The selectivity of human placenta in maternal-foetal transfer of plasma proteins: mechanisms and implications

This item was submitted to Loughborough University’s Institutional Repository by the/an author.

Additional Information:

- A Doctoral Thesis. Submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of Loughborough University.

Metadata Record: https://dspace.lboro.ac.uk/2134/14460

Publisher: © John Lubega

Please cite the published version.
This item was submitted to Loughborough University as a PhD thesis by the author and is made available in the Institutional Repository (https://dspace.lboro.ac.uk/) under the following Creative Commons Licence conditions.

For the full text of this licence, please go to:
http://creativecommons.org/licenses/by-nc-nd/2.5/
<table>
<thead>
<tr>
<th>AUTHOR/FILING TITLE</th>
<th>LUBEGA, J</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACCESSION/COPY NO.</td>
<td>007134/02</td>
</tr>
<tr>
<td>VOL. NO.</td>
<td></td>
</tr>
<tr>
<td>CLASS MARK</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LOAN COPY</td>
</tr>
</tbody>
</table>
THE SELECTIVITY OF HUMAN PLACENTA IN MATERNAL-FOETAL TRANSFER
OF PLASMA PROTEINS; MECHANISMS AND IMPLICATIONS.

by

John Lubega
MB ChB, LRCP (Lond), MRCS (Eng), M.Sc (Cantab), M.I.Biol.

a doctoral thesis submitted in partial fulfilment
of the requirements for the award of the degree of
DOCTOR OF PHILOSOPHY
of the Loughborough University of Technology

October 1984

*********

Supervisor: Professor J.N. Miller, M.A., Ph.D (Cambridge),
C.Chem., F.R.S.C.

© by John Lubega (1984)
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>(i)</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>(ii)</td>
</tr>
<tr>
<td>LIST OF SYMBOLS AND ABBREVIATIONS</td>
<td>(v)</td>
</tr>
</tbody>
</table>

## CHAPTER 1

### INTRODUCTION TO PLACENTAL MORPHOLOGY, DEVELOPMENT, AND FUNCTION.

1.1.1 Anatomical Aspects of the Placenta 1  
1.1.2 Primary Villi Formation 3  
1.1.3 Anchoring Villi 4  
1.2 Some Ultrastructural Microvillar Variations 5

### FUNCTIONAL ASPECTS OF THE VILLI

1.3.1 Vascular Syncytial Membranes 5  
1.3.2 Dynamic Anatomical Microvillar Changes; adaptation to function. 7  
1.3.3 Overall Placental Microvillar Function 9  
1.3.4a Microvillar Circulatory Arrangement 11  
1.3.4b Maternal Uterine Placental Circulation Models 13  
1.4 Placental Mesenchymal Cytoskeletal Elements 18  
1.5 Role of Trophoblastic Lysosomes 19  
1.6 Placental Coated Vesicles and Transfer 21

### THE PLASMA MEMBRANE

2.1 The Trophoblastic Plasma Membrane 25  
2.2.1 Trophoblastic Antigens 30  
2.2.2 Human Placental Chorionic Villar Matrix; Solubilisation of Plasma Membrane. 32  
2.2.3 Specific Placental Antigens or Proteins 35

### IMMUNOREGULATORS

2.3.1 Immunoregulation of Placental/Foetal Antigens 38  
2.3.2 Specific Immunoregulators 40
Chapter 1 Cont'd..

PROTEIN SYNTHESIS AND TRANSFER

3.1 Foetal Ontogeny and Plasma Protein Transfer or Synthesis 40

CHAPTER 2

GENERAL METHODS

PURIFICATION OF TROPHOBLASTIC MEMBRANE

1.1 Overview 46
1.2 Purification Procedure 49
1.3 Placental Processing 50

PROTEIN ASSAY METHODS

2.1 Micro-Lowry Technique 57
2.2 Bio-Rad Protein Assay Procedure 62

RADIOLABELLING PROCEDURE

3.1 Preparation of Reagents 68
3.2 Preparation of Column for Separation of labelled and unlabelled IgG-H\(^3\). 68
3.3 Preparation of LKB-K\(_9\) Column 69
3.4 Radiolabelling Procedure; Separation of Labelled Protein 70
3.5 Logistics of Delivery; Microgram Amounts for Reacting with NSP 72
3.6 Determination of Specific Activities 74
3.7 Assessment of Tricarb Scintillation Counter Efficiency 78
3.8 Filtration Techniques. 79

CHAPTER 3

IMMUNOCYTOCHEMICAL METHODS

Introduction 87
Chapter 3 Cont'd..

**Immunocytochemical Methods Cont'd.**

Materials and Methods

a) Immunofluorescence Method  
   b) Immunoperoxidase Method

**RESULTS**

Immunofluorescence 94
Immunoperoxidase 104
Summary 104
Display of Photographs 108

**CHAPTER 4**

**SELECTIVE ANTIBODY TRANSFER MECHANISMS; THE STUDY OF PLACENTAL IgG-Fcγ RECEPTOR.**

**Aims**

Introduction 115

4.1 Development of Foetal Immune System 115
4.2 Antibody Synthesis 116
4.3.1 Brambell's Hypothesis 117
4.3.2 Selectivity of Placenta 118
4.3.3 Coated Vesicles 119

**MATERIALS, METHODS AND RESULTS**

Establishment of Theoretical Methods for Receptor-Ligand Interaction.

A) Ligand/Receptor Theory 120
B) Log-dose Response Curves (LDR) 122
C) Stoichiometry of Binding; Theory 125
D(a) Data on Stoichiometry of IgG-Fcγ interaction from LDR 126
Chapter 4 - Establishment of Theoretical Methods Cont'd.

D(b)  Affinity Equilibrium Constant from LDR
      Transformation of LDR Curves into Inverse Scatchard Plots
      Data for LDR Plot and Inverse Scatchard

The IgG-Fcγ Receptor Kinetics

I  Optimisation of Reaction
II  Association Rate Constant (k_1)
III Determination of Receptor Concentrations
IV  Dissociation Rate Constant (k_2)

THERMODYNAMICS OF IgG-Fcγ RECEPTOR INTERACTION

Discussion of Effect of Temperature on Binding
Activation  Enthalpy and Entropy Changes
Discussion of Thermodynamic Results

CATIONIC MODULATION OF IgG-Fcγ BINDING

Modulation of IgG-Fcγ Binding by Mono and Divalent Cations
Materials and Methods
Results
Discussion

CHAPTER 5
PLACENTAL SELECTIVITY AND TRANSFER OF PLASMA PROTEINS; STUDIED BY
IN-VITRO PERFUSION SYSTEM.

5.1  Introduction
5.2  Transcellular and Paracellular Transfer Pathways
5.3  Receptor Recycling

Discussion of Effect of Temperature on Binding
Activation  Enthalpy and Entropy Changes
Discussion of Thermodynamic Results

CATIONIC MODULATION OF IgG-Fcγ BINDING

Modulation of IgG-Fcγ Binding by Mono and Divalent Cations
Materials and Methods
Results
Discussion

CHAPTER 5
PLACENTAL SELECTIVITY AND TRANSFER OF PLASMA PROTEINS; STUDIED BY
IN-VITRO PERFUSION SYSTEM.

5.1  Introduction
5.2  Transcellular and Paracellular Transfer Pathways
5.3  Receptor Recycling
Chapter 5. Cont'd.

THEORETICAL MODELS OF MEMBRANE TRANSFER

5.4.1 Diffusion Model
5.4.2 Zero-trans Procedure
5.4.3 Infinite Cis-Procedure
5.4.4 Infinite Trans-Procedure
5.5 Aim of Transfer Studies

MATERIALS AND METHODS

5.6 In-vitro Placental Perfusion System
5.7 The Placenta and Perfusion Experiments
5.8 Modifications Introduced into Placental Perfusion System

Results
Zero-trans Model applied to Uptake
Calculation of Affinity Constants
Conclusion
Display of Linearised Curves

CHAPTER 6

COLOIDAL GOLD PROTEIN CONJUGATES FOR ULTRASTRUCTURAL STUDY OF PLACENTAL RECEPTORS.

Aims of Study

MATERIALS AND METHODS

Preparation of Gold Sol
Preparation of Protein Gold Conjugates
Incubation of Au_{18}-Protein Conjugates with Placental Villi

Results
CHAPTER 7

AFFINITY CHROMATOGRAPHY OF TRITON-X_100 SOLUBILISED TROPHOBLASTIC MICROVILLAR MEMBRANE IN AN ATTEMPT TO ISOLATE RECEPTOR STRUCTURES FOR SOME SPECIFIC PLASMA PROTEINS.

Introduction 236

Methods;

Preparation of Placental Homogenate 238
Membrane Solubilisation 239
Coupling of Protein to CN Br-Activated Sepharose 4B 240
Affinity Isolation of Receptors using Specific Adsorbents 241
SDS-Polyacrylamide gel Electrophoresis 242

Results 246
Discussion 251

CHAPTER 8

RECEPTOR-PROTEIN BINDING ISOOTHERMS USING PURIFIED TROPHOBLASTIC MEMBRANE OR RECEPTOR DESORBATES FROM AFFINITY CHROMATOGRAPHY.

Introduction 256

Materials and Methods;

Isolation and Solubilisation of Placental Membrane 259
Radiolabelling of Plasma Proteins 260
Radio-Receptor Ligand Binding 260
Chapter 8 Cont'd..

Results 263
Scatchard Plots for Deriving Binding Parameters 265
Discussion 274
GENERAL: CONCLUSION 277
References 286
ACKNOWLEDGEMENTS

Work contained in this thesis would not have been possible without assistance from many people from various departments of the Leicester Royal Infirmary, the Leicester University Medical School and the University of Loughborough.

I wish to express my sincere gratitude to my Supervisor and Director of research, Professor J.N. Miller, Department of Chemistry, Loughborough University for his assistance which has enabled me to complete this study and particularly for his guidance and provision of laboratory facilities. I appreciate practical help received from Messrs. J.J. Swithenbank and M.K. Patel of Analytical Chemistry Section. I am also grateful to my Consultant, Dr. T.J. Davies, of the Department of Chemical Pathology, Leicester Royal Infirmary, where some of this work was performed. I wish to thank Dr. C.D. Ockleford, Department of Anatomy, Leicester University, for his help in some techniques related to placental study; the same goes for Professor J. McVicar, of the Department of Obstetrics and Gynaecology for allowing me to utilise Labour ward facilities, particularly in obtaining placentae.

My thanks go to Dr. W. Becker, Head of Research Department, Behring Institute, Marburg, West Germany, who made it possible to obtain some highly purified plasma proteins. I would also wish to thank Dr. H. Sewell and Mr. A. Willox, both of Immunopathology Department, Leicester Royal Infirmary, for help with various immunocytochemical studies. I appreciate help and encouragement received from my wife, Esther Lubega, whilst carrying out this research, and for her typing of this thesis.

Finally, I am indebted to the Trent Regional Health Authority Locally Organised Research Committee for the financial support of this project.
SUMMARY

The placenta is a highly organised and effective 'barrier' interposed between maternal and foetal circulations. Multiple functions normally requiring separate organs are condensed in this one organ and transport of various metabolites is of selective nature. Transfer of plasma proteins across the placenta is complex and has received little attention so far; this thesis sets out to establish existence of receptor molecules responsible for initial recognition and selective transfer of the following protein molecules; α₁ antitrypsin, haptoglobin, α₂ HS-glycoprotein, prealbumin, Gc-specific components, α₁ acid glycoprotein, thrombin, fibrinogen, in addition to IgG and transferrin.

Firstly, isolated syncytiotrophoblastic membranes were used to establish the topographical distribution of binding of these protein ligands by immunocytochemical methods. All proteins studied showed marked positive fluorescence but for immunoperoxidase method, prior aggregation of these protein ligands was necessary to elicit positive reactions. Secondly, an in-vitro placental perfusion system was utilised to monitor uptake of tritium radiolabelled derivatives of these proteins and uptake profiles in all cases showed saturable characteristics. Mathematical application of equations for describing assymmetric carriers linearised all these curves, a fact which was very suggestive of receptor mediated uptake as the main mechanism for placental protein transfer. Furthermore, uptake curves were very similar in nature to Michaelis-Menten type of kinetics; additional
evidence for 'carrier' mediated uptake.

The IgG antibody transfer which is also selectively transferred was studied in some detail. It was necessary to re-establish the kinetic association and dissociation characteristics of the IgG-receptor binding as well as any evidence of heterogeneity of receptor sites. $K_a = 2.2 \times 10^{8} M^{-1}$ with binding sites of $1.5 \times 10^{14}/mg$ membrane were obtained with an average rate constant of $2.29 \times 10^{7} M^{-1} \text{min}^{-1}$. Dissociation was heterogeneous at high displacement dose of 1000 µg of unlabelled IgG giving a curvilinear rate dissociation curve; the fast affinity site had $k_2 = 0.10 \text{min}^{-1}$, whilst the slow affinity site had $k_2 = 0.01 \text{min}^{-1}$.

Temperature effects led to derivation of thermodynamic binding parameters; binding was strongly exothermic with marked negative enthalpy ($\Delta H = -2.51 \text{KCal/mole}$) which was suggestive of hydrogen bonding between IgG and its receptor. There was a high entropy energy change when IgG associates to its receptor ($\Delta S = +29.3 \text{KCal. Mole}^{-1} \text{K}^{-1}$), typical of hydrophobic type of interaction. Furthermore, the stoichiometric ratio of IgG to Fc$_\gamma$ receptor was 4:1 as opposed to 1:1 as previously stated.

Monovalent and divalent cations strongly modulated the IgG-Fc$_\gamma$ receptor binding, (Na$^+$, 60-140 mM) caused a 32-50% drop in binding whilst Li$^+$, 1-4 mM gave a 50% depression in binding but no effect at therapeutic dose (Li$^+$ < 2mM) Mg$^{2+}$ (1-2mM) increasing binding by 8.4% and > 2mM depressed binding up to 30% at 10 mM. Ca$^{2+}$ (2.6mM) inhibited 13% of binding increasing to 33% by 10mM. Mn$^{2+}$ showed a 1.7%
depression at 1 mM which rose to 68% at 15 mM.

Colloidal gold adsorbed to various proteins offered a visualisable means of following the process of receptor binding and subsequent formation of coated vesicles which are responsible for transfer across the placenta. Affinity isolation of receptors from solubilised membrane was performed using protein coupled to CN Br-activated Sepharose 4B and enabling analysis of desorbed receptors on SDS-polyacrylamide gels. Molecular weights of 46,000, 48,000, 49,000 and 62,000 for $\alpha_1$ acid glycoprotein, haptoglobin, prealbumin and $\alpha_1$ anti-trypsin receptors were obtained respectively. IgG showed heterogeneity with three visible polymorphic bands with $M_r$ 72,000, 78,000 and 195,000.

Radiolabelled proteins were reacted with either the affinity isolated receptor fractions or the unsolubilised purified membrane and from binding isotherms and Scatchard plots, binding data such as the affinity, receptor site capacity, co-operative and homogeneous receptor effects were evaluated.
SYMBOLS AND ABBREVIATIONS

StMPM = Syncytiotrophoblastic Microvillar Plasma Membrane
PreAlb = Prealbumin
$\alpha_1$AT = $\alpha_1$antitrypsin
Hp = Haptoglobin
$\alpha_2$HS = $\alpha_2$HS-glycoprotein
Thr = Thrombin
$\alpha_1$AG = $\alpha_1$Acid glycoprotein
Fg = Fibrinogen
BSA = Bovine Serum Albumin
= greater than
= less than
EM = Electron Microscope
$M_r$ = Molecular Weight
SDS-PAGE = Sodium Dodecyl Sulphate - Polyacrylamide gel electrophoresis.
$\mu$l = Microlitre
l = litre
$\mu$g = Microgram
g = Gram
mg = Milligram
g = Acceleration due to gravity
$\Delta$H = Activation Enthalpy
$\Delta$G = Gibb's free energy
$\Delta$S = Entropy
R = Gas constant = 1.987Cal/mole/degree
T = Temperature
K = Degree Kelvin
$K_a$ = Equilibrium association constant
$K_d$ = Dissociation constant
Symbols and Abbreviations Cont'd.

\[ k_2 = \text{Dissociation rate constant} \]
\[ k_1 = \text{Association rate constant} \]
\[ C_t = \text{Uptake at time t} \]
\[ S_0 = \text{Maternal input in a Cis/trans exchange system} \]
\[ \text{Sol} = \text{Gold sol (or suspension of tiny gold colloid)} \]
CHAPTER 1

GENERAL REVIEW

INTRODUCTION TO PLACENTAL MORPHOLOGY, DEVELOPMENT AND FUNCTION.

1.1.1 Anatomical Aspects of the Placenta.

Knowledge of function of any organ's biological system is incomplete without discussing morphological considerations. It is even more imperative, for an organ like the placenta with highly specialised multiple but diverse functions and normally requiring separate organs (Burton et al 1976) to try to understand the anatomical arrangements that make it a very unique organ in the biological heirarchies. Although it can execute functions of an endocrine nature, excretion of foetal waste, gaseous exchange, transfer of nutrients, antibody transfer and other immunological roles, including cellular filtration, it nevertheless remains an effective barrier (Steven and Samuel 1976 and 1979). The term 'barrier' is classically applied to the blood - brain, blood cerebral spinal fluid interfaces as well as the choroid plexus system and of course the placenta. All these interesting tissue functions do form anatomical as well as functional barriers and attention must be drawn to the fact that they are all embryologically ectodermal derivatives (Davson 1976).

The placenta develops originally from a fertilised ovum, which after migration to the uterine wall implants, loses its outer layer, the zona pellucida to form a blastocyst. Subsequent proliferation of its outer layer forms the trophoblastic cell mass; this infiltrates into the endometrial epithelium to come into direct contact with the endometrial stroma. It is believed this process of implantation with
expression of trophoblastic cells is complete within tenth or eleventh day post ovulatory day (Fox 1978).

The trophoblastic cells proliferate into two prominent layers, the outer forming multi nuclear layer of syncytiotrophoblast whilst the inner mono-nucleated cells become the cytotrophoblast. It is now widely believed the inner cytotrophoblast gives rise to the outer syncytium; initially by fusion of its cells, then eliminating the cell wall membranes, to become the syncytiotrophoblast (Richart 1961; Galton 1962). Often remnants of the shed cell wall membranes can be seen by the electron microscope (Carter 1964; Enders 1965).

Mitotic activity is recordable only in the inner cytotrophoblast. The syncytium's DNA does not seem to replicate. Consequently, some investigators have teleologically, postulated (Contractor et al 1977) that this allows the syncytiotrophoblast, a layer in direct contact with maternal circulation, to direct its full metabolic energy towards transfer processes; to and from the foetus rather than being deployed in cell division.

Around the tenth to thirteenth post-ovulatory days, clefts appear in the trophoblastic layers and a series of these form intercommunicating valleys or lacunae which seem to incorporate maternal endometrial capillaries (Harris and Ramsay 1966). Some of these lacunae become confluent to form the crucial intervillous space. This space seems to be filled with maternal blood, which is important in facilitating exchange of materials between the trophoblast and maternal blood system.
1.1.2 Primary Villi Formation

Excrescences of the trophoblastic surface form structures which at first become the primary villi. These originally form from proliferation of cytotrophoblast which results in formation of central core of column of cells, called trabeculae, from which villi form within the first 25 days post ovulation (Fox 1978, Dearden et al 1983). The primary villi are solid, but afterwards their mesenchymal core becomes invaded by fibroblasts, the Hofbauer phagocytic cells (Enders and King 1970), as well as collagen fibres and hence become secondary villi. Further differentiation and acquisition of foetal capillaries within the villous core convert them into tertiary villi. This vascularisation or angiogenesis, it has been argued, is a function of the mesenchymal core (Dempsey 1972), although others contend the vessels are formed by the cytotrophoblastic cells (Hertig 1935).

Sprouts from the well formed tertiary villus go through all the stages described above, become vascularised too, and thus produce new villi and thus proliferation of villous tree is achieved and consequently amplification of absorptive surface is attained (Ludwig 1974, Aherne and Dunhill 1966, Aherne 1975). Some of these sprouting villi have been observed to pinch off at their bases, to enter the intervillous space (Boyd and Hamilton 1970). Somehow as Douglas reports (Douglas et al 1959), these bits of villi are often found in the maternal circulation. Its significance is ill understood, although it has a possible immunosuppressive role to maternal antibody that would otherwise cause host versus graft rejection (Thomas et al 1959).
1.1.3 Anchoring villi.

The placenta has to be firmly anchored to the maternal endometrium for it to perform its functions, without as it were "falling off". This is, at ultrastructural level achieved by specialisation amongst some villi, to the role of securing the placenta; and these are termed anchoring villi. They differ from other villi, in that their core is composed to solid trophoblast, and no mesenchymal core with its usual compliment of blood vessels (Dearden et al 1983). These authors point out that these type of villi are for purely mechanical support and even if they got torn off due to certain pregnancy stresses, the fact that they are devoid of blood vessels, no vascular exanguination takes place which is otherwise potentially dangerous.

It is also important to note that at this stage the distal part of the villus stem is formed by cytotrophoblast which is not yet invaded by the mesenchyme or blood vessels. These cells filling the whole villus are often referred to as 'cytotrophoblast cell columns', they do spread laterally to form a continuous cytotrophoblast shell, (Fox 1978) which splits the syncytium into two layers that lie on the foetal side and the other peripheral one between the shell and the decidua plate. This peripheral type degenerates to be replaced by a layer of fibrinoid material (Nitabuch's layer), whilst the inner syncytium persists to become the usual limiting layer or border between the trophoblast and the inter-villous space.
1.2 Some Ultrastructural Microvillar Variations.

The syncytial border referred to above is normally studded by microvilli formed to increase absorptive surface (Fox 1979), in a manner similar to the brush border of the gastrointestinal tract (King and Menton 1975) on the renal epithelium. However, there are some areas of the trophoblastic epithelium, namely the villus, which are devoid of microvilli (Ludwig 1974, Dearden et al 1983). Either these areas had them but they got denuded and thus lost microvilli or they never got formed at all.

There has been considerable electron microscopic interest in studying microvillar variations as reported by Dempsey and Luse 1971, Clint et al 1979. King and Menton, 1975 saw no such denuded areas, lacking microvilli, whilst Clint et al 1979, Panigel and Anh 1964, noted such areas. It has been noted that the distribution of these 'smooth' areas devoid of microvilli is entirely random and also the change from microvilli covering to lack of them is gradual (Dearden et al 1983). Areas which invariably lack microvilli are the syncytial vascular membranes (Fox and Agrofojo-Blanco 1974).

Functional Anatomy of the Villi

1.3.1 Vascular Syncytial Membranes.

Some areas of the chorionic villi are extremely thin morphologically, lack the cytotrophoblast, no nuclei and also have no microvilli. Light microscopic examination of these areas seem to show apparent fusion of the syncytial membrane
with the underlying endothelium of the foetal capillaries. Higher magnification of these areas reveal that there is no true fusion but merely close apposition of the thin syncytial membrane and the endothelium (Strauss et al 1965, Fox 1979). These areas are thus called "the syncytial vascular membranes" (VSMs), and Fox (1979), maintains that this peculiar morphological thinning, connected with blood vessels, have a functional role namely the transfer of those substances where membrane thickness is an important limiting factor, such as gaseous exchange; oxygen and carbon dioxide (Fox 1979, Baker et al 1944, Longo 1972). Transfer of gases or indeed simple diffusion is inversely proportional to the square of the distance separating two compartments, in this case the membrane, and so thinning of these syncytial-vascular membranes subserves a vital role. It is postulated that these areas are formed by lateral mechanical stretching as proposed by Pisarki and Topilko (1966), particularly by distended or dilated foetal vessels (Fox 1979). They are also randomly distributed, often occurring along the course of a vessel as a dome-shaped swelling protruding into the intervillous space from the lateral wall of the villus (Fox and Agrafojo-Blanco 1974).

These specialised areas, the vascular-syncytial membranes, quite apart from their gaseous transfer role, differ also histochemically (Burgos and Rodriguez 1966) from unthinned areas of the trophoblast. In first trimester placentae, the vascular-syncytial membranes are just discernible, however, as pregnancy continues, they become more prominent. This structural amplification correlates with functional integrity.
of the placentae and hence nutritional status of the foetus. This argument is ratified by finding that deficiency of VSMs in mature placentae is associated with foetal deprivation (Fox 1967). Fox argues that such paucity of VSMs can arise from lack of differentiation of the trophoblast, possibly by the stretching mechanisms to form these structures, which leads to poor transfer of those substances dependent on simple diffusional flux processes.

Although the membrane thinness of VSMs offers an attractive theory in respect of enhancing transfer, Longo 1972, seems to destroy the argument that this membrane thickness, and hence resistance, is positively the limiting constraints in placental transfer. From comparative studies, some species do not seem to exhibit VSMs, in fact some have thicker placental membranes, but evidently transfer seems to be adequate in these species. The clear resolution of why VSMs exist is far from complete. It is worth mentioning work of Ockleford et al 1981, who by immunofluorescent staining these VSM areas, for polymeric cytoskeletal protein, did not detect any reduction in thickness.

1.3.2 Dynamic anatomical microvillar changes; adaptation to function.

Placental villi with their microvilli impose a great absorptive surface for foetal-maternal exchange. There are constant changes in villar structure as pregnancy progresses. These maturation processes have been regarded as embodying the ageing process (Fox 1979). Probably a better way to
regard these villar changes is to depict them as maturation processes. Area/volume change increase whilst surface area changes from $5m^2$ at 28 weeks to $11m^2$ at term (Aherne and Dunhill 1966). Thickness of villi alter from 10 μm at early gestation to 1.7 μm in later pregnancy (Boyd and Hamilton 1967).

Furthermore, the increasing foetal capillary diameters as they shift from central to eccentric position have the effect of creating a steep concentration gradient of any substance from maternal to foetal side. In addition the crowding of flow lines or cris-crossing effect has also a bearing on increasing maternal-foetal transfer efficiency within the microvilli. As the placenta matures and thus alters in size, the importance of close apposition of maternal and foetal circulations must be maintained in the end. Therefore it can be construed that the microvillar maturation changes are adaptive features to attain the desired goal of better efficiency in transfer processes. Placental maturation and foetal growth should go hand in hand as it were.

However, it is not uncommon to find fully grown, mature foetus, but with immature villi as seen in diabetes mellitus complicated pregnancies. Conversely, too, mature microvilli have been associated with immature foetus as seen in 10% of prematurely delivered babies (Nezelof and Roussel 1954, Becker 1963, Fox 1969), and as much as 25% rate has been given in Aldjems series (1968). This foetal-placental asynchrony (Becker 1975, Fox 1978) has been observed which might therefore mean that there is independence between microvillar maturation, morphologically at least,
and foetal growth although obviously the two processes are strongly intertwined (Fox 1978). This imbalance presents even more difficulties in appreciating how immature villi can function so well in terms of transfer processes to produce a well 'fed' baby. This disparity or dissociation in microvillar morphology and function remains unresolved. A full term placenta is usually about 600gm and for many years various clinical work has associated low placental weight with low birth weight or even immaturity. From the above considerations of the growth of the placenta, its size and foetal maturity, it is hard to draw a hard and fast correlation between mere size and foetal outcome.

1.3.3 Overall Placental Microvillar Function.

Placental microvilli, because of their large surface area, are fundamental to all transfer processes. These include absorption of carbohydrates, amino acids, lipoproteins, gaseous exchange, vitamins and other small molecular weight nutrients, electrolytes, and proteins (Longo 1972, Hill and Longo 1980, Hill and Young 1973). Further mention of the exchange of these materials and modes of transport involved will be made later, under specific transfer of some substances. Microvilli are also involved in excretion of foetal waste products via maternal route (Salazar and Gonzalez-Angulo 1967). As for macromolecules whose transfer cannot be effectively achieved by simple diffusion, the syncytial trophoblast has surface receptors for these molecules. By micropinocytosis, bits of membrane containing these substances and still attached to their receptors are able to pinch off
to form coated vesicles which then transport them across the membrane to foetal capillaries (Brambell 1970, Dempsey and Luse 1971, Fox 1978, Ockleford and Whyte 1977). Detailed discussion of coated vesicles and how they perform a receptor and then transfer role, will be given later. Foetal nutrition and development also depends on the integrity of these microvilli on performing their various functions, and as such there have been studies which have correlated abnormal or pathological pregnancies with microvillus immaturity, a generalised failure of villous maturation has also been associated with a relatively high incidence of foetal hypoxia (Becker 1975) and foetal growth retardation (Busch 1972, 1974, Bender et al 1976). One must add that these complications are by no means constant accompaniment of villous immaturity because many infants whose placental villi are immature are born normal both for weight and gestational period.

The cause of this immaturity is unclear and one would have thought that vascular supply complications such as ischaemia in hypertension or preeclampsia would contribute to villous immaturity. This is not so because the incidence of villous immaturity is no higher in these conditions, and therefore ischaemic insults to the placenta cannot be a direct cause of villous immaturity.

Nutrients themselves stimulate microvillar growth, for if they are restricted there is a proliferation of microvilli (Jones and Fox 1979) as a kind of compensatory change. However mere proliferation has to be distinguished from accelerated villous maturation. The latter is seen in placentae from immature or prematurely delivered infants,
(Becker 1975). This is not unexpected as asynchrony between foetal and villous maturity is well recognised (Becker 1963, 1971, 1975) and therefore one finds fully mature villi from immature placentae and vice versa. Others like Schuhmann (1975) have argued that the finding of accelerated growth in immature placentae is more of a compensatory change to counter a deficit in villous numbers.

1.3.4a Microvillar Circulatory Arrangement.

For the unique uptake mechanisms of the trophoblastic villi, circulatory arrangements play a vital role and for our understanding of this and its connection to transfer mechanisms a brief mention of this circulatory anatomy will now be made.

Two umbilical arteries spiralling around a central umbilical vein enter the chorionic membrane, and each seems to supply one half of the placenta. Each runs parallel to the chorionic plate but dividing into sub-branches, which in turn give off straight cotyledonary arteries which enter the placental substance (see Fig. 3 depicting chorionic tree and vessels). These vessels still follow the subdivisions of the chorion and finally forming capillaries to enter each microvillous. The capillaries intertwine and cris-cross to form plexuses. It is those plexuses which run over each other that create a unique transfer amplification arrangement.

Longo and Power (1969) have considered a schematic arrangement whereby umbilical and maternal vessels of differing diameters are considered in close apposition so as to
facilitate an effective arrangement. See diagram (Fig.1) If small compartments A and B are considered, it will be seen that in A, with a large blood flow, maternal and foetal blood will equilibrate near the initial foetal value. However, at B, with larger maternal vessel relative to the foetal one, exchange will equilibrate near the initial maternal value. At D&H, after mixing, the blood leaving the placenta will have uterine vein and umbilical vein values which differ, even though equilibration was achieved in the separate A and B exchange compartments. This system is especially suitable for gaseous exchange and any other small molecular weight substances with rapid diffusional fluxes.

To a lesser extent, this is also true for macromolecules such as proteins, although existence of receptor or carriers on the trophoblastic membrane which separates the maternal and foetal circulation will obviously impose more complex kinetics of exchange. Consider the case of oxygen exchange as per arrangement in Fig.1. The mixture of maternal blood from compartments A and B having unequal maternal and foetal flow rates, results in uterine venous blood at C having a composition weighted heavily by unit B, namely closer to the inflowing maternal value as opposed to the umbilical vein which will be weighted heavily by unit A. The nett result becomes that of differing oxygen or carbon dioxide partial pressures in the umbilical and uterine veins, even though gaseous equilibration may be complete in each of the
placental segments A and B. Therefore if calculations are made, assuming that all capillaries are identical in size, results will not reflect the true situation imposed by

---

Fig. 1
the complexity of foetal/maternal capillary arrangements in terms of the overall exchange.

Therefore this complex geometry of vascular arrangements has forced several workers to drop assumptions of identical capillaries, and analyse the effect on transport in terms of different capillary orientations, countercurrent and cross-current patterns, multivillous and pool-type flow arrangements; all which show vast differences in the efficiency of exchange of materials between the placenta and mother. This has been done by Longo (1972), Faber and Hart (1967), Metcalfe et al (1967). Faber has taken the issue even further and considered it as analogous to the theory of heat exchange as applied to the transfer of inert materials in the placenta (Faber 1969), and here quite complex mathematical considerations have been presented.

1.3.4b Maternal Uterine Placental Circulation Models.

Ramsey and her colleagues (Ramsey 1954, 1956, 1962) have shown that arterial inlets from the uterine decidual plate sprinkle blood into the intervillous space in a funnel-shaped stream (See Fig.2). This stream hits the microvilli on the opposite side, which have the effect of dispersing the stream sideways. This forces the blood already in the intervillous space out through the basal decidual venous outlets which drain into larger uterine veins. The arterioles are coiled and spiral-shaped, which have the effect of modulating pressure as well as creating a jet flow effect (Borrel et al 1958). There has been an objection to the
use of the term 'jet', since cineangiography has shown that this term gives an undue impression of speed and

intermittency implied in a jet but rather, maternal blood seems to enter the intervillous space gently (Ramsey 1965). Whatever the true situation, the spiral arteries as they

Fig. 2
(After H. Fox, Pathology of the Placenta; W.B. Saunders, Lond. 1978).
reach the intervillous space create a pressure difference relative to the more straight vessels from which they are continuations; the nett effect being that the full arterial pressure is never felt at the level of the intervillous space. Once in this space, there is very little resistance to flow which further negates the need to have any high pressures in the first place in order to 'push round' the intervillous blood back into the venous returns. Since flow can be achieved at such low pressures, differences in the intervillous space, placental flow vascular must be very high (Moll et al 1975).

Ramsey's model of flow events within the intervillous space, leading to close apposition between chorionic villar circulation and that of the mother for purposes of exchange of materials, was found to be too simplistic. This was due to her system being based on random distribution of arterial inlets sprinkling blood into the intervillous space. Other models are available, namely that of Freese (1966) and Wigglesworth (1967) and that of Gruenwald (1973). In case of Freese and Wigglesworth, arterial inlets are not random but are centrally situated (Fig. 3), so as to inject their stream in villous-free space, in otherwords villi do not play a part in side streaming or dispersion of the blood; but blood flows laterally into the interlobular spaces from which it is drained.

Gruenwald's model (Fig.4), envisages the chorionic tissue with finger-like lobules which project into the intervillous space. Then two arterial spiral arteries are strategically placed in such a way that jet flow from one and the other forms a 'spiral' of fluid
flow which bathes these lobules, and finally the fluid
dropping centrally to collecting venous returns. Whichever
is the true circulatory description, is not yet resolved.

Fig. 3

(After H. Fox, Pathology of the Placenta; W.B. Saunders, Lond. 1978).
1.4 Placental mesenchymal and other cytoskeletal elements.

The chorionic mesenchyme is essentially the ground matrix containing connective tissue elements which offer support to the whole chorionic tree. These elements therefore
constitute the cytoskeleton of the whole placenta. These include fibroblasts, microtubules, microfilaments and polymeric cytoskeleton proteins. A review of their distribution has been given by Ockleford et al 1981, Dearden and Ockleford 1983, Ockleford and Wakely 1981, but suffice to add that fibroblasts project out fibrous like extensions to hold various trophoblastic elements together, whilst microfilaments 8nm in diameter and made of polymeric proteins, such as actin, are seen immediately beneath the plasma membrane and arranged in an open lattice network. It is just possible that micropinocytosis process might involve these actin filaments in eliciting the pinching off of these vesicles which are crucial to the transfer process. The other cytoskeletal elements are of purely supportive nature and may not be involved in transport at all.

1.5 Role of Trophoblastic Lysosomes.

Lysosomes are essentially bags of hydrolytic enzymes and for this reason many scavenger cells such as macrophages deploy the lysosomal enzymes to digest foreign material and organisms. Tumour cells are also able to invade tissues and destroy connective tissue and limiting membranes, by the power of lysosomal enzymes. With regards to the placenta, lysosomes play a very important role not only in controlling the catabolic hydrolytic activity of the placenta, but it is also thought that during ontogeny and development of the placenta, lysosomes play a vital role in its remodeling (Corash and Gross 1974). The loss of limiting membrane
of the cytotrophoblast is thought to be due to dissolution of the membrane by proteolytic, hydrolytic and lipolytic lysosomal activity and thus cause its transformation into the syncytiotrophoblast (Contractor et al 1977). The finding of membrane pieces in lysosomes (Rhodin and Terzakis 1962) as well as free membrane fragments in contiguity of secondary lysosomes (Contractor 1977) do lend support to the strong part lysosomes play in membrane breakdown and modification too. Their most important contribution to foetal well being is their role with regard to transport and transfer of materials from mother to foetus. Take a case of transfer of antibodies across the placenta; once receptors to IgG fix this antibody, there follows pinocytosis and formation of coated vesicles in which the IgG receptor with its ligand are enclosed (Brambell 1970, Hemmings and Williams 1976). Fusion of these vesicles with primary lysosomes leads to secondary lysosomes and destruction of antibody. Those vesicles which escape lysosomal fusion are able to transmit the IgG antibody intact to the foetus, and it is vital for the foetus to receive this antibody wholesome if it is going to retain full antibody function (Wild 1973).

The syncytium contains a large number of varying grades of multivesicular bodies. Essentially these are vesicular bodies with lots of inclusion of small vacuoles, and hence their name. Those with light translucent appearance are designated light multivesicular bodies (L-MVBs) and the much darker ones, the dense multivesicular bodies (D-MVBs) (Martin and Spicer 1973). There is evidence to show that these
L- and D-MVBs are involved in transport (Martin and Spicer, 1973) and that proteins for transplacental transport are selectively transported in these bodies. Furthermore Tighe (1967) has made an observation that lysosomes can contain large granules within themselves and it is possible to trace transitions from these into multivesicular bodies. Thus lysosomes and their counterparts the MVBs are important in transfer process and its control.

