THE IDENTIFICATION OF BIOLOGICAL ACTIVITIES OF PREGNANCY-ASSOCIATED PROTEINS OF THE HORSE

by

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A thesis submitted to the Council for National Academic Awards in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Sponsoring Establishment : Department of Biological Sciences
Sheffield City polytechnic

Collaborating Establishment : Equine Fertility Unit
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ABSTRACT

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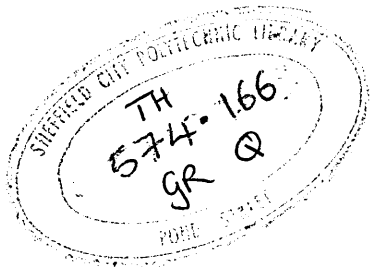
Equine epitheliochorial placental extracts, taken from 2 mares 60 days and 80 days after mating, were found to induce a dose-dependent inhibition of lymphocyte proliferation in the mixed lymphocyte reaction (MLR) and mitogen stimulated assay (MSA). MLR supernatant reversed the inhibition and the addition of foetal tissue extracts to previously activated lymphocytes suppressed lymphocyte proliferation. This suggests suppression at the IL-1/IL-2 level of the immune response. Commercially available eCG inhibited lymphocyte proliferation. The presence of endometrial cup secretion containing high levels of eCG had no additive effect on the inhibitory activity of an extract of allantochorion (day 80), previously shown to induce less than 100% inhibition. This suggests that the commercially available eCG may contain an immunosuppressive contaminant.

Equine and bovine epitheliochorial placental extracts (allantochorion) inhibited collagen and ADP-induced platelet aggregation respectively. The mode of action of the anti-aggregatory factors may be related to the vascularity of the placentae.

Overall, it appears that local immunomodulatory and haemostatic mechanisms are important in the maintenance of pregnancy in species with epitheliochorial placentation.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ADVANCED STUDIES, COURSES AND CONFERENCES UNDERTAKEN AND ATTENDED IN CONNECTION WITH THE PROGRAMME OF RESEARCH IN PARTIAL FULFILLMENT FOR THE REQUIREMENTS OF THE DEGREE OF Ph.D.

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- B.Sc. Applied Science (1985-1987).

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Overall, it appears that local immunomodulatory and haemostatic mechanisms are important in the maintenance of pregnancy in species with epitheliochorial placentation.

PAPERS WRITTEN AND PUBLISHED IN CONNECTION WITH THIS Ph.D. THESIS

1. Lea, R.G. and Bolton, A.E. (1988)
   An immunosuppressive factor from equine placental tissue.
   (Biochemical Society Transactions - In Press)
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2. Lea, R.G. and Bolton, A.E. (1988)
   An immunochemical demonstration of a pregnancy specific protein in the horse and its use in the serological detection of early pregnancy.
   Journal of Reproduction and Fertility (Submitted).

3. Lea, R.G. and Bolton, A.E. (1988)
   The inhibition of lymphocyte proliferation by equine endometrial tissue.
   British Equine Veterinary Association, 27th Annual Congress.
   Accepted for poster presentation and publication in Congress handbook.

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ABBREVIATIONS USED IN THE TEXT

ADP	Adenoside diphosphate
ADP-ase	Adenoside disphosphatase
AFP	Alpha foeto-protein
Ag	Antigen
ALS	Anti-lymphocyte serum
APC	Antigen presenting cell
Approx.	Approximately
ASF	Afferent suppressor factor
ATCS	Adoptive transfer of contact sensitivity
Bl antigen	Lymphocyte alloantigen (class 11)
B Cells	B lymphocytes
BCDF	B cell differentiation factor
BCGF	B cell growth factor
β_2-hors.PP	Beta$_2$ horse pregnancy protein
BSA	Bovine serum albumin
CBA/J, DBA/2, BALB/c	Strains of mice mus musculus mus caroli
CD antigens	Surface molecules on lymphocytes
CG	Chorionic gonadotrophin
Ci	Currie
CL	Corpus luteum
cm	Centimeter
CML	Cell-mediated lympholysis
Con.A	Concanavalin A
CPM	Counts per minute
CPSR-2	Controlled process serum replacement-2
CSF	Colony stimulating factor
CTL	Cytotoxic T lymphocyte

dCG	Donkey chorionic gonadotrophin
DMEM	Dulbecco's modification of Eagles medium
DMEM+	Supplemented DMEM
DNA	Deoxyribonucleic acid
eCG	Equine chorionic gonadotrophin
ECM	Embryo culture medium
EDPAF	Embryo derived platelet activating factor
EDTA	Ethylenediaminetetracetic acid
EEO	Electroendosmosis
ELA	Equine leucocyte antigen
ELISA	Enzyme linked immunosorbent assay
EPAP	Early pregnancy-associated protein
EPAT	Early pregnancy-associated thrombocytopenia
EPF	Early pregnancy factor
ES	Oestrone sulphate
FCS	Foetal calf serum
Fig.	Figure
FSH	Follicle stimulating hormone
γ_2-hors.P	Gamma$_2$-horse protein
g	Gram
GVH	Graft-versus-host reaction
h	Hour
H-2	Histocompatibility complex of mice
^{3}H-Tdr	Tritiated thymidine
hCG	Human chorionic gonadotrophin
HLA	Human leucocyte antigen
HMWGP	High molecular weight glycoprotein
HPLC	High performance liquid chromatography
-IF	Inhibiting factor

Ia antigens	Murine and human class II antigens
Ia region	Murine class II genes
Ig	Immunoglobulin
IL-1 (2,3,4)	Interleukin-1 (2,3,4)
IL-1R	Interleukin-1 receptor
IL-2R	Interleukin-2 receptor
iu	International units
kD	Kilo daltons
Km	Michaelis-Menton constant
l	Litre
LH	Luteinizing hormone
LPS	Lipo-polysaccharide
M	Molar
mA	Milliamps
mCi	Millicurrie
mg	Millagram (10^{-3} gram)
MHC	Major histocompatibiltiy complex
min	Minute
ml	Millilitre
MLR	Mixed lymphocyte reaction
mM	Millimole (10^{-3} mole)
Mol.wt	Molecular weight
MPP-1	Mare pregnancy protein-1
MO	Macrophage
Mr	Molecular range
MSA	Mitogen stimulation assay
NaCl	Sodium chloride
NK cell	Natural killer cell
nm	Nanometre (10^{-9} metre)

PAF	Platelet activating factor
pAP	Porcine acidic protein
PAPP-A	Pregnancy-associated plasma protein-A
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffer saline
PGE2	Prostaglandin E2
PGF	Prostaglandin F_2alpha
PGFM	Prostaglandin F_2alpha metabolite
PHA	Phytohaemagglutinin
Phase A cells	Hormone dependent uterine suppressor cells in the mouse
Phase B cells	Trophoblast dependent uterine suppressor cells in the mouse
PMSF	Phenylmethylsulphonyl fluoride
PMSG	Pregnant mare serum gonadotrophin
PP (5,12,14,15)	Placental protein (5,12,14,15)
PPP	Platelet poor placenta
PRP	Platelet rich placenta
RIA	Radioimmunoassay
RIT	Rosette inhibition titre
RNA	Ribonucleic acid
SDS PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
SIF	Suppressor inducer factor
T cells	T lymphocyte
T antigens	Surface molecules on human lymphocytes (old nomenclature)
Tc (cTL)	Cytotoxic T lymphocytes
TCA	Trichloroacetic acid

Th	Helper T lymphocyte
TLX	Trophoblast-lymphocyte cross-reactive antigens
TNF	Tumour necrosis factor
Ts	Suppressor T lymphocytes
TsF	T cell suppressor factor
U	Units
v/v	Volume to volume ratio
w/v	Weight to volume ratio
W1-W11	Lymphocytes alloantigens of the horse (class 1)
α	Alpha
β	Beta
γ	Gamma
l	Microlitre
M	Micromole
xg	Gravity (relative centrifugal force)
%	Per cent
^{o}C	Degrees centigrade
>	Greater than
<	Less than

CHAPTER -1-

MAMMALIAN PLACENTAL CLASSIFICATION

1.1 Introduction

In 1937, Mossman stated that the placenta consists of an "apposition of foetal and parental tissues for purposes of physiologic exchange". This is a broad definition, but necessarily so, such is the variety of structurally different placentae found within mammals. It must be remembered however that no matter how vastly placentae differ from one another, they all do the same thing. The 'what' is always the same, only the 'how' differs (Ramsey, 1982).

Research is presented in this thesis relating to the biological activities of the horse placenta. Data obtained has been compared to existing data on the more widely researched activities associated with the human placenta. In this regard, the project can be considered as a comparative study of placenta associated activities in the horse and human.

Placentae can be classified according to their origin, shape, internal structure, relation to maternal tissues and also by the composition of the placental membrane. When classified according to the composition of the placental membrane, the equine and human placentae can be considered as being at either end of the mammalian placental spectrum. When the placentae are classified according to their origin, however, both are chorioallantoic, that is, during placental development the allantois fuses with the chorion and vascularization is effected by the allantoic vessels (Bjorkman and Dantzer, 1987). When placentae are classified according to the composition of the placental membrane, the major determining factor

with respect to the type of placenta is the degree of 'trophoblastic invasive activity', that is the extent of implantation of trophoblast into the uterine wall. The other placental classifications, however, overlap in that the degree of trophoblastic invasive activity is a key determining factor in classifications based on shape, internal structure and relation to maternal tissues. The classification based on trophoblastic invasive activity only applies to chorioallantoic placentae. The differences in placental structure between the human, horse and cow, which has a similar placenta to the horse, are summarized in Table 1.1.

1.2 Classification of Placentae by the Composition of the Placental Membrane'

The trophoblast is a layer of large cells making up the blastocyst wall prior to implantation; it is the outermost layer of cells of the conceptus. The conceptus initially implants into the endometrium by the insertion of trophoblast cells (in small numbers) between surface epithelial cells into the stroma, and this usually occurs in the close proximity of a maternal capillary. These invading clumps of trophoblastic cells are known as the cytotrophoblast. A number of these areas of cytotrophoblast penetrate further into the uterine wall and lose their cell margins to become a multinucleated cytoplasmic mass, a synctium, the syncytiotrophoblast.

The syncytiotrophoblast lies external to the cytotrophoblast, nearer to the maternal tissue. Most if not all of the trophoblastic invasive activity comes from the syncytiotrophoblast. In villous placentae the syncytiotrophoblastnormally coats the villi except in haemochorial placentae (such as human) where it lines the intervillous space. In most mammals, at least one layer of trophoblast persists throughout the gestation period, the relative amounts, however, may vary.

Table 1.1

Placental Structure - Difference between Human, Horse and Cow

PLACENTAL CLASSIFICATION	HUMAN	HORSE	COW
1) Origin	Chorioallantoic	Chorioallantoic	Chorioallantoic
2) Shape	Discoid	Diffuse	Cotyledonary
3) Internal structure	Villous	Villous	Villous
4) Relation to maternal tissues	Deciduate	Adeciduate*	Adeciduate
5) Composition of placental membrane	Haemochorial	Epitheliochorial	Epitheliochorial

Trophoblastic invasive activity exhibits three variables, depth of penetration, manner of penetration and duration of invasive activity. The Grosser classification of placental structure (1927) is essentially concerned with the depth of penetration and is based on the number of layers of tissue separating the foetal and maternal blood streams thus comprising the placental membrane. Grosser originally proposed four basic categories of placental structure as outlined in Table 1.2.

