,1)<>"Y" THEN PRINT"RE-ENTER DATA"
     :GOTO 3040
3190 REM MEAN VALUE
3200 LET A=0
3210 FOR I=1 TO NUMSTAN
3220 LET A=A+STDPM(I)
3230 NEXT I
3240 LET A=A/(NUMSTAN)
3250 PRINT"NUMBER OF SAMPLES =  ";NUMSTAN
3260 PRINT
3270 A=INT((A*10)+0.5)/10
3280 PRINT"MEAN DPM =  ";A
3290 REM DIFF SQUARED BETWEEN EACH SAMPLE AND
     AVERAGE AND COLLECT IN D
3300 LET D=0
3310 FOR I=1 TO NUMSTAN
```

```
3320 LET D1=STDPM(I)-A
3330 LET D=D+(D1*D1)
3340 NEXT I
3350 REM VARIANCE V AND STANDARD DEVIATION S
3360 LET V=D/(NUMSTAN-1)
3370 PRINT'':PRINT"VARIANCE =  ",V
3380 LET S=SQR(V)
3390 PRINT'':PRINT"STANDARD DEVIATION =  ";S
3400 REM COEFFICIENT OF VARIATION
3410 CV=(S/A)*100
3420 PRINT'':PRINT"COEFFICIENT OF
     VARIATION  =  ";CV
3430 INPUT"VOLUME OF LIGAND IN STANDARD IN uL  ",VOL
3431 PRINT#X,VOL
3440 INPUT"CONCENTRATION OF LIGAND  ng/100uL",ng
3441 PRINT#X,ng
3450 numng=(ng/100)*VOL
3460 DPMNG=A/numng
3470 PRINT"DPM/ng = ";DPMNG
3480 INPUT"WHAT WEIGHT OF MEMBRANES ",umem
3481 PRINT#X,umem
3490 ENDPROC
```

REFERENCES

Alaupovic, P., Curry, M.D., McConathy, W.J., Fesmire, J.D. (1979), in Report on the High Density Lipoprotein Workshop, NIH, Publication No. 79 - 1661, pp 227-240, US Dept of Health, Education and Welfare, Bethesda, USA.

Alaupovic, P. (1980) in CRC Handbook of Electrophoresis. ed. Lewis, L.A., Opplt, J.J. Vol. 1, pp 27-46, CRC Press, Boca Raton, USA

Alaupovic, P., McConathy, W.J., Fesmire, J.D. (1983) in Report of Apo-Lipoprotein Methods Workshop. ed. Lippel, K. NIH Publication.

Alsat, E., Bouali, Y., Goldstein, S., Malassine, A., Laudat, M., Cedard, L. (1982) Characterization of specific low density lipoprotein binding sites in human term placental microvillous membranes. Mol. Cell. Endocrinol., 28: 439-453

Alsat, E., Bouali, Y., Goldstein, S., Malassine, A., Berthelier, M., Mondon, F. and Cedard, L. (1984) Low density lipoprotein binding sites in the microvillous membranes of human placenta at different stages of gestation. Mol. Cell. Endocrinol. 38: 197-203

Alsat, E., Mondon, F., Rebourcet, R., Berthelier, M., Erlich, D., Cedard, L. and Goldstein, S. (1985) Identification of specific binding sites for acylated low density lipoprotein in microvillous membranes from human placenta. Mol. Cell. Endocrinol. 41: 229-235

Anderson, N.G., Waters, D.A., Fisher, W.D., Cline, G.B., Nunley, C.E., Elrod, L.H., Rankin, C.T. Jr. (1967) Analytical techniques for cell fractions. V. Characteristics of the B-XIV and B-XV zonal centrifuge rotors. Anal. Biochem. 21: 235-252

Anderson, R.G.W., Goldstein, J.L., Browne, M.S. (1976) Localisation of low density lipoprotein receptors on plasma membrane of normal human fibroblasts and their absence in cells from a familial hypercholesterolaemia homozygote. Proc. Natal. Acad. Sci. USA. 73: 2434-38

Anderson, R.G.W., Browne, M.S., Goldstein, J.L. (1977) Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. Cell 10: 351-64

Applebaum-Bowden, D., McLean, P., Steinmetz, A., Fontana, D., Matthys, C., Warnick, G.R., Cheung, M., Albers, J.J., Hazzard, W.R. (1989) Lipoprotein, apolipoprotein, and lipolytic enzyme changes following estrogen administration in postmenopausal women. J. Lip. Res. 30: 1895-1905

Bagdade, J.D., Porte, D., Bierman, E.L. (1967) Diabetic Lipaemia: A form of acquired fat-induced lipaemia. N. Engl. J. Med. No. 276: 427-433