1.6 Placental coated vesicles and transfer.

The term 'coated vesicles' was first applied to organelles derived from internal invaginations of the plasma membrane by Rosenbluth and Wissig (1963, 1964). This was because of the appearance of these vesicles, having a microscopically dense coat on their outside which distinguished them from other vesicles in tissues (Pearse 1976, Ckkeford 1976). It would appear that from evolutionary point of view, more advanced cell types, the eukaryotes, transfer of membrane from one area of a cell to another, or for packaging of potentially dangerous enzymes, such as lysosomal enzymes, or secretory products of golgi apparatus, necessitated special organelles to do it (Navikoff and Navikoff 1977).

From cell chemistry point of view the enclosing of reactants from their substrates is achieved by membrane transfer and thus attain cell compartmentalisation. Membrane transfer is necessary in formation of lysosomes or presynaptic vesicles at nerve axonal junctions. In this way receptor-bound substances can cross various tissues.
The latter situation is particularly relevant in the placental transfer processes of material from mother to foetus and hence the importance of coated vesicles in this regard, (Ockleford 1976, Ockleford and Whyte 1977).

Comparative studies have revealed the ubiquitous distribution of coated vesicles in various species as well as different tissue types within the same species or animal (Navorotin 1980). This underlines the fundamental importance of coated vesicles in the animal kingdom (Ockleford 1980, Navorotin 1977) and are therefore intimately involved in transport mechanisms. They have two major points of origin, namely the plasma membrane and golgi apparatus membrane and once they are formed they have to be translocated in a process called vesicle motility, and then some which originate from plasma membrane carrying receptor bound material fuse with the opposite capillary membrane to release their contents therein.

A detailed review of possible mechanisms of this peculiar translocation, or motility from the time of formation is given in a book by Ockleford 1980, under the title of Dynamic aspects of coated vesicular function. He also discusses the problem of how two membranes fuse; that of the coated vesicle membrane and the target organelle to which it is directed. Even more interesting, in terms of cell economy, there seems to be membrane recycling; in otherwords, the vesicle membrane that fused with the target seems to return to the plasma membrane where it came from in the first place which is very intriguing although the exact mechanisms are still obscure.
Take the case of the frog's neuromuscular function; the coated vesicles have been shown to resorb membrane from presynaptic membrane for re-use after the secretion of acetylcholine by the synaptic vesicles. Evidence of this has been provided by studies of Hauser and Reese (1973). The case whereby the endocytosed extracellular protein needs to be transferred, coated vesicles seem to subserve this function (Roth and Porter 1964, Friend and Farquahar 1967) particularly when some protection against proteolysis whilst in transit is a critical factor for the intact functioning of that protein as in the case of transplacental immunoglobulin transfer (Wild 1973, Brambell 1970).

The dense coat which gives their characteristic appearance has been shown to be due to polymers of a protein called clathrin. Pearse (1975, 1976), coined this name after studying coated vesicles from pigs' brain and devising a procedure for isolating them, and thereby being able to study the coat on sodium dodecyl polyacrylamide gels for molecular weight characterisation of clathrin. She found that whether the coated vesicles were derived from pig or bullock brains, or adrenal medulla (a secretory tissue) (Pearse 1976), a molecular weight of 180,000 daltons was consistently obtained. Ockleford and Whyte 1977, and Pearse 1978, have extended the study to placental coated vesicles in which again 180,000 daltons molecular weight clathrin was obtained. Furthermore, Ockleford and Munn 1980, Ockleford 1976, have considered how clathrin molecules come to form a coat around the endocytosed bilipid plasma membrane. Figure 5 illustrates briefly this sequence. Ligand binding initiates a
sequence of conformational changes and free clathrin ends up in polymerisation and forms a 'coat' around the bilipid layer, internal to which is the inner glycocalyx layer.

Fig. 5


A fuller discussion of these changes is reviewed by Ockleford and Munn, 1980. The final shape of these dense clathrin coats is not spherical but form a lattice of polygons due to molecular rearrangements; in particular hexagons
coexisting with pentagons even on the same coat. The three dimensional construction of these shapes by electron microscopy is discussed fully by Ockleford (1976).

Quite apart from the intriguing molecular conformation of coated vesicles, their supreme role lies, for the placenta in selective uptake of protein (Roth and Porter 1964). IgG has been detected in such micropinocytic vesicles (Lin 1980). Also transferrin bound on its receptor has been detected enclosed in coated vesicles by Booth and Wilson (1981). These authors reported a rather surprising phenomenon, in that transferrin protein was apparently the only one which could be detected still receptor bound within these vesicles. There seemed to be no other serum proteins detected in these preparations from the material included within these vesicles. Evidently these other proteins are transported by coated vesicles, including well documented proteins with specific receptors on the placental microvilli plasma membrane. These are IgG (McNabb et al 1976), insulin (Whitsett and Lessurd 1978) and transcobalmin II-vitamin B₁₂ complexes (Friedman et al 1977). For other plasma proteins, it is not clear whether receptors do exist, and hence the reason for the study in this thesis to find out whether other plasma proteins indeed possess receptors as a prelude to being endocytosed.

2.1 The trophoblastic plasma membrane.

In many ways, from the foregone sections, some special properties of the trophoblastic membrane have been touched
upon; these include the existence of microvilli, syncytiovascular membranes, receptors, coated vesicles and the syncytium with its specialised multinuclear appearance with single cytoplasm due to absence of cell walls.

Under the above heading, it is intended to discuss more the biochemistry of the bilipid plasma membrane, lateral movements within this layer, and more about surface receptors. The latter, however, may fit in better in the next section whereby surface receptors are better regarded as simply part of the generalised discussion of 'antigens of the plasma membrane matrix'.

The double phospholipid layer of plasma membrane is now a well proven entity for all systems of eukaryotic cell systems; the phosphate terminals being the hydrophilic ends, whilst the rest of the long 2-carbon skeletons of lipid components are the hydrophilic ends, and these face inwards. Another identical arrangement constitutes the bilayer. Protein molecules are randomly dispersed within this bilipid matrix. They either span the whole length of the bilayer or stop part of the way. The whole arrangement is fluid-like according to Singer and Nicholson's fluid-mosaic plasma membrane model (Singer and Nicholson 1972). (see diagram, Fig. 6). This model is now widely accepted and in fact explains most of our available data on membrane structure.

For receptor-studies, the most critical molecules are the proteins, some constituting antigens of all kind, HLA system, and receptors to a host of ligands. The fact that this is a dynamic fluid model, these protein molecules are
A schematic three dimensional and cross-sectional view of a fluid mosaic model of a membrane. Solid bodies represent globular proteins in a matrix of phospholipid membrane bilayer. Small open circles represent the hydrophilic ionic phosphate groups. V-shaped lines represent hydrophobic fatty acid chains. (After Singer and Nicholson 1972)
constantly changing positions. Their lateral movements are so rapid that a single molecule can change positions several hundred thousands of times within say a time scale of 5 seconds. Furthermore, the dynamic fluidity state can change into a quasi-static state or gel as a function of temperature. This phase transition being dependent also on the type of membrane lipids. For example lipids with more unsaturated bonds have lower transition temperatures than saturated ones. Longer carbon chains have higher transition temperatures than short ones. In addition, cis-unsaturated fatty acids have lower transition temperatures than trans-forms. These phase transition with temperature considerations are extremely critical to consider whilst studying antigen-receptor binding, for if the operational temperature is not controlled, (usually all incubations are done at 0°C, ie. on ice), one can get a phenomenon of capping.

Here, as soon as the ligand binds to its receptor, aggregation occurs of other receptors in the vicinity or even the far off ones, around the site of initial binding (Pernis et al 1970, Pinto De Silva 1972). One therefore gets uneven distribution, namely capping due to shifting of receptors to one spot; all made possible because of the fluidity state within which these receptors have vast lateral movements. This is minimised at 4°C (Munro 1975). The phenomenon of capping is not a metabolic one, but purely a physical reaction. It can be enhanced at high temperatures or blocked entirely by metabolic inhibitors (Taylor et al 1971). Eventually the capped area is internalised and when this occurs, it can result in a cell or membrane surface losing
or being stripped of an entire antigenic determinant (Boyse et al 1967).

Many systems have been observed to behave in this manner, for example immunoglobulin molecules on mouse lymphocytes (Taylor et al 1971) and human B-lymphocytes (Loo et al 1972), cytophilic antibody on basophils and macrophages (Becker et al 1973, Ferranini et al 1973). In indirect immunofluorescence or peroxidase methods used to detect antigens on cell surfaces or in the case of my placental receptor study, the antigen bound to its receptor - one is using antibodies, directed against these antigens as divalent reagents. They are capable of multiple binding and enhance aggregation and hence capping. Therefore in my study and in all operations whilst looking for receptors against plasma protein antigens on the placenta, were carried out on ice (0°C) at all times to eliminate or minimise these possibilities which could disrupt topographical patterns.

There are times when the apparently undesirable capping phenomenon, with its disruptive effect on antigenic distribution, can be put to good use. For example when one seeks to know whether certain surface antigens are associated or related in their determinants, co-aggregation or co-capping upon use of the same reagents provides a useful clue. This has been used successfully to show lack of association between D and K regions of H-2 antigen in mice (Neauport et al 1972), as well as Ig antigen receptors and HLA antigen on B-lymphocytes (Taylor et al 1972).
2.2.1 **Trophoblastic Antigens.**

One of the fundamental puzzles in immunology is how the placental homograft survives for nine months, with complete avoidance of rejection by the maternal immune system and yet have its circulatory system in intimate contiguity to that of the mother (Billington 1979, Billington et al 1975). Medawar (1953) has suggested that the mechanism is entirely immunological. McComick et al, 1971 were able to demonstrate the presence of IgG complement and fibrinogen particularly on the trophoblastic basement membrane (TBM) and were also able to elute the TBM-IgG. These eluates have subsequently been shown to inhibit several blastogenic reactions by human lymphocytes in culture (Faulk 1972, Faulk et al 1974).

Consequently, these and other placental antigens are thought to be at the very heart of inducing immunological tolerance. Page-Faulk et al 1975 confined their research to immuno-proteins, fibrillar proteins and contractile proteins, studied by both direct and indirect immunofluorescence. Immuno-proteins like IgG, A, M, C₃ and C₄ were predominantly localised in trophoblasts and TBM. The stroma was negative for these reactions but areas of fibrinoid necrosis stained for fibrinogen, IgG and complement. These immuno-proteins are specifically deposited on TBM and are of maternal origin (Faulk et al 1974). They inhibit blastogenic reactions of lymphocytes in vitro (Faulk et al 1973) and are therefore thought to contribute to the survival of the placental homograft. It is also very likely that all these proteins possess receptors on the trophoblast for uptake. Contractile proteins,
actin, actinin, and meromyosin were localised in the trophoblasts and fibroblasts, whilst myosin, troponin and tropomyosin were found in the stroma. These contractile proteins are, as a whole, thought to subserve locomotion, endocytosis and exocytosis of cellular products (Allison 1973). These processes cause the formation of coated vesicles which in turn transport proteins and other substances. Fibrillar proteins on the other hand, namely collagen and fibrinogen were mainly in the stroma and TBM. Fibrinogen was very much associated with IgG areas lending some support that fibrinoid areas represent areas of immunological activity within the stroma.

In general, intervillous fibrinoid areas represent degenerative changes in the cytotrophoblast most evident in placentae from diabetes, and pre-eclamptic toxaemia (Fox, 1968). Concurrent finding of IgG, C₁q, and C₃ in both the first trimester and full term placenta (McComick et al 1971, Faulk et al 1980) might indicate that immunocomplex depository might also result in fibrinoid necrosis. On the other hand, the finding of fibrinoid areas is not always linked to IgG in every case, whereas fibrinogen/fibrin, plasminogen, α₂macroglobulin, and C₄ are consistently identified, signifying that clotting proteins play a very vital role in maternal/foetal relationship. Furthermore, α₂macroglobulin, a protease inhibitor might inactivate plasminogen and cause poor formation of plasmin, a major initiator of fibrinolysis, and so result in persistence or even an increase in fibrin deposits. Of the clotting proteins sought for
receptor mediated uptake, only fibrinogen and thrombin will be considered in this study and will be discussed later. This might amplify on how fibrinogen possibly reaches the TBM or intravillous stroma if it had a receptor on the plasma membrane.

2.2.2 Human placental chorionic villar matrix - solubilisation of membrane antigens.

The extraordinary characteristics of the syncytiotrophoblast which allow the placenta homograft to survive maternal rejection have prompted various workers to search for reasons as to why and how nature has achieved such an immunological fit. The "why" is a complicated philosophical and teleological question but one supposes that whatever the true answer, it is aimed at promoting reproduction and survival of the animal species. As to finding an answer to "how", an attempt has to be made to survey the function of the antigens which span the trophoblastic surface, which is the nearest we can get in answering this question (Whyte and Loke 1970, Billingham 1964, McCcomick et al 1971).

There are a number of approaches to topographing these surface antigens. One is to raise heterologous antisera to highly purified trophoblastic membrane and then use antisera directed to suspected antigens, as blocking antibodies which then inhibits the binding of the antitrophoblastic antisera, when re-applied. This technique has been used extensively by Page-Faulk et al (1975), Whyte and Loke (1979) and mention of the main findings will be detailed shortly. The second technique is more of biochemical analysis of
these antigens. Here the membrane antigens are solubilised either in 1% Triton-X100 or sodium deoxycholate which results in extraction of all membrane proteins. Thereafter analysing the immunological identity of the antigens by classical immunoelectrophoresis, Laurell rocket electro-immunoassay, or the molecular weight profile on sodium dodecyl polyacrylamide gels (Ogbimi et al 1979, Whyte and Loke 1979, Ohno et al 1980).

To go back to the first method, it is first of all essential that antisera raised against the trophoblast is specific and limited in its activity to the surface otherwise antigens picked up other than on the surface would simply confuse the picture. It is almost unanimously agreed that the trophoblast is immunogenic and when injected in heterologous animals, antibodies with high specificity for only the surface are obtained (Whyte and Loke 1979, Beer, Billingham and Yang 1972, Rigby and Cursen 1969). Results of Whyte and Loke (ibid) for example, showed that when antisera to IgG, A, M, E, $\beta_2$microglobulin, $\alpha_2$macroglobulin, and transferrin are preincubated with placental tissue, there was no blocking of fluorescence or peroxidase reactions when trophoblastic antiserum was subsequently applied (direct and indirect). This implied that there are still other antigen components other than those represented by the blocking antisera to which the original trophoblastic antibody is directed. Others believe that these could be partly due to a multiplicity of surface glycoproteins which are abundant on the placental plasma membrane (Bradbury et al 1970).
It was also found that secretory products of the placenta such as specific pregnancy protein (SP), human chorionic glycoprotein (hCG), and human placental lactogen contributed to the antigenic pool and stained the membrane. Also monospecific antisera to IgG, (probably maternal), IgM, and albumin reacted although weakly. These staining characteristics for secretory products or other antigens were reminiscent of similar findings envisaged using the trophoblastic antiserum. Conclusion from these experiments imply that an array of antigenic components contribute to the total composition of antigenic determinants to which the trophoblastic antiserum is directed.

By solubilising membrane antigens, Carlson et al (1976), first purified trophoblastic membrane fractions using differential and rate-zonal ultracentrifugation and then proceeded to identify the various glycoproteins after solubilisation in 2% (W/V) sodium dodecyl sulphate. 1% Triton-X100 or sodium deoxycholate has also been used for the same purpose by other workers (Ogbimi et al 1979, Ohno et al 1980). In Carlson et al's series, 16 major protein bands were obtained with molecular weight range of 20,000 to 330,000. Out of these, 10 bands stained with periodic acid shift, implying their glycoprotein subunits. These workers pointed out that SDS-PAGE bands could be artefacts of proteolysis induced during membrane purification. Therefore experiments were repeated in presence and absence of 1mM PMSF, (Phenyl methyl sulfonic fluoride), a protease inhibitor.
No qualitative change in band numbers were observed and identical results were obtained in four placentae. Other investigators have detected up to 21 discrete bands (Ogbimi et al 1979). Most of these surface proteins remain largely unidentified in terms of function.

2.2.3 Specific Placental Antigens or Proteins.

Out of the array of surface antigens only a few have been successfully localised in terms of surface distribution (Page-Faulk et al 1977, Ogbimi et al 1980). Some of the proteins produced by the placenta are secreted into the maternal circulation and are therefore detectable in maternal serum. They are either pregnancy specific or associated for a simple reason that they are not seen outside pregnancy and are therefore products of the placenta. Hans Bohn and his group at Behring Institute, Marburg, West Germany, have been responsible recently for over 20 soluble placental tissue proteins; at least 11 protein antigens have been identified by immunochemical methods (Bohn 1973, 1974, 1975, Bohn et al 1980, 1981). The pregnancy specific proteins have been designated SPs and three are of particular interest, namely SP₁, SP₂ and SP₃. The twenty or so soluble proteins are called PPs (PP₁ to PP₂₀) (Bohn 1982). SP₁ is of molecular weight 90,000, a $\beta_1$-globulin and is also called pregnancy associated plasma protein C (PAPP-C). SP₂ is also a $\beta_1$ globulin of molecular weight 65,000 but known as sex hormone binding globulin (SHBG), whilst SP₃ is an $\alpha_2$ globulin with molecular weight 360,000 and
synonymously called pregnancy associated. $\alpha_2$ glycoprotein ( $\alpha_2$ PAG) or pregnancy associated Macroglobulin (PAM). These SP series are pregnancy related since they are only found in barely detectable amounts in normal serum, but attain strongly elevated levels in pregnancy.

However only SP$_1$ is specific for pregnancy, whilst SP$_2$ and SP$_3$ are only associated due to the fact that high levels can be encountered in women taking contraceptives, in particular SP$_2$ turning out to be a sex hormone binding globulin. Therefore SP$_1$ is of some clinical usefulness in terms of early detection of pregnancy (Grudzinskas et al 1977), intrauterine growth retardation (Gordon et al 1977), a tumour marker in relation to monitoring trophoblastic tumour disease (Seppälä et al 1978).

Of the PP series, they are largely unknown functionally, and only tentative function has been recently designated to some of them (Bohn 1982), namely PP$_2$ as ferritin, an already known protein, PP$_5$ as an inhibitor for trypsin and plasma as well as complexing with heparin and hence a protease inhibitor of coagulation system importance (Obiekwe et al 1979, Salem et al 1980), whilst PP$_7$ is probably a glutathione reductase (Marcus et al 1978). PP$_{15}$ has immunosuppressive effects on mixed lymphocytic cultures (Bohn 1980) as well as exhibiting immunogenicity whilst trying to raise antisera to it. These studies have explored its immunosuppressive capabilities. Seppälä et al 1979 have reported clinically that PP$_5$ might have a very important differentiating role between pregnancy and invasive trophoblastic disease.
Here PP<sub>5</sub> is barely measurable in trophoblastic tumour, whilst SP<sub>1</sub> and hCG are extremely high in both pregnancy and trophoblastic disease.

Mention will now be made of one promisingly important pregnancy related protein and that is PAPP-A (pregnancy associated plasma protein-A). A Miami-Florida group led S.P. Halbert first isolated this protein in 1972 (Gall and Halbert 1972) as well as three other related, PAPP-B, PAPP-C, and PAPP-D. PAPP-B remains half-forgotten, whilst PAPP-C is SP<sub>1</sub> and PAPP-D is placental lactogen. Unfortunately this group withdrew from pursuing the clinical correlation. The Aberdeen group under Klapper and others (Klapper 1982, Ahmad, Klapper and Toop 1981, Klapper et al 1977), have pursued this clinical relationship.

Klapper et al (ibid) found that PAPP-A levels unlike SP<sub>1</sub>, hCG or hPL, are unrelated to placental weight and growth in terms of lack of characteristic increasing levels which taper off near term. This even led to speculation that PAPP-A is probably a non placental protein. PAPP-A is an \( \alpha_2 \) macroglobulin with MW = 750,000 according to the data by Bischof (Bischof et al 1979). A break-through came from the Aberdeen group who found that PAPP-A was not only significantly raised in preeclamptic taxaemia of pregnancy, but that it was even more pronounced if preeclampsia occurred early in pregnancy. Hence a possibility of putting PAPP-A to use as an early warning signal for the advent of overt disease. The striking similarity of PAPP-A to \( \alpha_2 \) macroglobulin, a serum protease inhibitor is more than a passing
coincidence. They have comparable molecular weights, \( \alpha_2 \) mobility and protease inhibitory roles as PAPP-A has been shown to inhibit plasmin (Bischof 1979). All these put together lend support to PAPP-A as a possible serine protease inhibitor.

2.3.1 **Immunoregulation of Placental/Foetal Antigens.**

The subject of the immunologically 'previleged' position of the placental homograft and the possible mediation of this by its extraordinary plasma membrane have been touched upon in preceding sections. Historically this 'previleged' position which we know now to be due to its elusion of both humoral and cell mediated responses, was recognised by Little (Little 1924) as early as 1924. In modern terms this has been explained away through its possible lack of foetal/placental HLA, B, C, and D histocompatability antigens as implied in Little's quotation; "Under a condition in which the embryo has no definite physiological characteristics which are individual enough to be recognised as foreign by the mother"; whilst this statement might be true or even make sense, histocompatability antigens do in fact appear during the immediate pre-implantation cleavage of mouse embryos (Miggleton et al 1976).

The picture is complicated by evidence of a further differential expression of both the major and minor histocompatability antigens following post-implantation and embryonic development (Sellens 1977, Sellens et al 1978).
Since these antigens are present, failure to reject them must be due to regulatory factors and speculation has ranged from hormones such as hCG, hPI, prolactin, oestrogens, pregnancy associated proteins and glycoproteins including \( \alpha_1 \) fetoprotein (Gusdon 1976, Murgita 1976). Whatever the mechanisms of these non-specific factors, they must in some way affect lymphocyte, afferent and effector functions to stop their adverse rejection sequelae by the way of initiating immunosuppression (Howe 1975) as tested in vitro and laboratory animals (Beer 1979). The role of hCG in particular, is interesting. It has been postulated that high secretions of hCG by the trophoblast could affect lymphocytes functionally or hinder them sterically from attacking trophoblastic antigens (Adcock et al 1973). Loke et al have found a protein of MW 49,000 and pI = 4.2 integrated in the isolated and purified trophoblastic membrane, and which showed homology with samples of hCG in polyacrylamide gel electrophoresis and isoelectricfocussing. They postulated that membrane integrated hCG may alter protein conformation of other surface antigens and thus modify their immunogenicity to the mother (Loke et al 1970).

The effect of PP\(_{15}\) and the other newly isolated pregnancy associated glycoproteins in immunoregulation is not yet thoroughlly explored.
2.3.2 Specific Immunoregulators.

The array of various factors outlined above are non-specific regulators in the immunological survival of the placenta. Whatever their role, they have in the final analysis to affect, influence or modify the specific effectors of immunological reaction namely, the lymphocytes and humoral antibodies of the mother. It has been postulated that an antibody binds on the target antigens in a non-cytotoxic way and thus prevent the host's immunocompetent lymphocytes from mounting a rejection. Current view is not in favour of this mode of action but that free antigen or antigen-antibody complexes block both the target antigens as well as the lymphocytic receptors and thereby prevent rejection (Price et al 1978). This is called immunological enhancement. The role of a subclass of lymphocytes, called the suppression T-cells, have also been found to play some role in modifying or inactivating primed maternal lymphocytes which would otherwise attack the placental antigens (Billington 1979). These T-cells have been detected in neonatal umbilical cord blood (Skowron-Cendrzak et al 1976) and there is mounting evidence that lymphocytes can be filtered from mother to the foetus by the placenta (Adinolfi 1970, 1979).

3.1 Foetal Ontogeny and Plasma Proteins transfer or synthesis.

In determining as to whether the foetus, which has immature synthetic systems, has to import all its serum plasma proteins as it were from mother, one has to test for
its protein synthetic potential at various gestations. This can clear some mist with regard to whether inspite of immaturity of systems, some of the plasma proteins are synthesised by foetal organs.

Gitlin and co-workers have addressed themselves to the task of culturing various foetal organs to test their synthetic capability (Gitlin and Biasucci 1969). They tested for $\gamma G$, $\gamma M$, $\gamma A$ immunoglobulins, $C_1$ esterase inhibitor, hemopexin, haptoglobin, fibrinogen, plasminogen, $\alpha_1$ antitrypsin, orosomucoid, $\alpha_2$-lipoprotein, $\alpha_2$ macroglobulin and pre-albumin. They were studied in 15 embryos and foetuses of 29 days to 18 weeks. In addition they studied yolk sacs of 4 selected embryos. Organs such as livers, spleens, thymuses were minced and incubated with $^{14}C$-amino acids and the incubation media were tested for the specific proteins by autoradiography of immunoelectrophoresed samples. They concluded that the human embryos as early as 29 days synthesised $C_1$ esterase inhibitor, transferrin, hemopexin, $\alpha_1$ antitrypsin, $\alpha_2$-lipoprotein, $\alpha_2$ macroglobulin and pre-albumin in culture. At 32 days caeruloplasmin and orosomucoid were synthesised but fibrinogen synthesis was not detectable before 5.5 weeks. $\gamma M$ and $\gamma G$ were synthesised after 10.5 weeks and 12 weeks gestation respectively. A synthesis was not detectable in any of the cultures. $\gamma G$ synthesis was highest in the spleen for 17 to 18 week foetuses although other sites were noted. They then studied serum concentrations of proteins. $\gamma G$ changed from 0.52 g/l (5.5 weeks) and 1.8 g/l (22 weeks), to 7 g/l (26 weeks) and 14 g/l (40 weeks), $\gamma M$ was hardly
detectable, γA, whilst undetectable early on, but near or at term it was detectable. Either the foetus acquires the capacity to synthesise this protein or else the placenta becomes permeable to the protein (Stielm et al 1966). A few words will now be said about each of a selected list of plasma proteins.

**Caeruloplasmin.**

Synthesis of this protein was not evident in any other organ except the liver cultures and from 4.5 weeks, embryo's serum levels change from 4 mg/l (6.5 weeks) to 46 mg/l at 27 weeks and 155 mg/l at term. There is a sharp drop post-natally probably due to an oestrogen drop which normally stimulates the synthesis of this protein (Gitlin and Biasucci 1969). Transplacental transfer mechanism, if any, is unknown.

**α₂ macroglobulin.**

This is synthesised predominantly by the foetal liver, although Adinolfi (1971) disputed this by suggesting that the apparent radiolabelling of the protein in cultures is simply picked up through radiolabelling of other proteins. However, Gitlin et al 1969 had earlier shown that radioactive hydrolytic peptides corresponded to those of unlabelled α₂ macroglobulin. Serum levels in conceptus rises exponentially from 17% of adult level (14 weeks) to 150% of adult level at 30 weeks.

**Haptoglobin.**

Serum levels are quite low, namely 1 - 2.7% of mean adult level for 7.5 to 41 weeks foetuses. Post-natal synthesis
is quite rapid. Low antenatal levels are thought to be due to haemolysis or increased catabolism of this protein. Phenotypes however show a mixture of maternal and foetal origins (Hirschfeld and Lunell 1962). The mode of transplacental transfer is unknown.

Hemopexin.

Hepatic and lung synthesis have been observed (Gitlin et al 1969) and serum conceptus levels start at 10 weeks at 1% of adult level to 30% at term and continue to rise, rather than fall in the immediate post natal period. Maternal levels are 1.5 times higher than non-pregnant levels.

\( \alpha_1 \) acid glycoprotein.

Hepatic synthesis starts at 4.5 weeks of gestation and reaches 32% of adult levels at term. \( \alpha_1 \) acid glycoprotein crosses the placenta from mother to foetus by process of first order kinetics (Gitlin et al 1964), and so a proportion of foetal concentrations is from mother. Mother's \( \alpha_1 \) acid glycoprotein is only 72% of non-pregnant levels and Laurell and Skanse (1963) have suggested this to be an oestrogen effect. Whether active receptor mediated transport exists is not clear. However, Johnson et al 1974 have showed that \( \alpha_1 \) acid glycoprotein in amniotic fluid shows phenotypes of maternal origin.

\( \alpha_1 \) antitrypsin.

The liver is the principal foetal synthetic site beginning as early as 4 weeks (Gitlin et al 1969). At 6 weeks
only 6.5% of adult level is attained. 70% of adult level is rapidly reached by 9.5 to 10.5 weeks and between 27 weeks to term levels between 110 – 175% of adult level are attained. Correspondingly maternal levels are around 200% of non-pregnant levels (Gourot and Bjerre 1967). Some is probably transferred from mother, though the mechanism is not clear (Gitlin and Gitlin 1975). Nonetheless, phenotypes are not only that of the mother, suggesting foetal synthesis (Adinolfi 1971). However, whatever stimulates the very high maternal and foetal levels way above adult levels could be oestrogens stimulating on both maternal and foetal sides.

**Gc-globulin.**

Gc-components or glycoprotein was discovered by Hirschfeld in 1959 and subject to inheritoblic polymorphic forms synthesised primarily by the liver. Its recently discovered function is that of vitamin D binding and transport (Review by Reinskou 1968). There is evidence of maternal origin in both foetal serum and amniotic fluid (Johnson 1974), but the transplacental modality is unknown and hence the interest of this protein together with several others in my study, as will be seen later on in examining receptor mediated uptake. Gc is synthesised by foetal liver at 4 weeks and rises to 70 mg/l at 12 weeks and to 230 mg/l at term (Gitlin et al 1969). Maternal level is twice this figure at 460 mg/l (Toivanen et al 1969).

**Fibrinogen.**

Synthesis of this protein starts at 5.5 weeks in the
liver. There is evidence to show that maternal transfer takes place although the rate of transfer is very small. At term foetal serum levels average 70% of adult level. Clotting proteins, including thrombin, plasminogen, and esterase inhibitors are important in placental physiology and it is just possible that transplacental passage of these proteins is carrier mediated as will be discussed later in this report.
CHAPTER 2

MATERIALS AND METHODS

General Methods.

Under this heading the following will be discussed; trophoblastic membrane purification, protein assay methods, radiolabelling techniques, filtration techniques for separation of bound from free radioactivity in radioreceptor assays. Other more specific methods will be mentioned in the respective chapters dealing with specific areas such as immunocytochemical methods, namely immunofluorescence and peroxidase study, and colloidal gold labelling of various proteins to obtain useful electron microscopic probes for following protein transport across the trophoblast. Furthermore, methods pertinent to transfer studies such as perfusion technique will likewise be mentioned in that study.

Purification of Trophoblastic membrane

1.1 Overview

In order to examine events occurring at membrane level, it is imperative to study these not only under in vivo conditions, but more subtle mechanisms can be unravelled if purified plasma membrane is obtained and then incubated with radiolabelled protein ligands. To isolate the trophoblastic membrane a number of methods have been used differing in detail but all apparently yielding reasonably purified syncytiotrophoblastic microvillar plasma membrane (StMPM) (Ogbimi et al 1979, Whyte and Loke 1970, Johnson and Brown 1980, Carlson et al 1976). All these methods involve some
means of grinding up cut pieces of the placenta to release villi and thereafter disrupt their membrane by osmotic shock (Smith et al. 1974) or by mechanical homogenisation using various tissue grinders such as the MSE homogeniser or Ultra-Turrax, or even the more gentle, Dounce glass homogenisers, to mention a few that have been used. Thereafter pooled homogenates are sequentially filtered through either one coarse nylon mesh cloth and then a finer one to get rid of large pieces and debris. Then the uniform microvillar preparations are subjected to differential ultracentrifugation either with or without sucrose gradients. This differentially gets rid of nuclei, microsomes, mitochondria, cytosol, and other cellular material in various fractions which are discarded, to leave an StMPM fraction greatly enriched in plasma membrane.

Some workers usually test for purity of fractions of interest with various enzyme markers which characterise various subcellular fractions; for example alkaline phosphatase and 5' nucleotidase are good markers of plasma membrane fractions (Martin and Spicer 1974). Isocitrate dehydrogenase is a marker of cytoplasmic membrane (Carlson et al. 1976) such as that of mitochondria, DPNH diaphorase as endoplasmic reticulum marker (Steck et al. 1970) and aryl sulphatase as a lysosomal marker. Often viewing the final membrane by light microscopy or phase contrast gives some rough indication of enrichment. Better still, the electron microscopic visualisation offers a more refined view.

On the initial steps, it is always wise to dissect off the chorionic membranes before grinding, wash the placenta
free of blood in a suitable buffer, and very often add EDTA and a protease inhibitor such as phenyl sulphoric fluoride to stop proteolysis. All operations should be carried out on ice or at 4°C and avoid any prolonged manipulations at room temperature.

Ogbimi et al (1979), have combined the cold saline extraction of microvillar membrane method of Smith et al (1974) which they slightly modified and then further purified these StMPM by phase centrifugation method of Hourani et al (1973). Electron microscopic visualisation showed by and large the preparations had membrane with plenty of microvilli projections. They also noticed some limited contamination by mitochondria and occasionally lysosomal structures. Hydroxyproline assays revealed no significant content of this enzyme and hence freedom of the membrane pellet from collagen contamination. These workers have also noted a marked absence of $\beta_2$ microglobulin from solubilized fractions in contrast to other cellular membranes and hence suggested that absence of $\beta_2$ microglobulin in StMPM could be a further marker of lack of contamination by other membranes. Furthermore they reacted lectins with the soluble fraction and found strong and the only reactivity to wheat germ agglutinin. They noted a high carbohydrate content (240 $\mu$g/mg total protein). They also suggested that immobilised supports containing this lectin, wheat germ agglutinin, can be a powerful adjunct in further membrane purification to already existing methods.
1.2 Purification Procedure Adopted in this Study.

Smith's original method (Smith et al 1974) to which other workers have subsequently added modifications is essentially based on his observation that when microvilli are exposed to cold 0.9% saline they pinch off and can be collected and then membrane is purified thereafter. This method has the advantage of minimising contamination from other types of intracellular membranes inherent in the use of more disruptive homogenisation methods which use various blenders and tissue of grinders. Smith's method nonetheless requires cutting up the placenta into tiny pieces or the whole tissue is minced to start with and before the start of saline extraction which inevitably still leads to the same problem of contamination.

Booth et al 1980, have recently devised and published a purification method based on that of Smith but with improvements and modifications. Here the non-microvillar plasma membrane if present, is preferentially aggregated by Mg$^{2+}$ ions. They have tested its yield, in terms of specific activities, enzyme markers, electron microspically and $\beta_2$ microglobulin content. Marked enrichment with membrane was achieved as exemplified by the ratio of final fraction to starting homogenate of 24:1 for alkaline phosphatase and 23.8:1 for 5' nucleotidase, both plasma membrane markers. For similar fractions, enrichment ratios were not significant for succinate dehydrogenase and NADPH-cytochrome C reductase which were not detected at all in the final fraction, whilst in a step before the application of Mg$^{2+}$ ions, there
were enrichments of 0.51 and 1:1 fold respectively. This confirmed the efficacy of \( \text{Mg}^{2+} \) in eliminating mitochondrial contaminants in the final membrane pellet. Booth et al's purification procedure was so detailed and seemed to be well vindicated. Consequently I have adopted their protocol step by step in preparation of StMPM and subsequently using these preparations for all radioreceptor assays as well as immunocytochemical methods in my attempts to study placental receptors to some plasma proteins of interest.

1.3 **Placenta and Processing Procedure.**

These were obtained from the Department of Obstetrics and Gynaecology labour ward at the Leicester Royal Infirmary. Placentae were from either elective caesarean or full term spontaneous deliveries. All placentae were given a quick wash with cold phosphate buffered saline pH 7.4 and transported to the laboratory on ice. Chorionic membranes were dissected off and blood vessels on the foetal surface were scraped off and then the whole placenta was cut into 15-20g pieces. These were minced using an MSE homogeniser (MSE Scientific Instruments, Crawley, West Sussex, England), with 100 ml glass container fitted into a Bakelite container. 40 mls of 0.15M NaCl were added to each 15-20g placental pieces and the MSE homogeniser was worked in 4-5, 3 minute bursts at half-maximal speed until the whole placenta was homogenised.

600 - 1000 ml of pooled homogenate was filtered through a fine mesh nylon gauze to remove large particles. All
solutions and buffers contained 2mM phenyl methyl sulphonic fluoride to inhibit proteolysis. The filtered homogenate was taken through all stages as outlined in the following flow-chart. (See Fig. 7).

The OTD-65 refrigerated ultracentrifuge (DuPont Instruments) set at 4°C and using a T.865 constant angle rotor was used for the 90,000g spin for 30 minutes to the final 15,000g spin for 30 minutes. For the initial spins at 800g for 10 minutes and 10,000g for 10 minutes, the Sorvall R C-5B (Du Pont Instruments) ultracentrifuge was used with a GSA rotor which could spin large volumes of say 250 ml per polycarbonate container (six in all on the GSA rotor) once again set at 4°C.

The final pellet was then suspended in 50mM Tris-HCl pH 7.4 buffer to give 5 mg of protein per ml or at other times 3 - 4 mls of buffer were added to the final pellet and vortexed then stored at -20°C till required. Fresh unfixed fractions P₃, P₄ and P₅ (see line diagrams in Fig. 7) were examined by light microscopy and also using phase contrast Normarski optics (Allen, David and Normarski 1969) fitted to a universal microscope (Carl-Zeiss). Figure 8 shows fractions P₃, P₄ and P₅, whilst figure 9 shows the Normarski contrast optics for viewing the same P₃, P₄ and P₅ fractions. In these fresh unfixed preparations the change from heterogenous to homogeneous appearance as one examines fractions P₃ to P₅, particularly with Normarski enhancement signifies a good final yield of plasma membrane enriched fractions (microvillar membranes seen as short uniform dark projections throughout the field).
Flow Chart for Membrane Preparation

FILTERED HOMOGENATE

4°C Centrifuge x 800g 10 min.