In the epitheliochorial placenta (eg horse), there are six layers of tissue: three maternal and three foetal, whereas the haemochorial (eg human) placental membrane is composed of three layers of tissue, all of which are foetal and the foetal trophoblast is bathed in maternal blood.

The type of placenta present depends on the number of placental tissue layers eroded by the trophoblast during placental development. The human is thus at one end of this mammalian placental spectrum with a haemochorial placenta, where all three maternal layers are eroded away by the trophoblast. The horse and cow are at the other end of this placental spectrum where no layers of maternal tissue have been eroded away and there is simple apposition of foetal to maternal tissue (Fig.1.1).

The cow was originally placed in the syndesmochorial group but electron microscopic findings have shown the presence of maternal epithelium in the bovine placenta and the placentas of other animals placed in this group, e.g sheep, goat and some deer. The basis of the syndesmochorial placenta has been so weakened that the Grosser classification has been narrowed to three categories:- epitheliochorial, endotheliochorial and haemochorial.

Both of these alternatives, however, have been disproved by examination of the proposed placentae with the electron microscope.

Table 1.2

The Grosser Classification

Designation	Tissues Separating Maternal and Fetal Blood: Maternal tissue: Endothelium	Maternal tissue: Connective tissue	Maternal tissue: Epithelium		Fetal tissue: Epithelium	Fetal tissue: Connective tissue	Fetal tissue: Endothelium	Gross form of placenta	Typical examples
Placenta epitheliochorialis	+	+	+	Uterine lumen	+	+	+	Diffuse	Pig Horse
Placenta syndesmochorialis	+	+	–		+	+	+	Multiplex or Cotyledonary	Ruminants
Placenta endotheliochorialis	+	–	–		+	+	+	Zonary	Carnivores
Placenta hemochorialis	–	–	–		+	+	+	Discoid	Rodents Insectivores Bats Mice Apes Man

Source: After Grosser: Frühentwicklung, Eihautbildung und Placentation des Menschen und der Saügetiere, 1927.

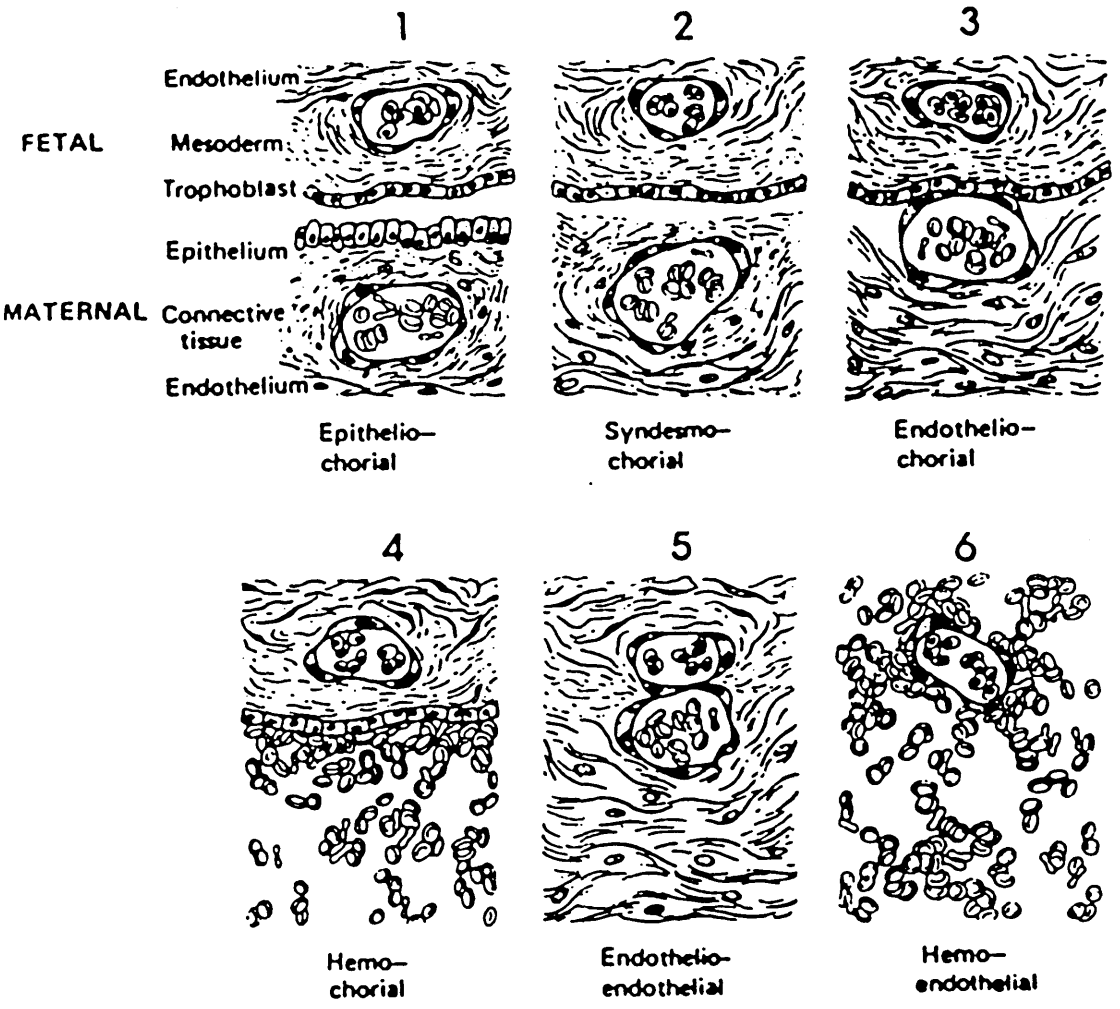

Fig.1.1 Possible types of placental membrane formed by differing degrees of trophoblastic invasive activity.

(Ramsey, 1982)

Differences also exist between placentae within a category, for example there are three recognised subdivisions of the haemochorial placenta. Electron microscopy has revealed that the trophoblast portion of the foetal membrane may be one, two or three layers thick resulting in a haemomonochorial, haemodichorial or haemotrichorial placenta respectively. The human placenta is haemomonochorial (Fig.1.2).

Although both the horse and cow have epitheliochorial placentation, there is a slight structural difference between the two in terms of the thickness of the barrier betweenthe maternal and foetal blood streams (Fig.1.3).

As illustrated in Fig.1.3, only slight differences exist between the equine and bovine placentae in terms of epitheliochorial placentation and these are as follows:

a) The maternal uterine epithelium in the cow is thicker than same layer in the horse between capillaries.
b) The distance between the uterine epithelium and the maternal capillaries is greater in the cow than in the horse.
c) Fibrocytes are found in the intervening space in the cow but not in the horse.
d) Only in the cow are large binucleate cells found on the foetal side. These, however, do not constitute part of the placental membrane.

The initial assumption accompanying Grosser's placental classification scheme was that the number of tissue layers assumed to be present could be directly related to the permeability of the placental barrier. As a consequence of this, the epitheliochorial placenta appeared to be the greatest obstacle to the transfer of diffusable materials from one

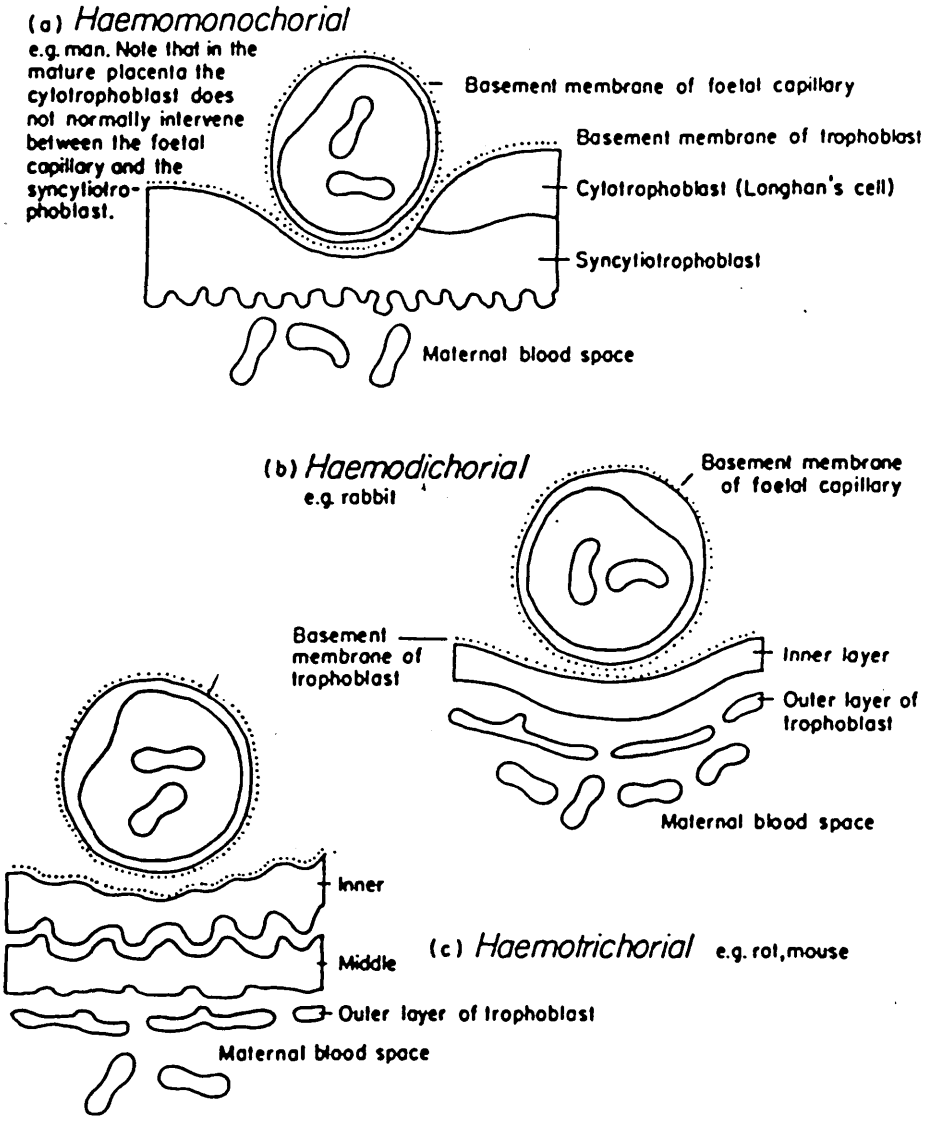

Fig.1.2 The haemochorial placenta. Variations in structure.