Basu, K.S., Goldstein, J.L., Brown, M.S. (1978) Characterization of the low density lipoprotein receptor in membranes prepared from human fibroblasts. The Journal of Biological Chemistry, 253, No. 11, 3852-3856

Battaglia, F.C., Meschia, G. (1978) Principal substrates of fetal metabolism. Physiol. Rev. 58: 499-527

Battaglia, F.C., Hay, W.W. Jr. (1984) Energy and substrate requirements for fetal and placental growth and metabolism. In Beard, R.W., Nathanielsz, P.W. Eds. Fetal Physiology and Medicine. New York, Dekker pp 601-628

Baxter, G.P. and Wallace, C.C. (1916) Changes in volume upon solutions in water of the halogen salts of the alkali metals. Part II. J. Amer. Chem. Soc. 38: 70-105

Bierman, E.L., Amaral, A.P., Belknap, B.H. (1966) Hyperlipidaemia in diabetes mellitus. Diabetes No. 15, 675-679

Bilheimer, D.W., Eisenberg, S. and Levy, R.I., (1972) The metabolism of Very Low Density Lipoproteins. I. Preliminary in vitro and in vivo observations. Biochim. Biophys. Acta 260: 212

Bowcott, J.E., Schulman, J.H. (1955) Emulsions: control of droplet size and phase continnuity in transparent oil-water dispersions stabilized with soap and alcohol. Elektrochem. 59: 283-290

Braunsberg, H. (1984) Mathematical Analysis of Data from Receptor Assays Recent Results in Cancer Research Vol 91: 18-31

Bretscher, M.S. (1984) Endocytosis: relation to capping and cell locomotion. Science, 244: 681-86

Bretscher, M.S., Pearse, B.M.F. (1984) Coated pits in action. Cell, 38: 3-4

Burstein, M., Scholnick, H.R., Morfin, R. (1970) Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. J. Lip. Res. 11: 583-595

Burstein, M., Scholnick, H.R. (1973) in Advances in Lipid Research eds. Paoletti, R., Kritchevsky, D. pp 67-108. Academic Press, New York

Carpenter, G., Cohen, S. (1979) Epidermal growth factor. Ann. Rev. Biochem. 48: 193-216

Cohn, E.J., Gurd, F.R.N., Surgenor, D.M., Barnes, B.A., Brown, R.K., Derouaux, G., Gillespie, J.M., Kahnt, F.W., Lever, W.F., Liu, C.H., Mittelman, D., Mouton, R.F., Schmid, K., Uroma, E. (1950) A system for the separation of the components of human blood: quantitative procedures for the separation of the protein components of human plasma. J. Amer. Chem. Soc. 72: 465-474

Coleman, R.A., Haynes, E.B. (1987) Synthesis and release of fatty acids by human trophoblast cells in culture. J. Lip. Res. 28: 1335-1341

Coleman, R.A. (1989) The role of the placenta in lipid metabolism and transport. Seminars in Perinatology, 13, No. 3, 180-191

Contractor, S.F., Pearlman, W.H. (1960) Progesterone metabolism in advanced pregnancy and in oophorectomized-hysterectomized women: urinary ketonic metabolites. Biochem.J. 76: 36-41

Contractor, S.F., Das, I., Oakey, N.P. (1982) Separation and characterization of different areas constituting the syncytiotrophoblast membrane. Biochem. Soc. Trans. 10: 39

Contractor, S.F., Stannard, P.J. (1983) The use of AIB transport to assess the suitability of a system of human placental perfusion for drug transport studies. Placenta, 4: 19-30)

Contractor, S.F., Eaton, B.M., Firth, J.A., Bauman, K.F. (1984) A comparison of the effects of different perfusion regimes on the structure of the isolated human placental lobule. Cell Tissue Res. 237: 609-617

Contractor, S.F., Eaton, B.M. (1986) Role of Transferrin in iron transport between maternal and fetal circulations of a perfused lobule of human placenta. Cell Biochemistry and Function 4: 69-74