PELLET

4°C Centrifuge 10,000g 10 min.

PELLET

4°C Centrifuge 90,000g 30 min.

PELLET

\[ P_3 \]

fraction

SUPERNATANT (discard)

PELLET \[ P_3 \] suspended in 50 ml of 10mM-mannitol, 2mM

- TRIS-HCl pH 7.1 with aid of loose fitting Dounce homogeniser to give fraction \( P_3 \).

- Solid \( MgCl_2\cdot6H_2O \) added to 10mM concentration stirred occasionally for 10 minutes on ice.

4°C Centrifuge 2200g 12 min.

PELLET \[ P_4 \]

4°C Centrifuge 15000g 30 min.

FINAL PELLET \[ P_3 \]

SUPERNATANT (discard)

Fig. 7
This shows Fractions $P_3$, $P_4$ and $P_5$; sequential purification of placental trophoblastic membrane as per purification schedule in Fig. 7. At the indicated stages unfixed portions were examined on a slide with a Carl-Zeiss microscope. $P_3$ shows dilute heterogenous preparation, which transforms into a more concentrated but homogenous pieces of membrane showing as dark spots throughout the field. (Magnification $X$ 110)
As in Fig. 8, the same $P_3$, $P_4$, and $P_5$ membrane purification stages are shown again in an unfixed state using Normarski interference contrast microscopy fitted to a photomicroscope (Carl-Zeiss).
Electron microscopy of each fixed piece of P₃, P₄ and the final pellet are shown in Figures 10a to 10c. P₃'s, the 90,000g pellets and prior to Mg²⁺ addition are shown in Figure 10a. This figure shows bits of microvilli which appear as rod-like, rounded and sometimes dumb-bell shaped structures depending on the plane of view. However, there is also marked contamination of other vesicular and much bigger membrane structures are seen in the central fields of view in each of these photographs. P₅'s the final pellets are shown in Figure 10c. In this figure the surface of StMPM is seen with microvilli and plenty of micropinocytic vesicles in the syncytium and a foetal blood vessel with a red blood cell, whilst Figure 10b shows again the surface of StMPM with microvilli.

The protein content of StMPM pellet in order to standardise amounts pipetted in radioreceptor assay incubations, were determined by the modified micro Lowry and Bio-Rad protein assay methods.

Protein Assay Methods.

2.1 Micro-Lowry technique.

The Lowry protein assay method (Lowry et al 1951) in principle involves the use of folin-ciocalteu's reagent (phosphomolybdic tungstic acid), with the phenolic tyrosine residues in a protein to give a blue colour. Micromodifications have been made to assay proteins at microgram level.

\[
\text{(Protein)} + \text{Phosphomolybdic} \quad \text{tungstic acid} \quad \rightarrow \quad \text{Blue} \quad \text{colour}
\]
Figs. 10 (a), (b) and (c).

Transmission electron micrographs of membrane purification stages P3, P4, and P5. Fig. 10(a) shows impure membrane pieces with rod-like microvilli, and at times circular depending on the plane of sectioning. Fig. 10(b) shows the limiting membrane surface with finger-like microvilli projections extending out into the maternal intervillous space from where nutrients are absorbed.

Fig. 10(c) again shows microvilli on the syncytiotrophoblast in the bottom half of the photograph. A multiplicity of rounded vesicles are seen scattered through the syncytium. In the top half is a semi-lunar wall of a foetal vessel with a dense body in the centre (Red blood cell). This arrangement, the contiguity of a blood vessel to the absorptive microvilli ensures rapid transfer of nutrients and proteins to the foetus.
This method is not sensitive in presence of other phenol containing compounds. As regards assaying membrane protein, modifications have to be made.

Soluble membrane protein is extracted by reacting the StMPM with 1M NaOH at 37°C for 2 hours, constantly stirring the incubation mixture every 15 minutes. Some proteins are then solubilised, others are not, and one has got to make a reasonable assumption that more membrane protein is substantially extracted. In the Micro-Lowry technique optimal pH is necessary for the Folin-ciocalteu's reaction. 1M NaOH hydroxide in solubilised membrane protein mixture would greatly alter the sensitivity of the assay and so neutralisation of NaOH with 3M HCl is carried out. The following protocol was followed:

MODIFIED LOWRY PROTEIN ESTIMATION ADAPTED FOR DETERMINATION OF MEMBRANE PROTEINS IN 1M NaOH.

Because the various colour development steps are sensitive to pH changes the presence of 1M NaOH in the sample shifts the reaction pH away from its optimum. By neutralising the excess alkali with acid, the buffers in the reagents can control the pH balance effectively.

STOCK SOLUTIONS

a. 1M NaOH : 40g/l

b. 3M HCl : Concentrated HCl is 11M, so 3.6 ml Conc. HCl is made up to 11ml with water.

c. Sodium Carbonate : 2g anhydrous Na₂CO₃ per 100 ml water.

d. Copper Sulphate : 1g CuSO₄·5H₂O per 100 ml water.
STOCK SOLUTIONS  Cont'd ..

e. Sodium Tartrate  : 2g \((\text{CH}_2\text{COO})_2\text{Na}\_2 \cdot 2\text{H}_2\text{O}\) per 100 ml water.

f. Bovine Serum Albumin  : 4.58 mg BSA/ml dissolved in 1M NaOH (Store at -20°C)
N.B. Takes about 1 hour to dissolve at room temperature.

g. Folin Ciocalteu's Reagent  : BDH (Store at 4°C)

REAGENTS.

Folin A  : Add 1 ml of d) and 1 ml of e) to 100 ml of c) (need 25 ml for 10 assays). Mix and prepare daily.

Folin B  : Dilute 1:1 with water (need 2.5 ml for 10 assays).

METHOD.

1. Place a piece of membrane pellet in 1ml 1M NaOH in a sealed tube and incubate at 37°C for 2 hours, whirlimixing every 10-15 minutes.

2. Add 0.145ml 3M HCl and mix.

3. Take 2 x 0.5ml aliquots into 9ml plastic tubes.

4. Add 2.5ml Folin A to each tube and leave at room temperature for 20 minutes.

5. Add 0.25ml Folin B and \text{MIX IMMEDIATELY} after each tube. (Seconds delay diminishes colour reaction).

6. Leave at room temperature for 45 minutes and read absorbance at 750nm against a distilled water blank.
STANDARDS

Take 0 - 50 µl (in 10 µl aliquots) of BSA Stock Solution and make up to 1ml with 1M NaOH.
Then proceed as from Stage 2 above.
The resultant amounts of standard protein in the 0.5ml will then range between 0 and 100 µg BSA.
Draw a standard curve (a straight line going through the origin) as shown in the graph. From the gradient one can calculate from \( y = a + bx \), the amount of protein per µl of membrane:

\[
\mu g \text{ PROTEIN PER } \mu l \text{ OF MEMBRANE} = \frac{C}{\text{GRADIENT}} \times 2.29
\]

where

- \( c = \) corrected absorbance (observed - blank)
- blank = 0µl BSA in standards
- 2.29 = 0.5ml sample from 1.145ml total.

2.2 Bio-Rad protein assay procedure.

The Bio-Rad protein assay is a dye binding assay based on the differential colour change to various concentrations of protein. Here the principle is based on the observation that absorbance for an acidic solution of Coomassie blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs (Bradford 1976). It is found that the extinction coefficient of dye-albumin complex was constant over a 10-fold concentration range and thus Beer's Law was obeyed. The beauty of this Bio-Rad method is in that essentially one reagent is required and five minutes to perform as compared
to three or more reagents and almost 1 hour in the micro-Lowry method and yet the sensitivity is comparable at 1 ug protein. Furthermore, chemical interference is very slight compared to the Lowry method where big effects are noted.

Standard Assay Procedure

Range: 20 - 140 µg protein.

Prepare several dilutions of protein standard supplied in the kit such that a range of 0.2 - 1.4 mg/ml is covered and then;

1. Place 0.1 ml of standards and appropriately diluted samples in clean dry test tubes. Place 0.1 ml sample buffer (supplied) in blank test tube.

2. Add 5.0 ml of diluted dye reagent (1:4 ratio of dye to deionised water).

3. Vortex or mix several times.

4. After 5 minutes to 1 hour measure OD _{595} versus reagent blank.

5. Plot OD _{595} versus concentration of standards - read unknowns therefrom.

Both the standard curves for the Lowry micro method and the Bio-Rad assays with respect to assaying membrane protein are shown in Figures 11 and 12 respectively.

Example: Comparison of two methods in accurately estimating membrane protein concentration.
Fig. 11: A typical standard curve using modified micro-Lowry protein assay capable of measuring 0-110 μg of protein.
Fig. 12: A typical standard curve for assaying membrane protein using a Bio-Rad micro-assay. This is based on maximum absorption at 595 nm when an acid extract of Coomasie-Blue G\textsubscript{250} dye combines with protein. Sensitivity is comparable to micro-Lowry.
Micro Lowry method

5μl of StMPM extracted solution

Mean absorbance at 750nm duplicated = 0.447

Gradient of standard curve = 0.50 x 10^{-2}

(ug) Membrane protein in 5μl StMPM = \frac{0.447}{0.50 \times 10^{-2} \times 2.29} = 202.06μg

whence

1μl = 40.40μg

Bio-Rad method

Same membrane used and;

Mean absorbance at 595nm of duplicates = 0.978

Corresponding to (0.1 ml sample) = 169.5μg

The reaction volume was made up of (0.5ml of 1M NaOH + 73μl 3M HCl + 25μl StMPM),

giving a total volume = 598μl giving Protein dilution of 25/598

Thus 100μl of 25/598 dilution of StMPM = 169.5μg

Therefore 100μl of undiluted StMPM = \frac{598}{25} \times 169.5μg = 4054.44μg

and so 1μl of StMPM = 40.54μg

Therefore both methods reliably gave identical results, the Bio-Rad being a much simpler one to use. Furthermore the
Bio-Rad procedure could be adjusted to span a range of 1 - 20 µg/ml, by not diluting the dye concentrate supplied (0.2 ml of it used) and adding 0.8 ml of standards or samples in Step 1, instead of 0.1 ml. The rest of the procedure is unaltered, this allowed assay of chromatography elution fractions of tritium labelled proteins to be measured where possible, namely where one used 1mg or more of protein to the labelling procedure otherwise other methods were used to calculate protein incorporation and from amounts used, the specific activities could be calculated.
Radiolabelling procedure for producing various tritiated protein ligand derivatives.

3.1 Preparation of reagents.

a) \textbf{0.1M Borate buffer} was made as follows:

- Boric acid 6.18gm
- Sodium tetraborate 9.5gm
- Sodium chloride 4.38gm

make up to 1000 mls with distilled water and adjust pH to 8.5 with HCl.

b) \textbf{Protein sample}

1mg of each protein was weighed out and added to 20 \textmu{l} of 0.1M borate buffer pH 8.5 in a plastic vial previously coated with gelatin (see preparation of column buffer).

c) \textbf{Glycine stop solution}.

0.2M glycine was made up in 0.1M borate buffer pH 8.5 (0.15g of glycine per 10 ml of borate buffer).

3.2 Preparation of column for separation of labelled and unlabelled IgG-H$^3$. 

a) \textbf{Pump speed and delay}; using an LKB 2120 vario perpex II pump the optimum speed was found to be 1.4. This did not compact the column or force air bubbles through it. An air bubble was used to measure the delay between the UV monitor and the fraction collector. Therefore
the number of drops counted between recorder deflection, when the bubble passes the UV monitor and reaching the dropper is the delay which was 40 drops. Therefore fraction collector was set at 20 drops and delay at 20 drops.

b) Column buffer

Phosphate buffer was made up as follows:

(i) Stock Solution A
0.2M sodium dihydrogen orthophosphate.

(ii) Stock Solution B
0.2M Disodium hydrogen orthophosphate anhydrous (MW = 141.96). Hydrous form (MW 177.99).

Column buffer was therefore made from 20 ml of solution A added to 80 mls of solution B adjusted pH = 7.5, and this gave a 0.05M phosphate buffer, pH 7.5. To 50 mls of this 0.125g of Gelatin was added. The latter was to minimise non-specific protein adsorption of the sample containing tritiated protein to the column.

3.3 Preparation of column LKB-K₉ column

1gm of Sephadex G-50 fine was added to 20 mls of 0.05M phosphate buffer pH 7.5, with 0.25% gelatin, and allowed to swell overnight at room temperature. The gel was poured on to the K₉ column and allowed to settle.
3.4 Radiolabelling procedure

250 μl of N-Succinimidyl (2,3 - $^{3}$H) propionate (NSP) (Radiochemical Centre Amersham Product, Code TRK 556) were added to a conical glass test tube. A jet of $N_{2}$ blowing gently through a fine glass pipette (Bilbao pipettes) was used to dry off the toluene solvent. The glass test tube had already been placed on ice (0°C) and then various amounts of protein (see table for exact amounts as per the desired NSP/protein ratio) previously dissolved in 20 μl of borate buffer pH 8.5, were reacted with dry NSP for 15 minutes. The reaction was stopped by addition of 500 μl of 0.2M glycine. Figure 13 shows the essentials of the protein labelling reaction.

Separation of labelled protein.

The radioactive protein sample was applied to the Sephadex G-50 column and run through at 1.9 ml fraction (20 drops per tube). Absorbance was monitored at 280 nm for column elution fractions. If they did not register any appreciable deflection, it was sufficient to monitor the radioactivity alone. Fractions were counted by aliquoting 20 μl of each tube in 5 ml Fisofluor I (Fisons, Loughborough, Leicestershire), and then counting in a Tricarb liquid scintillation spectrometer.

Control.

Each cold (non-labelled protein) at the same concentration as the labelled one was added to the 0.05M phosphate buffer pH 7.5 and then run through the column monitoring
N-SUCCINIMIDYL (2 3\(^{3}H\)) PROPIONATE

AMINO TERMINAL OF PROTEIN

TRITIATED PROTEIN DERIVATIVE
the elution fractions as before. This confirmed where the protein peaks were located in comparison to the protein and glycine peaks when labelled proteins were separated. This 'cold' control also helped in setting the amplitude of the deflection expected from a 1mg protein on to the chart recorder.

**Settings on IKB 2120 fraction collector were as follows:**

- **Absorbance Range**: 0.1
- **Chart speed**: 0.1mm/sec
- **Log scale**: 100mV
- **Linear scale**: 50mV
- **Flow rate**: 20 drops/tube

Table 2 gives NSP/protein ratio and amount utilised in 20 μl of borate buffer for reacting with 250 μl NSP. Incorporation of the radioactivity into the protein peak as well as specific activities are also indicated. All calculations are based on 250 μl NSP namely 5.812 x 10⁻⁶ mMoles and supplied as 1mg/ml with specific activity of 43Ci/mmole. The appropriate ratios are worked out by taking into account the molecular weight of each protein.

3.5 **Logistics of delivering microgram amounts for reacting with NSP.**

Where low NSP/protein ratios are required, this means more protein is delivered and in some cases, say 1:1 ratio NSP/IgG, 1mg of IgG was easily dissolved in 20 μl buffer. However, as shown in Table 2 where lower microgram amounts required to give 3:1 NSP/protein ratios there was a problem
<table>
<thead>
<tr>
<th>Protein</th>
<th>(NSP/Protein) ratio</th>
<th>1 mg of protein dissolved in; (µl) of buffer</th>
<th>20 µl aliquot of buffer delivers (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>1:1</td>
<td>20</td>
<td>1000</td>
</tr>
<tr>
<td>Transferrin</td>
<td>3:1</td>
<td>114</td>
<td>174.3</td>
</tr>
<tr>
<td>PreAlbumin</td>
<td>3:1</td>
<td>187</td>
<td>106.5</td>
</tr>
<tr>
<td>Gc-Glycoprotein</td>
<td>3:1</td>
<td>127</td>
<td>156.9</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>3:1</td>
<td>104</td>
<td>193.0</td>
</tr>
<tr>
<td>α₁-Acid-glycoprotein</td>
<td>3:1</td>
<td>240</td>
<td>85.2</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>3:1</td>
<td>35</td>
<td>571.4</td>
</tr>
<tr>
<td>α₁-Antitrypsin</td>
<td>3:1</td>
<td>191</td>
<td>104.0</td>
</tr>
<tr>
<td>Thrombin</td>
<td>2:1</td>
<td>186</td>
<td>107.5</td>
</tr>
<tr>
<td>α₂-HS-Glycoprotein</td>
<td>3:1</td>
<td>210</td>
<td>94.9</td>
</tr>
</tbody>
</table>

Table 1

During radio-labelling procedure, the Table shows the best way of delivering microgram amounts initially weighed out as 1 mg/appropriate volume of buffer and then 20 µl were added to the NSP-H³, to give the indicated labelling ratios.
of weighing these small amounts. A quicker way was to weigh out 1 mg for all proteins and then dissolve this in a calculated buffer volume such that when 20 μl of this solution is pipetted the desired amounts of protein to give appropriate NSP/protein ratios are delivered as per table 1.

3.6 Determination of Specific Activities.

a) Column method

From the elution profile of each protein the incorporation of the tritium label was calculated from the counts associated with the protein peak relative to the total counts, that is the protein and glycine peaks added together. Percentage of incorporation was then calculated as per table 2.

A worked example of incorporation of tritium into IgG will be considered here.

From elution profile on Sephadex G-50,

<table>
<thead>
<tr>
<th>Fraction</th>
<th>CPM (radioactive counts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24.0</td>
</tr>
<tr>
<td>2</td>
<td>24.3</td>
</tr>
<tr>
<td>3</td>
<td>24.5</td>
</tr>
<tr>
<td>4</td>
<td>22.0</td>
</tr>
<tr>
<td>5</td>
<td>36174.3</td>
</tr>
<tr>
<td>6</td>
<td>500266.8</td>
</tr>
<tr>
<td>7</td>
<td>228779.0</td>
</tr>
<tr>
<td>8</td>
<td>88023.0</td>
</tr>
<tr>
<td>9</td>
<td>41973.8</td>
</tr>
<tr>
<td>10</td>
<td>45634.3</td>
</tr>
<tr>
<td>11</td>
<td>512282.3</td>
</tr>
<tr>
<td>12</td>
<td>882009.5</td>
</tr>
<tr>
<td>13</td>
<td>509899.6</td>
</tr>
<tr>
<td>14</td>
<td>87932.8</td>
</tr>
<tr>
<td>15</td>
<td>16626.5</td>
</tr>
<tr>
<td>16</td>
<td>7120.0</td>
</tr>
<tr>
<td>17</td>
<td>4615.8</td>
</tr>
<tr>
<td>18</td>
<td>3847.8</td>
</tr>
<tr>
<td>19</td>
<td>2971.8</td>
</tr>
<tr>
<td>20</td>
<td>3420.0</td>
</tr>
</tbody>
</table>

Protein peak = 995312 cpm

Glycine peak = 2,076,360 cpm

Total = 3,071,672 cpm
Based on 20 µl aliquots of eluted fractions,

Protein yield \( = \frac{995312}{3071672} \times 100 \)
\( = 32.4\% \)

1 vial of NSP = 1 mCi/ml as supplied
But 1 Ci
\( = 3.7 \times 10^{10} \) dps
\( = (3.7 \times 10^{10}) \times 60 \text{ dpm (At 100\% efficiency)} \)
1 mCi
\( = 10^{-3} (3.7 \times 10^{10}) \times 60 \text{ dpm} \)

And 250 µl NSP = \( \frac{1}{4} \) mCi = 0.25 mCi

therefore this is equal to \( 0.25 \times 10^{-3} (3.7 \times 10^{10}) \) dpm
\( = 55.5 \times 10^{7} \text{ dpm} \)

Assuming no significant loss, 1mg of IgG that was reacted, will represent approximately 1mg in the fractionated protein peak.

If only 32.4% of the total counts are incorporated according to the yield then the counts in protein peak
\[ = 0.324 \times 55.5 \times 10^{7} \text{ dpm} \]

therefore 1000 µg = 179820000 dpm
1 µg = 179,820 dpm

b) 10% TCA (Trichloroacetic acid) precipitation method.

The reaction mixture in all instances consisted of 500 µl of 0.2M glycine and 20 µl of protein in buffer (total 520 µl) and prior to application of this mixture to the column specific activity was assessed using 10% TCA precipitation as shown in Fig. 14.
520 μl (original mixture)

5 μl mixture

+ 200 μl 0.2M Phosphate buffer pH 7.5

Take 20 μl of this + 10 ml Fusiflour scintillant

TOTAL COUNTS

5 μl mixture

+ 50 μl 1% BSA in Phosphate buffered saline pH 7.4

Add 1 ml 10% TCA

Centrifuge
discard supernatant

Pellet + 100 μl 0.2M NaOH

Take 20 μl + 10 ml scintillant

Specific counts
TOTAL COUNTS = % Yield

(Specific Counts) calculated

Fig. 14 - shows a 10% Trichloracetic acid precipitation method for working out specific activities of radio-labelled proteins.
<table>
<thead>
<tr>
<th>Protein</th>
<th>NSP/Protein ratio</th>
<th>Protein in 20ul buffer (µg)</th>
<th>Incorporated percentage of radioactivity in protein peak</th>
<th>Specific activity/ug protein (cpm/ug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 IgG</td>
<td>1:1</td>
<td>1000</td>
<td>32.4</td>
<td>179,820</td>
</tr>
<tr>
<td>2 Transferrin</td>
<td>3:1</td>
<td>174.4</td>
<td>69.8</td>
<td>81,847</td>
</tr>
<tr>
<td>3 Prealbumin</td>
<td>3:1</td>
<td>106.5</td>
<td>42.0</td>
<td>432,393</td>
</tr>
<tr>
<td>4 Gc-Glycoprotein</td>
<td>3:1</td>
<td>156.9</td>
<td>30.4</td>
<td>387,872</td>
</tr>
<tr>
<td>5 α1 Acid glycoprotein</td>
<td>3:1</td>
<td>85.2</td>
<td>30.6</td>
<td>583,103</td>
</tr>
<tr>
<td>6 Haptoglobin</td>
<td>3:1</td>
<td>193.0</td>
<td>59.8</td>
<td>336,836</td>
</tr>
<tr>
<td>7 Fibrinogen</td>
<td>3:1</td>
<td>581.2</td>
<td>14.5</td>
<td>10,718</td>
</tr>
<tr>
<td>8 α1 Antitrypsin</td>
<td>3:1</td>
<td>104.6</td>
<td>72.9</td>
<td>121,370</td>
</tr>
<tr>
<td>9 Thrombin</td>
<td>2:1</td>
<td>107.5</td>
<td>10.0</td>
<td>186,986</td>
</tr>
<tr>
<td>10 α2 HS-Glycoprotein</td>
<td>3:1</td>
<td>94.9</td>
<td>19.7</td>
<td>49,943</td>
</tr>
</tbody>
</table>

Table 2 - shows the labelling ratios, when the indicated micrograms of protein were added to 250 µl NSP-H\(^3\) (0.25 mCi NSP equal to 5.812 x 10\(^{-6}\) mmole for a 43 Ci/m mole batch or 4.23 x 10\(^{-6}\) mmole for a 59 Ci/m mole batch), to give the displayed percentage radioactivity incorporated and specific activities.
3.7 Assessment of counting efficiency of Tricarb scintillation spectrometer.

Take 1 µl of supplied NSP at 1 mCi/ml

\[ \text{i.e. } 1/1000 \text{ mCi taken} \]

\[ = 1/1000 \times 10^{-3} (3.7 \times 10^{10}) \text{ 60 dpm} \]

\[ \text{Theoretical activity is } 10^{10} (3.7 \times 10^{10}) \text{ 60 dpm} \]

\[ = 222 \times 10^4 \text{ dpm} \]

If 10 µl is added to 10 ml scintillant and counted as \((x)\)dpm.

\[ \text{Efficiency} = \left( \frac{x}{225 \times 10^4} \right) \times 100\% \]

**Worked example**

NSP was supplied as 1 mCi/ml and from which

\[ 1 \mu l = 10^{-3} \text{ mCi} \]

\[ = 10^{-3} \times 10^{-3} \times 60 (3.7 \times 10^{10}) \text{ dpm} \]

\[ \text{(since 1 Ci} = 3.7 \times 10^{10} \times 60 \text{ dpm)} \]

1 µl of same NSP was diluted 1/80 and

20 µl of this when counted on the Tricarb spectrometer gave \(= 165164 \text{ dpm} \)

Hence the total 80 µl contained \(\frac{80 \times 165164}{20} \text{ dpm} \)

But this is equal to the original 1 µl before it was diluted 1/80.

Therefore \(1\mu l = \frac{80 \times 165164}{20} = 660658 \text{ dpm} \)

\[ \text{Efficiency of scintillation spectrometer.} = \frac{\text{counts registered from 1µl}}{\text{calculated theoretical counts}} \]

\[ = \frac{660658}{222 \times 10^4} \]

\[ = 30\% \]
3.8 Filtration Techniques

All experiments involving labelled proteins and incubated with purified trophoblastic membrane, necessitated separation of bound from free radioactivity in order to assess the degree of label binding. There was a need to have a rapid, reliable and easy method to use. To this end, two methods were used, namely the Amicon Millipore filtration system and another one constructed by the author from improvised old bits of Technicon Auto-Analizer A_I or A_{II}. These included the flow pump linked by tubings to a system of three piece funnels that had been bought from Whatman (Maidenhead, Surrey, U.K). This latter method will be described first mainly because it was constructed at very little cost compared to a £400 Amicon Millipore system.

a) Improvised filtration technique.

There are three major components of this system; the flow pump linked to a system of pump tubings which in turn were connected to reservoir attached to 3 piece funnels through which samples were introduced. Figure 15 illustrates all the main parts diagramatically. Also the photograph in Fig. 16 shows the whole assembly capable of taking 12 filtration funnels with a filtration time of 5 minutes for all.
This shows some components of the improvised filtration system. It consists of a three-piece funnel (Whatman Products, Maidenhead, Surrey), connected to a 15ml reservoir, in turn connected by tubings to a Technicon A1 peristaltic pump, normally used for the Technicon A1 AutoAnalyzer (Technicon Instruments, Basingstoke, Hants). Up to 12 funnels could be connected to the pump as shown in Fig. 16.
Fig. 15

- Funnel
- Acrylic plate
- Tubings
- 15ml plastic reservoir
- Technicon Peristaltic pump

*Technicon A1*
Fig. 16

A photograph of the filtration system described in Fig. 15 is shown; consisting of filtration funnels and tubings connecting to the Technicon A₁ peristaltic pump. The pump's transformer on extreme right is shown too.
The following is a description of some of the components.

3 Piece-funnel

These were twelve in all to increase the number of filtrations that can be done simultaneously. The funnels were purchased from Whatman Ltd., Maidenhead, Surrey, and consisted of a 15ml funnel, a central acrylic perforated plate on which Whatman Glass fibre discs GF/C (Pore 4 mm diameter, 1.2 μM) filters were mounted, and the bottom receptacle attached to 15ml reservoirs (see photograph in Fig 16).

Tubings

These were pump tubes (white/purple and of 4 mm in diameter for 3 ml/min flow rates and supplied by Technicon Co. for the Technicon AutoAnalyzer A_I or A_II machines, (Technicon Co. Ltd., Basingstoke, Hants). There were 12 tubings in all and securely pressed under the chain driven rollers of the pump.

Pump

This was taken from a dismantled old AutoAnalyzer A_I and could pump through of tubings used at a rate of 3mls/minute. It must be emphasised that due to the way the tubings were attached to the reservoirs and then left open ended at the other end, only air was pumped and the fluid flow was restricted between the funnel and the collecting reservoirs. The system ran satisfactorily.

b) The Amicon Millipore Filtration System
Fig. 17 - shows an Amicon filtration chamber (Amicon Corporation) which takes a maximum of 12 glass-fibre filter discs (Whatman). This unit is connected to a vacuum generated by a pump on the right. The vacuum pressure should not exceed 15 Ib/in². The whole system costs about £400 in comparison to a cheap version that was constructed from various bits of old Technicon machinery shown in Fig.16.

In both systems filtration was rapid (12 filters every 3 minutes) when either a 1.2 um GF/C or 0.7 um GF/F Whatman glass-fibre disc was used.
this system which takes 12 GF/C filters. These were pre-soaked in 50 mM Tris HCl buffer pH 7.4 containing 5% BSA and then placed in appropriate groves designed to take these discs. The top cover was secured in position by a screwable top and then contents of reaction mixture are directly poured on to the wells containing the GF/C filters and suction created by vacuum pump assists filtration. Only 2 minutes is need for 12 filters.

Discussion.

In either case the filtration is rapid between 2 to 5 minutes depending on which system is used. This is contrasted to about 20 minutes if the reaction mixture was left to filter through by the weight of the fluid column, usually 15 mls. Filters were then dipped into 5 or 10ml of Fisoflour I scintillant and counted. Some manufacturers recommend the following;

- presoaking of filters in buffer containing 5 - 10% Bovine serum albumin.
- Dry filters after filtration to minimise quenching of scintillant.
- Choice of scintillant, that can usually dissolve the filter completely for effective release of bound ligand.

Whilst presoaking with 5-10% BSA helped to minimise non-specific binding the drying of filters was not found to be a critical factor. Very close counts were obtained with and without drying (usually at 100°C for 10 minutes) and therefore all the subsequent experiments drying was not
necessary and filters were dipped straight into the scintillant immediately after filtration. Duplicates were always done.
CHAPTER 3

IMMUNOCYTOCHEMICAL METHODS - THE USE OF IMMUNOFLUORESCENCE AND PEROXIDASE PROBES IN STUDYING THE TOPOGRAPHY OF PLACENTAL MEMBRANE RECEPTORS.

Introduction

Immunocytochemistry provides a rapid, usually very reproducible results particularly in localising antigens of cytosolic or membrane origin. To this end, various workers have employed immunofluorescent techniques to the study of the placenta for both the peri-implantation (Johnson 1975) and post-implantation antigens (Page-Faulk et al 1975). Other workers have been interested in delineating cytoskeletal elements which provide the backbone support for the placenta and have also used fluorescence to localise polymeric molecules such as tubulin (Ockleford and Wakely 1981), desmin, actin and polymeric proteins (Ockleford et al 1981).

Youtananukorn (Youtananukorn et al 1972) reported on cell mediated immunity to placental antigens by maternal lymphocytes. However, these antigens were not identified. Faulk et al (1975) in the search for these antigens on post implantation placenta were able to show that immunoproteins, fibrillar proteins, and contractile proteins were very important antigens. Antibodies directed to such antigens were found to have concormitant blocking effects to cell mediated lymphocytic reactions, which points to the immunologically protective role of these surface antigens. To study the distribution of these antigens,
these workers used both direct and the two stage indirect immunofluorescent assays. They raised they own antisera to isolated immunoproteins (IgG, A, M), complement (C₃, C₄), fibrillar proteins (collagen), contractile elements (actin, tropomyosin) and conjugated it with fluorescein isothiocyanate (FITC) by dialysis technique of Clark and Shepard (1963). Unreacted FITC being removed on Sephadex G25 column. Conjugates were then carried through staining reactions and specificity checks as described by Faulk and Hijmans (1972). Any antigen of interest can thus be studied in a manner analogous to the above study of Page-Faulk and others using placental tissue prepared by snap-freezing for cryostat sections or treatment with 3% Formalin in phosphate buffered saline, and then sectioning. For direct fluorescence FITC-labelled first antibody (usually Rabbit anti-human) can be applied. Where amplification or higher sensitivity is required the FITC label is attached to the second antibody layer (usually goat or swine anti-rabbit serum). For even higher sensitivity a third layer the peroxidase-anti peroxidase conjugated attached to the second antibody, can be utilised in the immunoperoxidase assay.

Instead of block cut sections, isolated purified placental microvillar membranes (StMPM) can be used to study membrane topography. In the present study StMPM was used with respect to delineating surface receptors to the proteins mentioned below.
Materials and Methods

Proteins Studied:

These included IgG, transferrin, haptoglobin, \( \alpha_1 \) acid glycoprotein, \( \alpha_1 \) antitrypsin, fibrinogen, \( \alpha_2 \) HS-glycoprotein, Gc-glycoprotein, and prealbumin.

Placental Membrane (StMPM), was prepared as per method of Booth et al (1981), as outlined in the protocol on page 52 Fig. 7. Membrane protein was adjusted to 40 \( \mu \)g/ml using a modified Lowry protein assay discussed on page 60.

Antisera

Antisera (rabbit anti-human) to IgG, transferrin, haptoglobin, \( \alpha_1 \) antitrypsin, \( \alpha_1 \) acid glycoprotein, fibrinogen, were purchased from Dakopatts (Mercia Brocades, Weybridge, Surrey). Antiserum to Gc-glycoprotein and \( \alpha_2 \) HS-glycoprotein were purchased from Behring Institute (Hoechst Ltd, Hounslow, Middlesex, U.K). FITC or peroxidase labelled antisera (Goat or Swine anti rabbit) were purchased from Miles (Miles research laboratories, Slough, Middlesex, U.K.)

Highly purified plasma proteins were purchased from Behring Research Institute (Marburg West Germany). My thanks to Dr. Wolfgang Becker, Head of Research Division, Behring Institute for assisting me in obtaining these proteins and for the one extra pack of Prealbumin and Gc-globulin which were actually donated. IgG and \( \alpha_1 \) acid glycoprotein were purchased from Miles (Miles research
laboratories, Slough, Middlesex).

Assay Protocol.

a) Immunofluorescence

(i) Tests

Each protein was dissolved in phosphate buffered saline (PBS) at 1 mg/ml concentration. 300 μl of each protein solution were added to 300 μl of membrane solution at 40 mg/ml of protein content in 1cm x 7.5cm plastic tubes (Starstedt Ltd, Beaumont Leys, Leicester).

(ii) Membrane Control

300 μl of membrane alone were added to the tube and this membrane was never incubated with any protein ligand.

Both test and membrane control, were left at room temperature for 1 hour constantly mixed by the roller and rocking actions of a mixer, then mixing and incubations were continued overnight at 4°C. The next morning both test and control were centrifuged with, washing three times with cold PBS. Membranes were re-suspended in the original volume, i.e. 600 μl (test) and 300 μl (for control), then all subsequent operations were done on ice at 0°C.

The following schedules A and B were followed for both membrane previously incubated with ligand, and membrane alone.
Schedule A - Membrane incubated with ligand, processed through steps 1 - 7 as follows:

1. Test (1)  
   200 µl

2. Test (2)  
   200 µl

3. LEAVE ON ICE FOR 1 HOUR

4. Add 50 µl of 1/20 FITC Goat-anti-rabbit serum - LEAVE for 30 minutes

5. Wash and centrifuge x 2

6. Add 1 ml of 1% BSA with 0.1% Azide in PBS
   Wash and centrifuge x 1

7. Add a drop of foetal calf serum via a pasteur pipette; gently mix and then a droplet is mounted on slides for fluorescence microscopy.
Schedule B - Membrane alone (Membrane without ligand, ie. Control).

300 μl of membrane.

CONTROL I

Step 1  150 μl aliquot

Step 2 Add 100 μl of 1/50 rabbit antiserum
          (any of the rabbit antiserum will do)

Step 3 Leave for 1 hour

Proceed as in step 4 to the end as in Schedule A.

CONTROL II

Step 1  150 μl aliquot

Step 2 Add 100 μl of 1/20 FITC goat anti-rabbit

Step 3 Leave for 1 hour

go to step 5 and to the end as in Schedule A.

These procedures completed the fluorescence experiments.

To summarise, the tests and test control were done with membrane pre-incubated with ligand. The test control helped to exclude non-specific reaction by FITC goat anti-rabbit serum since in this system everything was present except rabbit antiserum. The latter two controls (I and II) obtained when the ligand was excluded from the membrane also helped to exclude non-specific
binding to the membrane due to both the rabbit and goat FITC anti-rabbit serum.

(b) **Immunoperoxidase Method**

Here all steps were identical as in A and B schedules except in the following respects:

1. After an overnight mixing at 4°C, and before step I was embarked upon, endogenous peroxidase was first blocked by reacting membranes with 3% H₂O₂ in methanol at room temperature for 30 minutes. Then proceeding with steps 1 to end in both A and B schedules.

2. Rabbit anti human sera were used in 1:2.5 dilutions; whilst peroxidase-labelled goat-anti rabbit sera were used in 1/5 dilutions, this was applicable whenever these had to be applied in A and B schedules.

3. Step 6 was not necessary. Although three instead of two washings were done at step 5. Also step 7 was not needed.

4. **So from step 5** - Membranes were mounted on glass slides and left to dry and then slides were fixed in methanol for 10 minutes.

5. The peroxidase reaction was developed by dipping slides into 3-3' diaminobenzidine tetra hydrochloride (BDH Poole Dorset) at 0.2g in 300 ml in PBS that had been pre-filtered.

6. Dehydration via graded alcohols was done (xylols) and
then slides were ready for microscopy.

RESULTS

Immunofluorescence.

Figures 18a to 18k, illustrate various positive fluorescence for different protein ligands. Controls are also shown in Figs. 19(a), (b) and (c) are representative of negative controls, as described in the text and against the photographs. Generally all proteins studied gave positive reactions and prior aggregation of ligand (as was found necessary for peroxidase reaction, see below) was not necessary. This implied that fluorescence might be a more sensitive method when compared to the peroxidase method which depends on enzymatically catalysed reaction and this assay might thus have less sensitivity or resolution.

Table 3 shows more detailed fluorescence distribution around the purified microvillar placental membranes. For the sake of comparisons, some IgG was aggregated (see Fig. 18c), in order to see if there were any substantial differences with the non-aggregated IgG, and not because increased sensitivity was being sought.