(Steven, 1975)

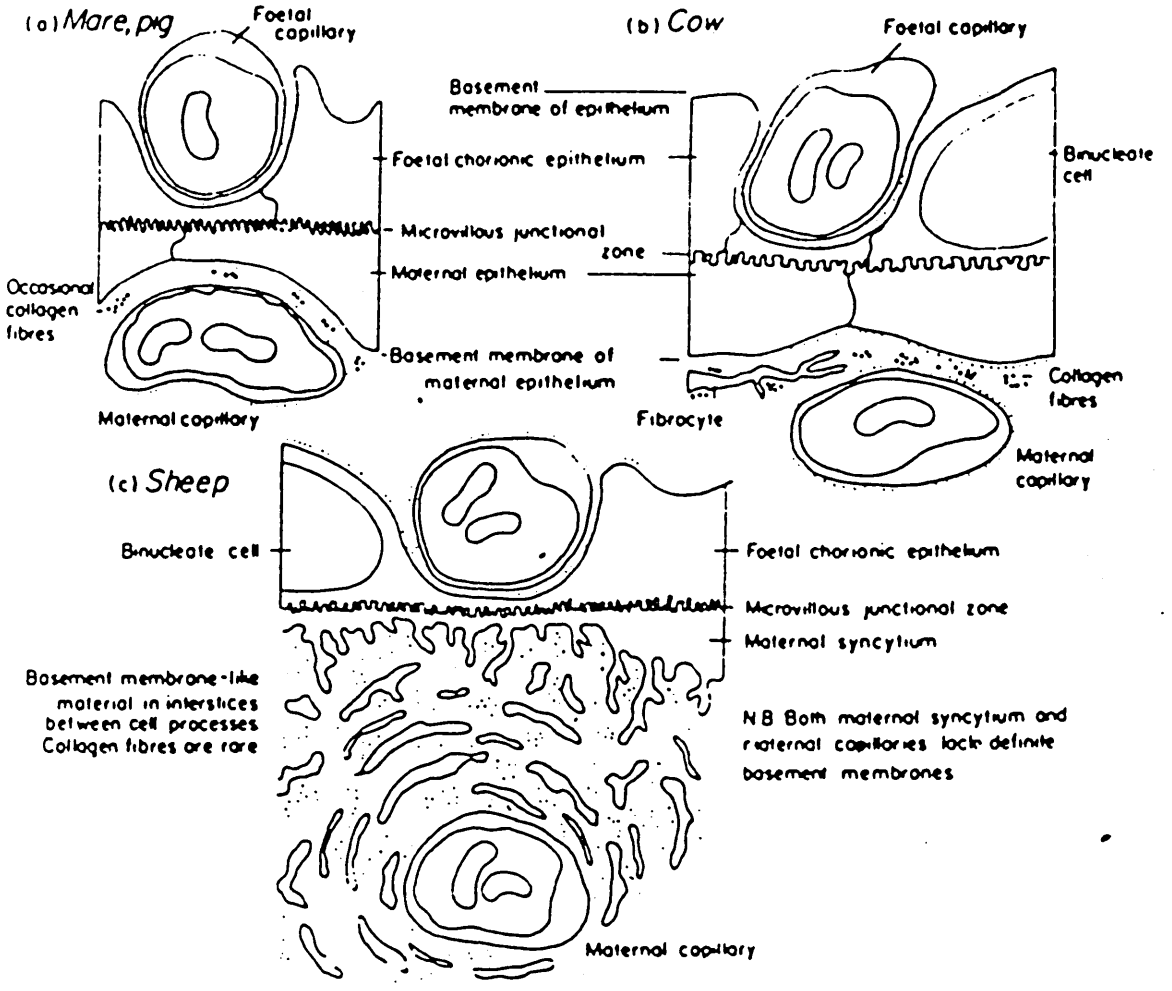

Fig.1.3 The epitheliochorial placenta. Variations in structure.

(Steven, 1975)

circulation to another. As early as 1946, however, reservations were expressed regarding such assumptions: Sir Joseph Barcroft in his 'Researches on Prenatal Life' pointed out that "with very few exceptions the greater the number of placental layers, the more fully developed the animal at birth". In Barcroft's publications, he gives an account of how Sir John Hammond and assistants spent some time trying to catch a newborn foal. Upon withstraining the animal, one exhausted knight remarked to the other, "not a bad advertisement for a six-layered placenta!". This illustrates that for such complete foetal development the passage of nutrients across the epitheliochorial placenta must occur with reasonable ease. It has in fact been shown that both maternal and foetal capillaries penetrate their coverings (connective tissue and epithelium on the maternal side and mesoderm plus trophoblast on the foetal side) to come very close to each other facilitating exchange of nutrients (Ramsey, 1982).

This demonstrates the dangers of assuming too much from initial observations of different placental structure. One objective of this project was to investigate some biological activities of placental samples taken from the equine epitheliochorial placenta and to compare the results to existing data relating to the human haemochorial placenta. The rational behind this work was that if the different placentae exhibit different biological activities then it may be possible to equate the results with differences in placental structure. This in turn may throw further light on the biological roles of placental membranes.

CHAPTER -2-

THE IMMUNOLOGY OF PREGNANCY

2.1 Introduction

The foetuses of all outbred mammalian species present an immunogenetic set of paternal antigens that are foreign to the mother. One enigma of pregnancy is that the foetus will survive throughout gestation despite the fact that the mother will reject foetal or paternal tissues grafted at any other site. Research into mechanisms by which the foetus is not rejected by the mother could have beneficial offspins in other research areas such as transplantation biology and cancer immunotherapy. Prior to assessing the validity of the most popular theories put forward to explain the enigma of the foetal allograft, the immunological mechanisms operating in graft rejection will be described briefly.

2.2 Graft Rejection and Pregnancy

Grafting or transplantation is defined as the transfer of living cells, tissues or organs from one part of the body to another or from one individual to another. The tranplanted cell, tissue or organ is the graft or transplant. There are essentially 4 different types of graft:

1. Allograft: Graft transplanted between genetically different individuals of the same species.
2. Autograft: Graft taken from and replaced in the same individual.
3. Syngraft (Isograft): Graft transplanted between genetically identical individuals.
4. Xenograft: Graft transplanted between individuals of two different species.

During normal pregnancy, the foetus is often referred to as the foetal allograft. Graft rejection exhibits the two major features of adaptive immunity, memory and specificity, and the major histocompatibility complex (MHC) plays a key role in specificity. The MHC is a group of closely linked gene loci, which code for molecules that restrict the specificity of antigen recognition by T lymphocytes. Although the MHC gene complex as a whole has a similar function in different species, the detailed arrangement of the genes differs. For example, in mice the H-2 complex is divided into 4 regions: K, I, S and D. The common terminology for the MHC loci of a number of species is outlined in Table 2.1. The MHC loci code for MHC proteins and these can be divided into three different generic types as determined by their structure and function. Class I antigens consist of 2 polypeptides, the larger of which is encoded by the MHC and is non-covalently linked with the polypeptide β_2-microglobulin which is encoded outside the MHC. In the human HLA complex, 3 loci, HLA-A, B and C, encode for the class I antigens The human HLA-A and -B regions are analogous to the murine H2 K and D antigens in that they code for cell surface molecules which can be identified by cytotoxic T cells.

In the horse, serological studies have revealed that lymphocyte alloantigens consist of a series of 11 codominant alleles (W1-W11) encoded by one locus (Lazary et al., 1980). It has been suggested that these ELA specificities are analogous to class I antigens (Lazary et al., 1986).

Class II antigens consist of 2 non-convalently associated peptides (α and β) both encoded by the MHC. In the human HLA, the D region contains genes for class II proteins which are involved in the cooperation and interaction between cells of the immune system. The analogous region in

Table 2.1

MHC Terminology

Species	MHC Loci
Mouse	Histocompatibility 2 (H-2)
Rat	Rat Locus 1 (RT1)
Guinea Pig	Guinea Pig Leucocyte Antigen Locus (GPLA)
Human	Human Leucocyte Antigen (HLA)
Horse	Equine Leucocyte Antigen (ELA)

the mouse is known as the I region and products of this region are termed Ia antigens, which is also the generic term of antigens encoded by the human D region.

In the horse, analogous structures appear to be present on membranes which are separate from the W1-W11 series. One such specificity, the B1 antigen, has been shown to govern the interactions occurring between cells in mixed lymphocyte reaction (Lazary et al., 1986). Indeed, the characterisation of the B1 antigen and anti-B1 antiserum respectively are identical to the behaviour of calss II antigens/antisera in humans.

Class II proteins are those complement components which are coded by the MHC. Class I antigens are found on all nucleated cells in varying amounts whereas those antigens encoded by the D region have a restricted distribution. Although the MHC was identified as a consequence of its role in graft rejection, its physiological role is the process of antigen recognition by T cells as outlined in the following section.

2.3 The Cellular Components of Graft Rejection

Adaptive immunity defined as exhibiting specificity and memory is effected primarily by lymphocytes and phagocytes which are derived from bone marrow stem cells. Phagocytes are primarily responsible for taking up foreign particles and breaking them down in phagolysosomes. Lymphocytes are responsible for recognizing and differentiating the various antigens which the immune system encounters. In the development of an immune response, both of these cells interact and cooperate with each other. For example, phagocytes process antigens such that they are recognised by lymphocytes (antigen presentation) and lymphocytes secrete lymphokines and antibody which enhance the ability of phagocytic cells to take up antigenic material and eliminate it.

Lymphocytes, comprising 20% of blood leucocytes, are of two types, B cells which differentiate into antibody producing cells and T cells which differentiate in the thymus and serve a number of functions. Some of the major T cell functions are helping B cells to make antibody, killing virally infected cells, regulating the level of the immune response and stimulating the microbicidal and cytotoxic activity of other immune effectors such as macrophages. Communication between cells is effected by cell/cell contact or by soluble factors.

T cells have a number of defined surface molecules on the cell surface some of which are used to distinguish between different subsets. Until recently, the cell surface markers of human T lymphocytes were designated a T number (T1, T2 etc.), however, to avoid confusion of nomenclature between species, all lymphocyte surface molecules are now referred to as CD antigens and this designation has been given to equivalent molecules in different species. Where CD antigens are described in this text, the old nomenclature will also be given in parentheses.

A major feature of T cells is that they will only recognize antigen when it is presented to them in association with molecules encoded by the MHC. B cells, however, can recognize an unmodified antigen free in solution or on the surface of a cell. The presentation of antigens to T cells is carried out by antigen presenting cells (APC).

T cells recognize antigen and MHC molecules via a receptor which is associated with the glycoprotein molecule CD3 (T3) identified on all mature human and murine T cells. In man, the marker antigens CD5 (T1) and CD2 (T11) are also present on all T cells, while in mice, Thy1 is the characteristic marker (the human equivalent of Thy1 is uncertain).

Activated T cells also carry MHC class II molecules and express interleukin-2 (IL-2) receptors. In terms of function and surface proteins, T cells can be divided into three main types, helper, suppressor and cytotoxic lymphocytes.

Helper T lymphocytes (T_h) are stimulated by an antigen in association with a particular class II MHC molecule on the surface of an antigen presenting cell. T_h cells cooperate with most B cells in the production of antibody in response to antigen although some B cells produce antibody without T cell help, stimulate cytotoxic lymphocytes to mature and interact with other cells by secreting lymphokines (see later). T_h cells are characterised by the surface marker CD4 (T4-human; L3T4-mouse) which appears to be involved in the recognition of MHC class II molecules.