Dana, S.E., Brown, M.S., Goldstein, J.L. (1977) Specific saturable and high affinity binding of ^{125}I-LDL to glass beads. Biochem. Biophys. Res. Commun. 74: 1369-1376

Dancis, J., Jansen, V., Kayden, H.J. et al (1973) Transfer across perfused human placenta ii. Free fatty acids. Paediat. Res. 7: 192-197

Driscoll, S.G., Benirschke, K., Curtis, G.W. (1960) Neonatal deaths among infants of diabetic mothers. Postmortem findings in ninety-five infants. AMA J. Dis. Child. 100: 818-835

Eaton, B.M., Browne, M.J., Contractor, S.F. (1985) Maternal to fetal movement of creatinine as a measure of perfusion efficiency and diffusional transfer in the isolated human placental lobule. Placenta, 6: 341-346

Elphick, M.C., Filshie, G.M., Hull, D. (1978) The passage of fat emulsion across the human placenta. British Journal of Obstetrics and Gynaecology. 85: 610-618

Elphick, M.C., Edson, J.L., Hull, D. (1978) Effect of maternal glucose infusions on fatty acid transport across the placenta in rabbits. Biol. Neonate 34: 231-237

Eriksson, U.J., Karlsson, M-G., Styrud, J. (1987) Mechanisms of congenital malformations in diabetic pregnancy. Biol. Neonate, 51: 113-118

Farhaeus, L., Larsson-cohn, U. and Wallentin, L.(1985) Plasma Lipoproteins Including High Density Lipoprotein Subfractions During Normal Pregnancy. Obstetrics & Gynaecology Vol 66 No.4. 468-472

Folkman, J., Cole, P., Zimmerman, S. (1966) Tumour behaviour in isolated perfused organs in vitro growth and metastases of biopsy material in rabbit thyroid and canine intestinal segment. Annals of Surgery 164: 491-502

Fredrickson, D.S., Gotto, A.M., Levy, R.I. (1972) Familial lipoprotein deficiency. The metabolic basis of inherited disease Ed. Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., McGraw-Hill, New York, 493-530

Gianturco, S.H., Gotto, A.M., Jackson, R.L., Patsch, J.R., Sybers, H.D., Taunton, O.D., Yeshurun, D.L., Smith, L.C. (1978) Control of 3-hydroxy-3-methylglutaryl-CoA reductase activity in cultured human fibroblasts by very low density lipoproteins of subjects with hypertriglyceridaemia. J. Clin. Invest. 61: 320-328

Gofman, J.W., De Lalla, F., Glazier, N.K. Freeman, F.T., Lindgren, A.V., Nichols, B., Strisower, B., Tamplin, A.R. (1954) Serum lipoprotein transport system in health, metabolic disorders, atherosclerosis and coronary heart disease. Plasma 2: 413-484

Goldstein, B., Wofsy, C., Bell, G. (1981) Interactions of low density lipoprotein receptors with coated pits on human fibroblasts: estimate of the forward rate constant and comparison with the diffusion limit Proc. Natal. Acad. Sci. USA, 78: 5695-98

Goldstein, J.L., Brown, M.S. (1974) Binding and degradation of low density lipoproteins by cultured human fibroblasts: comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolaemia. J. Biol. Chem. 249: 5153-62

Goldstein, J.L., Buja, L.M., Anderson, R.G.W., Brown, M.S. (1978) Receptor-mediated uptake of macromolecules and their delivery to lysosomes in human fibroblasts. in Protein turnover and lysosome function Ed. Segal, H.L., Doyle, D.F. p.455-77, New York: Academic Press

Goldstein, J.L., Brown, M.S., Anderson, R.G.W., Russell, D.W., Schneider, W.J. (1985) Receptor-mediated endocytosis: Concepts emerging from the LDL receptor system. Am. Rev. Cell Biol. 1: 1-39