Table 4, describing microscopical fluorescence appearances of placental membranes incubated with protein ligands in terms of impression of degree of positivity is also given.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Description of fluorescence topography</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 IgG</td>
<td>Heavy but diffuse distribution. Stippled and coarsely granular.</td>
</tr>
<tr>
<td>2 IgG</td>
<td>Same as in No.1</td>
</tr>
<tr>
<td>(Aggregated)</td>
<td></td>
</tr>
<tr>
<td>3 Fibrinogen</td>
<td>Moderate density but coarsely granular.</td>
</tr>
<tr>
<td>4 $\alpha_1$ antitrypsin</td>
<td>Diffuse and heavy distribution, stippled, linear in parts.</td>
</tr>
<tr>
<td>5 $\alpha_1$ acid glycoprotein</td>
<td>Granular and stippled distribution. Some areas free.</td>
</tr>
<tr>
<td>6 $\alpha_2$ HS-Glycoprotein</td>
<td>Moderate density, coarse granules and patchy, head on view showed trabeculated appearance.</td>
</tr>
<tr>
<td>7 Transferrin</td>
<td>Marked density, patchy with blobs of fluorescence in places.</td>
</tr>
<tr>
<td>8 Haptoglobin</td>
<td>Areas of linearity, interspersed with granular distribution.</td>
</tr>
<tr>
<td>9 Gc-glycoprotein</td>
<td>Granular fluorescence.</td>
</tr>
<tr>
<td>10 PreAlbumin</td>
<td>Very sparse distribution; mildly granular.</td>
</tr>
</tbody>
</table>

Table 3
Figs. 18 (a), (b), and (c).

(a) and (b) - Fluorescence positive trophoblastic membrane for monomeric IgG. Here marked positivity is shown for every available membrane in the field. The distribution is granular and coarse giving a stippled appearance.

(c) - Aggregated IgG shows more or less the same appearance as in (a) and (b), except that the granular stippling is even more marked.
Fig. 18(d) - Haptoglobin

Fig. 18(e) - $\alpha_2$HS-Glycoprotein
Fig. 18(f) - Gc-glycoprotein

Fig. 18(g) - $\alpha_1$ Antitrypsin
Fig. 18(h) - $\alpha_1$ Acid glycoprotein

Fig. 18(i) - Fibrinogen
Fig. 18(j) - Transferrin

Fig. 18(k) - Prealbumin
Fig. 19(a) - Test control

200 μl membrane + 50 μl 1/20 FITC goat anti rabbit.
+ ligand

Fig. 19(b) - Membrane control I

150 μl membrane without ligand + 100 μl of 1/20 rabbit anti human serum + 50 ul of 1/20 FITC goat anti rabbit.

Fig. 19(c) - Membrane control II

150 μl membrane without protein ligand + 100 μl of 1/20 FITC goat anti rabbit serum.
Immunoperoxidase Experiments

RESULTS were all negative except for Gc-glycoprotein, when rabbit and peroxidase antisera were each done at 1/20 and then at 1/2.5 and 1/5 dilutions respectively. The latter dilutions for both sets of antisera were considered very concentrated and should have been quite adequate to give positive results and were near to utilising neat antisera.

It was rather puzzling at first why fluorescence gave adequately positive results but not the peroxidase. It was then put down to a matter of sensitivity between the two assays. Prior aggregation of ligand was considered and this should step up sensitivity considerably. Therefore each 1 mg/ml of protein was treated to 60°C in a water bath for 1 hour, prior to incubating with placental membranes. When this was done and repeating the assay through A and B schedules, good, POSITIVE peroxidase reactions were obtained.

The importance of ligand aggregation and receptor binding was thus considered a very important factor and it presumably increased sensitivity through the numbers of available binding sites.

Figures 20a to 20g illustrate various peroxidase positive results for different protein ligands. All CONTROLS were negative as exemplified in Fig.21. Table 4 summarises results of these two techniques.

Summary

Considering that controls were all strongly negative,
<table>
<thead>
<tr>
<th>Protein</th>
<th>Ligand pretreatment</th>
<th>1st antibody</th>
<th>2nd antibody</th>
<th>Immunofluorescence Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>NIL</td>
<td>1/100</td>
<td>1/20</td>
<td>+ + + aggregated</td>
</tr>
<tr>
<td>IgG aggregated</td>
<td>1/100</td>
<td>1/50</td>
<td>+ + +</td>
<td></td>
</tr>
<tr>
<td>Tf</td>
<td>NIL</td>
<td>1/100</td>
<td>1/20</td>
<td>+ + aggregated</td>
</tr>
<tr>
<td>Hp</td>
<td>NIL</td>
<td>1/50</td>
<td>1/20</td>
<td>+ + aggregated</td>
</tr>
<tr>
<td>Fib</td>
<td>NIL</td>
<td>1/50</td>
<td>1/20</td>
<td>+ + aggregated</td>
</tr>
<tr>
<td>Gc</td>
<td>NIL</td>
<td>1/50</td>
<td>1/20</td>
<td>+ + aggregated</td>
</tr>
<tr>
<td>α1AG</td>
<td>NIL</td>
<td>1/50</td>
<td>1/20</td>
<td>+ + + aggregated</td>
</tr>
<tr>
<td>α1AT</td>
<td>NIL</td>
<td>1/50</td>
<td>1/20</td>
<td>+ + + aggregated</td>
</tr>
<tr>
<td>α2HS</td>
<td>NIL</td>
<td>1/50</td>
<td>1/20</td>
<td>+ + + aggregated</td>
</tr>
<tr>
<td>PreAlb</td>
<td>NIL</td>
<td>1/50</td>
<td>1/20</td>
<td>+ + + aggregated</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Immunoperoxidase</th>
<th>Pretreatment</th>
<th>1st antibody</th>
<th>2nd antibody</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>NIL</td>
<td>1/2.5</td>
<td>1/5</td>
<td>+ + +</td>
</tr>
<tr>
<td>IgG aggregated</td>
<td>1/2.5</td>
<td>1/5</td>
<td>+ + +</td>
<td></td>
</tr>
<tr>
<td>Tf</td>
<td>NIL</td>
<td>1/2.5</td>
<td>1/5</td>
<td>+ +</td>
</tr>
<tr>
<td>Hp</td>
<td>NIL</td>
<td>1/2.5</td>
<td>1/5</td>
<td>+ + +</td>
</tr>
<tr>
<td>Fib</td>
<td>NIL</td>
<td>1/2.5</td>
<td>1/5</td>
<td>+ +</td>
</tr>
<tr>
<td>Gc</td>
<td>NIL</td>
<td>1/2.5</td>
<td>1/5</td>
<td>+ +</td>
</tr>
<tr>
<td>α1AG</td>
<td>NIL</td>
<td>1/2.5</td>
<td>1/5</td>
<td>+ + +</td>
</tr>
<tr>
<td>α1AT</td>
<td>NIL</td>
<td>1/2.5</td>
<td>1/5</td>
<td>+ + +</td>
</tr>
<tr>
<td>α2HS</td>
<td>NIL</td>
<td>1/2.5</td>
<td>1/5</td>
<td>+ +</td>
</tr>
<tr>
<td>PreAlb</td>
<td>NIL</td>
<td>1/2.5</td>
<td>1/5</td>
<td>+ +</td>
</tr>
</tbody>
</table>

Key: +++ Marked reaction
++ Moderate reaction
+ Reasonable reaction
- No reaction

Table 4
this implied that non-specific binding due to rabbit antibody, or labelled goat antiserum were excluded. Furthermore, when preincubation of proteins with membrane was omitted, subsequent treatment of membranes with both rabbit and labelled goat antisera still gave negative results; implying the significance of protein binding to the membranes in the first instance. Therefore structures which are responsible for the initial protein binding are most likely to be the respective specific receptors on the surface of placental membranes.

Results of this study have shown positive fluorescence in all instances, but with varying degrees of intensity and density. Appearances of granular, stripped or at times coarse areas was also observed. Peroxidase reaction was less sensitive in that initially at several lower antisera dilutions, no positive results were seen. However, after prior ligand aggregation, good positive reactions were then observed.

Specificity of receptors is the other important check to do and this involves competitive incubation of ligand with excess 'cold' protein. The word 'cold' is likened in this context to competition between radio-labelled and unlabelled proteins for the same antibody binding sites in radioimmunoassays. This can be done by incubating the first antibody with excess protein prior to applying it to membranes originally incubated with the same ligand. This was done and negative results were obtained.
Other circumstantial evidence for receptor to these proteins will be obtained in the experiments using the isolated in-vitro placental perfusion system for dynamic uptake of these same proteins.
POSITIVE IMMUNOPEROXIDASE REACTIONS FOR SPECIFIC PROTEIN BINDING TO PLACENTAL MEMBRANES.

Fig. 20(a)1 - Transferrin

Fig. 20(a)2 - Transferrin; same as in (a)1 showing larger pieces of membrane with positive reaction.
Fig. 20(b) - Fibrinogen

Fig. 20(c) - $\alpha_1$ Acid glycoprotein
Fig. 20(d) - Gc-glycoprotein

Fig. 20(e) - $\alpha_1$ Antitrypsin
Fig. 20(f) - Haptoglobin

Fig. 20(g) - \( \alpha_2 \text{HS-glycoprotein} \)
Fig. 21

All the three controls as described below gave negative immunoperoxidase reaction similar to that shown in the above photograph. These controls were:

- Membrane alone (no ligand) + Rabbit anti human serum (1/2.5) + peroxidase labelled goat anti rabbit (1/5).

- Membrane + ligand as protein + peroxidase labelled goat anti rabbit (1/5).

- Membrane alone (no ligand) + peroxidase labelled goat anti rabbit (1/5).
Aims of the Study.

Of the placental receptors the IgG one is the most widely studied although its structure and some physicochemical characteristics are not clearly known. In this regard, the study of its characteristics have produced conflicting results due to different techniques used for binding studies, for example, fluorescein-conjugated IgG or adsorption of erythrocytes sensitised with IgG (Johnson et al 1976, Matre and Johnson 1977) or direct binding of $^{125}$-IgG ($^{125}$Nabb et al 1976, Balfour Jones 1978), cryostat sections of placenta (Matre et al 1975) and isolated membranes (Johnson and Brown 1981).

Therefore different binding affinity constants and binding sites have been obtained ($^{125}$Nabb et al 1976) with $(K_a = 4 \times 10^6 M^{-1}$ and $2 \times 10^{12}$ sites/mg membrane protein), (Johnson et al 1981), $(K_a = 4 \times 10^7 M^{-1}$ and $1.5 \times 10^{14}$ sites/mg membrane protein). Others have demonstrated biphasic nature of binding sites from Scatchard plots (Rees and Wallace 1979) with high and low affinity states. The latter authors proposed heterogeneity of sites but most subsequent data by others could not substantiate this heterogeneity. Most have demonstrated a homogenous nature of Fc$\gamma$-receptor sites.

There have also been controversy with regard to the effect of temperature on Fc$\gamma$-binding. Others argue (Balfour and Jones 1978) that there is no binding of pH 8.5, whereas Niezgodka et al (1981) showed binding even at pH 9.0.
It was therefore decided that IgG (Fc\(\gamma\)) receptor be re-studied with particular reference to:

1) Association and dissociation kinetics to re-assess the affinity constants.

2) Check as to whether there is heterogeneity of binding sites.

3) To test the stoichiometry of binding which has been thought to be a 1:1 binding for a hypothetical receptor of molecular weight 60,000 (Johnson and Brown 1981), although no specific experiments are mentioned to verify this binding ratio.

4) The effects of temperature were re-studied for a number of reasons. Rees and Wallace (1979) and Niezgodka et al 1981, have claimed preferential binding at 4°C rather than 37°C. The reasons for this, if any, have never been sought particularly in terms of whether lower temperature alters the total number of binding sites or affects the affinity constant. The study was therefore made with a different choice of temperatures namely 45°C, 20°C, and 0°C. These same temperatures were used to look at the thermodynamics of binding for Fc\(\gamma\) receptor in terms of energy changes, such as activation enthalpy and entropy changes over this temperature range which gives some clues as to the nature of binding.

5) The effect of monovalent and divalent cations in modulation of IgG-Fc\(\gamma\) receptor interaction, were studied with possible view that in vivo ionic changes can regulate IgG uptake. The role of cations in modulation of Fc\(\gamma\)
receptor has hardly been studied, just as the thermodynamics of binding in terms mentioned above has not been looked into previously.

Introduction

4.1 Development of Foetal Immune System

The foetus survives in its maternal host through an obviously complex immune adaptation in which there is suppression of maternal rejection, as well as prevention of its immunologically competent cells from damaging itself (self preservation). Furthermore the maternal tissues in contact with foetus, do not normally spark off severe foetal immune reactions capable of damaging maternal tissues. These three elements of maternal/foetal immunobiology are ill-understood but must be due to a multitude of fine immune regulators.

Moriya et al (1979) and Oldstone et al (1977) have demonstrated relatively high suppressor T-cells activity in cord blood and suggested that these function by suppressing maternal rejection. Siegel and Gleicher (1981) believe that these suppressor T-cells operate simultaneously in all the three tolerance areas mentioned above. The suppressor cells in eliminating or diminishing activity of immune competent cells must strike a balance between total suppression and thus cause undesirable antigens such as bacteria, to thrive and infect the foetus, and retaining some capability to recognise and deal with some otherwise dangerous antigens.
A review of the development of cellular immunity from stem cells in haemopoetic centres, through several other sites, to localising in lymphoreticular system, is given by Siegel et al (1981). On the other hand, the development of humoral immunity in relationship to ontogeny is much slower. The undifferentiated B cells migrate from their haematopoetic centres to the human foetal gastrointestinal tract equivalent of the bursa of Fabricius. There they mature, tempered by antigenic exposure or modulation, and finally form into antibody producing cells.

These B-lymphocytes express Ig-receptors as early as 9 - 10 weeks of gestation and at 14½ weeks (Siegel et al 1981), the proportion of Ig-bearing B-lymphocytes reaches an adult level. First, IgM then IgG, IgA, and IgD receptor bearing lymphocytes, in that order appear.

4.2 Antibody Synthesis.

Despite reaching adult levels for Ig-receptor bearing lymphocytes as mentioned above, their actual synthetic and production activity of antibodies remains very low (Bridges et al 1959). Most of foetal antibody activity is thus due to placental transfer, particularly of IgG. In agammaglobulinaemic mothers IgG is almost completely absent from their newborns (Zak and Good 1959), signifying the maternal origins of IgG (Gitlin and Biasucci 1969).

Nevertheless some foetal IgG synthesis still takes place as evident from studying Gm markers (Martensson et al 1964). Gitlin and Biasucci (1969), have observed, for
example, that foetal synthesis of IgG starts at around 10 - 12 weeks gestation, whilst IgM was shown at 10½ weeks, and IgA at 30 weeks gestation (Miller 1978). These experiments employed labelled aminoacids and detection of radiolabel incorporation into each immunoglobulin. IgD and IgE are almost at the limit of detection in terms of whether they are synthesised by the foetus.

Cord blood has been frequently utilised as indicative of foetal immunoglobulins status. It must be pointed out that whilst IgM and IgG increase with gestational age, IgA (and its subclasses IgA₁ and IgA₂), and IgD are not affected by gestational age, (Cederqvist et al 1978).

Furthermore, IgM and IgG are higher in foetuses weighing 2000 gm or more while IgA (IgA₁ and IgA₂) are hardly influenced by birth weight. Interpreting cord Ig levels is thus dependent on knowing the additional information. For example, an IgM level of 10mg/100ml is significantly elevated at 35 weeks gestation or if birth weight is less than 2000gm. The same value, however, is normal in a foetus of more advanced gestation and/or weighing more than 2000gm. Intrauterine infection is accompanied by raised IgG or IgA classes, say in congenital toxoplasmosis (Cederqvist et al 1977).

4.3.1 Brambell's hypothesis for maternal/foetal antibody transfer.

The year 1966 became a turning point in our knowledge of maternal transmission of antibodies to the foetus.
Brambell (1966), first focussed attention to prenatal antibody transfer through foetal membranes, such as the york sac for rabbits and rats. In animals such as cows and pigs, antibody transport via placentae does not occur; their young get antibodies via absorption from colostrum. In humans, because the haemochorial type of placentae exists, that is to say, a very thin type of placenta, antibody transport to the foetus readily takes place.

4.3.2 Selectivity of the Placenta in Antibody Transport.

In prenatal transport of immunoglobulins across the rabbit york sac endoderm as well as the postnatal transport across the gut enterocytes of rats and mice, selective events govern antibody uptake rather than molecular weight characteristics. Selection is in terms of antibody class, structure , and species. for example, only IgG is selectively transported whilst IgM, IgA, IgD and IgE are not; a fact attributable to lack of appropriate receptors (Wild 1975). Only IgG possessed receptors, as proposed by Brambell and their binding of IgG was a prelude to its transport (Brambell 1966, and 1970).

Even amongst IgG, its subclasses are differentially selected for binding and uptake due to minor stereospecificities; IgG\textsubscript{3} more than IgG\textsubscript{2} or IgG\textsubscript{1}. Molecular mass is of no importance with respect to uptake since IgA and IgG are virtually of similar mass but IgA is not taken. Also insulin with a very small mass has vast avidity for membrane binding.
4.3.3. **Coated Vesicles and Antibody Transfer and Protection.**

Already mention of these specialised organelles has been made in the review chapter (page 21). Brambell (1966, 1970), suggested the existence of IgG (Fc) receptor on the limiting membrane of endocytic vesicles for both yolk sac and placenta. Such receptors would thus provide selective protection against proteolysis during transport within endocytic vesicles in which non-selectively endocytosed proteins fused with lysosomes and then get degraded (in phagolysosomes).

Brambell's ideas were almost right though were lacking in meaningful detail. Wild (1979, 1975) 'paraphrased' in the scientific sense, Brambell's ideas. He postulated a different role for Fc-receptors, suggesting that they become associated with coated vesicles which form from the membrane surface and so serve to segregate the IgG selected for transport from immunoglobulins and other proteins destined for proteolytic phagolysosomes. Selection therefore would be a surface event and independent of proteolysis since the coated vesicles would not fuse with lysosomes in the first instance. Ultrastructural studies have lent support to Wild's ideas, as coated vesicles with IgG (rabbit and human but not bovine) have been traced from formation stage to transport across tissues and exocytosing their contents at the endothelial surface of foetal vessels, (Moxon et al 1976).
Materials and Methods

Establishing theoretical methods of receptor - ligand interaction.

Some background theoretical aspects are mandatory for discussion if the basis of methodology and calculations that are used in this section are to be appreciated. These methods will include a brief mention of:

a) Receptor-ligand interaction and use of certain equations for subsequent assessment of association and dissociation binding kinetics.

b) The basis of log-dose response curve and its application to the study of IgG-Fcγ receptor binding; with this goes mention of the theory of inverse Scatchard plots.

c) The Vant-Hoff equation and its respective plot was used to derive the activation ethalpy and enthropy as energies of Fcγ-receptor binding to its IgG ligand.

These theoretical aspects will be mentioned as and when appropriate sections are being dealt with.

A) Ligand/Receptor theory

\[
\begin{align*}
R + X & \xrightarrow{k_1} RX \xrightarrow{k_3} D \\
& \quad \underset{k_2}{=} \\
\end{align*}
\]

where

- \( R \) = Receptor
- \( X \) = Ligand
- \( RX \) = Receptor ligand complex
- \( D \) = Dissociation product of complex
\( k_1, k_2 \), are forward and backward reaction constants, \\
\( k_3 \) = dissociation constant of complex.

Therefore 
\[
\begin{bmatrix}
R \\
RX
\end{bmatrix}
\begin{bmatrix}
x \\
x
\end{bmatrix} = \frac{k_2}{k_1} = K_D \quad \text{Equilibrium Dissociation Constant} ...
\]

if \( R_T = \text{Total receptor concentration} \)

then \( R_T = R + RX \)

\( R_T - RX = R \) ..........................(2)

Substitute (2) in (1)

we have 
\[
\begin{bmatrix}
R_T - RX \\
RX
\end{bmatrix}
\begin{bmatrix}
x \\
x
\end{bmatrix} = K_D \quad ......................(3)
\]

Rearranging;

\[
\frac{RX}{R_T} = \frac{x}{K_D + x} \quad ......................(4)
\]

whence \( \frac{RX}{R_T} \) represents fractional binding of receptor or receptor occupancy. There is substantial evidence to prove that post-receptor membrane effects, in the internal milieu of a cell, depends on the degree of receptor occupancy, namely the fractional binding.

From enzyme-substrate kinetics, we have the well known Michaelis-Menten relationship;

\[
\frac{V}{V_{MAX}} = \frac{S}{K + S} \quad ......................(5)
\]
where \( V \), is velocity of reaction, \( V_{MAX} \) is the maximum velocity and \( S \), is substrate concentration whilst \( K \) is the equilibration constant. Equation (4) and (5) have marked similarities, and so for receptor/ligand interaction, Michaelis Menten type of kinetics do apply and consequently the Lineweaver-Burk double reciprocal plot, from this equation, is an extremely useful tool in deriving receptor kinetic data.

Thus from (4)

\[
\text{if } RX = B \quad \text{(observed binding)}
\]

\[
\text{and } RT = B_{MAX} \quad \text{(maximum binding)}
\]

\[
\text{then } \frac{1}{B} = \frac{K_D}{B_{MAX}} \cdot \frac{1 + \frac{X}{B_{MAX}}}{B_{MAX}} \quad \text{(6)}
\]

This is a very useful plot and \( K_D \), the dissociation constant can be derived from the gradient of the linear graph. Equations similar in form to this double reciprocal plot, but derived rather differently, will be discussed later in the section on placental perfusion, where foetal uptake of protein, gave characteristic rectangular hyperbolas of Michaelis-Menten kinetics although some minor modifications were made to the exact form of the equation. (see page 201).

B) Log-dose Response Curves (LDR)

Figs. 22 and 23 illustrate a very useful tool in studying receptor binding from various points of view and so binding can be monitored in terms of LDR curves (Avram Goldstein et al, Principles of Drug Action; The Basis of Pharmacology,
John Wiley & Sons, 1974, pp. 89-127). The LDR curve was used to calculate also the stoichiometry of Feγ binding as will be mentioned below. In most cases in this study, with the exception on data for stoichiometric assay, the horizontal axis (log dose) was modified in that instead of the dose representing increasing ligand concentration, and for the sake of economising on valuable radiolabel, the membrane (which is easy to prepare) concentration was varied whilst the amount of label was fixed. This was a kind of immunoradiometric assay. From such curves, inverse Scatchard plots were the only way of deriving binding parameters such as affinity and maximum binding sites as will be shortly shown. First of all the stoichiometry of binding.

Fig. 22
Log (dose) Response Curves (LDR)

- **a** = normal LDR
- **b** = shift to right due to competitive inhibitor
- **c** = even greater shift due to still high competitive dose
- **M** = maximum receptor binding
- **d** = same as (a)
- **e** = non-competitive inhibitor
- **f** = higher dose of non-competitive inhibitor

**M** and **M** = impaired maximum binding due to non-competitive interference.

Fig. 23. Log.dose Response Curves (LDR)
Derivation of the stoichiometry of binding from Log-Dose Response curves

From equation (4) on page 121

Let \( f = \frac{B_{\text{RX}}}{B_{\text{MAX}}} = \frac{RX}{RT} = \frac{X}{K_D + X} \) ....... (7)

rearranging;

\[ X = K_D \left( \frac{F}{1 - F} \right) \] .......... (8)

But \( RT = X + RX \) .................... (9)

\[ \text{Total ligand} = \text{Free} + \text{Bound} \]

\[ . \quad RT - RX = X \]

Under conditions of excess free ligand.

\( RT \approx X \)

Since the amount bound, \( RX \), becomes negligible in relation to high free ligand.

Therefore combining (8) and (9),

\[ K_D \left( \frac{F}{1 - F} \right) = X = RT \]

(i.e. Total ligand mostly existing as free)

\[ d \ln K_D \left( \frac{F}{1 - F} \right) = d \ln X = \frac{df}{f[1 - f]} \]

and so \( \frac{dF}{d \log X} = 2.303 f \left( 1 - f \right) \) .......... (10)
\[ \frac{df}{d \log X} = \text{GRADIENT of LDR curve} \]

at \( f = 50\% \) (Point A in Fig. 22), we have

\[ \frac{df}{d \log X} = 2.303 \times 0.5 \times 0.5 \]

\[ = 0.576 \]

Therefore for a 1:1 ligand/receptor interaction the gradient at 50\% of total binding must be 0.576.

Thus at 50\% binding

for (1:1 binding) \( X + R \rightleftharpoons XR \) \( \left( \frac{df}{d \log X} = 0.576 \right) \)

for (1:2 binding) \( X + 2R \rightleftharpoons XR_2 \) \( \left( \frac{df}{d \log X} = 0.288 \right) \)

for (2:1 binding) \( 2X + R \rightleftharpoons X_2R \) \( \left( \frac{df}{d \log X} = 1.15 \right) \)

and so multiples of 0.576, gave the appropriate binding ratio.

In the experiment that follows, attempts to assess the \( Fc_y-IgG \), interaction in terms of stonchiometric binding were made.

D) (a) Data on stoicheiometry of IgG-Fc\( y \) interaction.

A reaction volume of 0.5 ml per tube consisted of 350 \( \mu l \) of 50 mM Tris HCl pH 7.4, 100 \( \mu l \) of 5\% Bovine serum albumin, 660 \( \mu g \) of membrane protein, and varying amounts of IgG-H\( ^3 \). After incubation at 37\(^\circ\)C and gentle vortexing every 15 minutes, aliquots of 50 \( \mu l \) from the reaction mixture were rapidly filtered through GF/F (Whatman) fibre glass filter discs in a 3-piece funnel and under negative pressure.
Fig. 24 - shows a log dose response curve for labelled IgG-H3 binding on placental membranes. The horizontal arrow (→) gives the 50% binding; the point where the gradient df/d log x, (equal to 2.40) was taken. This gave a 4:1 stoichiometric binding ratio for IgG/receptor interaction as described in the text. The vertical arrow (↓) gave the corresponding affinity constant of $K_a = 2 \times 10^7 M^{-1}$ for IgG.
Duplicate assays were performed. Bound counts were expressed as percentage of maximum specific binding and then plotted against ligand concentration as shown in Fig. 24.

At 50% binding (Fig. 24)

\[
\frac{df}{d \log X} = \frac{72.5\%}{\log 5.0 - \log 2.5} = 2.40
\]

Since 1:1 binding gradient is 0.576
A gradient of 2.40, gives

\[
\frac{2.40}{0.576} = 4.1:1
\]

Thus from these experiments it would appear that 4 IgG molecules to 1 of receptor, interact and not 1:1 as previously assumed (Johnson and Brown 1981) and without concrete data to prove it.

Thus from the point of view of cell economy a 4:1 as opposed to 1:1, IgG-Fc binding would make subsequent uptake a relatively efficient system in animal systems inspite of available data to suggest that only 12% of injected labelled IgG is subsequently transferred to the foetal circulation (Wild 1973).

Comment on LDR-curve for stoichiometric determination; the horizontal axis in Fig. 24 shows IgG-\(^{3}H\) concentration expressed in \(\mu g/0.5ml\). It does not really matter whether these were expressed in \(\mu g/l\) or molar concentrations. When the change in \(\log X\) is considered, ie. \(\log X_2 - \log X_1\)
\[
\log \frac{X_2}{X_1}, \text{ and so it is the log of the respective ratio that counts which then is independent of units concentration for } X.
\]

D) (b) **Affinity equilibrium constant from the same LDR-curve.**

Furthermore, from Fig. 24 and at 50\% binding an arrow points to a value on the X-axis from which an approximate equilibrium constant can be derived for the Fcγ -IgG reaction.

\[\text{eg. } \frac{1}{K_a} = 3.6 \, \mu g/0.5ml \text{ Reaction volume} \]
\[= 7.2 \, \mu g/ml \]
\[= 7.2 \times 10^{-6} \times 10^3 \text{ g/l} \]

If MW IgG = 150000 daltons

\[\text{then } \frac{1}{K_a} = 7.2 \times 10^{-3}/150000 \text{ moles/litre} \]
\[K_a = 2 \times 10^7 M^{-1} \]

This value compares well with published affinities ranging from \(4 \times 10^6 - 5 \times 10^8 M^{-1}\) (Johnson and Brown 1981). This is a further check against the validity of the gradient derived at 50\% binding and subsequently used for stoichio-

metric determination. If the gradient point was erroneous, its corresponding point on the X-axis, for affinity constant calculation would also be wrong. However, as the X-axis value reasonably predicted the affinity constant, it must be assumed that the gradient was also meaningful.
Transformation of LDR-curves into Inverse Scatchard plots for assessment of Fcγ-binding parameters.

The ordinary Scatchard linear plot (Scatchard 1949) has the form of:

\[ yL = AB - \gamma / (1 - \gamma),K^{-1} \]

where \( b \) = Bound ligand concentration for both labelled and unlabelled; and \( L \) = Total ligand concentration, then \( y = b/L \), \( Ab \) = Total antibody or receptor binding sites, and \( K \), the equilibrium constant. The axes \( yL \) and \( y/(1-y) \) are linear.

This is a disguised quadratic equation in \( y \), with two roots \( y_1 = b/L \), \( y_2 = Ab/b \). The second root is particularly suitable for data from immunoradiometric assays where the antibody or receptor (Ab) is labelled (Addison 1974).

The inverse version of the Scatchard plot is obtained thus, by setting \( w = 1 - y \)

\[ wL = AB / \left( \frac{1 - w}{1 - w} \right) - K^{-1} \]

with ordinate \( w/(1 - w) \) and abscissa \( wL \), the plot is linear with slope of \( Ab^{-1} \) and intercept \( (-K^{-1}) \) on the \( wL \) axis.

There are still two roots in \( w \), since the equation is quadratic still, \( w_1 = L - b/L \) and \( w_2 = (b - Ab)/b \), \( w_2 \) is a better suited root in immunoradiometric assays and for that matter LDR-curves.

Therefore the abscissa is left at \( Lw_2 \) whilst ordinate is usefully transformed from \( w/(1 - w) \) by putting \( 1 + w/(1 - w) \)
Fig. 25  Schematic representation of an inverse Scatchard plot

Where

\[ L = \text{Concentration ligand bound} \]
\[ w_2 = (B - Ab) = \left[ \frac{\text{Bound counts}}{B} \right] - \left[ \frac{\text{Maximum Plateau counts}}{\text{(Bound counts)}} \right] \]
\[ B = \text{Bound counts} \]
\[ Ab = \text{Maximum bound counts} \]
\[ Ab = \text{Maximum binding capacity of receptor} \]
which changes ordinate limits to 0 and 1 (see full theoretical and practical details of how these plots are done in "An Approach to Immunoassay" by W.H.C.Walker, Clin. Chem. Vol.23, No.2, pp.384, 1977, and also see Walker 1977. Suffice it to use the final form of inverse Scatchard outlined in the above article, as illustrated in Fig. 25.

Data from LDR curves was easily converted into the format of the inverse Scatchard plot for deriving maximum binding capacity (Ab) and affinity constant K, both obtainable from values on the abscissa axis.

Data for LDR Plot and Inverse Scatchard.

1 µl of labelled IgG-H₃² (32224 cpm and 0.458 µg protein) was added to approximately 500 µl in a reaction tube made of 380 µl of 50mM Tris-HCl buffer pH 7.4, 100 µl of 5% bovine serum albumin in the same buffer and varying amounts of placental membrane protein (1 - 1000 µg). GF/C glass fibre rapid filters (Whatman) were used to separate bound from free radioactivity. Radioactivity on filters was counted by dipping them in 5 ml fisofluor 1 (Fisons) scintillant and from specific activities, bound IgG at each StMPM concentration was calculated. Specific binding was assessed in presence of a 200-fold excess of unlabelled IgG. A typical experiment is shown in Figure 26 for LDR plot and Figure 27 for the inverse Scatchard transformation of data. A number of experiments were performed to determine these constants as per Table 5. In this table also published affinities and binding sites for Fcγ -IgG system are also given for making comparisons.
Fig. 26

Log dose response curve for specific IgG-H^3 binding. 1 µl of H^3-IgG (32224 cpm) was incubated with increasing amounts of membrane protein in a total reaction volume of 500 µl of phosphate buffered saline pH 7.4 (PBS), made up of 100 µl of 5% BSA, 380 µl PBS, and a variable volume of membrane protein. Non-specific binding was subtracted when the experiment was repeated in presence of 91.2 µg of unlabelled IgG.
Fig. 27 - shows an inverse Scatchard of data from Fig. 26. The horizontal axis represents bound membrane protein $Lw_2$ where $L =$ membrane bound protein (μg/l), and $w_2 = (1 - AB/B)$. $AB =$ maximum binding capacity (maximum counts), and $B =$ bound $H^3$-IgG (bound counts). The intercept on the abscissa = $(AB + K^{-1})$ where $K =$ equilibrium affinity constant and a molecular weight of IgG = 150,000 daltons was used in its calculation.

The affinity and total binding sites per mg membrane protein are shown too.
<table>
<thead>
<tr>
<th>Expt.</th>
<th>Affinity Constant (Ka)</th>
<th>Binding sites /mg StMPM</th>
<th>Published Constants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ref</td>
</tr>
<tr>
<td>1</td>
<td>$8.56 \times 10^8 \text{M}^{-1}$</td>
<td>$0.69 \times 10^{14}$</td>
<td>Johnson et al 1981</td>
</tr>
<tr>
<td>2</td>
<td>$1.46 \times 10^8 \text{M}^{-1}$</td>
<td>$1.32 \times 10^{14}$</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>$2.1 \times 10^7 \text{M}^{-1}$</td>
<td>$0.30 \times 10^{13}$</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>$5.86 \times 10^7 \text{M}^{-1}$</td>
<td>$2.837 \times 10^{13}$</td>
<td>Niezgodka et al 1981</td>
</tr>
<tr>
<td>5</td>
<td>$0.53 \times 10^8 \text{M}^{-1}$</td>
<td>$5.23 \times 10^{14}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Average</strong></td>
<td><strong>1.50 \times 10^{14}</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table 5
As will be seen there are close similarities of this data to that published. In the experimental system, using purified membrane, an average of \( K_a = 2.2 \times 10^8 \text{M}^{-1} \) and the number of sites of \( 3.6 \times 10^{14} / \text{mg} \) were obtained. It would thus appear that the overall affinity constant, pooling all available data, is in the order of \( 10^7 - 10^8 \text{M}^{-1} \) whilst total number of binding sites are in the order of \( 10^{13} - 10^{14} \) sites/mg of membrane. The variation seen for these constants amongst different workers, is a reflection, in part, of differences in the system used. For example, Balfour and Jones 1978, used whole placental membranes, and so did McNabb et al 1976, and Rees and Wallace 1979. Johnson and Brown 1981, used Chaotrope washed membrane preparations, whilst Niezgodka et al 1981, used purified membranes too, but these were subsequently solubilised in lithium iodosalicylate.

The IgG-Fcγ-Receptor Kinetics; Association and Dissociation Rate Constants.

(1) Optimisation of aliquoting of reaction mixture for kinetic data.

For both association and dissociation rate constant determination, it is not practicable to filter the whole reaction volume, for purposes of determining bound ligand at specified time intervals. This is not because it is impossible but it would be too laborious. Aliquoting is necessary and because of rapid time sequences with short
In the kinetic experiments rapid aliquots of membrane at timed intervals were filtered through GF/C (Whatman) discs. It was necessary therefore that aliquots contained sufficient membrane to give optimal results for the sampled fractions. Data shows log dose response curves (LDRs) for incubating 300 µg of membrane and aliquoting 100 µl (●●●), 300 µg of membrane and filtering 50 µl (■■■), and 75 µg of membrane and filtering 50 µl (▲▲▲) to assess binding. From the LDRs, utilising 300 µg and sampling 100 µl gave a more sensitive curve (curve shifted to the left) and this was used for all kinetic experiments.
intervals, it was therefore necessary to find the optimal amounts to aliquot which contained sufficient membrane to get decent results.

Initial experiments were performed using 300 µg of membrane in 0.5 ml reaction volume and filtering only 100 µl through GF/F filters (Whatman), then using 300 µg membrane and filtering 50 µl or 75 µg membrane and filtering 50 µl. Data is shown in Fig. 28 where varying amounts of IgG-H\textsuperscript{3} were incubated with either of the above membrane amounts and then aliquoting in the manner outlined. From counts of bound ligand for aliquots an estimate of total binding due to whole membrane in each tube was made. From the three dose response curves in Fig.28, it will be seen that using 300 µg of membrane and filtering 100 µl gave better sensitivity on the LDR-curve as judged by this curve shifting more to the left relative to the others. Therefore it was decided to use these conditions in the subsequent kinetic experiments, with the provision that a fixed amount of ligand was used usually 2 µl of H\textsuperscript{3}-IgG (0.458 µg/µl).