To date, no surface molecule has been identified which differentiates cytotoxic cells (T_c) and suppressor cells (T_s). The two populations are, however, distinct in terms of function. Both T_c and T_s cell sub-populations express the CD8 molecule (T8-human; Ly2,3-mouse) which is involved in the recognition of class I MHC molecules. Tc cells kill other cells by changing the permeability of the target cell membranes. In order to become effective killing cells, Tc precursor cells must be stimulated by an antigen in association with class I MHC molecules. Allogeneic class I MHC molecules can activate cytotoxic T cells in the absence of other antigenic differences. Ts cells suppress the function of other cells either directly or via suppressor factors. Precursors of Ts cells are activated in a similar way to Tc cell precursors by antigen in association with class I MHC molecules.

2.4 Interaction of the Cellular Components

The interaction of cells of the immune system is effected by a combination of direct cell/cell contacts and by the release of various mediators. Of greater relevance to graft rejection are those soluble substances produced by stimulated lymphocytes known as lymphokines which regulate the immune response, largely by affecting the behaviour of other cells. Lymphokines are produced from both B and T cells. A wide range of activities have been attributed to lymphokines but few can be associated with single molecules. Some of the better characterized activities are listed in Table 2.2.

2.5 The Proposed Mechanism of Graft Rejection

As described above, the type of T cell stimulated by antigen is dependent on the MHC molecule presented to the T cell in association with the antigen. The antigen presenting cells thus play a key role in the induction of an immune response. B lymphocytes can also be stimulated by APC's as shown by follicular dendritic cells which take up immune complexes via their complement C_3b receptors. It is apparent that an individual MHC haplotype plays a major part in determining the level and specificity of the immune response.

The observation of small lymphocyte infiltration into an allogeneic skin graft led to the assumption that these cells were responsible for graft rejection and that cytotoxic T cells were the main effectors. Further investigations, however, have indicated that T_h cells are the effectors in rejection reactions elicited by MHC gene differences. This led to the second theory in which graft rejection was likened to a specialized form of delayed type hypersensitivity where monocytes and macrophages were recruited to the site. These two hypotheses are outlined in Fig.2.1.

Table 2.2

Lymphokines and their Activities

1. Regulation of other lymphocytes (non-antigen specific factors)

interleukin-1 (α and β)	(IL-1)
interleukin-2	(IL-2)
interleukin-3	(IL-3)
interferon (α and γ)	(IFN)
soluble immune response suppressor	(SIRS)
inhibitor of DNA synthesis	(IDS)
allogeneic effector factor	(AEF)
T cell replacing factor	(TRF)
B cell growth factor 11	(BCGF11) or (IL-5)
B cell stimulation factor 1	(BSF-1) or (IL-4)
B cell differentiation factor	(BCDF) or (IL-6)

2. Regulation of other lymphocytes (antigen specific factors)

assorted antigen specific helper factors	(T_HF)
assorted antigen specific suppressor factors	(T_SF)

3. Induction of inflammation and mononuclear cell infiltration

skin reactive factor	(SRF)

4. Modulation of the function of phagocytes

migration inhibition factor	(MIF)
leucocyte migration inhibition factor	(LIF)
chemotactic factor	(CF)
interferons (α and γ)	(IFN)
colony stimulating factor	(CSF)
macrophage fusion factor	(MFF) or (IL-4)

5. Regulation of other tissues

colony stimulating factor (I,II,III)	(CSF)
osteoclast activating factor	(OAF)

6. Destruction of non-leucocyte target cells

lymphotoxins (heterogenous)	(LT)
tumour necrosis factor (α and γ)	(TNF)

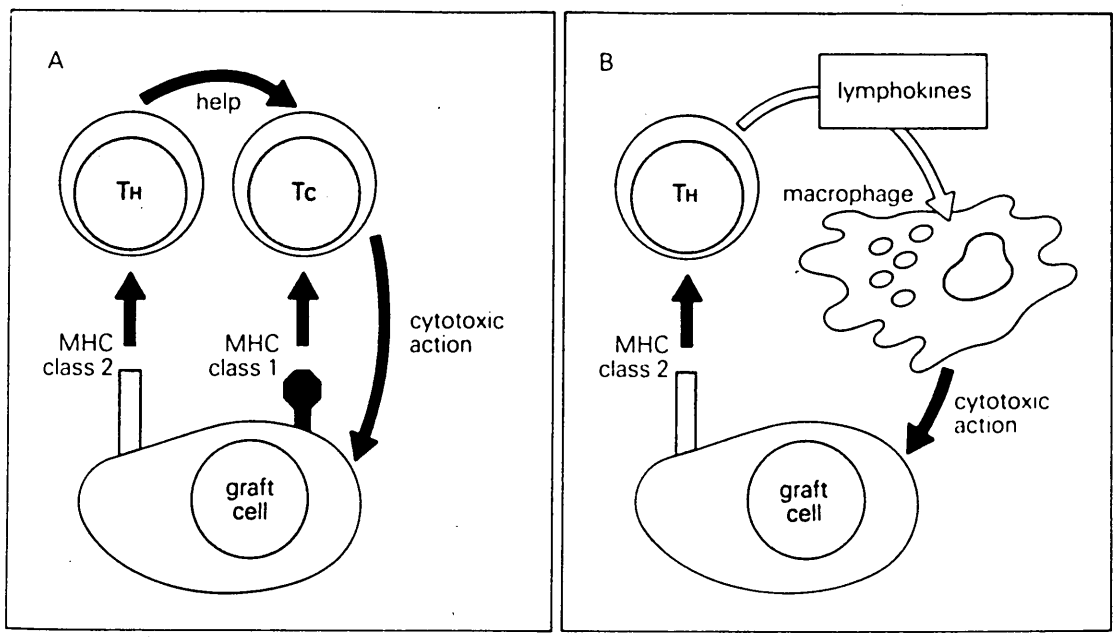

Fig.2.1 Two ways in which the graft cell antigens may induce cytotoxic activity.

(Roitt et al., 1986)

A/ MHC class II antigens in association with antigen on the graft cell stimulate Th cells to secrete a lymphokine(s) which stimulates Tc cells to destroy the tissue. Tc cells recognize the graft via foreign MHC class I antigens.

B/ Th cells reacting to the graft release lymphokines which stimulate macrophages to enter the graft and destroy it.

It must be stressed that the above mechanisms relate to the generation of cytotoxic activity which will, in turn, lead to graft rejection. In response to these reactions and to the presence of foreign antigens, other immune responses will also be occurring but will have a lesser role in graft rejection. Thus, T_h cells will stimulate B cells to synthesize antibody by direct interactions with the B cells across an antigen bridge and/or by releasing factors which may act by first binding to APC's. The interaction between the Th cells and B cells is also affected by the lymphokines IL-2, B cell growth factor (BCGF) and differentiation factor (BCDF).

2.6 Interleukins and Graft Rejection

The proposed mechanisms of graft rejection outlined in Fig.2.1 include cell/cellinteraction involving the secretion and activity of lymphokines, particularly the interleukins. The two interleukins of prime importance in the mechanisms of T cell interactions are interleukin-1 (IL-1) and interleukin-2 (IL-2).

2.6.1 Interleukin-1 (IL-1)

IL-1 is a peptide of 17,500 daltons produced by cells of monocyte/macrophage series and which acts on T cells. It is also produced by a number of other cell types such as skin keratinocytes, endothelial cells and kidney mesangial cells. Most normal cell types and a number of cell lines only produce IL-1 in response to a variety of stimulants. It is believed that reports of a constitutive production of IL-1 by normal cells can be attributed to a continuation *in vitro* of a response to prior stimulation *in vivo* or alternatively, are due to the presence of a contaminating stimulant (Oppenheim *et al.*, 1986). In terms of graft rejection, the most important stimulus to the macrophage for IL-1

production comes from activated T cells. Two basic mechanisms have been proposed whereby T cells induce macrophages to produce IL-1 during the immune response.

1. Cell contact occurs in the MHC class II (Ia) restricted region between activated T cells and macrophages (Fig.2.2) (Farr et al., 1977). IL-1 is released due to a releasing stimulus from Ia molecules on the macrophage membrane.

2. Chao et al. (1977) have suggested that IL-1 release occurs in response to lymphokines such as colony stimulating factor. IL-1-inducing/releasing agents have been found: antigen-antibody complexes are weak inducers but stronger in the presence of complement, C5a is also a potent IL-1 inducer as is platelet activating factor (PAF). Furthermore, a number of IL-1 inducing agents have been partially purified from human mixed lymphocyte culture supernatants. This latter mechanism is the most widely accepted but it is possible that both mechanisms act together.

It is thought that IL-1 is synthesized as a larger molecular weight product (35 kD) which is subsequently subjected to post-translational modification (March et al., 1985). Furthermore, there appears to be a number of genes coding for an IL-1 family as illustrated by the development of two human cDNA clones which code for products termed IL-1α and IL-1β (March et al., 1985). IL-1α and β have similar molecular weights, only about 30% amino acid sequence common, but apparently have similar activities.

IL-1 evokes a variety of responses as a short range mediator in the immune response. The known effects of IL-1 on T and B cells is listed in Table 2.3.

Table 2.3

Effects of IL-1 on Immune Cells

I.	T cells:	Co-mitogenic with lectins or Ag + Ia
		Increased lymphokine secretion (IL-2, BCGF, CSF, etc.)
		Increased expression of IL-2 receptors
		Chemotaxis
		Radioprotection
II.	B cells:	Induces maturation of pre-B cells
		Cofactor in clonal expansion

Oppenheim et al. (1986)

In terms of lymphoproliferation, the major effects of IL-1 are on T_h cells resulting in an increase in IL-2 release and IL-2 receptor expression. In addition, IL-1 enhances the activity of Th cells by raising body temperature and it also protects Th cells from T cell suppressor factor (TsF) (Durum at al., 1985).

In response to IL-1, macrophages release prostaglandin E2 (PGE2) and produce tumour necrosis factor (TNFα). It is uncertain, however, whether the cells that respond to IL-1 also produce it. Since PGE2 inhibits IL-1 production by macrophages and decreases class II MHC expression, the process of IL-1 production appears to be self limiting (Durum et al., 1985).

Little is known about the IL-1 receptor although initial investigations indicate a structure of 80 kDA. Furthermore, all forms of IL-1 (IL-1 α and β and the precursor molecule) have been shown to bind to the same receptor (Dower et al., 1985).

2.6.2 Interleukin-2 (IL-2)

IL-2 is a mitogenic lymphokine as it induces T cell proliferation and provides a means by which antigen-triggered T cells can be clonally expanded in vitro. IL-2 is a single peptide (15.4 kD) produced by T cells within hours of stimulation with antigen (Smith, 1984). IL-2 exhibits variable glycosylation but the carbohydrate has been shown to play no part in the growth promoting activity of variably sialylated IL-2 molecules (Robb and Smith, 1981). IL-2 is also produced by natural killer (NK) cells which are known to produce a variety of cytokines such as IL-1, BCGF and interferon i following stimulation with lectin or antigen (Smith, 1984).

Activated T cells express both high affinity and low affinity IL-2 receptors (Robb et al., 1984). Binding studies using radiolabelled IL-2 and radiolabelled monoclonal antibody against an IL-2 receptor protein (anti-Tac) have revealed that the major proportion of IL-2 receptors are of low affinity (Robb et al., 1981; Depper et al., 1984). In general, the high affinity receptors comprise 5-10% of the total number of IL-2 receptors (Robb et al., 1984). The function of the low affinity receptors is uncertain but IL-2 appears to bring about its growth-promoting effects by interaction with the high affinity receptor. In addition, the high affinity but not the low affinity receptor undergoes receptor-mediated endocytosis (Weismann et al., 1986). It is thought that the internalization of the high affinity receptor is involved in signal transduction. Monoclonal antibodies have been used to isolate and characterize the IL-2 receptor (Tac protein) and it appears to be a membrane glycoprotein of 55 kD.