Gwynne, J.T. and Strauss, J.F. 3d (1982) The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. Endocrine. Rev. 82 Summer; 3(3): 299-329

Havel, R.J., Eder, H.A., Bragdon, J.H. (1955) The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest. 34: 1345-1356

Heiss, G., Johnson, N.J., Reiland, S., Davis, C.E. and Tyroler, H.A. (1980) Epidemiology of plasma high density lipoprotein cholesterol levels: The Lipid Research Clinic's programme prevalence study. Circulation, 62: Suppl IV, 116-136

Hollingsworth, D.R., Grundy, S.M. (1982) Pregnancy-associated hypertriglyceridaemia in normal and diabetic women. Diabetes, 31: 1092

Howard, B.V. (1987) Lipoprotein metabolism in diabetes mellitus. J. Lip. Res. 28: 613-628

Hull, D., Elphick, M.C. (1979) Evidence for fatty acid transfer across the human placenta; in Beard, Hoet, Pregnancy metabolism, diabetes and the fetus. Ciba Fdn. Symp. 75-86 (Excerpta Medica, Amsterdam)

Hummel, L., Schirrmeister, W. and Zimmerman, T. (1975) Quantitative studies on the metabolism of placental triglycerides and phospholipids in the rat. Acta Biol. Med. Ger. 32 (4): 311-314

Hummel, L., Schinckmann, R., Zimmerman, T. (1983) Maternal and fetal transfer of free fatty acids during late gestation in the rat. Biomed. Biochim. Acta 42 (1): 143-145

Hytten, F., Chamberlain, G. (1980) Eds. Clinical Physiology in Obstetrics. Blackwell Scientific Publications, Oxford, UK.

Innerarity, T.L., Arnold, K.S., Weisgraber, K.H., Mahley, R.W. (1986) Apolipoprotein-E is the determinant that mediates the receptor uptake of β-very low density lipoproteins by mouse macrophages. Arteriosclerosis. 6: 114-122

Ishibashi, S., Yamada, N., Shimano, H., Takaku, F., Akanuma, Y., Murase, T. (1989) Composition of Very Low Density Lipoproteins in Non-Insulin Dependent Diabetes Mellitus. Clin. Chem. 35: No. 5, 808-812

Jones, C.T. (1976) Fetal metabolism and fetal growth. J. Reprod. Fert. 47: 189-201

Jones, C.T., Rolph, T.P. (1985) Metabolism during fetal life: a functional assessment of metabolic development. Physiol. Rev. 65: 356-430

Joven, J., Vilella, E., Costa, B., Turner, P.R., Richart, C., Masana, L. (1989) Concentrations of lipids and apo-lipoproteins with patients with clinically well-controlled insulin-dependent and non-insulin-dependent diabetes. Clin. Chem. 35: No.5, 813-816

Knopp, R.H., Warth, M.R. and Carroll, C.J. (1973) Lipid metabolism in pregnancy. I. Changes in lipoprotein triglyceride and cholesterol in normal pregnancy and the effects of diabetes mellitus. J. Reproductive Med. 10: 95

Knopp, R.H., Bergelin, R.O., Wahl, P.W., Walden, C.E., Chapman, M., Irvine, S. (1982) Population-based lipoprotein lipid reference values for pregnant women compared to non-pregnant women classified by sex hormone usage. Am. J. Obstet. Gynaecol. 143: 6, 627-637

Knopp, R.H., Bergelin, R.O., Wahl, P.W. and Walden, C.E. (1985) Relationships of infant birth size to maternal lipoproteins, apo-proteins, fuels, hormones, clinical chemistries, and body weight at 36 weeks gestation. Diabetes 34: Suppl. 2, 71-77

Knopp, R.H., Warth, M.R., Charles, D., Childs, M., Li, J.R., Mabuchi, H., Van Allen, M.I. (1986) Lipoprotein metabolism in pregnancy, fat transport to the fetus and the effects of diabetes. Review Biol. Neonate, 50: 297-317