(ii) Association rate constant (k\textsubscript{1})

2 µl of IgG-H\textsuperscript{3} (0.458 µg/µl and 70358 cpm/ug) were incubated with membrane at 37°C in a total volume of 0.5 ml. This consisted of about 378 µl of 50mM Tris-HCl pH 7.4 buffer, 100 µl of 5% BSA in same buffer and 20 µl containing 300 µg of membrane (StMPM). At intervals of 30 seconds, 1, 2, 3, 5, 7, 10, 15, 25, 30, and 45 minutes. Aliquots of 100 µl were rapidly filtered through GF/F filters, that had
previously been pre-soaked in 5% BSA. Radioactivity on duplicate assay filters was measured using a Tricarb scintillation spectrometer in 5 ml of Fisofluor 1 (Fisons, Loughborough). Specific binding was obtained in excess cold IgG (x 200 fold). Fig. 29 shows the association curve for IgG-H<sup>3</sup> binding on Fc<sub>γ</sub> receptor. The second order rate constant, k<sub>1</sub>, is given by:

\[
k_1 = \frac{1}{t} \left\{ \ln \frac{B_t}{B_0} - \frac{B_t}{B_{eq}^2 - B_0 L_0} \right\} + \ln \frac{B_0 L_0}{B_{eq}^2 - B_t B_{eq}}
\]

where 
- \( k_1 \) = Second order rate constant 
- \( t \) = Time 
- \( B_{eq} \) = Maximum binding at equilibrium 
- \( B_0 \) = Initial target (Receptor) concentration expressed in terms of IgG bound at maximal capacity. This is usually done in Receptor kinetic work, since actual receptor molecular weight and amount are usually unknown and reasonable results are usually obtained when receptor concentration is expressed in terms of maximal ligand binding capacity. 
- \( L_0 \) = Initial ligand concentration 
- \( B_t \) = Bound ligand at time \( t \)

By plotting \( \ln \left( \frac{B_{eq} - B_t}{B_0 L_0} - \frac{B_t}{B_{eq}} \right) \) versus time a negative linear regression line is usually obtained. By calculating the slope of this line, \( k_1 \), the rate constant can be derived.
Fig. 29 Association of IgG-H³ to Fcγ-receptor; time course of IgG-H³ binding.

2 µl of IgG-H³ (0.916 µg) were incubated at 37°C with 300 µg of placenta membrane protein in a reaction volume of 500 µl as described in the text. At various times, 100 µl of reaction mixture were rapidly filtered through Whatman GF/F filters and bound radioactivity (counts/mg membrane protein) was plotted as a function of time.

A typical saturable hyperbolic curve was obtained (Note - Non-specific binding was subtracted in 200-fold excess cold IgG)
Data from Fig. 29 was plotted in a manner described in the text using a second order rate constant equation whereby
\[ \ln \left( \frac{B_{eq} - B_t}{B_0 L_0 - BeqB_t} \right) \]
is plotted against time. This gave a negative linear regression whose gradient yielded the rate constant \( k_1 = 0.676 \times 10^7 \text{M}^{-1} \text{min}^{-1} \). In this system \( L_0 \) (initial ligand concn) = 6.0 nM of IgG-H³ and \( B_0 \) (initial receptor concn) = 8 nM.
This data from a similar experiment to that in Fig. 29 was plotted as shown to determine the second order constant as in Fig. 30. In this experiment $L_0 = 4.44$ nM (initial ligand) and $B_0 = 5.33$ nM (initial receptor concn), a rate constant $k_1 = 3.91 \times 10^7 M^{-1} \text{ min}^{-1}$ was obtained. The pooled rate constant from two experiments was $k_1 = 2.29 \times 10^7 M^{-1} \text{ min}^{-1}$.
whence

\[ k_1 = \text{slope} \times \frac{\text{Beq}}{\text{Beq}^2 - \text{BoLo}} \quad \ldots \ldots . \quad (12) \]

For full details of derivation of these second order kinetics refer to Maelicke et al 1977.

**Determination of Total Receptor Concentration, Bo, for use in kinetic experiments.**

In equations (11) and (12), \( k_1 \), cannot be calculated unless Bo is known - A prior determination of the total receptor concentration Bo, must be made. One of the fundamental difficulties lies in not knowing the molecular weight and amount of membrane bound receptors, but whose actual concentrations cannot be calculated and so an indirect estimate has to be made in terms of their maximum ligand binding capacity.

This is usually done in two major ways; a) membrane is varied with a fixed amount of ligand counts; here the amount of membrane protein is varied so as to absorb 10% of total counts. This amount is usually evaluated from specific activities of ligand and becomes the receptor concentration. b) With membrane fixed the ligand is varied till maximum binding takes place and from Scatchard plot of bound/free versus free ligand concentration, the maximum binding capacity (depicting receptor concentration) can be calculated. The latter system (b), was utilised to determine
For data displayed in Figs. 29 and 30, determination of receptor concentration \( (B_0) \) was done in a separate experiment. Here increasing ligand IgG-H\(^3\) (70358 cpm/\( \mu \)g) was added to a reaction mixture of 1ml consisting of 756\( \mu \)l of 50mM Tris-HCl, 200 \( \mu \)l 5\% BSA, IgG-H\(^3\) and 660 \( \mu \)g of membrane protein, till saturation occurred. (Note; 4 \( \mu \)l IgG-H\(^3\) = 1.83 \( \mu \)g IgG)

A Scatchard plot is shown from which the maximum receptor concentration \( (B_0) = 8.0 \text{ nM (intercept)} \) was calculated using IgG MW = 150,000.
receptor concentration $B_0$, in the following kinetic assays. Fig. 32 shows one such assay to establish, $B_0$, which was equal to 8nM receptor concentration.

Data to determine $k_1$ association rate constant.

Fig. 30 shows a plot of $\ln \left( \frac{B_{eq} - B_t}{B_0L_0 - B_{eq}B_t} \right)$ versus time for data shown in Fig. 29. A rate association constant, $k_1 = 0.676 \times 10^7 \text{M}^{-1}\text{min}^{-1}$ was obtained using equations (11) and (12). In this assay $L_0 = 6.0\text{nM}$ of $H^3$-IgG and $B_0 = 8\text{nM}$ of receptor concentration and 2 $\mu\text{l}$ of IgG-$H^3$ at 0.458 $\mu\text{g/ul}$ in a reaction volume of 0.5 ml.

Fig. 31 shows another similar experiment whereby $L_0 = 4.44\text{nM}$ of IgG-$H^3$ and $B_0 = 5.33\text{nM}$, 'receptor concentration'. The rate constant, $k_1 = 3.91 \times 10^7 \text{M}^{-1}\text{min}^{-1}$ was obtained. An average of $k_1 = 2.29 \times 10^7 \text{M}^{-1}\text{min}^{-1}$, was achieved from the two types of experiments.

Dissociation of IgG-Fcγ Rate Constant ($k_2$)

2 $\mu\text{l}$ of $^3\text{H}$-IgG at 0.5 $\mu\text{g/ul}$ was incubated with 20 $\mu\text{l}$ of StMPM (membrane) representing 660 $\mu\text{g}$ membrane protein; 200 $\mu\text{l}$ of 5% BSA in 50mM Tris-HCl pH 7.4 buffer and 756 $\mu\text{l}$ of buffer to make up to 1 ml of incubation volume. Reaction proceeded at 37°C for 1 hour to attain equilibrium. Then 1000 $\mu\text{g}$ unlabelled IgG ( x 1000-fold excess) were added, and kinetic measurements of dissociation commenced at 0, 30 seconds, 1, 3, 5, 10, 15, 25 and 55 minutes, and rapidly filtering through GF/F filter discs.
Fig. 33 shows a dissociation curve plotted as percentage inhibition of maximum binding at $t = 0$, versus time from first order kinetics.

$$-k_2t = \ln \frac{B_t}{B_{eq}}$$

$$k_2 = -\left(\frac{\ln B_t}{B_{eq}}\right) \frac{1}{t}$$

Therefore a plot of $\ln \frac{B_t}{B_{eq}}$, versus $t$ (time), a negative linear regression curve was obtained as shown in Fig. 34. The gradient $(s)$, gave $k_2 (s)$. The regression in Fig. 34 showed two linear segments, one occurring within the first 10 minutes of the dissociation and having a half life $(t_{1/2}) = 6.5$ minutes and $k_2 = 0.10 \text{ min}^{-1}$ and a slower component $k_2 = 0.010 \text{ min}^{-1}$, ten times less than the first, and which characterised the rest of the dissociation curve. This experiment seems to suggest two receptor sites, with different dissociation rates, the first with low affinity (high $k_2$), and second high affinity (low $k_2$). Using $k_1 = 3.91 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ (from association data) and $k_2 = 0.100 \text{ min}^{-1}$ a $K_D$ ($k_2/k_1$) of $2.55 \times 10^{-9} \text{M}$ was obtained.

This extraordinary finding seemed to suggest heterogeneity of Feγ-receptor sites, at least on the dissociation curve. The association curve did not show this - It is possible, as has been shown in other receptor systems that the kinetics of receptors is dose dependent and sometimes, activation or recruitment of other receptor sites can be manifested depending on the activating or inhibitory ligand concentration. Thus some sites can be inhibited or activated in a dose dependent manner. This allosterism has been shown
2 µl of IgG-H³ (1 µg) was incubated with 660 µg of placental membrane protein in a reaction volume of 1ml consisting of 778 µl of 50 mM Tris-HCl pH 7.4 and 200 µl of 5% BSA. The reaction was allowed to proceed to equilibrium at 37°C for 1 hour and then an excess unlabelled IgG (x1000 fold, ie. 1000 µg) was added and then 100 µl aliquots sampled and filtered through GF/F filters. The dissociation curve is shown with arrows depicting 50% binding (t½ = 6½ min).
Fig. 34  
A plot of First order dissociation to derive dissociation rate constant(s) \((k_2)\).

Data in Fig. 33 was subjected to \(\log_e (B_t/Beq)\) against time, where \(B_t\) is the ligand still bound at time \(t\) and \(Beq\) is the steady state bound IgG prior to displacement. 1000 \(\mu\)g of unlabelled IgG had been used. Data here shows a heterogeneous curve with the first linear initial fast component \((t_1 = 6\frac{1}{2} \text{ min}, \text{ arrowed})\) with \(k_2 = 0.10 \text{ min}^{-1}\) and a second slower component \((k_2 = 0.010 \text{ min}^{-1})\). Overall the high displacing dose revealed heterogeneity of binding sites on the Fc\(\gamma\)-receptor.
to occur, for example, with the acetylcholine receptors which may exist in different forms.

It is difficult to draw hard and fast conclusions regarding the $\text{Fc} \gamma$-receptor in the light of this apparent heterogeneity from the dissociation data. It is noteworthy with regard to the deployed unlabelled displacing dose (1000-fold excess) that this might affect the kinetics of dissociation to expose heterogeneity of receptor sites. On available data from literature the inhibiting doses have normally been around 200-fold excess against $1 \mu g$ of $I^{125}$-IgG (Brown and Johnson 1981), 160 $\mu g$ against 0.082 - 18 $\mu g$ $I^{125}$-IgG (Niezgodka et al 1981), and 50 $\mu g$ cold IgG against $1 \mu g$ $I^{125}$-IgG (Johnson and Brown 1980). In none of many of these experiments was dissociation kinetics specifically measured particularly with high unlabelled doses. The main concern was in inhibiting non-specific binding.

Heterogeneity of IgG receptor binding has been only reported once by Rees and Wallace (1979), who showed a biphasic Scatchard plot. At two temperatures of $4^\circ C$ and $37^\circ C$, they showed $K_a$ of $5 \times 10^8 M^{-1}$ and $1 \times 10^8 M^{-1}$ ($4^\circ C$) as well as $K_a$ of $4 \times 10^7 M^{-1}$ and $5 \times 10^6 M^{-1}$ at $37^\circ C$. Thus the second lower affinity component varied 5 to 8 times less than its higher affinity counterpart at both $4$ and $37^\circ C$ respectively. In the present study, the lower affinity component was 10-times less than the high affinity one, which agrees closely to Rees and Wallace's findings.

To summarise, it appears there is a need to investigate
as to whether the Fcγ-receptor can exist in different states. The more reported homogeneous population of receptor sites is only a limited view particularly when high ligand or displacing doses have not been sufficiently used to this end.

Thermodynamics of IgG and Fcγ-receptor Interaction.

Introduction.

Temperature effects on binding of IgG to placental receptors have been studied by Balfour and Jones (1976), who found greater binding at 37°C than at 4°C. Niezgodka et al (1981), using isolated membranes found no differences at all in specific binding at 4°C to 45°C. Ockleford and Clint (1980) however, used incubated placental tissue and found depressed IgG uptake at 4°C in contrast to enhanced uptake at 37°C.

These workers differentiated between mere binding of IgG to its receptor and subsequent uptake which is modulated by endocytosis (Ockleford and Clint 1980, Arend and Mannik 1973). Thus temperature might exert different effects on these two interrelated but independent processes of binding and uptake. Ockleford and Clint stated that lower temperature principally inhibited uptake and not binding, whilst higher temperatures enhanced both binding and uptake. They found uptake at 4°C to be 20% of that found at 37°C. Therefore temperature studies have generated both conflicting and at times complimentary results.
In the present study temperature effects were re-appraised with respect to binding of IgG on isolated placental membranes. A choice of 0, 20, and 45°C temperatures was made. Any differences in binding were used to calculate some thermodynamic parameters such as the activation enthalpy and entropy energy changes. These would give some clues as to the molecular nature of the IgG-receptor interaction.

Assay and Assessment of IgG Binding at 0, 20, and 45°C.

0 - 1400 µg of placental membrane protein were incubated with 2 µl of 3H-IgG (138258 cpm and specific activity 27651 cpm/µg), in a total volume of 0.5 ml per tube made up of 350 µl of 50 mM Tris-HCl pH 7.4 buffer, 100 µl of 5% BSA in same buffer, and a variable volume of membrane (0 - 35 µl at 40 µg/µl). One set of duplicate assays was incubated at 0°C for 3 hours, another at 20°C for 2 hours and the last at 45°C for 1 hour.

At the end of incubation the duplicate reaction mixtures were filtered through GF/C glass fibre filters (Whatman) to assess bound radioactivity which was subsequently plotted against membrane concentration as shown in Fig. 36. From the log dose response curves, it would appear that there is more binding at 0°C than at 20°C and than at 45°C. However, the reason for increased binding with respect to lowered temperature can be due to either a change in affinity or total binding capacity (number of sites). To find out, data from Fig. 36 were transformed into inverse Scatchard plots to check on affinities and binding capacities as shown in
**Fig. 36**  \( \text{IgG-H}^3 \) binding at 0\(^\circ\), 20\(^\circ\) and 45\(^\circ\)C.

Log dose response curves for \( \text{IgG-H}^3 \) binding at different temperatures. Conditions of the assay are as described in the text.
Fig. 37  Scatchard plot of saturation data.

Binding data in Fig. 36 depicting the effect of temperature on IgG-H\(^3\) were subjected to the inverse form of the Scatchard plot to find out whether temperature affects the equilibrium constant (\(K_a\)) and the total number of binding sites (\(AB\)) for the Fc\(\gamma\)-receptor. Results are given and discussed in the text.

- \(L\) = amount of IgG bound in \(\mu g\) per 50 \(\mu l\) of reaction volume containing a specific amount of membrane.
- \(A\) = amount or number of counts of IgG bound.
- \(AB\) = maximum number of counts or amount of IgG bound.
- \(W = (1 - AB/A)\)
Fig. 37
There were slight changes in affinity as per;

\[ Ka = 2.66 \times 10^8 \text{M}^{-1} \text{ at } 0^\circ C, \]
\[ Ka = 1.77 \times 10^8 \text{M}^{-1} \text{ at } 20^\circ C, \]
\[ Ka = 1.45 \times 10^8 \text{M}^{-1} \text{ at } 45^\circ C. \]

Clearly the affinity went up with respect to lowering the temperature. It increased by 1.8 times at 0°C, which was almost twice the value at 45°C.

Likewise, the binding capacities were \( 0.118 \times 10^{-7} \text{M} \) at 0°C, \( 0.107 \times 10^{-7} \text{M} \) at 20°C, and \( 0.108 \times 10^{-7} \text{M} \) at 45°C. There was a slight increase in binding capacity at 0°C although by 20°C, right through to 45°C similar binding capacities were obtained. The slight increase in capacity at lower temperatures could be significant for the placenta taken as a whole.

The changes are small and little wonder that Niezgodka et al (1981), who did not look for any possible alterations in affinity or binding capacity concluded that there were no differences in specific binding between 4°C and 45°C.

Nonetheless, the slight increases in both affinity and binding capacity at low temperatures make sense in terms of the fluid mosaic model of plasma membrane proposed by Singer and Nicholson (1972). The lower temperature leads to diminished membrane fluidity and this restricts lateral movements of receptor molecules which increases the apparent nett binding capacity. At higher temperatures, the overall affinity drops as there is more kinetic movements leading to dissociation of ligand and increased fluidity and so both the affinity and to a much lesser extent, the capacity may drop.
Discussion of effect of Temperature on Binding

In essence this data could be interpreted that the primary change is in the affinity rather than the capacity. In fact one could go further to add that the capacity over this temperature range is presumably not significantly altered. A very comparable situation has been observed in a very recent study, by Ciaraldi and Olefsky (1983), who specifically examined the effect of temperature on the coupling of insulin receptors to stimulation of glucose transport in the isolated rat adipocytes. Their temperature range included 16°C, 24°C and 37°C.

They found that both total and cell surface binding varied in an inverse linear manner over this temperature range. Scatchard analysis of their data suggested that diminished binding was caused by altered affinity as temperature rose with no concormitant change in the overall receptor numbers. They added that the drop in affinity was caused by an increase in the dissociation rate as temperature rose but with no change in the association rate. In these two respects there seems to be close similarities between the insulin receptor behaviour with respect to temperature and the Fcγ-receptor data in the present study, where the IgG binding capacity was slightly greater at 0°C than say at 20°C and 45°C. This was judged from the log dose response curves; the half-maximal binding (at 50% or the IC_{50}) is for example, greater at 0°C than at 45°C. It must therefore be assumed, as in the insulin receptor study that the dissociation rate for IgG from its receptor increases
with rising temperature, and hence the fall in binding. This is evidenced by corresponding association constant (Ka) to drop by 1.8 times between 0 and 45°C as has been demonstrated.

This data contrasts that of Watabe et al (1980), who showed IgG binding to 60,000g placental pellet to be less at 4°C than at 37°C.

Other receptor systems have been studied with respect to temperature. Maes et al (1981), who studied the Warfarin-albumin binding system also noted the decrease in binding affinity with the rise of temperature, although these workers did not investigate on what happens to total binding capacity as has been done for the IgG-Fcγ receptor system in the present study, where temperature causes measurable changes to the affinity and total number of binding sites. Niezgodka et al (1981) denied any effects of temperature on binding between 0 and 45°C, although in their paper no data is given for either the effect on affinity or binding capacity.

Ockleford and Clint (1980) tried to speculate on diminished uptake at 4°C as opposed to 37°C to be due to possible depression of function of cytoskeletal elements presumably actin filaments which mediate endocytic vesicle formation and hence uptake. These workers too stated that there was no temperature effect on binding although in the same paper they proceeded to speculate that both uptake and binding were enhanced at higher temperatures. As for binding per se, they did not produce any data to prove that there was no effect on binding with respect to temperature, particularly
as they utilised placental tissues to measure uptake rather than isolated membranes which measure binding.

**Thermodynamic Activation Enthalpy and Entropy changes.**

The changes in affinities between 0 and 45°C were submitted to a Vant-Hoff plot as shown in Fig.35 where loge K (where K is the association equilibrium constant) is plotted against the reciprocal of temperature (1/T) as deduced from the following relationships;

\[ \Delta G = -RT \ln K \quad \ldots \quad (13) \]

or

\[ \ln K = -\frac{\Delta G}{RT} \]

But

\[ \Delta G = \Delta H - T \Delta S \quad \ldots \quad (14) \]

Therefore

\[ \ln K = -\left( \frac{\Delta H - T \Delta S}{RT} \right) \]

or

\[ \ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad \text{(Vant-Hoff equation)} \ldots \quad (15) \]

Therefore a plot of ln K (log_e of equilibrium association constant ,K) against 1/T (degrees Kelvin ^{-1}) is linear IVant-Hoff plot), with gradient of -\Delta H/R, where

\[ K = \text{Equilibrium association constant at a particular temperature.} \]
G = Gibb's free energy
H = Activation enthalpy
S = Activation entropy
T = Temperature in degrees Kelvin
R = Gas constant = 1.987 Cal/Mole/degree

Fig. 35 shows results of a Vant-Hoff plot for three specific temperatures, namely, 0.20 and 45°C.

Table 6 summarises all the thermodynamic parameters obtained with use of equations 13, 14, and 15 in addition to the affinity and total number of binding sites, with respect to different temperatures.

Discussion of Thermodynamics results.

Thus the binding of IgG to its Fcγ receptor is exothermic with \( \Delta H = -2.51 \text{ Kcal/Mole} \) as well as associated with favourable \( \Delta G \) of \(-10.52\) to \(-11.87 \text{ Kcal/Mole} \). Even more important is the associated large positive entropy changes of \( +29.4 \text{ Cal.Mole}^{-1} \text{ degree K}^{-1} \) between \( 0^0 \) and \( 45^0 \text{C} \). Thus the binding has unfavourable enthalpy change but a very favourable large entropy changes which are typical of hydrophobic interactions.

Therefore at a molecular level the binding IgG area includes or extends to the hydrophobic region of the receptor and the reaction is entropy driven. Furthermore, the marked exothermy of \( \Delta H = -2.51 \text{ Kcal.Mole}^{-1} \) is also typical of reactions ascribed to involvement of hydrogen bonding. O'Rielly et al (1967) studied the anticoagulant Warfarin's
Fig. 35 - shows a Vant-Hoff plot. For calculation of activation enthalpy ($\Delta H$) from the gradient of $\log_e K_a$ against $1/T^0$ (per degree Kelvin), where $K_a$ is the equilibrium constant at 0°C, 20°C, and 45°C. From the gradient ($\Delta H/R$) and gas constant, $R = 1.987$ Cal. Mole$^{-1}$ deg$^{-1}$, $\Delta H = -2.51$ KCal.Mole$^{-1}$ was obtained for IgG-$H^3$ - Fc$\gamma$ receptor interaction.
Table 6 - shows various thermodynamic parameters for interaction between the IgG and its Fcγ-receptor at various temperatures. The changes in equilibrium constant and total numbers of binding sites are also given. The reaction is associated with high negative enthalpy (ΔH = -2.51 KCal/Mole) which is exothermic and is entropy driven (ΔS = + 29.3 Cal. Mole⁻¹ K⁻¹) due to hydrophobic binding.
binding on albumin and noted $\Delta H$ of $-3.14 \text{ Kcal.Mole}^{-1}$ which was a very pronounced exothermy and they ascribed it to hydrogen bonding. Still on similar studies, many workers have also noted the high entropy changes of $\Delta S = +21 \text{ Cals.Mole}^{-1}\text{deg}^{-1}$ (Maes et al 1982) and $\Delta S = +16 \text{ Cals.Mole}^{-1}\text{deg}^{-1}$ (Oester et al 1976) which they too ascribed to hydrophobic interactions. Thus the IgG-Fc$\gamma$ system has close similarities to other binding systems.

Dumphy et al (1981), also studied specific binding of $^3$H-phorbol, 12, 13-dibutyrate by mouse brain at 4°, 13°, 23° and 37°C. They found slight increase in endothermy ($\Delta H = +0.4 \text{ Kcal/Mole}$) associated with large entropy changes of $\Delta S = +38 \text{ Cal/Mole/deg}$, which they too attributed to hydrophobic nature of binding.

Modulation of IgG-Fc$\gamma$-receptor Binding by Monovalent and Divalent Cations.

Various workers have studied effects of pH, ionic strength, and even temperature on binding of IgG to its receptor (Balfour and Jones 1976, Brown and Johnson 1981, Watabe and Gitlin 1979, Niezgodka et al 1981). In these experiments, ranges of pH (6.6 – 8.8), 0°C – 45°C for temperature and ionic strength 0.05 – 0.5M NaCl from Niezgodka et al were studied whilst Brown and Johnson investigated
pH (5.4 - 9.4), ionic strength 1 - 200mM Tris-HCl pH 7.4. Others have used 4°C - 37°C temperature gradients.

It must be emphasised that whilst drastic changes in binding or even uptake have been noted, these pH, ionic strength and temperature gradients are non-physiological, and more of mere in-vitro laboratory assays. This is the major criticism of all these experiments in that these drastic changes in these variables are hardly encountered in vivo-physiological milieu. Body temperature is constantly kept at 37°C, and the drastic temperature differences at say 4°C for which altered binding and uptake have been observed, are not applicable to in vivo situations. Therefore in-vivo modulators of binding or even uptake must be sought.

Possible in-vivo modulators of binding have been examined with respect to oestrogen receptor, having also shown that in vitro activators such as temperature, ammonium sulphate precipitation, addition of salts for the oestrogen receptor are not operative in vivo. The search for others resulted in discovery that phosphorylation (addition of ATP to the system), induced increased nuclear (King et al, 1971) or DNA (Nawata et al, 1981) binding to the oestrogen receptor. Also it increased binding of glucocorticoid receptor (John and Mondgil, 1979). Proteases also have stimulatory effects on oestrogen receptor-DNA binding, therefore it emerged that phosphorylation and/or proteolysis led to in vivo activation of oestrogen and glucocorticoid receptors. There is a need to study these factors with respect to the IgG receptor.
In the present study univalent and divalent cations were selected for their modulation of IgG binding. These included cations normally found in vivo such as sodium, calcium, and magnesium, others of interest included lithium (this cation is not physiological except in therapeutic doses) and manganese. Physiological doses were in some instances exceeded to observe their effects on IgG-receptor binding.

Materials and Methods.

Membrane (StMPM) was prepared as described before and had protein content 33 mg/ml. Therefore 20 μl of StMPM which was used in incubations delivered 0.66 mg StMPM. However, in cases of Ca, Mg, and Mn incubations membranes were first treated with ethylene glycol tetraacetic acid (EGTA) or ethylene diamine tetraacetic acid (EDTA), as follows to get rid of endogenous Ca, Mn, and Mg;

Suspend membrane pellet in 50mM EGTA in 50mM Tris-HCl pH 7.4

Stir 30 min
Centrifuge x 100,000g for 35 min
resuspend pellet in 50mM EGTA 50mM Tris-HCl pH 7.4 and homogenise in Dounce HOMOGENIZER
Centrifuge x 100,000g for 30 min
PELLET

FREE Pellet of EGTA by washing TWICE in 50mM Tris-HCl pH 7.4 in Dounce Homogeniser and centrifuge at 100,000g. for 20 minutes.
The membrane is now ready for use in the experiments with cations.

Table 7 shows the details of relative concentration ranges prepared for each cation for incubation with placental membranes.

**Assay of Binding with respect to Cations.**

20 μl of non-EGTA treated StMPM (0.66 mg protein) was incubated with 2 μl of IgG-\(^3\) (5μg) in a series of reaction volumes of 0.5 ml per tube made of the above membrane and ligand plus 369 to 299 μl of 50mM Tris-HCl pH 7.4 buffer corresponding to 10 to 80 μl of added univalent cations (Na\(^+\) and Li\(^+\)). The dispensed volumes were made up such that concentrations in a reaction volumes of 0.5 ml spanned the range of 10 - 160mM as per table 7. Each tube also contained 100 μl of 5% bovine serum albumin to minimise non-specific binding.

For other cations such as high dose of Li\(^+\)(1 - 4M), or Ca\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\) (1 - 15mM each) appropriate volumes of these were dispensed as per table 7, and added to adjusted buffer volumes, plus 100 μl of 5% BSA, to give a total reaction volume of 0.5 ml. For Ca\(^{2+}\), Mg\(^{2+}\), and Mn\(^{2+}\) incubations, 20 μl of EGTA treated membrane were used in each case.

At the end of 1 hour at 37°C and at constant mixing, each of the duplicate assay tubes of 0.5 ml were rapidly filtered through GF/C glass fibre filters (Whatman), and the radioactivity retained on filters was measured in vials of 5 ml Fisoflour 1 (Fisons) scintillant with a Tricarb
### Monovalent Cations

Note. Concentrations are based on a Total Reaction Volume = 0.5 ml

<table>
<thead>
<tr>
<th>Monovalent Cations</th>
<th>Volume dispensed (µl)</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (5.84g/100ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vol. dispensed</td>
<td>10 20 30 40 50 60 70 80</td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td>20 40 60 80 100 120 140 160</td>
<td></td>
</tr>
<tr>
<td>(Low) LiCl (4.24g/100ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vol. dispensed</td>
<td>10 20 30 40 50 60 70 80</td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td>20 40 60 80 100 120 140 160</td>
<td></td>
</tr>
<tr>
<td>(High) LiCl (21.2g/10ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vol. dispensed</td>
<td>10 20 30 40</td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td>1 2 3 4</td>
<td></td>
</tr>
</tbody>
</table>

### Divalent Cations

<table>
<thead>
<tr>
<th>Divalent Cations</th>
<th>Volume dispensed (µl)</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂ (1.095g/100ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vol. dispensed</td>
<td>10 20 30 50 90 120 150</td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td>1 2 3 5 9 12 15</td>
<td></td>
</tr>
<tr>
<td>MgCl₂ (1.016g/100ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vol. dispensed</td>
<td>10 20 30 50 90 120 150</td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td>1 2 3 5 9 12 15</td>
<td></td>
</tr>
<tr>
<td>MnCl₂ (0.989g/100ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vol. dispensed</td>
<td>10 20 30 50 90 120 150</td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td>1 2 3 5 9 12 15</td>
<td></td>
</tr>
</tbody>
</table>

Table 7
scintillation spectrometer.

Specific binding was assessed in a third tube at each concentration where 1mg excess unlabelled 'cold' IgG was added. Specific binding in tubes incubated with cations was then related to the 'zero' tubes where no cations were added and percentage change in binding was plotted as a function of concentration of added cations.

Results

Fig. 38 shows the results for Na\(^+\), and Li\(^+\) (0 - 160mM) and high dose Li\(^+\) (1 - 4M) whilst Fig. 39 shows data for Mg\(^{2+}\), Ca\(^{2+}\) and Mn\(^{2+}\) (1 - 15mM). For both Na\(^+\) and Li\(^+\) (0 - 160mM) there is a steady but marked drop in specific binding at 100mM concentration for both cations; there was a 35% drop for Na\(^+\) and a 36% drop for Li\(^+\) which were practically identical depressions for both at this concentration.

At 2mM Li\(^+\) no depression occurs, this is important because Li\(^+\) is not a physiological cation and patients receiving Li\(^+\) for treatment, the therapeutic range should not exceed 2mM otherwise toxicity occurs. If therefore less than 2mM were to be given in pregnant women, no effect should be observed in vivo for IgG binding. High in-vitro Li\(^+\) concentration (1 - 4M) resulted in severe depression of IgG binding and by 1M and right up to 4M, a plateau of depression of 50% binding was achieved.

Na\(^+\) ion has a normal physiological range of (135 - 145 mMoles/litre). This ion showed significant modulating effect
Fig. 38 - shows inhibition of binding of IgG to its receptor by monovalent cations. Physiological concentrations of sodium caused marked reduction in binding. Similar depression was caused by an equivalent concentration of Li⁺. For higher doses of Li⁺ inhibition plateaued off and any further Li⁺ did not cause inhibition in excess of 50%.
Fig. 39 - shows the effect of divalent cations on IgG-binding. All cations (Mg$^{2+}$, Ca$^{2+}$, and Mn$^{2+}$) caused marked inhibition of binding as described in the text.
on IgG-binding since between this reference range some 45 - 50% inhibition of binding was observed.

Mg$^{2+}$, Ca$^{2+}$, and Mn$^{2+}$, also resulted in marked inhibition of binding as the dose of ions was increased in each incubating medium. Mg$^{2+}$ reference physiological range is 0.7 - 0.95mMoles/litre. However, at 1 - 2mM, there was some increase of 8.4% in binding. Beyond 2mM, increasing inhibition of binding was observed and by 10mM there was a 30% drop in IgG binding.

Ca$^{2+}$ reference normal range is 2 - 2.6mMoles/litre. At 2.6mM there was 13% inhibition, and by 10mM there was 33.3% depression in binding similar to that of Mg$^{2+}$.

Mn$^{2+}$ is an essential trace metal and present in serum at 10.7 ± 2.2nmole/litre (0.0107 ± 0.0022mM). There are very small quantities and for purposes of the experiment millimolar quantities were used. There was a steady dose dependent inhibition of binding, of 1.7% by 1mM and 45% at 10mM, and which continued to fall to 68% by 15mM Mn$^{2+}$.

Of the three divalent cations Mn$^{2+}$ seemed to have more inhibitory potential, and it is just possible that at the receptor level normal physiological levels could still regulate IgG binding.

Discussion

Niezgodka et al 1981, studied ionic strength 0.05 - 0.5M NaCl and claimed no effect on $^{125}$I-IgG binding for 0.1 - 0.5 range whilst a decrease to 0.05 increased binding by 40%.
Watabe et al 1980, in contrast to Niezgodka et al, found significantly marked inhibition of binding. For both Na\(^+\) and Ca\(^{2+}\) at 0.1M more than 50% inhibition was attained and by 0.2M for either cation, binding was reduced to 20% of control value.

The above papers are practically the only specific references to IgG binding and the effect of cations. The present study was an attempt to delve a bit further and also expand the range to include Li\(^+\), Mn\(^{2+}\), and Mg\(^{2+}\) from Na\(^+\) and Ca\(^{2+}\) mentioned in the above studies. Results are unequivocal in terms of showing marked effects of these cations in depressing IgG binding and more or less in line with what Watabe et al 1979, observed for Na\(^+\) and Ca\(^{2+}\).

Cations have been extensively studied in some receptor systems and a brief mention of a few will be made. Shiu and Friesen (1974) studied Na\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\) ions with respect to \(^{125}\)I-labelled Ovine prolactin binding on mammary gland prolactin receptors. NaCl at 50mM enhanced binding of prolactin whilst 10 - 25mM CaCl\(_2\) increased binding three-fold. Higher concentrations of all salts started to inhibit binding. MgCl\(_2\) (1 - 10mM) increased binding by 1.5 - 2.6 fold over the concentration range.

Roscher et al (1983) have studied bradykinin receptors in cultured human fibroblasts. Na\(^+\) at physiological concentrations and Ca\(^{2+}\) or Mg\(^{2+}\) at 3 - 10mM reduced bradykinin binding by 25%.

ACTH hormone binding to its receptor is likewise very
sensitive to Ca\(^{2+}\) ions as has been shown by Lefkowitz et al (1970). Here even within the physiological ranges of Ca\(^{2+}\) marked fall in binding was noted. However, by 10mM binding was totally inhibited. Summers (1980), studied \(^{3}\)H-clonidine binding to the \(\alpha_1\)-adrenergic receptors in kidney membranes as affected by Na\(^{+}\), K\(^{+}\), Mg\(^{2+}\) and Mn\(^{2+}\). Both Na\(^{+}\) and K\(^{+}\) produced concentration dependent decreases and 50% reductions were achieved by 25mM and 37mM respectively. Mn\(^{2+}\) (0.1mM) and Mg\(^{2+}\) (1mM) produced marked enhancement of binding followed by dose dependent inhibition at higher concentrations.

Summers was able to show from Scatchard plots that Na\(^{+}\) inhibited binding by reducing the apparent affinity and not the agonist number of receptor sites. Mg\(^{2+}\) and Mn\(^{2+}\) at low concentrations increased the apparent affinity. He also speculated on cations acting as physiological modulators with respect to \(\alpha\)-adrenergic receptors.

It is tempting to formulate a unified theory to explain these ionic effects on receptor function, since many similarities and sometimes identical effects have been observed on receptors of different types. Summers, for example, speculated on the possible link of a receptor to Na\(^{+}\) transport system and since Na\(^{+}\)/K\(^{+}\) ATP'ase pump system exists ubiquitously local concentration of these ions at membrane surfaces or through Na\(^{+}\)/K\(^{+}\) channels could indeed have significant controlling effects on contiguous receptor molecules.

In the present study profound inhibiting effects of IgG and Fc\(\gamma\)-receptor binding lend support to a possible but
significant local control or regulation, particularly when physiological levels exhibited marked alterations in binding of IgG. All cations investigated inhibited IgG binding at physiological levels for Na$^+$, Ca$^{2+}$ and high dose for Mg$^{2+}$, Li$^+$ and Mn$^{2+}$. 
CHAPTER 5

PLACENTAL SELECTIVITY AND TRANSFER OF PLASMA PROTEINS; STUDIED BY IN-VITRO PERFUSION SYSTEM.

5.1 Introduction

The placenta is a remarkable organ in that it can perform multiple complex functions condensed into a single organ but which would normally require several separate organs. Amongst its prime functions are gaseous exchange, endocrine function, selective transfer of amino acids, antibodies, monosaccharides, lipids and proteins. Salt and water transfer are also of major importance. The placenta can perform all these functions and yet remain an effective 'barrier'.

There are general principles governing placental exchange. These include concentration gradients, exchange area, thickness, and permeability, molecular size, lipid solubility, degree of ionisation and the molecular conformation of transported material (Hill and Longo 1980). These are a formidable array of variables and whilst the placenta in-vivo manages to modulate them, it imposes an almost impossible task for in-vitro or indeed in-vivo experimental design to study transfer.

The placenta also transfers red blood cells from maternal to the foetal circulation during pregnancy and at delivery (Lewi et al 1961). Likewise foetal red cells are evidenced by HbF (Foetal haemoglobin), have also been detected
in maternal circulation (Cohen et al 1964). Clint, Wakely and Ockleford (1979), have shown that there are depressions or pits on the syncytiotrophoblast with correct size to engulf red blood cells or several of them and these workers have proposed that these 'pits' are differentiated regions with receptors for red cells. It could be that these enclosures transfer red cells eventually to foetal circulation. The importance of these tiny red cell leakages into the foetus and vice-versa, is not clear.

The placenta also transfers maternal lymphocytes, although the potential dangers of exposing maternal antigens expressed on these cells to the foetus are evident. The problem and tolerance to these cells has been reviewed by Adinolfi (1970).

5.2 Transcellular and Paracellular Placental Transfer Pathways and the Selectivity of Uptake.

The transfer of macromolecules across the placenta poses some problems. Two pathways have been proposed, the transcellular and paracellular pathways. There is little morphological evidence as a basis for assessment for presence or absence of paracellular form of transport. In contrast, the syncytiotrophoblast is in direct contact with maternal blood, any uptake is mostly, if not all, by transcellular route (Stevens and Samuel 1979). For transcellular route to be more effective, areas of the surface epithelium have differentiated to subserve a function of molecular recognition by specific adherence or adsorption, as a
prelude to membrane invagination and formation of transport vesicles.