IL-2 receptors are only transiently expressed on T cells following antigen activation but there is evidence to suggest that after cell division, antigen triggering must occur again before the IL-2 receptors are expressed at high density (Depper et al., 1984). The response to IL-2 is therefore restricted by the availability of IL-2 receptors. Since the density of IL-2 receptor expression is dependent on stimulation of the responding cells by antigen, the process remains antigen specific although IL-2 is itself a non-specific effector molecule. All three functional T cell subsets (Th, Tc and Ts) can be induced to develop IL-2 receptors and will proliferate in the presence of IL-2. It is, however, unclear whether a given T cell can both produce and respond to IL-2 at the same time, or

whether production and response to this lymphokine is restricted to certain phases of the cell cycle. The events that occur during T cell activation are summarised in Fig.2.2.

To summarise, antigen presenting cells process and present antigens to T cells (T_h in Fig.2.2) together with class II MHC antigens. IL-1 produced by antigen-presenting cells augments the response of some T cells. In response to these stimuli, Th cells produce IL-2 which allows those T cells with IL-2 receptors to proliferate. IL-2 receptor expression is increased following antigen presentation (shown in Fig.2.2 for class II-restricted T cells) and it is therefore probable that the same cell can both produce and respond to IL-2 as well as trigger other cells.

The above mechanisms are not the only immune responses that occur during graft rejection although they are of prime importance. For example, IL-2 is reported to induce interferon-γ production from T cells which augments T cell expression of IL-2 receptors (Kasahara et al., 1983). IL-2 receptors are also found on B cells and thus IL-2 acts as a growth factor (Tsudo et al., 1984). Tumour necrosis factor (TNFβ) is also released by T cells in response to IL-2 which has a synergistic effect with interferon γ in killing target cells (Male et al., 1987).

2.6.3 Lymphocyte Proliferation Assays

During pregnancy, many factors/proteins, produced by the foeto-placental unit, are described as being able to suppress the maternal immune response (Section 2.7.6.1). The activity of these factors are often assessed by their inhibitory activity on in vitro lymphocyte stimulation assays. One of these assays is the mixed lymphocyte reaction (MLR) in which two populations of peripheral blood lymphocytes from two donors are incubated

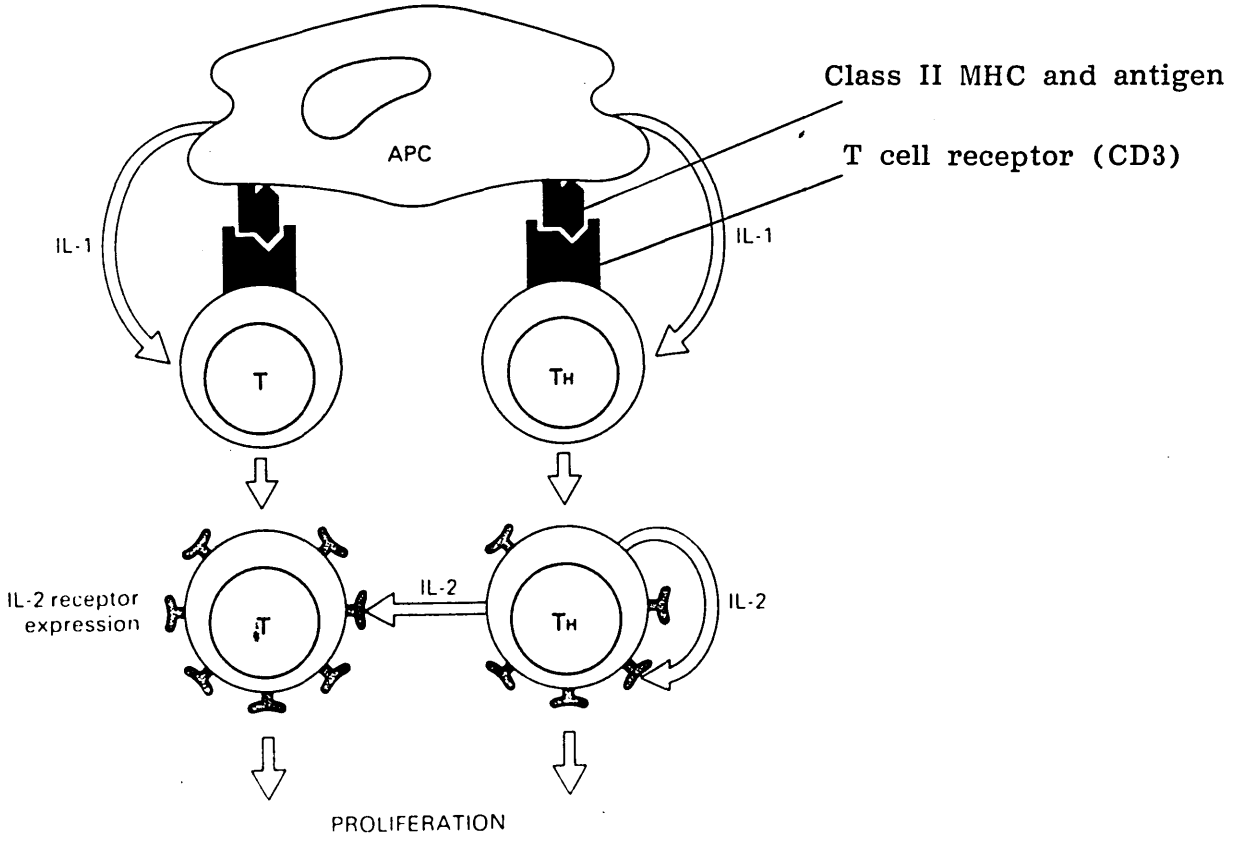

Fig.2.2 T_h, T_c and T_s respond to IL-2 and proliferate in its presence.

Male et al. (1987)

together. The cells are stimulated to grow in the presence of foreign class II antigens, the cells must differ at the HLA-D region (human), ELA-B1 region (horse) or I region (mouse) for stimulation to occur. Consequently, both populations of cells proliferate in response to each other. The other type of lymphocyte proliferation assay is the mitogen stimulation assay (MSA) in which lymphocytes are stimulated to grow and proliferate by lectins. A lectin is a protein which binds and cross-links specific cell surface carbohydrate determinants and will polyclonally stimulate lymphocytes. Lectin-induced lymphoproliferation is not therefore dependent on the presentation of MHC molecules.

2.7 Immunomodulatory Activity during Pregnancy

During mating, implantation, placental development and throughout gestation, the maternal immune system is being challenged by foetal and MHC antigens and the efferent arm of the immune reaction is often activated but does not lead to a rejection reaction. It has been suggested that the immune responses to the foetus following a recognition of its foreign status, may play an important or even essential role in the immunological protection of the foetus from the time of implantation to parturition (Beer & Sio, 1982). The actual process of parturition has been likened by some to the rejection of a previously tolerated graft. The many changes in hormone and protein levels at birth are well documented and thus it is proposed that these changes remove the immunoregulatory influences which have maintained the foetal allograft throughout gestation.

There has been much theoretical speculation to explain the lack of rejection of the histoincompatible foetus and placenta and each theory has been investigated to a greater or lesser extent in a number of mammalian species. As a consequence of this , it must be emphasized that in an

interspecies comparative study such as this, there is need for caution when extrapolating immunologic and reproductive events in the human to mammals with different placentation and gestational length. An immunological interpretation of these differences between animals with haemochorial placentation may lead to a further understanding of the functions of different types of placentation and the maintenance of mammalian pregnancy.

Recent research findings indicate that there is more than one mechanism involved in the establishment of an immunological endocrine equilibrium necessary for the maintenance of pregnancy. The following is a summary of the proposed mechanisms relating to the non-rejection of the foetal allograft.

The cell-mediated immunological mechanisms of graft rejection (as previously described) are summarised in Fig.2.3.

The early theories put forward to explain the non-rejection of the foetal allograft were based on the idea that the mother did not react immunologically to the embryo.

2.7.1 The Uterus is an Immunologically Privileged Site

The poor lymphatic drainage of the endometrial epithelium as compared to the rapid drainage of the myometrium suggested that the uterus may be an immunologically privileged site. It has been demonstrated in rodents that allogeneic skin transplants were rejected from the uterus as quickly as those placed elsewhere but show prolonged survival in the pregnant uterus (Kaye et al., 1974). Prior active sensitization to the donor

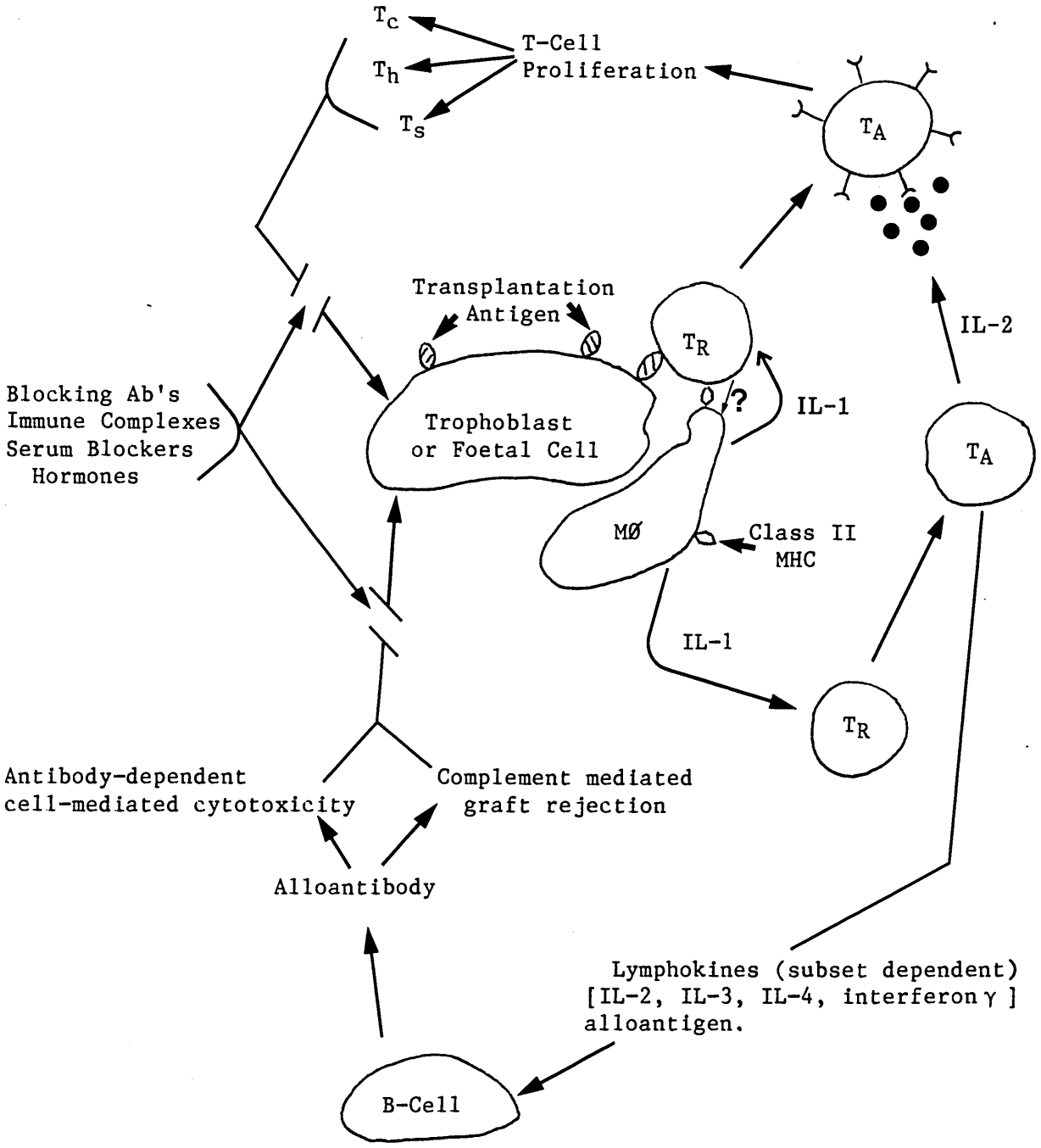

Fig.2.3 Immunologic Events in Pregnancy Relative to Foeto-Placenta Rejection

(T_c, T_h, T_s: cytotoxic, helper, suppressor T cell; T_A: activated T cell, T_R: resting T cell; MO: macrophage; IL-1,2,3,4: interleukins-1,2,3,4).