Krantz, K.E., Panos, T.C., Evans, J. (1962) Physiology of maternal fetal relationships through the extracorporeal circulation of the human placenta. Am. J. Obstet. Gynecol. 83: 1214-1228

Lindgren, F.T. (1975) in Analysis of lipids and lipoproteins. ed. Perkins, E.G. in American Oil Chemists Soc., Champaign, IL, USA, pp 204-224

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265

McFarlane, A.S., (1958) Efficient trace labelling of proteins with iodine. Nature (London), 182: 53

Maguire, G.F., Lee, M., Connelly, P.W. (1989) Sodium dodecyl sulphate-glycerol polyacrylamide slab gel electrophoresis for the resolution of apo-lipoproteins J.Lip.Res. 30: 757-761

Mahley, R.W. (1981) Cellular and molecular biology of lipoprotein metabolism in atherosclerosis. Diabetes 30: (Suppl), 60-65

Mahley, R.W. (1982) Medical Clinics of North America: Lipid Disorders (Havel, R.J. Ed.) Volume 66, 375-402 W.B. Saunders, Philadelphia

Mahley, R.W., Innerarity, T.L. (1983) Lipoprotein receptors and cholesterol homoeostasis (Biochemica et Biophysica Acta, 737: 197-222

Miller, R.K., Koszalka, T.R., Brent, R.L. (1976) The transport of molecules across placental membranes in: Poste G, Nicolson, G.L. (Eds) The Cell Surface in Animal Embryogenesis and Development. Elsevier, Amsterdam pp 145-223

Mills, G.L., Lane, P.A., Weech, P.K. (1984) in Laboratory Techniques in Biochemistry and Molecular Biology - A Guidebook to Lipoprotein Technique eds. Burdon, R.H., van Knippenberg, P.H., Elsevier, Amsterdam

Mills, J.L. (1982) Malformations in infants of diabetic mothers. Teratology. 25: 385-394

Mintz, D.H., Chez, R.A., Hutchison, D.L. (1972) Sub-human primate pregnancy complicated by streptozotocin-induced diabetes mellitus. J. Clin. Invest. 51: 837-847

Mostov, K.E., Friedlander, M., Blobel, G. (1984) The receptor for transepithelial transport of IgA and IgM contains multiple immunoglobin-like domaines. Nature, 308: 37-43

Munro, H.N., Pilistine, S.J., Fant, M.E. (1983) The placenta in nutrition. Ann. Rev. Nutr. 3: 97-124

Nikkila, E.A. (1984) in Plasma lipid and lipoprotein abnormalities in diabetes in Diabetes and Heart Disease Ed. Jarrett, R.J. Elsevier, Amsterdam 133-167

Noble, R. (1968) Electrophoretic separation of plasma lipoproteins in agarose gel. J.Lipid Res. 9: 693-700

Norum, K.R., Berg, T., Helgerud, P., Drevon, C.A. (1983) Transport of cholesterol. Physiol. Review. 63: 1343-1419

Octave, J-N., Schneider, Y.-J., Trouet, A., Crichton, R.R. (1983) Iron uptake and utilisation by mammalian cells. I: Cellular uptake of transferrin and iron. Trends Biochem. Sci. 8: 217-220

Osborne, J.C. and Brewer, H.B. (1977) Advances in protein chemistry. ed. Anfinsen, C.B., Edsall, J.T., Richards, F.M. Vol.31, p 253-337, Academic Press, New York

Ouchterlony, O. (1970) Handbook of Immunodiffusion and Immunoelectrophoresis. Ann Arbor Science Publications Inc. USA.

Paavola, L.G., Strauss, J.F. III, Boyd, C.O., Nestler, J.E. (1985) Uptake of Gold- and (^{3}H)Cholesteryl Linoleate-labelled Human Low Density Lipoprotein by Cultured Rat Granulosa Cells: Cellular Mechanisms Involved in Lipoprotein Metabolism and Their Importance to Steroidogenesis. J. Cell Biol. 100: 1235-1247

Panigel, M. (1971) Morphological evaluation of perfused tissues. In: Diczsalusy, E. (Ed) Karolinska Symposia on Research Methods in Reproductive Physiology. 4th Symposium: Perfusion Techniques. Karolinska Institute, Stockholm, p 74-94.