These are of two types, the smooth and coated endocytic vesicles. These organelles are thought to mediate transcellular transfer of materials for both local needs (for placental use and synthesis) and foetal uptake. The coated vesicle route is the basis of selective and protected transport across cells by which proteins normally required intact, must be transported.

In the pictorial words of Hemming and Williams (1976), "The protein enters the cell; some is passed on through and some is degraded. But the fascinating thing is that in those cells there dwells a Maxwell's demon or perhaps a troop of slightly specialised demons, which picks over the molecules as they enter, deciding what is acceptable and may be passed on and what is not and must be degraded; that is selection".

In a nutshell this entails the whole concept of selective transfer of molecules particularly macromolecules, as first proposed by the Late F.W. Rogers Brambell (1966, 1970). Brambell, accepted that primary entry into the micropinocytic vesicles was random, and that was non-selective, but from thereon, the walls of these vesicles contained specific receptors which recognised the Fc-portion of the homologous IgG.
On fusing with lysosomes, the encapsulated IgG was protected against proteolysis by steric hindrance, so that the vacuole at last contains only the receptor-selected protein. What is true of IgG could be true for many other proteins or peptides and hormones with respect to receptor selected uptake.

Brambell's ideas have since been refined, and instead of one vesicle containing both non-selected and selected protein at first, the existence of smooth and coated vesicles has simplified matters; it is now thought that the coated vesicles rather than the smooth vesicles are the ones with receptors and transfer protein molecules protected against proteolysis, whilst smooth ones are for stuff destined for placental digestion (Wild 1973, Wild 1979, Hemming and Williams 1976, Gitlin and Gitlin 1976).

5.3 Receptor Recycling after Transfer.

\[ \begin{align*}
L & \downarrow \\
L + R & \xrightarrow{RL} \text{Membrane} \\
L & \leftarrow \text{recycling} \\
R & \uparrow \\
\end{align*} \]

\[ \text{L = ligand} \]
\[ \text{R = receptor} \]

Coated vesicles have received vigorous study to characterise their structure and particularly as they are the major vehicles of endocytically protected transcellular uptake.
Their inner periphery is thought to have the specific receptors to various molecules including proteins, polysaccharides, fats, certain vitamins and inorganic ions (Llewellyn-Jones 1969, Ockleford and Whyte 1977).

The mechanics of vesicle formation involve transformation of particular hexagons existing as hexagonal lattice under the cytoplasm surface of the membrane into pentagons with the same length of side; this produces curvature in the membrane and eventually causes the vesicle to pinch off.

Numbers of coated vesicles at the membrane surface do not seem to diminish with time although they are continually being internalised and carry membrane. From cell economy considerations, it follows that re-cycling (depicted in the above diagram) of membrane and hence receptors must be taking place (Ockleford and Whyte 1977), or else new receptors are continually being re-synthesised.

Theoretical Models of Membrane Transfer; possible application to Placental Uptake.

5.4.1 Diffusion Model.

The general flux across membrane by diffusion forces is given by;

$$ J = -C A \frac{w \, DU}{DX} \quad \text{(Leaf 1981)} $$

where, $J$ = flux of solute across unit area of membrane, is
proportional to force \( \frac{du}{dx} \) exerted on solute, concentration, \( c \), of solute, to fraction \( A \) of membrane area available to penetration by the solute in question and the mobility, \( w \), of solute in membrane.

This equation gives linear uptake fluxes. Many substances cannot fit this model and sometimes permeation is faster than is predictable by diffusional uptake, and is inhibited by excess of substrate. The curves of uptake for such substances are not linear, and more usually are hyperbolas. This is true of uptake by placenta for macromolecules and certain sugars, which imply a carrier or receptor mediated uptake which warrants the use of Michaelis-Menten kinetics, for models such as the simple zero-trans-uptake procedure, infinite Cis- and infinite Trans-uptake.

5.4.2 Zero Trans Uptake Procedure.

Consider two sides of the membrane, the Cis and Trans, (the uptake side) when uptake is initially zero, the system becomes the zero-trans procedure. At variable substrate inputs on
the Cis side one monitors the trans side uptake. The rate of uptake reaches a maximum limiting velocity, \( v_{zt}^{2t} \), as substrate concentration is increased. The substrate concentration at half-maximal velocity, is \( K_m \), then the uptake hyperbola is described by:

\[
v_{zt}^{2t} = \frac{v_1 \to 2}{S_1} \frac{S_1}{K_m + S_1}
\]

where \( zt = \text{zero trans} \) and \( 1 \to 2 \), denotes uptake from face 1 (Cis) to face 2 (Trans), an \( S_1 \) = substrate concentration in face 1. This equation has the form of Michaelis-Menten kinetics (Stein 1981). The zero-Trans uptake model has been used extensively to study carrier mediated uptake, for example, galactose uptake by human red blood cells (Ginsburg and Stein 1975), to characterise kinetics of glucose efflux from red blood cells (Karlish et al 1972).

5.4.3 Infinite Cis-procedure.

Here one sets the substrate concentration on the Cis face at limitingly high level and then measure the nett Cis to trans flow of substrate, when the substrate concentrations at the trans face are set at various levels. In absence of substrate on the trans side, uptake is at maximal, initial rate velocity, but as trans substrate levels build up, uptake slows down and reaches saturation once again. It can be shown that flux velocity \( 1 \to 2 \) face, \( v^{1c}_{1 \to 2} \);
i_c = infinite Cis, Km is the half-saturation concentration of substrate at the trans face which is just sufficient to reduce the net flow of substrate (S_2) in the Cis to trans direction to one half of the maximal value.

5.4.4 Infinite Trans-procedure.

The procedure here is infinite Cis in reverse. The substrate concentration at the trans face is set at a limitingly high level. Concentrations of substrate (S_1) at the Cis face are set at various levels, usually labelled, and the unidirectional flux( Cis-trans (1→2) ) is measured; the flux has the form;

\[
\frac{V_{1c}}{1\rightarrow2} = \frac{Km \cdot V_{1c}}{Km + S_2}
\]

\[
\frac{V_{1t}}{1\rightarrow2} = \frac{S_1 \cdot V_{max}}{Km + S_1}
\]

it = infinite trans, V_{max} is the maximum velocity and Km is the concentration at which half maximal flux is obtained.

Each of these models are capable of describing receptor or carrier mediated fluxes across membranes. As will be seen later, the first model of zero trans procedure, was slightly modified for application to uptake of radiolabelled proteins in an in-vitro isolated placental perfusion system.
5.5 Aims of Transfer Studies.

In vivo-studies of placental uptake particularly when radioligands are used are for ethical considerations, difficult to perform, let alone the large body compartments which would dilute the label before even the placenta begins to take it up. Quite satisfactory results can be obtained by using an in-vitro perfusion system, whereby controlled inputs of labelled ligand to mimic maternal conditions can be utilised and uptake via the foetal aspect of the placenta is measured.

In-vitro perfusion have been studied with respect to placental amino acid transfer (Young 1981), oxygen consumption (Challier et al 1976), glucose and lactate transfer and metabolism (Schneider et al 1972).

However, this technique has not been widely applied to the study of transfer of serum proteins. Therefore it was felt that the system is well suited to this sort of study particularly when uptake of radiolabelled proteins is easy to measure. Quite apart from application of this technique to study of IgG-\(^3\) uptake (Ockleford, personal communication), other plasma proteins have not yet been subjected to this useful assay.

The primary aim was to see if uptake was saturable in the first instance and subsequently whether it was relatable to an asymmetrical carrier system for each protein and thus raising the possibility of this carrier being a receptor. Testing for the carrier, involved utilisation of
the Zero-trans model of uptake. Uptake curves were then subsequently tested with Ginsburg and Stein's (1975) equation for asymmetric carriers in an attempt to see if this equation could linearise the uptake curves. If it did, then placental protein uptake is most probably a receptor mediated transfer process. The other supportive evidence from these curves would be any appearance of saturability.

Materials and Methods.

Proteins: Nine proteins were studied: IgG, $\alpha_1$-antitrypsin, $\alpha_1$-acid glycoprotein, haptoglobin, $\alpha_2$-HS-glycoprotein, transferrin, prealbumin, fibrinogen, and Gc-glycoprotein. These proteins were radiolabelled with tritium in a similar manner to that outlined on page 77. Table 8 gives a summary of the doses infused on the maternal aspect of the isolated perfusion system and the specific activities of the radioligands.

5.6 The In-vitro Placental Perfusion System

The apparatus:

The apparatus was designed such that it had two major components; the maternal and foetal compartments. Fig. 40 shows the overall plan of all the major components; the whole assembly is also shown in the photograph in Fig. 41.

The Foetal Circuit.

This consisted of a reservoir, an inverted conical flask, in which a hole was artificially bored in the middle of its bottom-end (now facing upwards), for introducing solutions and additives. The exit glass piece was
connected to a flow meter by a rubber tubing which then connected to a 'heat exchanger' (Trevanol miniprime heat exchanger unit)). Water at 37°C was pumped in and out of the heat exchanger from a water tank reservoir. The conical flask reservoir was also partially submerged in the water tank to keep it at 37°C. A peristaltic pump (set to pump 3 - 5mls/min) was usually either an LKB 2120 vario-perpex peristaltic pump or a Water-Marlow infusion pump. The tubing from the pump was finally connected to a nylon portex microcannula (diameter 1.3 mm), via a three-way sampling tap to the foetal artery. This had previously been identified by stripping or peeling the chorionic membranes off the placenta. Usually a small peripheral cotyledon was selected for perfusion as this was usually small and gave good flow characteristics.

A return circuit was completed by first letting fluid perfuse the foetal vein for about 5 minutes so as to identify the cotyledonous vein through which the return was flowing. This was likewise cannulated with a similar Portex 1.3mm nylon microcannula and connected via another three-way-sampling tap to a mercury manometer and back to the reservoir. The foetal reservoir was gassed with 40% oxygen, 5% carbon dioxide, and 55% nitrogen.

The Maternal 'Circuit'

This was an 'open' system in that whilst it is called a circuit, it really is a one way system and no return was needed. Once again like the foetal side, a conical flask
reservoir containing buffer with additives and for introducing maternal radiolabelled protein, was partially submerged in a water tank bath kept at 37°C. This reservoir was also gassed with 40% O₂, 5% CO₂ and 55% N₂. The flow rate was kept between 2 - 5mls/min and the pressure kept at 10 - 20mmHg as shown in the line diagram in Fig. 40. The flow through the pump tubes was effected by a Water-Marlow automatic infusion pump connected to a flow meter and at the end was attached a glass microcannula with a very thin end, for insertion into the maternal decidual cotyledon as shown in the diagram. The photograph in Fig.41 shows the laboratory arrangement of the various components.

Reservoirs.

Hartman's solutions (Trevanol, Thetford, East Anglia) were used for both foetal and maternal reservoirs and contained 100mM glucose, 4% dextran 70 (MW = 70000) as additives. Appropriate volumes (usually 1ml) of radiolabelled proteins were added to the maternal reservoir as shown in Table 8, and allowed to warm to 37°C prior to the start of maternal perfusion.

5.7 The Placenta and Perfusion experiments.

Placentae were obtained from the Department of Obstetrics and Gynaecology at the Leicester Royal Infirmary (Labour Ward), soon after full term normal deliveries. The placentae were rapidly washed with cold Hartman's solution and rushed to the laboratory but carried on ice.
MATERNAL CIRCUIT
TEMPERATURE 37°C
FLOW RATE 2-5 ML/MIN
PRESSURE OPEN CIRCUIT/10-20 MMHG

FETAL CIRCUIT
TEMPERATURE 37°C
FLOW RATE 5 ML/MIN
PRESSURE 60-80 MMHG

Fig. 40: The diagrammatic arrangement of the in-vitro placental perfusion system.
This shows the laboratory set up of the main components of the in-vitro placental perfusion system outlined in Fig.40. The two tanks on either side kept the perfusates (in inverted conical flasks) on maternal and foetal sides at 37°C, then the peristaltic pumps, tubings and microcannulae inserted into the placenta on the tray. Detailed description is given in the text.
This shows a closer view of the in-vitro perfusion in progress. The foetal side is being perfused with 'inlet' and 'outlet' microcannulae in place. Sampling taps are clearly visible; the exit one for monitoring radioligand uptake.

On the left is a maternal microcannula entering the underside (the maternal decidua) to perfuse a corresponding selected cotyledon.
Chorionic membranes were stripped off and cannulation of the foetal artery and veins was done. Fig. 42 shows the foetal circuit completed, with microcannulae in place and preliminary perfusion to 'flush out' blood, in progress. At the end of about 20 minutes, the reverse side corresponding to the perfused cotyledon, when the placenta was turned on its back, became pale and swollen (see Fig. 43). The paleness implied near or completed flushing out of blood and blood clots as well as a good flow circuit had been attained.

In most instances, no anticoagulant was found necessary. Some workers usually add 50 - 60ug/ml of heparin in the perfusion fluids. The placenta was kept moist throughout the experiment by dripping of warm buffer at 37°C from an overhead reservoir (Fig. 40). The maternal microcannula was then gently inserted into the pale area (Fig. 43) and secured into place. Radioligand was then perfused into the maternal aspect at a rate of 2 - 5mls/min. Monitoring of uptake was immediately started by drawing 0.5 - 1ml aliquots through the exit three-way tap on the foetal aspect at 0,1,2,3,5,7,10,15,25,30,45,60,70 and 80 minutes. These aliquots were added to 5 - 10ml Pisoftluor 1 scintillant and counted for 4 minutes each. Counts were expressed per ml of aliquot.
After about 30 minutes of perfusing the foetal side, the placenta was turned on its back (maternal side up) to see which cotyledon was being perfused as judged by the paleness. The photograph shows such a pale swollen area in which the maternal microcannula was inserted and then timing of the experiment commenced.
5.8 Modifications introduced into the perfusion system

a) Maternal circuit

After several experiments, it was found that the maternal circuit could be modified, without loss of experimental detail. Since ligand quantities administered were usually small, there was a big dilutional effect caused by long tubings between the maternal reservoir and the microcannula. It was decided that the radiolabel after equilibrating at 37°C in the maternal water-bath, a 1ml syringe attached to the microcannula would be used to aspirate and deliver the radioligand directly into the perfused pale area shown in Fig. 44.

b) Foetal circuit.

Once again, it was found that the foetal return line to the reservoir was too long and if circulation of foetal uptake were allowed to flow back to the reservoir and then back into the placenta, it would take a very long time for equilibration to occur. In any case, equilibration was not important for the short period of perfusion 0 - 30min or up to 1 hour. Secondly, it was found that the exit fluid line from a single and usually small cotyledon, very often stagnated with poor flow because of a long fluid column to push along and often causing significant back pressure between the exit and the reservoir.

Therefore the extensive tubings leading back to the reservoir were removed and the exit cannula was allowed to drain directly into a collecting flask. This improved the
Fig. 44: Modifications in the original perfusion system for effective delivery of maternal radioligand and relief of column back pressure on the foetal circuit.
flow characteristics substantially since the dumping down of flow due to the fluid column ahead was no longer present. This arrangement converted the foetal circuit into an 'open' one, just as the maternal circuit was. These modifications are diagramatically depicted in Fig. 44. Satisfactory results were achieved by these changes. Consequently, there was no need to monitor back pressure as in the closed system where pressures in excess of 60mmHg led to rupture of placental capillaries.

Results

Counts/ml of uptake were plotted against time for each protein. Fig. 45 shows one graph reproduced by kind permission (Dr. C.D. Ockleford of University of Leicester) aimed at showing the uptake of IgG-\(\text{H}^3\) when both 'inlet' and exit lines were sampled when a completed foetal circuit was used. Saturable uptake is evident. Radioactivity in the inlet samples signifies uptake mixing in the reservoir and then returning to the cotyledon. In the present study interest was only in the uptake exit line which was 'open' so that no radioactivity in the inlet was encountered. Data for uptake of radiolabelled proteins over the period of 60 - 80 minutes is presented in Figs. 46 to 50.

Without exception all uptake curves showed high initial rate of uptake, which reached equilibrium as saturation was reached. Times at which maximum uptake commenced are given in Table 8. The curves have the characteristic rectangular hyperbolas, typical of Michaelis-Menten kinetics.
This shows uptake when both the input and output of foetal microcannulae were sampled at various times. This was in a closed foetal circuit when the uptake equilibrated with the reservoir and returned to the placenta. Other perfusion conditions are shown in the diagram (Reproduced with kind permission of Dr. C.D. Ockleford, University of Leicester).
Expt 4 Maternal side $1.4 \times 10^3 \text{cpm}^3 \text{H IgG}$
Open circuit 2.5 ml/min
Fig. 46 - shows uptake of transferrin, haptoglobin, and $\alpha_1$ acid glycoprotein.
Fig. 47

Foetal uptake of fibrinogen, Gc-glycoprotein and IgG.
Fig. 48: In-vitro foetal uptake of $\alpha_2$HS-glycoprotein
Fig. 49: In-vitro foetal uptake of $\alpha_1$ antitrypsin
Fig. 50: In-vitro foetal uptake of PreAlbumin.
This signifies or points to these protein ligands binding on to carriers of some sort which have saturable characteristics. It is therefore implicitly proposed that this putative carrier is a specific receptor for each of the test-proteins.

However, certain other characteristics of a receptor quite apart from saturability, must be fulfilled such as possession of high affinity sites or constants. In the following sections, attempts will be made to use equations for asymmetric carriers that is to say for unidirectional nett fluxes, to see if these uptake curves could be linearised; and if so, do they have the form of Lineweaver-Burk like plots (mentioned earlier on page 122) from which affinity constants can be calculated?

Zero-trans model of uptake applied to placental uptake curves.

Of the membrane models discussed in this chapter, the zero-trans model was thought to be the most appropriate for placental work, although in-vivo transport could be more complex. A diagramatic representation is shown in Fig. 51. Here the maternal side is regarded as the Cis (side 1) whilst the uptake foetal side is the trans (side 2). So, is the maternal input substrate or ligand concentration. $C_t$, is the uptake with respect to time (t), $R_1$, is reciprocal of maximum velocity of transfer from 1$\rightarrow$2 direction, $R_2$, is the corresponding quantity in 2$\rightarrow$1 direction. $K$ = half saturation concentration.
Fig. 51: The Zero-Trans uptake procedure.
Eilam and Stein (1974) have produced such an integrated kinetic equation, taking into account all these parameters for an asymmetric carrier and it is applicable to the placenta where uptake is generally in favour of the foetal side (asymmetrical) and here regarded as the **Trans** side of maternal input.

Ginsburg and Stein (1979), with minor modifications, re-stated the integrated time course equation as;

$$\left( A + BS_0 \right) \ln \left( \frac{S - C}{S_0} \right) + B = -t \quad \ldots \ldots (16)$$

where, $A$ and $B$ are constants, which take into account $K$, $R_1$, $R_2$ and $K$, constants (see Ginsburg and Stein 1979).

These workers have transformed algebraically the above equation into a better usable form, namely;

$$\left( A + BS_0 \right) \left[ \ln \left( \frac{1 - C/S_0}{S_0/C} \right) + \frac{C/S_0}{C} \right] = -t \quad \ldots \ldots (17)$$

where, in this context,

- $C$ = uptake
- $S_0$ = maternal input
- $t$ = time

Uptake (cpm/ml) was converted to (cpm/min) by multiplying with the **foetal flow rate** shown in Table 8 for each protein. From specific activities (cpm/ug protein),
the perfused counts were converted into molar units, $S_0$, all shown in Table 8.

Plots as per Ginsburg and Stein's integrated equation (17), are shown in Figs. 52(a) and 52(b) for all proteins. There is a striking similarity to the well known Lineweaver-Burk double reciprocal plot as discussed on page 122. Thus, this equation for asymmetric carriers, predicts placental protein uptake.

Calculation of affinity constants

From equation 16, the intercepts are therefore

$$Y{-\text{cept}} = \frac{A}{S_0}$$

$$X{-\text{cept}} = \frac{A}{S_0} \left( \frac{S_0}{A + BS_0} \right)$$

under conditions, where $S_0$ is reasonably high

$$S^2 x{-\text{cept}} \approx \frac{A}{B}$$

$$\approx \frac{\Pi}{S_0 \cdot K}$$ (details as per Ginsburg and Stein, 1979).

Therefore,

$$K \text{ (affinity constant)} = S_0^2 \cdot X{-\text{cept}} \times S_0 \frac{\Pi}{\Pi}$$

where, $\Pi$ = equivalent concentration of an impermeable solute, and $S_0$, the perfused concentration. Assumption is made that $S_0/\Pi = 1$, that is to say, the theoretical concentration of an impermeable solute, $\Pi$, and $S_0$, are set the same then,
Table 8

This table summarises some of the in-vitro placental perfusion conditions, such as the maternal input of radio-labelled proteins, the corresponding specific activities, the maximum rate of foetal uptake (C) and the time ($T_{max}$) required for this at a given foetal perfusion flow rate.

Furthermore, the affinity of each perfused protein expressed as ($K_D$) for membrane carrier is shown in the last column, as estimated by the use of Ginsburg and Stein's integrated equation for asymmetric carriers for flux across plasma membranes.
<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>LIGAND INPUT VOL (MLs)</th>
<th>CPM/μL (SPECIFIC ACTIVITY)</th>
<th>CPM/μG</th>
<th>MOLAR CONC NsO (NM)</th>
<th>FOETAL FLOW RATE (MLs/MIN)</th>
<th>tMAX (MIN)</th>
<th>CALCULATED AFFINITY (Kd) (NM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIBRINOGEN</td>
<td>0.9</td>
<td>5401</td>
<td>10718</td>
<td>1677</td>
<td>3</td>
<td>23.33</td>
<td>40</td>
</tr>
<tr>
<td>TRANSFERRIN</td>
<td>1</td>
<td>19273</td>
<td>24558</td>
<td>8718</td>
<td>3</td>
<td>4.74</td>
<td>50</td>
</tr>
<tr>
<td>PreALB</td>
<td>1</td>
<td>36140</td>
<td>87272</td>
<td>6788</td>
<td>2</td>
<td>2.63</td>
<td>85</td>
</tr>
<tr>
<td>IgG</td>
<td>1</td>
<td>9801</td>
<td>43344</td>
<td>1215</td>
<td>2</td>
<td>1.39</td>
<td>60</td>
</tr>
<tr>
<td>HAPTOGLOBIN</td>
<td>1</td>
<td>13900</td>
<td>48165</td>
<td>2886</td>
<td>5</td>
<td>2.75</td>
<td>60</td>
</tr>
<tr>
<td>α1 AT</td>
<td>0.6</td>
<td>6260</td>
<td>121370</td>
<td>573</td>
<td>3</td>
<td>0.688</td>
<td>65</td>
</tr>
<tr>
<td>α1 AG</td>
<td>0.9</td>
<td>9515</td>
<td>174930</td>
<td>1113</td>
<td>3</td>
<td>0.623</td>
<td>65</td>
</tr>
<tr>
<td>Gc</td>
<td>1</td>
<td>18150</td>
<td>52606</td>
<td>6757</td>
<td>3</td>
<td>13.42</td>
<td>55</td>
</tr>
<tr>
<td>α2 HS-GLY-COPROTEIN</td>
<td>1</td>
<td>6559</td>
<td>49942</td>
<td>2680</td>
<td>3</td>
<td>10.99</td>
<td>70</td>
</tr>
</tbody>
</table>

Table 8
Affinities ($K_D$ values) have been calculated as displayed in Table 8, bearing in mind that by the very nature of perfusion experiment, non-specific binding cannot be eliminated. Therefore any estimate of the affinity constants will be affected by this inherent problem. In the case of dissociation constants the true values must be less than those in Table 8. Nonetheless, $K_D$'s obtained were very reasonable approximation and in chapter 8 better values will be derived via radioreceptor assays.

Conclusion

It must be appreciated that there were many uptake factors to consider in a perfusion study in terms of those of theoretical interest and those which are practicable in order to obtain good estimates of uptake parameters. Good data was obtained in this study and pointed to possibilities of receptor mediated mechanism for protein uptake in the human placenta since uptake profiles showed similarities to Michaelis-Menten kinetics. In conclusion, placental uptake of proteins was clearly a saturable entity and when a zero-trans model of uptake was considered, particularly the use of Ginsburg and Stein's integrated equation, which when applied to uptake data linearised these curves. The linear graphs were strikingly similar in shape to the classical Lineweaver-Burk plots. This has been observed also for receptor mediated glucose and galactose influx and efflux in erythrocytes (Eilam and Stein 1974; and Ginsburg and Stein 1979).
Figs. 52(a) to (i) - show linearisation of uptake curves shown in Figs. 47 and 50. This was done by use of Ginsburg and Stein's equation for assymetric carriers (1979) as applied in this instance to foetal uptake of various radio-labelled plasma proteins in an in-vitro placental perfusion. Here \( t/C \) (where \( t \) is time and the corresponding \( C \), uptake), plotted against \( \ln \left( 1 - \frac{C}{S_o} + \frac{C}{S_o} \right)/C \) where \( S_o \) is the maternal or \( C_i \)s input. The fact that curves are linearised proves that a Zero-trans uptake model and hence equation (17) describe placental transfer well. Furthermore, the saturable character of uptake curves coupled to this subsequent linear transformation is strongly suggestive of a receptor mediated uptake. The intercept on the horizontal axis (X-cept) and the initial maternal input are shown for each graph.
\( \alpha_1 \) ACID GLYCOPROTEIN

\[ X_{CEPT} = 5 \times 10^{-8} \]

\[ S_0 = 1113 \text{ nM} \]

\[ \frac{\ln (1 - C/S_0) + C/S_0}{C \times 10^8} \]

Fig. 52(a)
$\alpha_1$ ANTITRYPSIN

$X_{\text{CEPT}} = 2.0 \times 10^{-7}$

$S_0 = 573 \text{ nM}$

Fig. 52(b)
HAPTOGLOBIN

X-CEPT = 2.3 x 10^{-8}

S₀ = 2886 nM

\[
\frac{\ln \left(1 + \frac{C}{S₀} \right)}{C \times 10^8}
\]

Fig. 52(c)
Fig. 52(d)
Fig. 52(e)

$\alpha_2$ HS-Glycoprotein

X-CEPT = $2.2 \times 10^{-7}$

$S_0 = 2580$ nM
**Fig. 52(f)**

**IGG**

\[
X_{-\text{CEPT}} = 1.5 \times 10^{-7}
\]

\[
S_0 = 1215 \text{ nM}
\]
TRANSFERRIN

$X_{CEPT} = 0.4 \times 10^{-7}$

$S_0 = 8718 \text{ nM}$

Fig. 52(g)
FIBRINOGEN

$X_{\text{CEPT}} = 100 \times 10^{-7}$

$S_0 = 1677 \text{ nM}$

$(\ln(1 - C \cdot S_0) + C \cdot S_0) / C \times 10^2$

Fig. 52(h)
**Gc-Glycoprotein**

- **X-cp** = $2.7 \times 10^{-8}$
- $S_0 = 6.757 \text{ M}$

**Graph**: Logarithmic plot with points and a linear trend line. The x-axis is labeled with values and the y-axis is labeled as $Gc$ (MIN.-L. FRETAL UPTAKE / MOL).

*Fig. 52(i)*
CHAPTER 6

COLLOIDAL GOLD - PROTEIN CONJUGATES FOR ULTRASTRUCTURAL ELECTRON MICROSCOPIC STUDY OF PLACENTAL RECEPTORS.

Gold particles in a colloidal form are very electron dense, and can be conjugated to various protein ligands for electron microscopic study. Colloidal gold protein conjugates are thus well suited to receptor binding investigations since the position of the protein adsorbant can be accurately located. Colloidal gold adsorbed immunoglobulins and lectins have been widely studied to localise surface antigens (Geoghagen 1977) and(Dickson et al 1981), have used the technique to visualise $\alpha_2$macroglobulin-gold uptake by endocytic vesicles.

Since colloidal gold is easily conjugated to immunoglobulins, the conjugation can be done directly or indirectly in a manner analogous, for example, to indirect and direct fluorescence or peroxidase immunocytochemistry. Furthermore, colloidal gold is non-cytotoxic and is a stable cytochemical marker, readily prepared and is fit for various modes of microscopy (review by Horisberger 1979, Goodman et al 1980). Multiple labelling experiments can be performed by use of different particle sizes, which are easily prepared and recognised (Horisberger and Vonlanthen 1979). Gold markers can be prepared in the size range of 5 - 150 nm with size dependent characteristics and absorption spectra (Goodman et al 1981).

Colloidal gold is a negatively charged hydrophobic sol maintained in solution by electrostatic repulsion. Its
negative charge can be put to use when a positively charged ligand is reacted with the gold sol. Proteins being amphoteric macromolecules with polarity, they can be made to acquire net positive charge if the buffer pH in which they are dissolved falls below or near to the protein's isoelectric point (pI). The electrostatic bonding that results, can be stabilised using polyethylene glycol (PEG). This interaction is therefore dependent on pH, protein concentration, as well as ionic concentration (Goodman et al 1980).

In a detailed study, Goodman et al 1981, have investigated the optimal conditions for these various factors which influence gold-protein interaction. They further elucidated on bioviability of these conjugates, absorption spectra, stability as influenced by pH, dialysis, and suitability of various buffers in the process of conjugation. They were also able to measure by radioimmunoassay the total amount of ligand bound on gold particles. These radioimmunoassays also permit the calculation of the numbers of molecules of label per gold particle. Dickson et al (1981), using Χ₂-macroglobulin-gold probe found ~400 molecules bound to each gold particle.

Aims of this Study.

The colloidal gold probe because of its simplicity to prepare as well as easy attachment to proteins if the right conditions are set, particularly extensive dialysis of protein solution, knowledge of pI values and titration of amounts that can be conjugated, then a very useful marker is in hand
and can be used to probe receptors on the placenta. This particular technique, unfortunately has not yet been applied extensively to the study of plasma proteins with respect to possible existence of receptors, particularly on the placenta.

It was therefore felt that some proteins be labelled with this marker and then the conjugates subjected to isolated placental membranes where receptors are suspected, since this is the layer normally in direct contact with the maternal circulation.

**Materials and Methods.**

**Reagents**

Chloroauric acid (HAuCl₂) (H. Lamb, London), trisodium citrate (BDH Poole Dorset), polyethylene glycol (PEG) (MW 20,000), 0.2 μm membrane filters, dialysis membranes.

**Dialysis of protein ligands**

A visking tubing (32/32) was soaked in distilled water overnight prior to dialysis. It was checked for any leaks. Proteins in PBS and at 3mg/ml were placed in the visking tubing, both ends tied, but one end left with sufficient string to hang in a beaker containing 0.005M sodium chloride like a 'tea-bag'. Dialysis continued for 48 hours under constant stirring and several changes of dialysis fluid. The solutions were left in a fridge to avoid moulds growing on the tubing.
Solutions

All solutions were centrifuged at 3000 rpm and filtered through 0.22 μm filter after being prepared. All glassware was kept thoroughly clean as any dusts or improper cleaning would cause flocculation of gold sols.

The following solutions were needed:

1) 200 mls of distilled water in a 250 ml round bottomed flask
2) 4% chloroauric acid
3) 1% sodium citrate
4) 1% PEG
5) 0.2 M \( \text{K}_2\text{CO}_3 \) or 0.2 M HCl
6) Phosphate buffered saline or Hanks buffer with PEG.

Relationship between colloidal gold particle size and formative factors;

Different gold particle sizes can be prepared depending on what is required, by the use of various combinations of sodium citrate, PEG, and total sol. volume as per the following schedule:

<table>
<thead>
<tr>
<th>Factor</th>
<th>18 nm</th>
<th>35 nm</th>
<th>50 nm</th>
<th>100 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total No. of particles</td>
<td>200 ( \times 10^{12} )</td>
<td>40 ( \times 10^{12} )</td>
<td>1 ( \times 10^{13} )</td>
<td>1.5 ( \times 10^{12} )</td>
</tr>
<tr>
<td>1% sodium citrate (mls)</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>1% PEG (mls)</td>
<td>6</td>
<td>3</td>
<td>2.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Hanks buffer or PBS with (1% PEG)</td>
<td>0.6mg/ml</td>
<td>0.3mg/ml</td>
<td>0.24mg/ml</td>
<td>0.12mg/ml</td>
</tr>
</tbody>
</table>

This table is formulated on the basis of total volume of 200 ml of sol. initially prepared.
Preparation of Gold Sol.

200 mls of distilled water were added to a round bottomed flask. For 18 nm particles, used in this study, 0.5 ml of 4% chloroauric acid (HAuCl₄) was added with mixing, then the solution was boiled under constant reflux till the yellow colour turned black and then wine red or dark red, as shown in Fig. 53.

The solution was cooled, and filtered. The pH of this solution was adjusted to below or near enough to the pI of the protein to be conjugated. This will result into adsorption of protein by electrostatic forces. Care was taken not to coat the pH meter electrode with gold, by taking 1ml aliquots to which drops of 1% PEG were added and then the electrode directly immersed into this to record the pH. Adjustments of pH with either way of the pI value were made by titration of the gold sol. with 0.2 M K₂CO₃ or 0.2 M HCl.

Preparation of protein gold conjugates

This was a trial and error process; the aim was to find the optimal level of protein that can be added to the pH adjusted gold sol. such that no flocculation occurred.

In order to do this, a series of 1ml aliquots of gold sols. in LP₃ tubes was prepared. Increasing microlitre volumes of the protein standards of each protein were added to each 1ml of the colloid till flocculation occurred. The volume of protein just before floucculation occurred, was taken as the titration point. Table 9 shows the various titration volumes which were used to work out how much of protein should be
Reflux condensing apparatus was required to prepare colloidal gold. 0.5ml of $\text{H}_4\text{AuCl}_4$ was added to 200mls of distilled water. For 18nM colloidal gold particles, 5mls of 1% sodium citrate were added and the solution boiled with refluxing till it assumed a red wine-like colour as shown here, and then cooled. Subsequent steps are discussed in the text.
added to a standard 200 ml of the originally prepared colloidal gold solution.

After adding the protein, the colloidal solution was stabilised by addition of PBS containing 0.6 mg/ml of PEG (for the 18 nm particles which were used in this study). The pH was then adjusted to neutrality for all gold protein solutions. The labelled colloid was concentrated by spinning at 28,000g (17000 rpm) for 30 minutes at 4°C in a Sorvall OTD-65 refrigerated ultracentrifuge (Du Pont Instruments) and using 35 ml polycarbamate tubes and an AH 625 rotor. The unlabelled colloid is discarded in the supernatant.

The resultant pellet was re-suspended in a suitable buffer (PBS at pH 7.4 and containing PEG at 0.6 mg/ml). The colloidal protein conjugate is now ready for incubating in placental tissue to assess receptor binding.

**Incubation of Au (18 nm) – protein conjugates with placental villi.**

Tiny villi were dissected (5mg pieces) from the placenta and then incubated in 1ml of Au-protein conjugates at 37°C for 1 hour and gassed with 5% CO₂, 40% O₂, and 55% N₂. Control samples containing 1mg of unlabelled protein were pre-incubated with placental membranes for 30 minutes before protein ligands were subsequently introduced for 1 hour incubation. Here, the idea was to competitively inhibit binding and thereby obtain controls for each protein.
<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>pI</th>
<th>Conjugating pH</th>
<th>Titrations; max. vol. (ul) of protein to 1ml of colloid without flocculation</th>
<th>Total vol. (µl) of protein per 200ml of gold sol</th>
<th>Equivalent in (µg) protein/200ml gold sol.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hp</td>
<td>4.5</td>
<td>3.6</td>
<td>1 µl at 0.33µg/µl</td>
<td>200</td>
<td>66</td>
</tr>
<tr>
<td>Tf</td>
<td>5.9</td>
<td>5.0</td>
<td>3 µl at 0.33µg/µl</td>
<td>600</td>
<td>198</td>
</tr>
<tr>
<td>Fg</td>
<td>5.8</td>
<td>4.6</td>
<td>10 µl at 1.5µg/µl</td>
<td>2000</td>
<td>3000</td>
</tr>
<tr>
<td>PreAlb</td>
<td>4.7</td>
<td>3.6</td>
<td>0.5 µl at 0.066µg/µl</td>
<td>100</td>
<td>6.6</td>
</tr>
<tr>
<td>Gc</td>
<td>Unknown</td>
<td>Tried pH 3.8</td>
<td>1 µl at 0.22 µg/µl</td>
<td>200</td>
<td>44</td>
</tr>
<tr>
<td>α₂HS</td>
<td>Unknown</td>
<td>Tried pH 3.8</td>
<td>25 µl at 0.33 µg/µl</td>
<td>5000</td>
<td>1650</td>
</tr>
<tr>
<td>α₁AT</td>
<td>4.0</td>
<td>3.6</td>
<td>1 µl at 0.066µg/µl</td>
<td>200</td>
<td>13</td>
</tr>
<tr>
<td>IgG</td>
<td>5.8</td>
<td>4.7</td>
<td>1 µl at 0.33 µg/µl</td>
<td>200</td>
<td>66</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Unknown</td>
<td>Tried pH 4.0</td>
<td>2 µl at 0.25 µg/µl</td>
<td>400</td>
<td>100</td>
</tr>
<tr>
<td>α₁AG</td>
<td>2.7</td>
<td>3.0</td>
<td>5 µl at 0.05 µg/µl</td>
<td>1000</td>
<td>50</td>
</tr>
<tr>
<td>hCG</td>
<td>Unknown</td>
<td>Tried pH 3.6</td>
<td>4 µl at 0.33 µg/µl</td>
<td>400</td>
<td>132</td>
</tr>
</tbody>
</table>

Table 9
At the end of the incubations, unbound Au-protein were rapidly washed off by two rapid washings of placental tissues in PBS or Hanks buffer and then fixed the tissue in 3% gluteraldehyde for 2 hours. Post-fixation of tissue was done in Karnovsky's fixative for 4 hours at 4°C and thereafter the tissue was washed in two changes of cacodylate buffer for 10 minutes each. The tissue was fixed also in 1% osmium tetroxide in cacodylate buffer at 4°C for 1 hour. Thereafter the tissue was washed in two changes of cacodylate for 10 minutes each, and then block stained in fresh 1% uranyl acetate in distilled water for 30 - 45 minutes.