[Combination of Greene and Leonard, 1986 and Siiteri and Stites, 1982)

alloantigens resulted in prompt rejection of the allograft in utero. It was subsequently concluded that pregnancy in no way blocks the efferent limb of the uterine immunological reflex arc (Kaye et al., 1974).

2.7.2 The Placenta Acts as a Mechanical Barrier

If the uterus responds immunologically to the presence of a foetus then perhaps the graft has some physical characteristic which prevents it reacting with the maternal immune system. In the haemochorial placenta, there is a fibrinoid layer around the syncytiotrophoblast (Bardawil & Toy, 1959). Bradbury et al. (1969) demonstrated that this layer consisted of highly sulphated glycoprotein (sialoprotein) which could electrostatically repulse negatively charged lymphocytes. Recent investigations however have shown that there is no consistent fibrinoid layer between the decidua and the trophoblast (Tekelioglu-Uysal et al., 1975). The concept is further weakened by numerous observations of the exchange of maternal and foetal cells throughout gestation (Ikle, 1964).

2.7.3 Lack of MHC Antigen Expression on Foeto-Placental Tissue

It was suggested that the trophoblast may be poorly immunogenic in vivo due to the lack of class I and class II antigens.

2.7.3.1 Class I MHC Antigen Expression Associated with Foeto-Placental Tissue

The expression of MHC antigens by human and murine placental tissue has been extensively investigated but some confusion still remains. MHC expression by the human morula or blastocyst is unknown. Classical MHC class I antigens have not been detected on the non-villous trophoblast, villous trophoblast or syncytiotrophoblast (Sunderland et al., 1981). The non-villous trophoblast, however, exhibits a class I-like antigen

throughout gestation (Redman et al., 1984). Similar unusual class I antigens have been detected in the mouse and rat (Cook et al., 1983; Smith et al., 1982).

Implantation in horses occurs 37 days after mating (Allen et al., 1973). This is typified by the invasion of the uterine endometrium by specialized gonadotrophin-secreting trophoblast cells of the annulate chorionic girdle region of the foetal membranes (Section 3.6). These cells develop into endometrial cups. Two to four weeks after the development of the cups, more than 90% of primigravid mares exhibit high titres of anti-paternal cytotoxic antibody in their serum (De Weck et al., 1978). Cytotoxic antibody is reported to persist throughout gestation, show strong specificity for the mating stallion and is absent in mares carrying foetuses that are histocompatible with regard to class I MHC antigens (Antczak et al., 1984). In addition, throughout the 80-100 day lifespan of the cups, increasing numbers of lymphocytes accumulate in the stroma around each cup. This maternal response occurs in both MHC class I compatible and incompatible pregnancies and in hybrid matings between equine species (Allen et al., 1984). No similar reaction, however, occurs at the border between endometrium and non-invasive trophoblast.

These and other observations suggest that the invasive trophoblast may express MHC antigens. It has in fact been suggested that cytotoxic antibody production and the endometrial cup leucocyte reaction represent respectively the humoral and cell-mediated arms of the maternal immune response to paternally-derived foetal alloantigens on endometrial cup cells (Kydd et al., 1982).

Recent investigations in this area have involved the use of well characterised anti-MHC monoclonal antibodies in an immunoperoxidase labelling system. Crump et al. (1987) were unable to demonstrate class I MHC antigen expression on mature endometrial cup cells or non-invasive trophoblast, although the progenitor tissue of the cups, the chorionic girdle, stained heavily. Moreover, it appears from antibody absorption and elution experiments that endometrial cups might express very low levels of paternal MHC antigen. Crump et al. (1987) also tested the immunogenecity of endometrial cup tissue by immunising naive recipients with the tissue and also by giving booster immunisation to a mare with endometrial cup tissue from her own pregnancy. No increase in cytotoxic anti-paternal antibody occurred suggesting that if endometrial cup cells express paternal MHC antigen, it is not expressed in a form that is highly immunogenic.

Despite the fact class I MHC antigens cannot be detected on mature endometrial cup cells histochemically, class I antigen expression by chorionic girdle tissue and the close temporal association between cytotoxic antibody production and endometrial cup formation still imply that the invasive component of the trophoblast is the source of alloantigen in equine pregnancy. In 1986, Zuckerman and Head found that when murine trophoblast cells were cultured in vitro with interferon γ, the level of expression of class I MHC antigens increased. It has been postulated that an equivalent type of stimulus may modulate temporarily the expression of MHC antigens on chorionic girdle cells in equids (Crump et al., 1987). Further speculation by Crump led to the idea that the proposed modulation of MHC antigens on chorionic girdle and endometrial

cup cells could also regulate the composition of maternal leucocytes that surround the endometrial cups, uterine suppressor cells may have an important role as described in the mouse (Clark et al., 1984).

2.7.3.2 Class II MHC Antigen Expression Associated with Foeto-Placental Tissue

The expression of class II MHC products in the placenta is of particular interest because these are a major stimulus for the rejection of allografts. Research groups generally agree that class II antigens are not expressed on trophoblast derived tissues at any stage throughout gestation both in the mouse (Chatterjee-Hasrouni and Lala, 1981) and in the human (Faulk and Temple, 1976; Sunderland et al., 1981; Bulmer et al., 1984). To date, no data is available regarding the expression of class II MHC antigens on equine foeto-placental tissue.

HLA-DR positive cells have been found in the decidualised endometrium (Bulmer and Sunderland, 1984), the stroma of term chorionic villi (Sutton et al., 1986) and the foetal mesenchyme of the term amniochorion (Sutton et al., 1983). These cells label with monoclonal antibodies specific for monocytes and macrophages (Bulmer and Johnson, 1984).

2.7.3.3 Non-Histocompatibility Antigen Systems

The body of evidence implicating important immunological roles of non-MHC antigen systems in the foeto-maternal relationship is rapidly increasing. Of these, the trophoblast-specific and trophoblast-lymphocyte cross-reactive TLX antigens have attracted most attention.

The human TLX antigen system refers to antigens shared by both trophoblast and lymphocytes and is described as a possible modification of the class I MHC antigen recognition system (Bulmer and Johnson, 1985). The TLX

antigen system is believed to be involved in the maintenance of normal pregnancy by virtue of maternal recognition and initiation of protective blocking antibodies but there is no direct evidence for this (Section 2.7.5).

In the horse, the slow deterioration of endometrial cups over a 60 day period (Section 3.6) has been likened to the rejection of skin grafts across minor histocompatibility antigen barriers (Crump et al., 1987). One possibility is that the endometrial cups express the TLX antigen (Section 2.7.5) (Allen et al., 1987). A further suggestion, however, is that antigens specific for trophoblast tissue are more likely to be expressed on cup cells than minor histocompatibility antigens. That a great increase in the leucocyte response to endometrial cups occurs in hybrid equine pregnancies (Allen et al., 1984) suggest that species related antigens may also be expressed on at least the invasive portion of the trophoblast (Allen et al., 1987).

2.7.4 Maternal Immunoregulation

Generally speaking, there are essentially two possible mechanisms by which the maternal immune response may be altered resulting in the failure of foeto-placental rejection. One way is the production of immunomodulatory substances in the placenta with non-specific effects on maternal cellular immunity, e.g. hormones/proteins. The other way involves circulating specific antipaternal immunosuppressant substances of which the main suppressants have become known as 'blocking antibodies'.

2.7.5 Circulating Blocking Factors

A humoral response to mammalian pregnancy has been demonstrated in many species. 15 to 25% of primiparous women and 40 to 60% of multiparous women produce anticlass I and class II HLA agglutinating and cytotoxic

antibodies (Doughty and Gelsthorpe, 1976; Terasaki et al., 1970). Anti HLA (Faulk et al., 1974) and anti-H_2 (Bell and Billington, 1981) can be detected in placental eluates. In contrast, 90% of primiparous mares develop high titre antipaternal alloantibody (Antczak et al., 1984).

Pregnancy sera is well known to contain certain blocking factors which inhibit a variety of in vitro assays of T lymphocyte proliferation and function and a number of these effects have been attributed to blocking antibodies. Antipaternal antibodies in parous serum (Stimson et al., 1979) or eluted from placenta (Stewart et al., 1984) have been shown to block lymphokine release, MLR against paternal lymphocytes and the generation of cytotoxic killers in vitro (Rocklin et al., 1979).

The three most likely mechanisms by which blocking antibodies are believed to prevent lymphocyte-target cell recognition are represented in diagramatic form below (Fig.2.4).

A deficiency in blocking antibodies in women suffering from recurrent spontaneous abortion has been described suggesting that a maternal humoral response may be important for the survival of the foetal allograft (Rocklin et al., 1976; Power at al., 1983). Indeed, it has been shown that when women suffering from spontaneous abortion are pre-immunized with paternal lymphocytes, the rate of abortion is significantly decreased compared to a group of women treated with their own lymphocytes (Mowbray et al., 1985). The apparent benefit of pre-immunization with paternal HLA has not been adequately explained but has been related to the appearance of anti-HLA antibodies.