Pastan, I.H., Willingham, M.C. (1982) Journey to the centre of the cell: role of the receptosome Science, 214: 504-9

Patsch, J.R., Sailer, S., Costner, G., Sandhofer, F., Holasek, A., Braunsteiner, H. (1974) Separation of the main lipoprotein density classes from human plasma by rate zonal ultracentrifugation. J. Lipid Res. 15: 356-366

Patsch, W., Schonfeld, G., Gotto, A.M. Jnr., Patsch, J.R. (1980) Characterization of human high density lipoproteins by zonal ultracentrifugation. The J. of Biological Chemistry, 255: No.7, 3178-3185

Pearse, B.M.F. (1975) Coated vesicles from pig brain: purification and biochemical characterization. J. Mol. Biol. 97: 93-98

Pederson, J. (1954) Weight and length at birth of infants of diabetic mothers. Acta Endocr. 16: 330-342

Pederson, J. (1954) Fetal mortality in diabetics in relation to management during latter part of pregnancy. Acta Endocr. 15: 282-294

Pederson, J. (1954) Glucose content of amniotic fluid in diabetic pregnancies; correlations with maternal blood sugar. Acta Endocr. 15, 342-350

Pederson, J., Osler, M. (1961) Hyperglycaemia as the cause of characteristic features of the fetus and newborn of diabetic mothers. Dan. Med. Bull. 8: 78-83

Pederson, J. (1977) The pregnant diabetic and her newborn. 2nd Ed. Pp 1-280 (Munksgaard, Copenhagen)

Penfold, P., Drury, L., Symonds, R., Hytten, F. (1981) Studies on a single placental cotyledon in vitro. 1. The preparation and its viability. Placenta 2: 149-154

Pernis, B. (1985) Internalisation of lymphocyte membrane components. Immunol.Today, 6: 45-49

Pernis, B., Tse, D.B. (1985) Dynamics of MHC molecules in lymphoid cells: facts and speculations (The Cell Biology of the Major Histocompatibility Complex Ed. B. Pernis, H.J. Vogel, New York:Academic. P & S Biomedical Sciences Symposium pp 152-64

Potter, J.M., Nestel, J. (1979) The hyperlipidaemia of pregnancy in normal and complicated pregnancies. Am. J. Obs. Gynecol. 1: No. 2, 165-170

Radding, C.M., Steinberg, D. (1960) Studies on the synthesis and secretion of serum lipoproteins by rat liver slices. J. Clin. Invest. 39: 1560-1569

Rigg, L., Cousins, L., Hollingsworth, D., Brink, G., Yen, S.S.C. (1980) Effects of exogenous insulin on excursions and diurnal rhythm of plasma glucose in pregnant diabetic patients with and without residual β-cell function. Am. J. Obstet. Gynecol. 136: 537-544

Roberts, D.C.K., Miller, N.E., Price, S.G.L., Crook, D., Cortese, C., La Ville, A., Masana L., Lewis, B. (1985) An alternative procedure for incorporating radio-labelled cholesteryl ester into human plasma lipoproteins in vitro. Biochem. J. 226: 319-322

Roth, T.F., Porter, K.R. (1964) Yolk protein uptake in the oocyte of the mosquito Aedes Aegypti. J. Cell Biol. 20: 313-32

Rudling, M.J., Peterson, C.O. (1985) A simple binding assay for the determination of low-density lipoprotein receptors in cell homogenates. Biochimica et Biophysica Acta, 833: 359-365

Scatchard, G. (1949) The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51: 660-672

Schneider, H., Panigel, M., Dancis, J. (1972) Transfer across the perfused human placenta of antipyrine, sodium and leucine. Am. J. Obstet. Gynecol. 144: 822-828

Shafrir, E., Barash, V. (1987) Placental function in maternal-fetal fat transport in diabetes. Biol. Neonate 51: 102-112