Tissue was dehydrated through 50, 70, and 90% alcohols 10 minutes each, and thereafter for two changes, 10 minutes each. Further treatment in 50:50 mixture of ethanol/propylene oxide for 10 minutes and then two changes of propylene oxide, for 10 minutes each.

The tissue was then transferred in fresh mixture of propylene oxide/Spurr's resin (50:50), and then placed in an incubator with lids removed from pots for 30 minutes.

The tissue was placed in fresh Spurr's resin at 37°C for 1 hour, then embedded in fresh resin in capsules or moulds and allowed to polymerise at 60°C for at least 16 hours or overnight.

Then sections were cut and then picked up on formvar/carbon coated 200 mesh grids and then subsequently counterstained with Reynolds lead acetate and uranyl acetate and then examined thereafter using a Jeol 100CX or Phillips electron microscope.
RESULTS

Figs. 54 to 58 show some of the proteins with Au-specific protein binding to the microvillar plasma membrane and some with particles endocytosed into specialised transport vesicles. For proteins to bind on to the surface of the gold particles, there are many variables to consider, such as pI, the configuration of the protein concerned, particle size of the gold sol, and the optimal binding conditions have to be right. In this study results are shown for transferrin, haptoglobin, hCG and α1 acid glycoprotein as shown in the photographs. Other proteins did not show colloidal gold adsorption.

It is possible also that during the processing for electron microscopy some of the conjugated protein or gold became desorbed and as stated above, there are critical conditions that have to be met for gold particles to adsorb on proteins. For some of the proteins it was a trial and error procedure to get optimal pH for purposes of conjugating gold to the protein since pI's for some proteins could not be found in the reference texts consulted. One significant point about this technique is that it provides a direct visualisation of the process of surface events from binding, formation of coated vesicles and inclusion of ligand, and then the vesicle dynamically transfers itself across the syncytio and cytotrophoblastic layers to exocytose its contents into a foetal capillary and thus effect a complete transfer of protein ligand.

This process has been observed for IgG antibodies in particular, and Huxham and Beck (1981) studied receptor
mediated transport of rat IgG across the 11.5 day in-vitro-rat york sac endoderm and demonstrated well the whole pathway from binding to extrusion of the gold-IgG in the york sac. Dickson et al (1981) adsorbed $\alpha_2$macroglobulin to colloidal gold particles. Binding was demonstrated in rat kidney (NRK-2T) and swiss mouse 3T3 cells at 0°C. On warming the cells for 30 minutes, gold was rapidly internalised into uncoated vesicles previously called receptosomes and with longer incubations the particles were visualised in lysosomes. This technique offers a useful new tool in the study of receptor mediated uptake.
Fig. 54(a) shows colloidal gold-labelled transferrin (Au-Tf), 18 nm particles bound to the surface of the trophoblastic membrane. The picture also shows the binding process of three gold particles (arrowed) leading to curving in of the membrane to form a caveola, which will eventually seal off as a coated vesicle. Coated vesicles are the specialised modalities of transcellular protein transport, protected against proteolysis, to the foetus.

Fig. 54(b) shows microvillus membrane. The arrow shows the process of coated vesicle formation with the caveola sealing off to enclose the gold-protein conjugate (Au-Tf), for uptake (arrowed).
Fig. 54(a)

Fig. 54(b)
Legend for Figures 55(a) and (b)

Fig. 55(a)

Competitive inhibition of Au-Tf (gold-transferrin) by unlabelled transferrin, 1mg in 1ml colloidal gold sol by a preincubation with membranes before Au-Tf was added.

The picture shows complete inhibition of binding as evidenced by absence of any gold particle on the microvillous placental membrane. This is suggestive of existence of transferrin receptors.

Fig. 55(b)

In another similar competitive inhibition experiment using unlabelled transferrin there is no Au-Tf binding anywhere on the surface or in the syncytio and cytotrophoblastic areas.
Fig. 55(a)

Fig. 55(b)
Fig. 56

Fig. 56(a) - Colloidal gold - haptoglobin conjugate, adsorption to the surface of syncytiotrophoblastic microvilli. One particle is in closed vesicle, (arrowed). (35 nm particles were prepared)

Fig. 56(b) - Microvilli surface in the left half of the picture. A colloidal gold (Au-Hp) particle appears taken up in a coated vesicle, (arrowed). (35 nm particles were prepared)
Fig. 57 - shows adsorbed Au-$\alpha_1$ acid glycoprotein adsorption to the surface of the placental microvillar membrane.
Fig. 58 - shows some gold particles conjugated to human chorionic gonadotrophin (hCG) binding to the apical surface of the trophoblastic membrane.
Introduction

The plasma membrane of the placental trophoblastic is particularly unique in that multiple functions normally requiring separate specialised organs are all carried out by the same membrane. These functions include phagocytosis, transfer of gases, excretion of foetal waste products, absorption of nutrients by either simple or facilitated diffusion or indeed by active transport. (Steven and Samuel, and Fox 1967).

Active transport is of great interest since macromolecules such as plasma proteins are too large to be effectively transported across the placenta by simple diffusion. (Reynolds 1979). Consequently the existence of specialised membrane receptors plays a vital role in recognition and active transfer of these macromolecular proteins to the growing foetus. (Brambell 1970, Wild 1976, Fox 1979). Therefore attempts must be made to identify human syncytiotrophoblastic plasma membrane (StMPM) associated components which are central to our concepts regarding successful maternal acceptance of the foetus as a homograft during pregnancy. These components normally form the interface between the maternal blood's immune system and the foetus.

Previous work has shown existence of numerous proteins associated with the StMPM (Carlson et al 1976, Smith et al 1977,
Johnson and Faulk 1978). These proteins include transferrin, $\alpha_2$macroglobulin, alkaline phosphatase, HLA determinants and $\beta_2$microglobulin (Ogbimi et al 1979). Cytoskeletal microvillar core proteins such as actin of ($M_T$ 38,000); fimbrin ($M_T$ 68,000) and villin ($M_T$ 95,000) have also been identified (Brown and Johnson 1981; Bretscher and Weber 1979; Matsudaira and Burgess 1979). More important are those proteins which act as recepients of others for purposes of selective uptake and transfer; namely receptors. With regards to the placenta, IgG-Fc$\gamma$ receptor, it has been successfully isolated from solubilised placental membrane extracts (Brown and Johnson, 1981; Ogbimi et al., 1979; Balfour and Jones 1978), although controversy still exists as to the actual structural make up of the receptor area. This will be discussed in chapter 8.

Transferrin receptors on the placenta have also been successfully identified as having $M_T$ 70,000 using gel filtration and $M_T$ 65,000 by SDS-PAGE (Brown and Johnson, 1981). Transcobalamin II, a vitamin $B_{12}$ transporting protein in plasma has been shown to have a specific high affinity receptor on the human placenta by which the vitamin $B_{12}$ is transferred to the foetus. (Seligman and Allen, 1978). It has a molecular weight, $M_T$ of 50,000. Apart from these three types of receptors, very little else is known as to whether other plasma proteins possess specific receptors on the human placenta. As already mentioned, a choice of proteins were studied in the preceeding chapters with respect to immunofluorescence and peroxidase, placental perfusion of radiolabelled ligands, colloidal gold, and in the case of IgG, by kinetics and saturation analysis; all these separate
studies jointly gave strong evidence as to the existence of specific saturable receptors.

In this chapter another step has been taken to try to isolate the actual receptor structure using affinity chromatography as the major purification step. This technique combined with SDS-PAGE provide a powerful tool for identifying receptors using cyanogen bromide Sepharose 4B linked to a specific purified plasma protein as the adsorbent to extract the receptor from solubilised placental membrane. In the articles quoted above with reference to IgG, transferrin and transcobalamin II receptors, these two techniques were the major steps in isolation of the receptors, although gel filtration was also used in molecular weight calculation of the receptor protein or in saturation analysis to find out other binding data such as receptor homogeneity and affinity constants. The following plasma proteins were conjugated to the cyanogen bromide Sepharose 4B and then used to try to isolate receptors from the soluble fraction of the StMPM; IgG, α₁-acid-glycoprotein, prealbumin, haptoglobin, thrombin, α₁-antitrypsin, Gc-glycoprotein and α₂-HS-glycoprotein.

Methods
Step 1: Preparation of placental homogenate

Four placentae, each weighing about 600g were stripped of chorionic membrane and cut into small pieces. These were homogenised at top speed in an MSE-blendor (Fisons) for 45 seconds in 2.0 volumes of 10mM Tris-HCl pH 8.2 buffer
containing 150mM of NaCl. At times a food homogeniser; a Multichef (Curry's electric store) proved to be the best to use since a whole placenta could be minced in about 2 minutes. The mince was filtered through cheesecloth and the homogenate of about 1.5 litre was centrifuged at 800g for 10 minutes, then at 10,000g for 10 minutes, each time discarding the pellet. The supernatant was then spun at 100,000g for 30 minutes and discarding the supernatant. The pellet was homogenised in 50 mls of a 10mM mannitol in 2mM Tris/HCl pH 7.1 with a loose fitting Dounce homogeniser and the rest of the purification steps are outlined in Fig. 7 on page 52. The final purified membrane was adjusted to 5mg/ml concentration.

Step 2: Membrane solubilisation

The membrane prepared above was solubilised in 10mM Tris/HCl pH 8.2 buffer containing 1% Triton-X<sub>100</sub> and 150mM NaCl. Solubilisation was continued overnight with homogenate constantly stirred, then sonicated on ice for 5-8 minutes. After stirring for 45 minutes, the mixture was re-homogenised using a Potter-Elveljeim homogeniser and then spun at 36,000g for 30 minutes and the supernatant removed.

Step 3: Ammonium Sulphate precipitation:

31.5gms of solid ammonium sulphate was added to every 100mls of supernatant from the previous step, stirred for 10 minutes on ice and centrifuged in portions of 400mls using 50ml x 8 polycarbonate centrifuge tubes, and spinning at 36,000g for 20 minutes. The pellet was resuspended in 100mls of 10mM Tris/HCl pH 8.2 containing 150mM NaCl and 0.15% Triton-X<sub>100</sub> and then dialysed for 24 hours. The dialysate
was changed at 6 and 20 hours. The solubilised membrane was stored at -20°C till required.

Step 4: Coupling of specific protein to CN Br-activated Sepharose 4B for affinity chromatography.

3gms of freeze dried CN Br-activated Sepharose (for a 0.9 x 15cm column) were swollen from 15 minutes to 1 hour or at times overnight in a beaker with 1mM HCl, and washed on sintered glass or Whatman filter paper (Whatman) with 600mls of 1mM HCl (200mls per 1gm of gel).

The protein to be coupled was then dissolved in a coupling buffer of 0.1M NaHCO₃ containing 0.5M NaCl. About 5-10mg protein per ml of gel can be coupled. Depending on the amounts of protein available the following quantities of protein were coupled to the 3g gel: 30mg for IgG, 44mg for α₁-acid-glycoprotein, 10.5mg for α₁-antitrypsin, 10mg for prealbumin, 7mg of Gc-glycoprotein, 10mg of α₂HS-glycoprotein, 3.4mg of haptoglobin, and 15mg of thrombin. Proteins were mixed with gel suspension in a 250ml beaker on a rocking mixer for 2 hours at room temperature.

After coupling unbound protein was washed away twice using coupling buffer and then any remaining active groups on the gel were blocked by reacting the gel suspension with 0.2M glycine pH 8.0. Excess adsorbed protein was washed away using a cycle of coupling buffer followed by acetate buffer (0.1M, pH 4.0) each containing 0.5M NaCl and repeated four to five times. Each Sepharose protein conjugate was then ready for use and was stored at 4°C till required.
Step 5: **Affinity isolation of receptors using protein-CN Br-activated Sepharose 4B adsorbents.**

Each gel adsorbent was poured on a 0.9 x 12cm column (Pharmacia Uppsala, Sweden). The dialysed solubilised receptor preparation was spun at 16,000g for 20 minutes and then applied to the column at flow rate of 12mls/hr. The system was linked to a Cecil 2010 UV-spectrophotometer, (Cecil Instruments, Cambridge) with scanning attachment for monitoring absorbance at 280nm.

The following washing and elution sequence was applied at 12mls/hr:

1) Wash with **100mls** of equilibration buffer
   
   \((10\text{mM Tris-HCl pH 8.2} + 0.5M \text{NaCl})\)

2) Wash with **50mls** equilibration buffer + 10mM EDTA.

3) Wash with **30mls** equilibration buffer.

4) Wash with **40mls** of 0.1M glycine/HCl pH 2.3 and collection of 6ml fractions.

Stage 4 gave single peaks, in each case, of varying sizes. The absorbance range was set at 0.2. It is important to note that the placental homogenate contained Triton-\(\times_{100}\) which itself absorbs at 280nm thus interfering with monitoring of elution protein peaks. Therefore thorough washing was done till the recorder set back to the base of the chart before the desorbing 0.2M glycine/HCl pH 2.3 was applied. The receptor eluate was immediately buffered to pH 7.4 by 1M Tris pH 11.0 and then dialysed against 10mM Tris-HCl pH 8.2 containing 0.15M NaCl and 0.15% Triton before freeze drying the receptor material.
SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The receptor preparation in each case was applied to SDS-PAGE which was carried out on a 9% polyacrylamide running gel incorporating a 3% stacking gel. The whole gel was cast as a slab of 0.75mm x 10cm x 14cm using Laemmli's buffer system (Laemmli 1970) which required stock solutions of acrylamide: N'N'-Bis methylene acrylamide (30:0:8), 1.5M Tris-HCl pH 8.8, and a 0.5M Tris-HCl pH 6.8. A running gel of 9% was cast in 0.375M Tris-HCl pH 8.8 containing 0.1% SDS. After polymerisation a 3% stacking gel was cast on top of the running gel in 0.125M Tris-HCl pH 6.8 containing 0.1% SDS. (See below for details of reagents and preparation of solutions for the Laemmli's system).

Samples were prepared in 0.0625M Tris-HCl pH 6.8 containing 2% SDS and 10% glycerol. 5% mercaptoethanol was added to the sample and the mixture immersed for 3 minutes in a boiling water bath. Bromophenol Blue (0.001%) was then added and electrophoresed using Protean Cell electrophoretic apparatus with a cooling facility (Fig. 59 shows the apparatus supplied by Bio-Rad Laboratories, Watford, U.K). The Protean Cell is a vertical slab cell instrument that virtually eliminates instrument variability with sharp separations and eliminates edge effects due to uneven cooling.

Electrophoresis was performed at 30-40mA constant current till the dye front was nearly reaching the bottom edge and gels were stained with 0.05% Coomassie Blue R-250 in acetic acid/isopropanol/water (1:1:8) overnight and destained with methanol, water and acetic acid (4:4:1).
Fig. 59: Bio-Rad Protean Cell for slab SDS-Polyacrylamide gel electrophoresis.
Molecular weight standards were purchased from Sigma (Sigma Chemical Co., London) for calibration purposes. Log $M_r$ was plotted against relative mobility of bands which included myosin, $M_r$ 205,000, $\beta$-galactosidase, $M_r$ 116,000, phosphorylase B, $M_r$ 97,400, albumin bovine, $M_r$ 66,000, and albumin egg, $M_r$ 45,000.

Reagents and gels for SDS electrophoresis (Laemmli's buffer System).

Stock solutions:

A. Acrylamide:BIS (30:0:8)
   - 30g acrylamide
   - 0.8g N'N' BIS methylene acrylamide
   - q.s. 100ml with distilled water
   - Filter and store at 4°C in the dark

B. 1.5M Tris-HCl, pH 8.8
   - 18.15g Tris base
   - 50ml distilled water
   - Adjust to pH 8.8 with 1 N HCl
   - q.s 100ml with distilled water

C. 0.5M Tris-HCl, pH 6.8
   - 3.0g Tris base
   - 50ml distilled water
   - Adjust to pH 6.8 with 1 N HCl

Separating gel preparation - 9% gel, 0.375M Tris, pH 8.8 (Sufficient for two 0.75mm x 10cm x 14cm slabs)

- Distilled water 13.1ml
- 1.5M Tris-HCl, pH 8.8 7.5ml
10% (w/v) SDS 0.3ml
Acrylamide: BIS (30:0:8) 9.0ml
10% ammonium persulfate (fresh) 0.1ml
TEMED 0.0075ml
30.0ml

Stacking gel preparation - 3% gel, 0.125M Tris, pH 6.8
(Sufficient for two 0.75mm thick stacking gels)

Distilled water 6.3ml
0.5M Tris-HCl, pH 6.8 2.5ml
10% (w/v) SDS 0.1ml
Acrylamide: BIS (30:0:8) 1.0ml
10% ammonium persulfate (fresh) 0.1ml
TEMED 0.005ml
10.0ml

1. Prepare the gel solutions on ice. Add all reagents, except the TEMED, in order and deaerate under vacuum for 15 minutes (while in an ice bucket). To initiate polymerisation, add TEMED and swirl gently to mix.

2. Dilute the sample 1:5 with sample buffer and heat on a boiling water bath for 2 minutes.

Sample Buffer
Distilled water 4.7ml
0.5M Tris-HCl, pH 6.8 1.0ml
Glycerol 1.0ml
10% (w/v) SDS 1.0ml
2-Mercaptoethanol 0.1ml
0.05% (w/v) Bromophenol Blue 0.2ml
Electrode Buffer, pH 8.3

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>6.0g</td>
</tr>
<tr>
<td>Glycine</td>
<td>28.8g</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>10.0ml</td>
</tr>
</tbody>
</table>

q.s. to 1 litre with distilled water

RESULTS

Fig. 60 shows the calibration curve for molecular weight standards used in molecular weight assessment of receptor extract bands. Fig. 61 shows sample wells 1 to 14. The band running at the centre of gel in all wells was albumin at $M_r$ 66,000 and regarded as contaminant during the extraction of receptor. It is a well documented fact that albumin is usually a problem in affinity chromatography even when thorough washing of the column is done. Bio-Rad's Cibacron Blue-F 3G-A affinity gel can be used to adsorb the albumin (Travis et al 1976), and thus remove it from contaminating affinity purification of proteins (Bio-Rad Technical Bulletin, No.36E). Also Blue Sepharose CL-6B has been used to remove albumin. Since the albumin band could be identified on the gels, it caused no confusion at all with regard to identifying receptor bands.

From the calibration plot for molecular weight markers, receptor bands were assigned molecular weights according to their relative mobility. Lanes 0 and 1 in Fig. 61 corresponded to low and high molecular weight markers respectively.
Fig. 60

\[ M_r \times 10^3 \]

Myosin (205,000)

\( \beta \)-Galactosidase (116,000)

Phosphorylase (97,400)

Bovine albumin (66,000)

Egg albumin (45,000)

RELATIVE BAND MOBILITY
Lane 0 is low molecular weight marker with (Bovine albumin and egg albumin). Lane 1, is high molecular weights and lanes 2 and 3 are solubilised membranes.

**Figure 61:** SDS-polyacrylamide slab gel for Triton-X$_{100}$ solubilised placental membrane and affinity isolated receptor structures to specific plasma proteins.
Figure 62

SDS-polyacrylamide slab gel electrophoresis of a 10-fold concentrated membrane extract initially solubilised with Triton-X$_{100}$ in lanes 2 and 3. Eight protein bands can be seen. Lanes 0 and 1 represent low and high molecular weight markers respectively.
Lanes 2 and 3 were duplicate runs of the solubilised membrane extract. Lane 5 for haptoglobin receptor gave a band of $M_r$ 48,000, lane 6 for $\alpha_1$ acid glycoprotein gave $M_r$ of 46,000, whilst lane 9 for prealbumin gave $M_r$ of 49,000. Lanes 13 and 14 for $\alpha_1$ antitrypsin receptor gave $M_r$ of 62,000. The $\alpha_1$ antitrypsin band showed a reasonably concentrated receptor extract. Whilst protein quantities conjugated to the CN Br-activated Sepharose 4B differed, the actual amount of receptor adsorbed was also a function of receptor density in the first place and so the amounts of receptor extracted differs greatly.

The solubilised receptor material in lanes 2 and 3 had bands of $M_r$ 54,000, 66,000, 74,000, 78,000, 140,000 and 195,000, and there were no co-migrating bands to correspond to haptoglobin, $\alpha_1$ acid glycoprotein and prealbumin receptor bands. Demarcation of bands on SDS-PAGE is usually difficult due to presence of Triton in the solubilised fraction, added to the fact that the receptor material was relatively dilute in comparison to the affinity isolated receptors. When the solubilised material was concentrated ten times in the Amicon chambers, some previously invisible bands appeared as shown in Fig. 62 although the staining was still weak. There were now eight visible bands as compared to six in Fig. 61 and had molecular weights of 46,000, 48,000, 66,000, 74,000, 78,000, 95,000, 105,000 and 195,000, some of which co-migrated with $\alpha_1$ acid glycoprotein, haptoglobin and IgG receptor bands (see below). Ogbimi et al. (1979) showed molecular weights of 114,000, 108,000, 95,000, 81,000, 62,000, 61,000, 48,500, 40,000, 30,000, 27.500, and 18,000. Those underlined corresponding closely either to those in lanes 1 and 2 in fig 61 for the putative receptor bands.

Lane 12 for the IgG receptor gave four bands with $M_r$ of;
(from top to bottom), band 1 (Mr 195,000) band 2 (Mr 78,000), band 3 (Mr 74,000) and band 4 (Mr 66,000). Band 4 was the albumin contaminant. The other 1 to 3, IgG receptor bands in lane 12 had co-migrating bands of Mr 74,000, 78,000 and 195,000 in the solubilised receptor preparation in lanes 2 and 3. It would therefore appear that IgG receptor could be heterogeneous as will be pointed out in the discussion.

**DISCUSSION**

In the SDS-PAGE analysis of affinity isolated receptors positive results were obtained for haptoglobin, $\alpha_1$ acid glycoprotein, prealbumin and $\alpha_1$ antitrypsin with molecular weights of 48,000, 46,000, 49,000 and 62,000 respectively. For reasons not quite clear, there were no receptor bands in the $\alpha_2$ HS-glycoprotein, Gc-glycoprotein and thrombin lanes. This is not to suggest that they have no receptors since there are a number of factors which affect the appearance of a band in SDS-PAGE; for example, the prevalence or density of a particular membrane protein or receptor in the purified preparation. It is also well known that Coomassie Blue R-250 is insensitive in staining clearly low concentrations of some proteins, for which reason Bio-Rad Laboratories (Watford, U.K) have developed a silver stain with a 10 to 50-fold increase in sensitivity (Technical Bulletin No. 39EG). There could also have been insufficient extraction of the receptor itself.

It should be noted that for some of the proteins studied there was only a small supply of 10mg per vial and either the whole amount or even less than this was conjugated to the affinity gel. Here manufactures recommend at best, 5-10mg/ml of gel and 9.5mls of gel were used for the 0.9 x 15cm affinity column.
Some workers go round these problems by radiolabelling the extract itself and thereafter try to isolate membrane antigens or receptors. By autoradiographing the SDS-PAGE gel, receptor bands could be identified. Alternately when gel-filtration is performed on the desorbed receptor from the affinity column and which had previously been incubated with radiolabelled protein, the monitoring of eluted radioactivity helps to identify the free or bound receptor fraction. (Seligman et al., 1979, Seligman et al., 1978, Brown and Johnson, 1981). In the present study, financial constraints limited the exploration of these alternatives due to shortage of highly purified proteins particularly as each 10mg vial for some of these proteins costs around £200.

The three molecular forms of IgG of Mr 195,000, 78,000, and 74,000 obtained in this study are reminiscent of other reports that the IgG receptor has molecular heterogeneity. Balfour and Jones (1978) were the first to attempt the isolation and characterisation of IgG placental receptor. They applied a Triton-X_100 solubilised extract on to IgG-Sepharose 4B column and the eluted receptor analysed on SDS-PAGE. They found three major bands with molecular weights of 37,000, 45,000 and 60,000 and six minor bands with higher molecular weights which they did not state.

Niezgodka et al. (1981) also noted this heterogeneity when they isolated the IgG receptor using lithium dicodoscilate to solubilise the placental membrane and found two major bands on SDS-PAGE with Mr 74,000 and 104,000 and three minor peaks with Mr 56,000, 144,000 and 163,000. They also commented on
the weak binding of Coomassie Blue to receptor glycoproteins which limits proper visualisation. In the present study only higher molecular weights of 74,000, 78,000 and 195,000 were seen for IgG receptor. It is quite possible that low ones were present but not discernible due to weak binding of Coomassie Blue. More significantly, most of the membrane antigens appear as very small bands and at times barely visible.

The IgG bands of $M_r$ 74,000 and 195,000 are in the same order of magnitude with those of $M_r$ 74,000 and 163,000 seen by Niezgodka et al (1981). The explanation for IgG's heterogeneity has not been conclusively worked out. Balfour and Jones (1978) suggested that the solubilised membrane inevitably contained membranes of other subcellular organelles or cell types that make up the placenta but which could conceivably possess IgG receptors. Also erythrocytes and white cell membranes which have IgG receptors (Balfour and Jones 1977, Abrahamson et al 1970), always contaminate placental membrane isolation.

Balfour and Jones continued to assert that their three major IgG receptor bands could represent three subtypes; one being found on the epithelial syncytiotrophoblastic membrane which is in direct contact with maternal blood, and is inhibitable or by monomeric IgG (Elson et al 1975). The other two are found on the endothelium of foetal stem vessels which take up IgG picked up from the maternal serum by the first receptor subtype. The endothelial IgG receptor is not blocked by monomeric IgG. This differential sensitivity to IgG itself by the receptor subtypes ensures delivery of IgG to the foetus and demonstrates a complicated control mechanism. Furthermore one endothelial receptor subtype can bind immunocomplexes containing IgG,
whilst the other binds aggregated IgG which is blocked by IgM-pretreatment (Johnson et al 1973).

Johnson and Brown (1981) have comprehensively reviewed the IgG-Fcγ receptor and seem to acknowledge the diverse molecular weights that have been obtained in detergent solubilised Fcγ-receptor. They point out strong evidence implicating a $M_r$ 115,000 globular protein with five globular units linked loosely and that other molecular weights could be proteolysis products of this molecule. The controversy is still unresolved. Nonetheless in the present study, there were bands in the solubilised membrane fraction co-migrating with the IgG-receptor fraction and agreeing with Niezgodka et al's (1981) findings. Information regarding other plasma protein receptors is rather novel as there is no reference to any other study that has attempted to identify receptors for haptoglobin, prealbumin, $\alpha_1$ acid glycoprotein and $\alpha_1$ antitrypsin on human placenta. Insufficient receptor material disallowed identification of the Gc-glycoprotein, $\alpha_2$HS-glycoprotein and thrombin receptors.

Thrombin receptors on other cell systems such as fibroblasts have demonstrated clearly that thrombin has a cell surface receptor of about $M_r$ 35,000 and fibroblasts possess about $2 \times 10^5$ thrombin receptors/cell and a high affinity of $1 \times 10^{-9} M$. Human foreskin also possesses these receptors. (Baker et al 1979). Glenn and Cunningham (1979) have also identified a thrombin cell receptor of $M_r$ 43,000 to which thrombin attaches and cleaves it before its mitogenic
stimulation of cells is expressed. This was consistently shown in chick embryo cells and mouse-embryo dividing cells. Platelets also have thrombin receptor but Scatchard analysis has revealed high affinity ($K_d = 1.8-2\text{nM}$) and low affinity sites ($K_d = 200\text{nM}$) on the receptor.

Prealbumin, as the main vehicle for transporting retinol (Vitamin A) bound to retinol-binding globulin has been shown by Vahlquist et al (1975) to be present in foetal cord blood but which is immunologically indistinguishable from that of the paired maternal blood. Evidence from their study suggested that prealbumin and retinol-binding globulin must be transferred across the placenta, the principle route by which Vitamin A reaches the foetus. A prealbumin receptor of $M_r = 49,000$ has been identified in this study in conjunction with other previous supportive evidence. Gc-glycoprotein has also been shown to cross the placenta readily (Johnson et al, 1974) although no receptor band was identified in the preparation used for SDS-PAGE.

Finally Carlson et al (1976) analysed the placental plasma membrane and obtained sixteen bands with $M_r$ of 330,000, 295,000, 240,000, 215,000, 210,000, 89,000, 77,000, 73,000, 67,000, 51,000, 49,000, 45,000, 33,000, 31,000, 26,000 and 21,500. The underlined molecular weights are close to those obtained in Fig. 62. Ogbimi et al (1979) also obtained twenty one bands. Of these, only six stained strongly, the rest stained very weakly.
CHAPTER 8

RECEPTOR-PROTEIN BINDING ISOTHERMS USING PURIFIED SYNCTIOTROPHOBLASTIC MEMBRANE OR RECEPTOR DESORBATES FROM AFFINITY CHROMATOGRAPHY.

Introduction

Once receptor molecules are isolated or while still intact on the plasma membrane, it becomes imperative to explore their functional and physical characteristics. It is these which impart iniqueness to these molecules and thus enable them to mediate cellular and extra-cellular functions with unparalleled precision.

One of the problems of studying isolated receptors is the fact that their natural environment on the plasma membrane is disrupted. This may render the receptor inactive or even lose its natural conformation. The interaction between the agent and its receptor by which an effect is achieved must be thought of as a dynamic event more than the static classical picture of lock and key. The receptor causes an effect by a series of biochemical and biophysical events (Ariens 1964, Ariens and Simonis 1976). The chemical nature of the agent also affects the nature of the receptor - certain protein molecules (the ligand) have been shown to have structural similarities to the receptor molecule; for example, the transcobalamin II molecule has complimentary structural similarity to the transcobalamin II receptor on the placenta, (Seligman and Allen, 1978). This implies that the agent and its receptor must have evolved from the same ancestral molecule.
Enzyme-substrate interaction has parallels in agent-receptor binding - the differentiation between an active site on the enzyme molecule and the enzyme molecule as a whole. Under certain circumstances the receptor site might be constituted from an interface between different molecules. Furthermore, there are accessory binding sites, not located on the receptor molecule itself, as in the cases of agonist and competitive antagonists for cholinergic-anticholinergic, histamine/antihistaminic, adrenergic and antiadrenergic relationships to the receptor. For these systems the agonist binds on the polar area of the receptor, whilst its antagonist binds on the surrounding hydrophobic area made up of the lipid membrane (Ariens 1967, 1974, Ariens and Simonis 1960). These points all emphasise the problem encountered in studying isolated receptors removed from the plasma membrane. These other structural relationships pertinent to the functioning of the receptor molecule would be removed in the isolated receptor.

Nonetheless, binding studies using radiolabelled ligands have permitted useful information to be obtained on isolated receptors, which is in fact more accurate than when studied on intact membranes. This presupposes that during isolation gentle methods have to be used to avoid destruction of the receptor molecule, which is usually a protein. Equally useful information can be derived by incubating radiolabelled ligands with purified membrane preparations so long as other contaminating membranes of subcellular organelles are removed.

Effective solubilising agents for extraction of receptor molecule are usually detergents which disperse the lipid
bilayer and thus cause release of embedded protein molecules. These range from strong ionic detergents such as sodium deoxycholate (NaDOC) and sodium dodecyl sulphate (SDS) to non-ionic ones like Triton-X100 and Nonidet-P40. These non-ionic detergents are both polyoxoethylene-octyl phenols and are extremely effective in solubilising membrane antigens, added to their non-disruptive nature with respect to the receptor molecule, (Miller 1970, Duchon and Collier 1971). NaDOC and SDS, whilst useful too, can degrade the protein molecule.

In the following study Triton-X100 solubilised membrane extracts were subjected to affinity chromatography using CN Br-activated Sepharose 4B coupled to a specific protein. The receptor desorbed from the affinity column was utilised as substrate for radiolabelled protein binding studies. It was seen in the previous chapter that when the receptor desorbates were analysed on SDS-polyacrylamide gels, some of the bands stain with varying intensity when Coomassie Blue is used, whilst others were not visualisable at all due to very low protein concentration amongst other factors.

The problem of eliciting good bands on SDS-PAGE can be resolved if radiolabelled proteins are used to locate the receptor-protein conjugate by autoradiography or if the desorbed receptor from the affinity chromatography is incubated with the respective radiolabelled ligand. In this chapter, these incubations have been performed and from binding isotherms, it was possible to analyse the data using Scatchard plots (Scatchard, 1949) to assess some properties
of these receptors. These included affinity constants, total binding capacity, and from the shape of the Scatchard plot, heterogeneity or homogeneity of binding sites could be assessed. In some instances intact purified plasma membrane instead of the receptor fraction was used.

**Materials and Methods.**

a) **Isolation and Solubilisation of Syncytiotrophoblastic Membrane (StMPM).**

This was done essentially as outlined on page 52 and following steps in Fig. 7 for isolation of purified StMPM using the method of Booth et al. 1981 by a combination of differential ultracentrifugation and precipitation of non-trophoblastic membrane with Mg$^{2+}$ ions. Once prepared, the StMPM was assayed for protein using Micro-Lowry protein assay (Lowry et al. 1951) or the Bio-Rad protein assay kit using Bradford's method based on the use of acidified Coomassie Blue G$_{250}$ (Bradford 1976). The membrane was solubilised using 1% Triton-X$_{100}$ as outlined in chapter 7, pages 238-239.

b) **Affinity Isolation of Receptors.**

The solubilised fraction was then passed over a column of CN Br-activated Sepharose 4B linked to a specific protein (eg. thrombin, Gc-glycoprotein, $\alpha_2$HS-glycoprotein, prealbumin, $\alpha_1$ acid glycoprotein and haptoglobin), as outlined in detail in chapter 7, pages 240-241. Receptors were desorbed using 0.1M glycine/HCl pH 2.3 which was immediately buffered to pH 7.4 using 1M Tris pH 11.0. Those receptor fractions were then freeze-dried and stored till required.
C) *Radiolabelling of Plasma Proteins.*

125 ul of N-Succinimidyl 2,3,\( ^3\)H\(^3\) propionate (NSP), (Amersham International, Product Code TRK 556 1mCi/ml), was placed into a 10ml conical glass test tube. Toluene was blown off by a gentle \(N_2\) stream. The dry NSP was reacted on ice with appropriate amounts of protein (see Table 10), dissolved in 0.2M Borate buffer pH 8.0. The mixture was chromatographed on Sephadex G\(_{50}\) column (0.9 x 15cm Pharmacia Fine Chemicals, London) and eluted with 50mM Tris/HCl pH 7.4. Fractions were monitored for radioactivity and protein labelled peaks identified. Percentage incorporation of label was calculated in each case and from counts incorporated amounts of protein used, and efficiency of the Scintillation counter, the specific activities were calculated, (see Table 10).

d) *Radio Receptor-Ligand Binding.*

Table 11 shows incubation conditions for reacting receptor preparations or intact StMPM, with radiolabelled proteins in order to construct binding isotherms. Incubations were carried out in 50mM Tris/HCl pH 7.4 containing 1% Bovine serum albumin (BSA). BSA minimises non-specific binding. Incubations were carried out for 1 hour at 37°C, shaking the tubes every 15 minutes. Separation of bound from free radioactivity was done by filtering the total test tube contents through GF/C glass fibre discs (Whatman) using the Amicon filtering chamber capable of taking twelve discs at a time and attached to a vacuum pump (Amicon Stoneygate, Gloucs). For solubilised receptor fraction, Sephacryl S 200
<table>
<thead>
<tr>
<th>Protein</th>
<th>NSP/Protein ratio</th>
<th>Molecular weight</th>
<th>Amount of protein labelled (ug)</th>
<th>Specific activity (cpm/ug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prealbumin</td>
<td>2:1</td>
<td>61,000</td>
<td>72.65</td>
<td>$1.2 \times 10^6$</td>
</tr>
<tr>
<td>Gc-glycoprotein</td>
<td>1:1</td>
<td>50,000</td>
<td>59.55</td>
<td>$6.43 \times 10^5$</td>
</tr>
<tr>
<td>$\alpha_2$HS-glycoprotein</td>
<td>1:1</td>
<td>49,000</td>
<td>58.35</td>
<td>$3.13 \times 10^4$</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>1:1</td>
<td>100,000</td>
<td>119.00</td>
<td>$5.55 \times 10^4$</td>
</tr>
<tr>
<td>Thrombin</td>
<td>2:1</td>
<td>39,000</td>
<td>100.00</td>
<td>$6.53 \times 10^4$</td>
</tr>
<tr>
<td>$\alpha_1$ acid glycoprotein</td>
<td>1:1</td>
<td>44,000</td>
<td>52.40</td>
<td>$3.24 \times 10^4$</td>
</tr>
</tbody>
</table>

Table 10: Radiolabelling of Plasma Proteins.
<table>
<thead>
<tr>
<th></th>
<th>Acid glycoprotein</th>
<th>Gc-glycoprotein</th>
<th>Prealbumin</th>
<th>(\alpha_2)HS-glycoprotein</th>
<th>Haptoglobin</th>
<th>Thrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% BSA per ((\mu l))</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>50mM Tris buffer pH 7.4 ((\mu l))</td>
<td>tt (1 - 4)</td>
<td>tt (1-4)</td>
<td>tt (1-4)</td>
<td>tt (1 - 4)</td>
<td>tt (1 - 4)</td>
<td>tt (1 - 5)</td>
</tr>
<tr>
<td>(H^3)-Protein ((\mu l))</td>
<td>10, 50, 100, 500</td>
<td>10, 50, 100, 500</td>
<td>10, 50, 100, 500</td>
<td>10, 50, 100, 500</td>
<td>10, 50, 100, 500</td>
<td>20(\mu l) of (H^3)-thrombin, (6043 cpn)</td>
</tr>
<tr>
<td>StMPM ((\mu l))</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1, 2, 6, 12, 18</td>
</tr>
<tr>
<td>Or Solubilised Receptor ((\mu l)) Preparation</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total volume ((\mu l))</td>
<td>650, 650, 650, 850</td>
<td>650, 650, 650, 850</td>
<td>650, 650, 650, 850</td>
<td>650, 650, 650, 850</td>
<td>650, 650, 650, 850</td>
<td>500</td>
</tr>
</tbody>
</table>

**Table 11:** Assay Procedure for Receptor-radioligand Incubations. tt = test tube
gel filtration was used on a 1.5 x 30cm column to separate bound from free radiolabel. Non-specific binding was always subtracted when experiments were repeated in 100-fold excess unlabelled protein.