Similar results to these have been obtained from studies of the extra-species donkey-in-horse pregnancy, that is the transfer of donkey embryos to horse mares. Endometrial cups do not develop in this type of

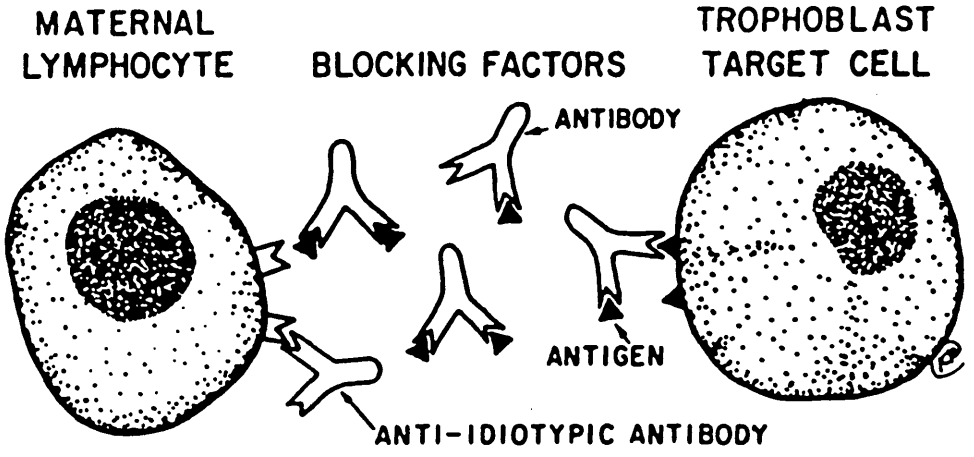

Fig.2.4 The potential role of blocking factors in preventing lymphocyte-target cell recognition.

Scott et al. (1987)

pregnancy and 80% of donkey foetuses are aborted between days 80 and 100 of gestation (Allen, 1982). Furthermore, a dense leucocyte response in the mare's endometrium is associated with the death of the foetus. In 1987, Allen et al. reported that the immunization of mares carrying donkey conceptuses with washed donkey peripheral blood lymphocytes increased the survival rate from 20% to 67%. These results were consistent when the immunized lymphocytes were taken from the genetic sire and dam of the donkey embryo or from unrelated male and female donkeys. The immunization of mares carrying donkey foetuses with serum from mares carrying normal intra-species pregnancies at a similar stage of gestation had a similar effect. These results suggest that similar immunoprotective systems operate in the human and the horse, possibly due to the development of anti-TLX antibodies (Faulk and McIntyre, 1986; Allen et al., 1987). Alternatively or in conjunction with blocking antibodies, specific suppressor cells may be induced in the uterus as described in the mouse (Clark et al., 1984) and in the human (Clark et al., 1986e) (Section 2.7.6.2).

2.7.6 Maternal Immunosuppression

The response of maternal lymphocytes to mitogen stimulation during pregnancy has shown normal or decreased T and B lymphocyte function (Lawrence et al., 1980). It is proposed that there is an involved suppression of the maternal response by various hormones and pregnancy proteins during pregnancy. This theory has been proved with respect to many hormones and pregnancy proteins but the mechanisms by which they interact with the maternal immune system is less clear. Any hormone/protein ascribed an immunoregulatory role could influence any one of a complex series of regulatory events, the net result being suppression or enhancement of the immune response. At the humoral level, regulatory

factors include antibody and immune complexes in addition to a large number of less well-characterised substances. At the cell-mediated level, immuno-regulation could result from the release of helper or suppressor factors which interact with other cells either in the afferent or efferent limb of the immune system or from the generation of a cell population which suppress the maternal immune response. It is thus clear that there is much overlap between the production of blocking antibodies and other immunoregulatory substances such as immunosuppressants.

2.7.6.1 Human Immunoregulatory Hormones and Proteins

In the human, a number of proteins have been identified as being secreted either specifically during pregnancy (pregnancy-specific) or at an enhanced rate (pregnancy-associated). The pattern of secretion of these proteins falls into 2 groups.

Pattern A: Those proteins at maximal levels during the first few weeks of gestation and thus are likely to have an important role during the implantation and placentation processes such as immunoregulation, e.g. human chorionic gonadotrophin (hCG) (Braunstein et al., 1976)

Pattern B: Those proteins the secretion of which appear to relate to the overall size of the placenta and whose levels increase throughout gestation, e.g. anticoagulants such as placental protein-5 (Soma et al., 1985).

There has been much interest in those proteins/factors produced locally in the foeto-placental unit which may have an immunomodulatory activity during the critical early stages of pregnancy. In this regard, the more important hormones and proteins secreted during this time are described below.

(a) Progesterone

Progesterone is described as the essential hormone for the maintenance of pregnancy and subsequently, much attention has been focused on it as a potential immunosuppressant. Placental progesterone production appears to be (in part) dependent on the type of placental structure with high concentrations produced by haemochorial placentae and relatively low concentrations produced by epitheliochorial placentae (Stites and Siiteri, 1983).

A number of *in vivo* studies using mice have illustrated that progesterone has a pronounced local immunosuppressive effect but little effect on systemic immunity (Siiteri *et al.*, 1977; Van Vlasselaer and Vandeputte, 1985).

Progesterone *in vitro* has been shown to inhibit T cell activation by mitogens, antigens and allogeneic cells and the concentrations required were equivalent to levels found at the placenta/decidua border (Kumar *et al.*, 1963; Clemens *et al.*, 1979). Stites and Siiteri (1983) confirmed the above findings and reported that progesterone inhibited mainly the recognitive and proliferative phases of the allogeneic T cell response. Further investigations by Stites and Siiteri revealed the following *in vitro* effects of progesterone:

1/ Progesterone has no inhibitory effect on monocyte proliferation but has a relatively selective action on T lymphocytes.

2/ Progesterone is effective in blocking the mitogenic activity of IL-1. It was not shown, however, that IL-1 production was blocked.

3/ Oxygen consumption by monocytes is inhibited by progesterone in phagocytosing culture. This may account for the *in vivo* anti-inflammatory action of progesterone.

Progesterone has also been reported to block cytotoxic T cell induction (Van Vlasselaer and Vandeputte, 1985) and to independently depress the function of alloreactive suppressor cells generated in the MLR (O'Hearn and Stites, 1983).

An important step in the process of antigen recognition and lymphocyte proliferation is the capacity of lymphocytes to form aggregates and neither antigen or major histocompatibility differences are involved in this. This property is believed to allow the local exchange of immunologic information by the presentation of antigens, by the secretion of lymphokines, or both. In 1980, Nussenzwaig and Steinmann reported the capacity of dendritic cells to aggregate with lymphocytes around their cellular protrusions enabling the exchange of immunologic information with neighbouring cells. Lymphocyte populations depleted of dendritic cells are unable to form clusters. In 1984, Hamann et al. reported that after 3-5 days stimulation by PHA, blast formation and ^{3}H-Tdr incorporation occurred only in the clustered lymphocytes. In 1986, Van Vlasselaer et al. examined the effect of progesterone and trophoblast culture supernatants on lymphocyte aggregation and found that diminished cell-cell contact occurred when progesterone was added to autologous lymphocytes. Trophoblast supernatants were found to have an identical effect to progesterone and the inhibition of lymphocyte clustering was counteracted by the addition of anti-progesterone serum. Furthermore, when a preparation containing lymphokines with a definite IL-2 activity was added, this effect was reversed (Gillis et al., 1979). Progesterone does not inhibit IL-2 production and thus Van Vlasselaer suggests that the decreased cluster formation may be due to an interaction of progesterone with the lymphocyte membrane leading to a decreased sensitivity for IL-2 molecules. This may have some significance during pregnancy in that once

maternal lymphocytes leave the placental bed, they regain their immune competence. Although the above experiments suggested that progesterone was the major immunosuppressant in trophoblast supernatant, the progesterone concentration in the supernatant was found to be too low to cause immunosuppression in vitro and it has subsequently been suggested that progesterone is bound to another factor which increases its immunosuppressive effect (Van Vlasselaer et al., 1986).

b) Human Chorionic Gonadotrophin (hCG)

Human chorionic gonadotrophin is a glycoprotein of molecular weight 38,000 with a carbohydrate content of 30%. The molecule consists of two dissimilar non-covalently linked subunits, alpha and beta, and these are located in the cytotrophoblast and syncytiotrophoblast respectively (Tojo et al., 1982). The detection of hCG in serum or urine is used for the early diagnosis of pregnancy. hCG can first be detected in the maternal blood at 8-12 days post conception which is the time of implantation. Levels then rise rapidly and reach a maximum at the tenth week of pregnancy after which the concentration declines rapidly and remains constant from the fourth month onwards. A similar pattern is found in the urine.

As a consequence of the early appearance of hCG in pregnancy, much attention has been focussed on the hormone as a potential immunosuppressant (Braunstein et al., 1976). All of the evidence indicating an immuno-suppressive role for hCG was based on work using a crude commercially available hCG extract from pregnancy urine. hCG-mediated immunosuppression has been reported in PHA-induced lymphocyte transformation, mixed lymphocyte reactions and antigen induced blastogenesis using doses of 50-100 iu/ml (Siiteri and Stites, 1982).

More recently ,however, hCG has been further purified from the crude extracts to 10,000-15,000 iu/ml. This more highly purified hCG is not immunosuppressive (Rolfe et al., 1983). Since this discovery, research has been carried out to try and find the immunosuppressive substance(s) in crude hCG preparations. One finding of interest in this area is that crude hCG preparations contain an immunosuppressive pregnancy protein known as early pregnancy factor [see (c) below] and it has been suggested that this may be entirely responsible for the immune-suppression observed (Rolfe et al., 1983).

hCG has thus been shown not to be immunosuppressive but this does not rule out an immunological role, indeed membrane-bound hCG may play an important role in blocking certain events in cellular recognition which could play an important role in foetal-maternal tolerance.

c Early Pregnancy Factor (EPF)

One development that has received much attention over a number of years has been the detection of an early pregnancy factor (EPF) in the serum within hours of fertilisation and before implantation of the embryo.

EPF is an activity detected by an in vitro rosette inhibition assay which measures the capacity of antilymphocyte serum (ALS) to inhibit rosette formation between T cells and heterologous erythrocytes, a high rosette inhibition titre (RIT) being indicative of a high concentration of specific antibody (Smart et al., 1981). Morton et al.(1976) adapted this assay to look for the presence of suppressor activity in pregnant mice and found that the activity of the ALS to inhibit rosette formation (RIT) in the test could be enhanced by a factor (EPF) present in pregnant mouse serum.

EPF has been detected by this technique in all species so far studied which include the mouse, sheep, pig (Morton et al., 1983), horse (Morton et al., unpublished), human and wallaby (unpublished) and the sensitivity of the system has been shown to vary between species. The mechanism of action of the EPF assay has recently been further investigated by Morton (1985) and it appears to measure soluble suppressor substances released by EPF-bound lymphocytes. It is these suppressors rather than EPF per se which are believed to increase the RIT of ALS. The lymphocytes involved in this assay are T helper cells and Morton suggests that these suppressors affect receptor mobility thus reducing red blood cell receptor availability resulting in a decrease in rosette formation.

EPF is as yet uncharacterised but it may be a unique pregnancy protein worthy of further investigation. EPF activity is detectable very early in pregnancy. In the mouse, the RIT is increased six hours after fertilisation, returning to normal four to six days before parturition. Thus far, research of this nature has been limited in other species but preliminary studies have shown that in humans, pigs and sheep, EPF is present in serum within 24 to 48 hours of a fertile mating (Morton, 1985). EPF production has also been shown to be dependent on the presence of a viable conceptus.

In sheep, it has been shown that embryonic death is preceded by a decline in EPF activity. Abortion leads to a decline in activity with no EPF activity detectable within 8 to 24 hours (Morton et al., 1979). Similar results have been obtained in humans and pigs (Morton, 1985).