Simpson, E.R., MacDonald, P.C. (1981) Endocrine physiology of the placenta. Ann. Rev. Physiol. 43: 163-188

Smith, N.C., Brush, M.G. (1978) Preparation and characterization of human syncytiotrophoblast placental membrane. Med.Biol., 56: 272-276

Solari, R., Kraehenbuhl, J.P. (1984) Biosynthesis of the IgA antibody receptor: a model for the transepithelial sorting of a membrane glycoprotein. Cell, 36: 61-71

Spady, D.K., Huettinger, M., Bilheimer, D.W., Dietschy, J.M. (1987) Role of receptor-independent low density lipoprotein transport in the maintenance of tissue cholesterol balance in the normal and WHHL rabbit. J.Lip.Res. 28: 32-41

Sparks, J.D., Sparks, C.E., (1985) Apolipoprotein B and lipoprotein metabolism. Adv. Lipid Res. 21: 1-46

Stone, M.C., Thorpe, J.M., Mills, G.L., Dick, T.B.S. (1970) Comparison of membrane filtration and nephelometry with analytical ultracentrifugation for the quantitative analysis of low density lipoprotein fractions. Clin. Chim. Acta. 30: 809-828

Stone, M.C., Thorpe, J.M., Mills, G.L., Dick, T.B.S. (1971) Diagnosis and classification of abnormal lipoprotein patterns. Clin. Chim. Acta. 31: 333-354

Szabo, A.J., Szabo, O., (1974) Placental free-fatty acid transfer and fetal adipose tissue development: an explanation of fetal adiposity in infants of diabetic mothers. Lancet, 2: 498-499

Thomas, C.R. (1987) Placental transfer of non-esterified fatty acids in normal and diabetic pregnancy. Bio. Neonate 51: 94-101

Unanue, E.R. (1984) Antigen presenting function of the macrophage. Ann. Rev. Immunol. 2: 395-428

Warth, M.R., Arky, R.A., Knopp, R.H., (1975) Lipid Metabolism in Pregnancy 3, J.C. E & M, 41: No 4

Weber, K., Osborn, M. (1969) The reliability of molecular weight determinations by dodecyl sulphate - polyacrylamide gel electrophoresis. J. Biol.Chem. 244: 4406-4412

Wilcox, H.G., Heimberg, M. (1970) Isolation of plasma lipoproteins by zonal ultracentrifugation in the B14 and B15 titanium rotors. J. Lip. Res. 11: 7-22

Winkel, C.A., Gilmore, J., and MacDonald, P.C. (1980) Uptake and degradation of lipoproteins by human trophoblastic cells in primary culture. Endocrinol. 107: 1892-1898

Winkel, C.A., MacDonald, P.C. and Simpson, E.R. (1981) The role of receptor mediated low density lipoprotein uptake and degradation in the regulation of progesterone biosynthesis and cholesterol metabolism by human trophoblast. Placenta Supp.3, 133-144

Yamamoto, M., Ranganathan, S., Kottke, B.A. (1985) Structure and function of human low density lipoproteins. Studies using proteolytic cleavage by plasma kallikrein. J. Biol. Chem. July No. 15; 260 (14): 8509-8513

ADDENDUM

Cummings, S.W., Hatley, W., Simpson, E.R., Ohashi, M. (1982) The binding of high and low density lipoproteins to human placental membrane fractions. J. Clin. Endocrinol. Metab. 54: 903-908

Freinkel, N. (1980) Banting Lecture on pregnancy and progeny. Diabetes 29: 1023

Knopp, R.H., Warth, M.R., Carroll, C.J. (1973) Lipid metabolism in pregnancy. 1. Changes in lipoprotein triglyceride and cholesterol in normal pregnancy and the effects of diabetes mellitus. J. Reprod. Med. 10: 95-101

Knopp, R.H., Montes, A., Childs, M., Li, J.R., Mabuchi, H. (1981) Metabolic adjustments in normal and diabetic pregnancy. Clin. Obstet. Gynec. 24: 21-49

Pastan, I.H., Willingham, M.C. (1981) Receptor-mediated endocytosis of hormones in cultured cells. Annu. Rev. Physiol. 43: 239-50