It should be noted that for thrombin, purified membrane (StMPM) was used; fixed number of counts were placed in each tube plus buffer and 1% BSA, and membrane was varied. (Membrane protein was 54 µg/µl StMPM). To evaluate binding parameters the inverse form of the Scatchard plot must be used as explained previously (Walker 1977) on pages 130-132. When solubilised receptor fraction was used (200 µl), the variable was the ³H-labelled protein. There was one persistent problem with regards to quantitating receptor protein which was at the lower end of sensitivity for the Lowry assay method and even more difficult was the fact that 0.15% Triton-X₁₀₀ which is necessary to add to the buffer to keep receptors (generally hydrophobic) in solution, is well known to absorb at 280nm and thus interferes grossly with protein quantitation. It was therefore a difficult problem to sort out but by adding constant amounts of receptor fraction, whatever protein was there, was assessed indirectly by being able to absorb sufficient counts of the radiolabel. (> 5% was sufficient although proteins with high receptor density such as IgG distribution can absorb up to 10% of added counts quite easily).

Results

Data from binding isotherms was appropriately transformed to ordinary Scatchard plots with the exception of thrombin,
where the inverse Scatchard was used. Figures 63 to 69 display these results. In all these plots, the bound/free radioactivity was plotted against bound counts. (Y and X-axes respectively) as per Scatchard equation;

\[
\frac{B}{F} = K_A \left( n - B \right)
\]

where \( B \) = number of moles of ligand bound per mole of protein (receptor)
\( F \) = molar concentration of free ligand
\( K_A \) = association constant
\( n \) = total number of binding sites (in moles) per mole of receptor protein.

The intercept on X-axis gives the maximum binding capacity (number of sites), whilst the gradient or slope gives the affinity constant \( K_a \).

Table 12 summarises these results in terms of affinity constants, binding capacity and existence of homogeneity or heterogeneity of binding sites. Scatchard plots for \( \alpha_1 \) acid glycoprotein, Gc-glycoprotein, prealbumin, and thrombin were linear, whilst those of \( \alpha_2 \) HS-glycoprotein and haptoglobin were non-linear. This implied non-homogeneity of receptor sites for these two sets of receptor proteins.

Consequently, \( \alpha_2 \) HS-glycoprotein exhibited a high affinity (\( K_a = 1.23 \times 10^{-8} \text{M}^{-1} \)), low capacity (capacity = \( 8.15 \times 10^{-11} \text{M} \)) and low affinity (\( K_a = 0.46 \times 10^7 \text{M}^{-1} \)), high capacity (capacity = \( 4.68 \times 10^{-10} \text{M} \)) receptor sites. Likewise haptoglobin possesses a high affinity (\( K_a = 8.89 \times 10^7 \text{M}^{-1} \)),...
Specific binding of $H^3$-prealbumin was transformed into Scatchard plot as per incubation conditions described in the text. The affinity of $K_a = 2.47 \times 10^8 M^{-1}$, with a total binding capacity of $1.736 \times 10^{-11} M$ was obtained. The receptor sites were homogeneous due to linear plot.
Fig. 64 - $\alpha_2$HS-Glycoprotein.

Scatchard plot of $^3$H- $\alpha_2$HS-glycoprotein binding on its isolated receptor fraction as described in the methods. The curvilinear shape implied two binding sites on the $\alpha_2$HS-glycoprotein receptor and exhibited co-operative effects. One site had high affinity, low capacity ($K_a = 1.23 \times 10^8 \text{M}^{-1}$; capacity $= 8.15 \times 10^{-11} \text{M}$) whilst the other had low affinity and high capacity ($K_a = 0.46 \times 10^7 \text{M}^{-1}$, capacity $= 4.68 \times 10^{-10} \text{M}$).

Fig. 65 - Haptoglobin

A Scatchard plot of $^3$H-haptoglobin interacting with its putative receptor. Non-linearity of the plot suggests heterogeneity of receptor sites on the receptor molecule; one site with high affinity, low capacity ($K_a = 8.89 \times 10^7 \text{M}^{-1}$, capacity $= 3.71 \times 10^{-11} \text{M}$), whilst the other site had low affinity and high capacity ($K_a = 0.55 \times 10^7 \text{M}^{-1}$, capacity $= 1.44 \times 10^{-10} \text{M}$).
Fig. 64

\[ \alpha_2 \text{HS-GLYCOPROTEIN} \]

Fig. 65

\[ \text{HAPTOGLOBIN} \]
Fig. 66 - Gc-glycoprotein

Specific binding of $^3$H-Gc-glycoprotein to its receptor was linear on Scatchard plotting with a high affinity homogeneous receptor site ($K_a = 8.95 \times 10^8 \text{M}^{-1}$).

Fig. 67 - $\alpha_1$Acid glycoprotein

Specific binding of $^3$H-$\alpha_1$acid glycoprotein interacting with its receptor also produced linear Scatchard plot, implying a homogeneous receptor population with a moderately high affinity constant of $0.76 \times 10^7 \text{M}^{-1}$ and capacity of $5.90 \times 10^{-10} \text{M}$. 
This shows an interaction of $^3$H-thrombin with purified trophoblastic membrane (StMPM); 6043 cpm in 20 µl of $^3$H-thrombin was placed in each tube (six in all), in presence of 100 µl of 1% bovine serum albumin, varying amounts of 50mM Tris-HCl pH 7.4 buffer as shown in Table 11, and varying amounts of membrane, (54 µg/µl StMPM). After 1 hour at 37°C separation of bound from free counts was achieved by vacuum filtration through glass fibre GF/C discs (Whatman). Non-specific binding was subtracted when the experiments were repeated in presence of 100-fold unlabelled thrombin. Saturation was reached at 648 µg of StMPM.
Data from Fig. 68 on specific binding of $^3$H-thrombin was transformed according to the inverse Scatchard plot, in order to assess the affinity binding sites on the putative thrombin receptor. A linear relationship implied homogeneous receptor sites with high affinity of $3.0 \times 10^{10}$ M$^{-1}$ and capacity of $3.05 \times 10^{11}$ sites/mg of syncytiotrophoblast was calculated. This was equivalent to a binding capacity of 24.7 $\mu$g/l per 1.296 mg of membrane.
Inverse Scatchard Plot for Thrombin/Receptor interaction

\[(AB + K^{-1}) = 26 \mu g/l\]

\(: AB \text{ (Binding capacity)} = 24.7 \mu g/l\]

per 1.296mg of membrane

\[K^{-1} = 1.3 \mu g/l\]
\[K = 3 \times 10^{10} M^{-1}\]

Fig. 69
| **Table 12**: Binding Parameters for Placental Receptors to Specific Plasma Proteins.  
**Key**: + present
- absent |  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha_1$ Acid glycoprotein</td>
<td>Gc-glycoprotein</td>
<td>Haptoglobin</td>
<td>$\alpha_2$ HS-glycoprotein</td>
<td>Prealbumin</td>
<td>Thrombin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>Affinity ($K_a$)</strong></td>
<td>$0.76 \times 10^7 \text{M}^{-1}$</td>
<td>$8.95 \times 10^8 \text{M}^{-1}$ and $0.55 \times 10^7 \text{M}^{-1}$</td>
<td>$8.89 \times 10^7 \text{M}^{-1}$ and $0.46 \times 10^7 \text{M}^{-1}$</td>
<td>$1.23 \times 10^8 \text{M}^{-1}$ and $0.46 \times 10^7 \text{M}^{-1}$</td>
<td>$2.47 \times 10^8 \text{M}^{-1}$</td>
<td>$3.0 \times 10^{10} \text{M}^{-1}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Binding Capacity</strong></td>
<td>$5.90 \times 10^{-10} \text{M}$</td>
<td>$1.6 \times 10^{-11} \text{M}$ and $1.44 \times 10^{-10} \text{M}$</td>
<td>$3.71 \times 10^{-11} \text{M}$ and $1.44 \times 10^{-10} \text{M}$</td>
<td>$8.15 \times 10^{-11} \text{M}$ and $4.68 \times 10^{-10} \text{M}$</td>
<td>$1.73 \times 10^{-11} \text{M}$</td>
<td>$3.05 \times 10^{11}$ Sites/mg StMPM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Heterogeneity of sites</strong></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Homogeneity of sites</strong></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
low capacity \( (3.71 \times 10^{-11} \text{M}) \) site as well as low affinity \( (K_a = 0.55 \times 10^7 \text{M}^{-1}) \), high capacity \( (\text{capacity} = 1.44 \times 10^{-10} \text{M}) \) site.

Thrombin on the other hand showed a homogeneous high affinity site with \( K_a = 3 \times 10^{10} \text{M}^{-1} \) with \( 3.05 \times 10^{11} \text{sites/mg StMPM} \). Thrombin was the only protein studied using unsolubilised intact membrane and was studied by the varying membrane amount at a fixed radioligand concentration. Fig. 68 shows the binding curve which reaches equilibrium or saturation and its inverse Scatchard plot in Fig. 69 for assessment of the affinity and total number of binding sites/mg of membrane.

Discussion

For the proteins chosen, this thesis reports for the first time on the binding characteristics of these receptors other than that of IgG which was separately studied in chapter 4. All along in this thesis evidence has been accumulated through immunochemical, in vitro perfusion of the placenta, colloidal gold study, the affinity chromatography for isolation of solubilised receptors, and now the saturation analysis which all point to existence of receptor molecules on the placental membrane for selective transfer of these plasma proteins from mother to the foetus.

From literature survey, it appears that there has been little interest in the subject of placental receptors and this explains why there is a paucity of information with regards to mechanisms of protein transport across the placenta.
On the other hand, there has been a number of studies which have established that some plasma proteins with genetic and immunochemical characteristics are indistinguishable from those of the mother and thus confirming their maternal origin (Sutcliffe 1981, Adinolfi 1970, Johnson et al 1974) and the widespread belief that they somehow cross the placenta.

Data presented in this chapter takes the story a little further and isolates the receptor structures to perform radioligand/receptor incubations so as to establish existence of high affinity receptor sites. High affinity constants in the order of $1 \times 10^7 \text{M}^{-1}$ to $1 \times 10^{10} \text{M}^{-1}$ implicate a receptor site as it is impossible for non-specific binding to have constants of this magnitude. The heterogeneity of haptoglobin and $\alpha_2\text{HS-glycoprotein}$ receptors is quite interesting although its implication in terms of binding of these proteins is far from clear as yet.

Thrombin, a proteolytic enzyme, when added to cultures of non-proliferating cells, leads to rapid cell division. Carney and Cunningham (1978) have studied thrombin induced cell division in chick and mouse embryo cells and human foreskin cells and have shown that thrombin possesses a specific receptor on these cells. By binding to it, it splits a 43,000 dalton receptor and then initiate mitogenesis (Glenn and Cunningham 1979). $^{125}\text{I}$-labelled thrombin incubations with these cells yielded linear Scatchard plots over a wide range of $^{125}\text{I}$-thrombin concentrations. This implies a single class of thrombin receptor sites with a high affinity constant of $1 \times 10^9 \text{M}^{-1}$ and $2 \times 10^5$ receptor sites/cell,
Platelets also possess thrombin receptors but their non-linear plots suggest multiple classes of receptors with co-operative interactions (De Meyts, 1976). Martin et al, 1976, have also confirmed two thrombin receptor sites on platelets, with non-linear Scatchard plots; one low capacity 390 sites/platelet and high affinity ($K_d = 1.9\text{nM}$) and a high capacity 2500 sites/platelet and low affinity ($K_d = 200\text{nM}$).

Macrophages are chemotactically attracted to thrombin (a useful role in the clotting process) by their possession of thrombin receptors. Bar-Shavit et al, 1983, have demonstrated (using a macrophage cell line J 774 cells) that a saturable receptor exists on these cells, and has a $K_d = 7.51 \times 10^{-9}\text{M}$. Thrombin concentrations eliciting chemotaxis range from $10^{-10}$ to $10^{-6}\text{M}$ with optimum of $10^{-8}\text{M}$ which is very near to its $K_d$ value. Existence of the thrombin receptor on the human placenta might be of an important role in coagulation control or indeed initiating mitogenesis; - mitogenetic role on the syncytiotrophoblast is of obscure importance since this layer does not divide except the layer below it, the cytotrophoblast. The existence of the receptor is therefore of as yet unclear significance. The high affinity obtained in other studies is of the order of $10^9\text{M}^{-1}$ and it compares well with a $K_a$ of $3 \times 10^{10}\text{M}^{-1}$ obtained for the placental receptor.
CONCLUSION

A choice was made of a few plasma proteins so as to explore the mechanism by which they cross the placenta with receptor mediated uptake as the major modality. These included $\alpha_1$ antitrypsin, $\alpha_1$ acid glycoprotein, haptoglobin, thrombin, fibrinogen, $\alpha_2$HS-glycoprotein, Gc-glycoprotein and prealbumin. IgG has also been studied with regards to re-establishing binding characteristics as well as the effect of temperature and cations on modulation of binding. In the following paragraphs a brief mention of each protein will be made and the conclusion made in the light of evidence accumulated in this study.

$\alpha_1$ antitrypsin is responsible for the bulk of (90%) antiprotease action in addition to other inhibitors such as $\alpha_2$ macroglobulin. This protein might therefore be required to protect the foetus against excessive proteolytic enzymes. Its mode of transport across the placenta was not clear, but from experiments in this study, there was strong adsorption on the syncytiotrophoblastic membrane as shown by both peroxidase and fluorescence experiments. Furthermore, placental in-vitro perfusion showed an active uptake profile. In addition, colloidal gold $\alpha_1$ antitrypsin conjugate showed some adsorption on the StMPM. This data would seem to indicate receptor mediated binding and possibly transport. When affinity chromatography of solubilised receptor fraction was performed using CN Br-activated Sepharose 4B linked to $\alpha_1$ antitrypsin, a band of $M_r$ 60,000 was desorbed. This was assumed to be the solubilised $\alpha_1$ antitrypsin receptor. Receptor-radiolabelled
α₁antitrypsin interaction demonstrated a homogeneous receptor site with the affinity of $K_a = 0.76 \times 10^7 \text{M}^{-1}$.

It is therefore not unexpected that by 26 weeks to birth, up to 175% of adult maternal level exists in the foetal circulation, an extremely high gradient. The placenta must therefore be responsible for maintaining this gradient, of course only active transport can explain this. The finding of α₁antitrypsin of maternal origin in foetal circulation by Biasucci and Gitlin (1969) lends support to placental transfer. It must be remembered that de-novo synthesis also takes place to some extent.

α₁acid glycoprotein is another acute phase protein, with no clear-cut function although there is some evidence that it is involved in inactivation of progesterone. If this is its major role, then it is reasonable to suppose that it would have a vital role to play in a milieu of foetal-maternal progesterone exchange, particularly as this hormone exerts an anabolic role as well as a precursor of other steroid hormones, and therefore requires a carefully controlled balance. The present study shows evidence that this protein too is actively transferred from in-vitro placental perfusion. Fluorescence, peroxidase and colloidal gold experiments also showed marked membrane binding.

Furthermore, Triton-X₁₀₀ solubilised placental membrane was subjected to the affinity isolation of α₁acid glycoprotein receptor using CN Br-activated Sepharose 4B coupled to α₁acid glycoprotein. This adsorbent extracted a receptor of $M_r 47,000$ on SDS-PAGE. Radioligand/receptor binding
studies also gave a homogeneus receptor site with high affinity of $K_a = 0.76 \times 10^7 \text{M}^{-1}$.

Gc-glycoprotein was chosen because of current evidence which shows that it is a major carrier of vitamin D and presumably its metabolites (Constans et al 1983). Vitamin D is crucial to the rapid skeletal development of the foetus, and therefore its plasma carrier, the Gc-glycoprotein was of interest from this point of view. Both immunocytochemistry and the perfusion experiments showed binding as well as a saturable uptake profile.

Due to insufficient quantities of purified Gc-glycoprotein, it was not possible to prepare a sufficiently strong CN Br-activated Swpharose 4B-Gc adsorbent in order to visibly see the Gc-receptor band on SDS-PAGE. However, the same receptor fraction was used in receptor-radioligand binding studies and due to the high sensitivity of radiolabelled protein, saturable binding enabled calculation of affinity constant of the receptor/Gc interaction ($K_a = 8.95 \times 10^8 \text{M}^{-1}$), saturable binding and high affinity are strong evidence for existence of Gc-receptor; Gc proteins have been found in foetal cord blood and phenotypic studies have indicated maternal origin of this protein, although foetal de-novo synthesis is known to take place (Johnson et al 1974, Adnolfi, 1970).

Transferrin carries iron in plasma which would otherwise be toxic in free state. There is much evidence to show equilibrium between this mobile binder of iron and ferritin,
another iron storage protein, both in plasma and in the liver, where both proteins are synthesised. The placenta has specific receptors to bind and subsequently transfer this protein across the placenta and from work of Brown and Johnson (1980), it has been shown from co-migration experiments of deoxycholate solubilised membrane preparation that there are no coincident protein bands between transferrin and this preparation. This led Ogbimi and Johnson (1980) to show from immunoelectrophoresis, using an anti-transferrin antibody that most of transferrin in this soluble fraction migrates as a complex with its receptor.

Brown and Johnson (1981) have isolated the transferrin receptor structure from StMPM and from affinity chromatography and gel filtration they found that the receptor is a 65,000 dalton protein with a 1:1 binding with transferrin. In the present study, immunohistological placental uptake and colloidal gold studies seem to support a receptor mediated transferrin transplacental transport.

The IgG receptor has been extensively studied (Matre and Johnson 1979, Brown and Johnson 1980, Johnson et al 1976, Balfour and Jones 1978), although the homogeneity of the receptor molecule is still a subject of some controversy. In this report, some effort was made to re-measure binding affinity and dissociation constants as various workers in the past have obtained different values because of differing radio-receptor assay systems.

For the association an average $K_a = 2.2 \times 10^{8} \text{M}^{-1}$ was obtained and total number of binding sites on the Fc
receptor of $1.5 \times 10^{14}$ were also observed. Furthermore, a rate constant of $2.29 \times 10^7 \text{M}^{-1} \text{min}^{-1}$ was obtained. Both being in close agreement with what some workers have obtained.

On the dissociation aspect, high displacing dose (x1000 fold) was used and this seemed to indicate some heterogeneity on the dissociation curve, with one fast component with $0.10 \text{ min}^{-1}$ and another slow one ten times smaller, at $0.010 \text{ min}^{-1}$.

From the dissociation rate data, a parallel has been shown from work of Rees and Wallace (1978), who showed a high and low affinity equilibrium constants for IgG-Fcγ receptor.

Furthermore, this work has differed from previous reports in that the IgG-Fcγ interaction may not be a 1:1 as stated before, but a 4:1 as data in this report suggests. The main criticism being that previous work does not specifically show reasonable data to prove a 1:1 relation. Thermodynamic activation energies for this system have also been presented for the first time as no previous work has reported on this aspect. In this report it is shown that the interaction is associated with a $\Delta H = -2.56 \text{ KCal/Mole}$, a markedly exothermic reaction and typical of hydrogen bonding interactions, as well as a large positive entropy change of $\Delta S = +29 \text{ Cal/ Mol}^{-1}\text{deg}^{-1}$, typical of hydrophobic associations.

The effect of monovalent and divalent cations were investigated and it was of interest to notice that Na⁺ ions in physiological range markedly inhibits binding by as much as 36% which might imply a fine physiological regulator as discussed before. Ca²⁺ ions also inhibited binding in physiological range. Li⁺, Mn²⁺ and Mg²⁺ also showed marked inhibitory modulating effects although in unphysiological concentrations.
Affinity chromatographic isolation of IgG receptor using CN-Br-activated Sepharose 4B-IgG adsorbent showed three bands on SDS-PAGE of \( M_r 72,000, 78,000 \) and 165,000). This implied that the IgG receptor is heterogeneous, presumably due to multiple number of antigenically similar molecules, but which differ in molecular weight. Work in this study has demonstrated this multiplicity of IgG-receptor subtypes and agrees with other investigators who have also showed multiple IgG-Fce receptors (Balfour and Jones 1978, Niezgodka et al, 1981, Johnson and Brown, 1981).

For fibrinogen, an existence of a receptor for it, quite apart from crossing the placenta, might have marked local clotting regulatory consequences. Both in-vitro uptake and immunohistological studies seemed to indicate binding to the trophoblastic membrane. The saturable uptake seen in the in-vitro perfusion experiment seems to suggest that this protein crosses the placenta; presumably by a specific receptor.

Thrombin that was radiolabelled showed marked membrane absorption in the region of 16% of added counts. This avid binding is too high to be due to non-specific binding, only a specific receptor to thrombin can explain this. From incubations of purified placental membrane with \(^{3}H\)-labelled thrombin, saturable uptake was shown and the inverse Scatchard plots showed a homogeneous receptor site with a high affinity constant of \( 3 \times 10^{10} \text{M}^{-1} \). Other workers have demonstrated existence of thrombin receptor on mouse and chick embryo cells, and platelets (Carney and Cunningham 1978, Glenn and
Cunningham 1979, Bar-Shavit 1983).

In the present study existence of placental thrombin receptor could be of vital importance. If one speculates that fibrinogen has receptor on the placenta (it could be locally immobilised by its receptor) and this acts as a substrate for thrombin (also immobilised by its receptor) to initiate clotting at a particular site in the placenta. This could be a very fine regulatory mechanism in clotting control.

\( \alpha_2 \text{HS-glycoprotein} \) has little subscribed function to it, although recent studies have implicated it as a major bone matrix protein. It is produced in the liver but settles in bone matrix. If it has a receptor mediated uptake, it then becomes obviously vital for the foetus with a rapidly growing skeletal system that requires this protein, as its own synthetic capacity is low in early gestation. In this study saturable uptake in the placental perfusion experiment has been shown as well as binding from immunocytochemical studies.

From receptor-radiolabelled \( ^3 \text{H-} \alpha_2 \text{HS-glycoprotein} \) binding, Scatchard transformation of the data showed heterogeneity of receptor sites. There were two sites; one with high affinity, low capacity and the other with low affinity, high capacity. Co-operative effects were therefore present for the \( \alpha_2 \text{HS-glycoprotein receptor}. \)

\textit{Haptoglobin} is the major binder of free haemoglobin and haptoglobin levels drop sharply in presence of increased
haemolysis. However, very little haptoglobin is detectable in foetal cord blood (the level is 1% of adult concentration) and only in 10% of cord bloods. The percentage rapidly rises to 46.5% with detectable levels by the age of 2 months. This suggests that the foetal liver has the capacity to synthesise this protein at birth (Adinolfi 1970, Gitlin and Gitlin 1975).

In spite of lack of evidence that this protein is transferred across the placenta, the present study has clearly showed marked membrane binding by immunocytochemical methods. Furthermore, there was saturable uptake in the placenta perfusion. Affinity chromatography of solubilised membrane gave a band with Mr 46,000, presumed to be a haptoglobin receptor. In addition there were co-operative receptor sites when isolated receptor fraction was incubated with 3H-haptoglobin. Both receptor sites exhibited high and low affinity constants \((K_a = 8.89 \times 10^{-7} \text{M}^{-1}, 0.55 \times 10^{-7} \text{M}^{-1})\). All this data is very suggestive of a haptoglobin receptor capable of transferring haptoglobin. As to whether it is detectable subsequently in cord blood, is a different argument. Gitlin and Gitlin, 1975, seem to think that this lack of detection of haptoglobin might be due to increased haemolysis in the conceptus but hastily add that this is not yet proven.

Receptor characteristics have been observed for prealbumin. Prealbumin is a major transporter of retinol, a vitamin A carotenoid which is required by the foetus. Also prealbumin is one of the transporters of thyroxine in addition to thyroxine binding globulin. Both these different but vital
functions of prealbumin might become very important if indeed a receptor exists on the placenta to transfer this protein to the foetus with its load of vitamin A and thyroxine, akin to transferrin and iron.
REFERENCES

J. Expt Med. 132, 1207.

Science. 181, 845.

New Developments in Immunoradiometric assay,
in Radioimmunoassay and related assay - in medicine

Adinolfi, M. 1970.

in Immunobiology of Trophoblast (Eds) R.G. Edwards,

Placenta. 2, 45.

Aherne, W., and Dunhill, M.S. 1966.
J. Path and Bact. 91, 123.

Aherne, W. 1975.
in The Placenta and its maternal supply line.
(Ed) Gruenwald, M.T.P., Lancaster, pp.80.

Aldjems, S. 1968.
Am. J. Obs & Gynaec, 102, 311.

Fur Wissenschaftliche Mikroskope and Mikroskopische
Technik, 69, 193.


Ariens, E.J. 1964.

Molecular Pharmacology Vol. 1
Academic Press, N.Y.


Receptors and Receptor Mechanisms in \(-\)adrenoceptor Blocking Agents.
P. R. Saxena and R. P. Forsyth (Eds) pp. 3-27
North Holland, Amsterdam.


Structure Activity Relationships of \(-\)adrenergic drugs and \(-\)adrenergic blocking drugs.
Ann. N.Y. Acad Sci. 139, 606.

Ariens, E.J. 1974

Clin Pharmacol Ther. 16, 155.


Arch. Int Pharmacodyr Ther. 127, 479.


Am. J. Anatomy 74, 291.


Nature 278, 743.


Int. Arch. of Allergy and Applied Immunol. 56, 435.


The Binding of IgG to Human Placental Membranes in Maternal-Foetal Transmission of Immunoglobulins.
(Ed) W. A. Hemmings, pp. 61, C.U.P., Cambridge.

Lab Invest. 49, 702.

Becker, V. 1963. 
Arch Fur Gynakologie 198, 3.

Becker, V. 1975. 
The Placenta and its Maternal Supply Line. 

Becker, V. 1971. 
Deutsche Medizinsiche Wochenschrift 96, 1845.


Archiv fur Gynaecologle 221, 187.

Billingham, R.E. 1964. 

in Early Development of Mammals (Ed) K. Balls and 

in Placenta, A neglected Experimental Animal 

Arch Gynec 227, 315.
Blut, 26, 205.

Bohn, H. 1974.
Arch Gynak, 217, 219.

Bohn, H. 1975.
Arch Gynak, 218, 331.

Arch Gynec 230, 167.


Bohn, H. 1982.

Bohn, H. 1980.
Arch Gynec. 229, 279.

Placenta 1, 327.

Biochem J. 196, 355.

Geburtshilfe and Fraenenschilkunde 18, 1.


Histochem. J., 2, 263.

Anal. Bioch. 72, 248.

The Transmission of Passive Immunity From Mother to Young.
Frontiers of Biology Vol. 18, Amsterdam, North Holland.

Lancet 2, 1087.


Proc. Nat. Acad. Sci., USA 76, 2321-2325.

J. Lab & Gen Med. 53, 331.

Brown, P.J., and Johnson, P.M. 1981.
Placenta 2, 1-10.

Brown, P.J., and Johnson, P.M. 1981.
Immunology 42, 313-319.

Am. J. Obs & Gynaecol 96, 342.

Quart J. Expt. Physiol 61, 275.

Archiv Fur Gynaecologie 216, 167.
    Archiv Fur Gynaecologie 212, 333.

    J. Biol. Chem. 251, 4139.

Carter, J.E. 1964.
    Obstet & Gynaecol 23, 647.

    Am. J. Obst & Gynaecol 131, 520.

    Obst & Gynaecol 50, 200.

    In-vitro Perfusion of Human Placenta, Oxygen Consumption.
    Am J. Obst & Gynaecol. 126, 216.

    Virology 20, 642.


    Blood. 23, 621.


    Cell and Tissue Research, 178, 411.

    Paed. Res. 8, 724.

    J. Physiol (Lond) 255, 1.
De Meyts, P. 1976.

Dempsey, E.W. 1972
   Am. J. Anatomy 134, 221.


   Am. J. Obst & Gynaec 78, 862.


   J. Membrane Biol 6, 138.

   Cancer Research, 41, 2640.

   Nature Lond. 255, 412.

   Obst & Gynaec. 25, 378.

   Anatomical Record 167, 231.

Faber, J., and Hart, F.M. 1967.
   Am. J. Physiol. 213, 890.

Faber, J. 1969.
   Circ. Res. 24, 221.
Prog Allergy 16, 9.

Faulk, P. 1972.
see discussion after chapter by Beer, A.E and Billingham, R.E.
in Ontogeny of acquired immunity (Ed. R. Porter and

J. Reprod. Fert Supplement.

in Immunobiology of Trophoblast (Ed. R.G. Edwards,

in Immunology of reproduction (Eds K. Bratanov, R.G. Edwards,
V.H. Vulchanov, V. Dikov, and B. Somlev). pp.405,
Bulgarian Acad. Sciences, Sofia.

Faulk, P and McIntyre, J.A. 1980.
in Pregnancy Hypertension (Ed) Bonnar, J. Chapter 57,
pp. 423-432, Lancaster M.T.P.


Freese, V.E. 1966.
Am. J. Obs & Gynaec 94, 354.

J. Cell Biol. 35, 357.

Fox, H. 1968.  
J. Obs & Gynaec Brit. Cwth. 75, 448.

Fox, H. 1978.  
Pathology of Placenta. W.B. Saunders.

Fox, H. 1979.  
Placental transfer (Eds) Chamberlain, C.V.P. and Wilkinson, A.W. Pitman Medical 1979, pp.19.


Fox, H. 1967.  
J. Obs & Gynaecol Brit Cwth. 74, 28.

Fox, H. 1969.  
J. Obs & Gynaecol Brit. Cwth. 76, 240.

Int. Arch. Allergy 42, 503.

Galton, M. 1962.  

J. Histochem. Cytochem 25, 1187.

Biochem et Biophysica Acta 382, 369.

Nature 278, 711,
Gitlin, D and Biasucci, A. 1969.


Gitlin, D, and Gitlin, J. 1975.
    in Plasma Proteins (Ed) F. Putnam.

Gonrot, P.O. and Bjerre, B. 1967.

    Lancet 1, 331.

    A review of colloidal gold marker system
    in Scanning Electron Microscopy 1980/II (Ed) O. Johari,
    pp. 133. S.E.M. inc. A.H.F O'Hare, Chicago.

    J. Microscopy 123, 201.

    Lancet 1, 333.

Harris, J.W.S. and Ramsey 1966.


The attachment of IgG to components of transporting membranes, in Maternal-Foetal Transmission of immunoglobulins (Ed) W.A. Hemmings, pp.91, C.U.P.


in Maternal-Foetal Transmission of immunoglobulins.
(Ed) W.A. Hemmings, C.U.P. pp.91-111.


J. Physiol 235, 409.


Nature (Lond) 196, 1220.

Horisberger, M. 1979.


J. Microsc. 115, 97.


Biochim. Biophys. Acta. 328, 520.

Howe, C. 1975.

Cell Biol Int. Reports vol.5 No.12, pp.1073.


J. Paediatrics 84, 588.

Antigens of Peri-implantation Trophoblast,


Placenta 2, 355.

Immunology 31, 659.


Immunology 34, 1027-1035.

J. Pathol, 126, 173.

Biochim et Biophysica Acta 255, 126.

Localisation and Nature of the Sex, Steroid Receptor in Basic Actions of Steroids on Target Organs, pp.21.

King, B.F., and Mentou, D.N. 1975.
Am. J. Obst & Gynaecol 122, 824.


Leaf, A. 1981.

Transport properties of cell membranes in Placental transfer, methods and interpretations. Chapter 6, pp.79.


J. Histochem. Cytochem 28, 339.

Little, C.C. 1924.

J. Cancer Res. 8, 75.

Llewellyn-Jones. 1969.

in Fundamentals of Obstetrics & Gynaecology.

pp. 216, Lond. Faber & Faber.


Nature (Lond) 228, 561.

J. Appl. Physiol. 26, 48.

Loor, F, et al 1972

J. Biol. Chem. 193, 265.

in The Placenta; Biological and Clinical Aspects.
(Ed) Moghissi, K.S., and Hafez, E.S.E., Charles C. Thomas.

Maelicke, A, et al 1977
J. Biol. Chem 252, 4811.

Molecular Pharmacology 21, 100

Arch Biochem. 188, 287.

J. Immunol. 94, 514.


J. Ultrastructure Res. 43, 133.
Biochemistry 15, 4886.


Matre, R., and Johnson, P. M. 1977.


J. Immunol. 117, 880.

J. Exp. Med. 133, 1.

Medawar, P. B. 1953.

Physiol. Rev. 47, 782.


Miller, M. E. 1978.
Host Defences in the Human Neonate,
N.Y. Grune & Stratton.


Cell Tissue Res. 171, 175.

Munro, A.J. 1975.
Immunobiology of Trophoblast (Ed) Edwards, R.G., Howe, C.W.S., and Johnson, M.H.
C.U.P. pp. 1-12.

Navikoff, A.B., and Navikoff, P.M. 1977.

in Coated vesicles. C.D. Ockleford and A. Whyte (Eds).
pp. 25-54, Cambridge University Press.

J. Biol. Chem. 256, 6593.

J. Exp. Med. 137, 511.

Semaine des Hopitaux de Paris, 30, 3163.

Molecular Immunol Vol. 18, pp. 163.


J, Cell Science 25, 293.


Proc R. Soc. (Lond) Ser. B. 212, 305.

Placenta. 1, 91.

J. Cell Science. 25, 293.


J. Cell Science 21, 83.

J. Pharmacol Science 65, 1673.


Biol Research in Pregnancy vol.1 No.2, pp.79.

Nature 269, 333.


Pearse, B.M.F. 1975.
J. Mol. Biol. 97, 93.

Pearse, B.M.F. 1978.
J. Mol. Biol. 126, 803.

Pearse, B.M.F. 1976.

J. Exp. Med. 132, 511.

Pinto De Silva, P. 1972.


Immunological Aspects of Cancer, pp. 155,

Am.J. Obs & Gynae 67, 1.
    Am. J. Anatomy 98, 159.

    Am. J. Obs & Gynae 82, 1649.


    Approaches to Structural Studies on Fc-receptors in Protein Transmission through Living Membranes,
    (Ed) Hemmings, W.A. pp.119, Amsterdam, Elsevier.

Reinskou, T. 1968.
    Ser. Haematol (N.S) 1, 21.

Reynolds, F. 1979
    in Placental Transfer. (Eds) Chamberlain, G.P., and
    Wilkinson, A.W.
    Pitman Medical, London pp.166.

    J. Ultrastruct. Res. 6, 88.

Richart, R. 1961.

    Nature (Lond) 222, 1062.

    J. Clin. Invest. 72, 626.

    J Cell Biol. 19, 914.

    J. Cell Biol. 20, 313.

    Am. J. Obs & Gynaecol 87, 851.


Scatchard, G. 1949.

    Am. J. Obst & Gynaecol 126, 261.

Schuhmann, R. 1975.
    Archiv Fur Gynaecologie 219, 357.

    J. Biol. Chem 253, 1766.

    J. Biol. Chem 254, 9943.

    Transplantation 25, 173.

    Nature, 269, 60.

Seppala, M. 1979.
    Int. J. Cancer, 24, 6.

    Int. J. Cancer 21, 265.
Siegel, I., and Gleicher, N. 1981.
Development of the Foetal Immune System.
in Reproductive Immunology, Alan R Liss Incorp, 156 Fifth Av. N.Y. pp. 31-40.

Biochem J. 140, 301.

Science 175, 720.


Nature (Lond) 252, 302.


Stein, W.D. 1981.
Concepts in mediated transport,


N. Eng. J. Med, 275, 971.

Summers, R.J. 1980.
B.J. Pharmacol 71, 57.

Sutcliffe, R.G. 1981.
Proteins in Human Amniotic Fluid,
in Amniotic Fluid and its Clinical Significance,


Scand J. Haematol 6, 113.

Travis, J., and Bowen, J. 1976.
Bioch J. 157, 301.


Scatchard Plot, in Immunometric Assay.
Clin Chem 23, 588.

J. Biochem (Tokyo) 87, 393.

Endocrinology, 103, 1458.

Wigglesworth, J.S. 1967,

in Lysosomes on Biology and Pathology.

Phil. Trans. R. Soc. Lond.B, 271.

Transport of Immunoglobulins and other Proteins From
Mother to Young - in Lysosomes in Biology and Pathology,
(Ed) J.T.Dingle, pp.425, Amsterdam, North Holland.

in Placenta - A Neglected Experimental Animal,

in Maternal-foetal Transmission of Immunoglobulins.

Young, M. 1981.
Placental Amino Acid Transfer,
in Placental Transfer, Methods and Interpretations,
pp.177, W.B. Saunders Co. Ltd.

Clin Exp. Immunol 11, 549.