In 1981, Nancarrow et al. found that EPF in cows could be detected 3 to 4 dys after artificial insemination. The use of EPF as a bovine pregnancy test has been problematic although the testing sensitivity has been

improved by adoptive transfer of contact sensitivity (ATCS) (Klima, 1985). In 1987, Klima et al. described a pregnancy specific serum component identified by crossed immunoelectrophoresis using rabbit antiserum produced against bovine early pregnancy serum and intensively absorbed with non-pregnancy serum. The early pregnancy-associated protein could be identified within 8 days of artificial insemination and was also shown to behave like EPF in sheep (Noonan et al., 1979) in suppressing the delayed cutaneous hypersensitivity in mice (Klima et al., 1987). It appears that the new protein described by Klima is very closely related to EPF.

During human pregnancy, levels of EPF activity have been reported to be very high during early pregnancy and then to decrease with continuing gestation, finally disappearing from the maternal blood 8 weeks before delivery (Zhihai and Zhen-Qun, 1987).

Despite these findings, the inability of some laboratories to detect EPF reliably in pregnant sera has made EPF a controversial topic (Cooper and Aitzen, 1981).

From the EPF studies reported thus far, it is clear that EPF in serum provides the earliest indication that fertilization has taken place. The following is a brief account of the production of EPF, as this may have considerable bearing on the further development of the research outlined later.

Prior to implantation, the fertilized egg is unable to produce EPF until it reaches the blastocyst stage. Before this, EPF is produced from maternal tissue as two components, EPF-A and EPF-B.

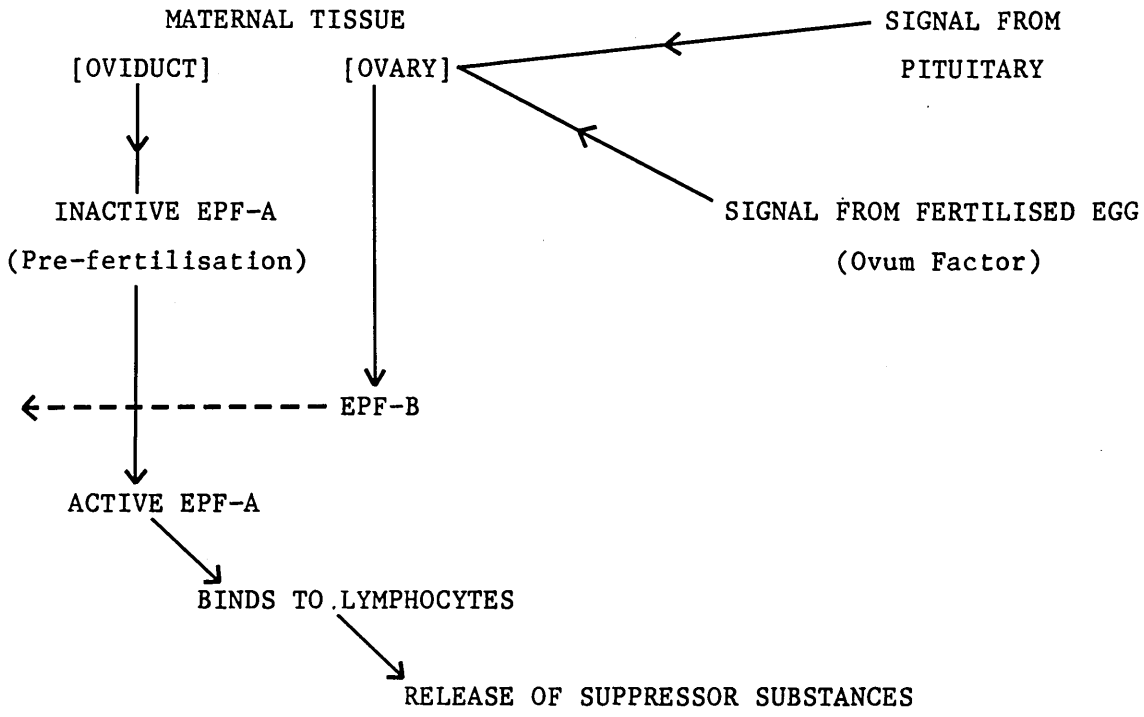

Fig.2.5 EPF secretion and activation.

EPF-A produced by the maternal oviduct is not pregnancy dependent and is produced prior to fertilisation. EPF-A is the component of EPF which binds to lymphocytes but it will only do so after activation by EPF-B. The activation process is poorly understood but once activated it binds to lymphocytes either on its own or in combination with EPF-B (Smart et al., 1981).

EPF-B is the pregnancy dependent component of EPF and is produced by the ovary in response to a signal from a fertilized egg. This signal, called ovum factor, is produced by the ovum on penetration of a fertilizing spermatozoon and production continues until at least blastulation. Thus far, it has been found that ovum factor is produced from human ova (Cavanagh, unpublished data), pig and sheep ova (Morton, unpublished) and mouse ova. Furthermore, the activity of the substance has been found not to be species specific; ovum factor from cultured human or pig embryos acts in the mouse similarly to mouse ovum factor (Cavanagh et al., 1982).

In addition, there is evidence that in the mouse the release of EPF-B by ovum factor is dependent on the presence of prolactin (Morton et al., 1982; Cavanagh et al., 1982). This has yet to be demonstrated in other species.

After implantation, foetal tissue replaces maternal tissue as a source of EPF. The cross-over point is not detectable in the species studied with the exception of the pig where biphasic production is clear (Morton et al., 1983).

All of the work quoted thus far has involved the use of the RIT in EPF detection. The RIT is, however, a non-quantitative assay, the value of the rosette inhibition titre is more a reflection of the quality of EPF rather than the quantity.

In conclusion, the capacity of EPF to enhance the activity of ALS in inhibiting rosette formation has resulted in the factor being classified as immunosuppressive. The pre and post implantation reactions involving EPF form a cascade initiated by the conceptus leading to the immunosuppression of the maternal immune system; thus it appears that the conceptus ensures its own defence against immunological rejection.

d) Alpha Foeto-Protein (AFP)

Alpha foeto-protein is a glycoprotein synthesized in the yolk sac and foetal liver and present in high concentrations in the blood and amniotic fluid of the foetus. Concentrations of AFP increase in relation to the size of the foeto-placental unit.

AFP is a well documented inhibitor of cellular and humoral immunity (Tomasi, 1978). Research to date indicates that AFP acts by preventing the cell surface expression of Ia antigens (Lu et al., 1984) and/or the generation of suppressor cells (Murgita et al., 1981).

Initially, Murgita and Tomasi (1975) reported that AFP isolated from murine amniotic fluid inhibited mitogen induced and mixed lymphocyte proliferation as well as primary and secondary humoral antibody responses. In 1977, Murgita et al. reported that AFP suppresses antibody production in vitro in mouse spleen cells by activation of a suppressor cell population. Yachin and Lester (1979) speculated that human AFP acts to inhibit mitogen stimulated human lymphocyte cultures through a similar mechanism. In 1985, Hoskin et al. demonstrated that AFP induces the generation of one population of non-T suppressor cells.

Finally, it has been observed that AFP, which is present at high concentrations in the placenta, down-regulates class II MHC expression on macrophages and dendritic cell lines, both at the cell surface (Lu et al., 1984) and at the messenger RNA level (Craimie et al., unpublished). It is thought that such cells which lack the surface expression of class II MHC molecules may prepare the antigen specifically for suppressor cells (Streilin and Wegmann, 1987).

e) Placental Protein 15 (PP15)

Very little has been published on this particular placental protein. Bohn et al. (1980) isolated PP15 from human term placentae and found that it had a significant inhibitory effect on the mixed lymphocyte reaction (Bohn et al., 1980).

f) Placental Protein 14 (PP14)

PP14 is a glycoprotein with a molecular weight of 42,000 to 43,000 daltons containing 17.5% carbohydrate. The isoelectric point of PP14 is 4.6 and it has an electrophoretic mobility between the alpha-1 and alpha-2 globulins (Bohn et al., 1982). A number of independent groups have described proteins which are immunologically similar to PP14 some of which may be identical.

PP14 was first isolated from human term placenta (Bohn et al., 1982). Since then, the development of a radioimmunoassay for PP14 (Bolton et al., 1983) has revealed that PP14 is not placenta specific and the culture of endometrial and decidual tissues has shown that these tissues secrete PP14 (Julkunen et al., 1986c).

During pregnancy, PP14 levels rise rapidly, particularly in the deciduum where a 100 fold increase has been measured during the first 10-12 weeks of gestation. After this period, PP14 levels decrease throughout gestation (Julkunen et al., 1985b). In non-pregnant women, circulatory levels of PP14 show a cyclic variation (Julkunen et al., 1986b), and it is probable that the synthesis and/or release of PP14 is controlled by progesterone.

The localisation and pattern of secretion of PP14 suggests a possible local immunosuppressive activity. PP14 has been shown to suppress both the allogeneic and mitogenic stimulation of peripheral blood lymphocytes (Bolton et al., 1986; Pockley et al., 1987). It has been demonstrated that the immunosuppressive activity of decidual tissue extracts is closely related to the PP14 content as measured by radioimmunoassay (Bolton et al., 1986). Furthermore, it was found that when the decidual extracts were immunoadsorbed with a monoclonal anti-PP14, there was a significant decrease in immunosuppression as measured by the allogeneic mixed lymphocyte reaction. This effect was also observed using purified PP14 and seminal plasma (Bolton et al., 1987).

More recent work has focussed on the action of PP14 on the production/action of lymphokines from mitogenically stimulated lymphocytes (Pockley and Bolton, unpublished). It was found that the immunosuppressive activity of decidual extracts and purified PP14 was partially reversed by the addition of both recombinent-IL-2 and culture supernatant from allogeneically stimulated lymphocytes. Pockley and Bolton suggest that PP14 may exert its suppressive activity at the IL-2 level of the immune response.

Work on PP14 to date suggests that PP14 may be an important local immunosuppressant in the human reproductive process, being present in seminal plasma, uterine luminal fluid, cervical mucous and decidual tissue. The levels of PP14 in all of these tissues/fluids are high enough to mediate such an immunoregulatory role.

2.7.6.2 Endometrial/Decidual Immunoregulatory Activity

During normal pregnancy in mice and humans, suppressor cells accumulate at the implantation site in the uterine decidua (Clark et al., 1986a). These cells have been shown to suppress the ability of maternal T cells to proliferate in response to concanavalin A. In the mouse, these cells are predominently small granule containing non-T lymphocytes from which a soluble factor can be obtained which blocks the action of IL-2 (Clark et al., 1985). In addition, these cells appear to be trophoblast dependent. A similar population of cells appears to be present in the human (Clark et al., 1986e; Clark et al., 1987). A second population of large sized, probably hormone dependent cells have been isolated from the endometrium taken during normal menstrual cycles. The suppressive activity associated with this cell is reported to occur only in the luteal phase with maximal activity occurring at the time when implantation would normally take place. These cells prevent the generation of cytotoxic T lymphocytes (CTL's) from precursors (pCTL), but do not affect antibody responses. As these larger cells appear during the luteal phase of the menstrual cycle, they appear before the smaller granular suppressor cells during pregnancy. Consequently, local active suppression is described as being bimodal and accomplished by two populations of suppressors, phase A cells (hormone dependent) and phase B cells (trophoblast dependent) (Clark et al., 1986).

Analogous research in species with epitheliochorial placentation has been limited. In the horse, techniques have been developed to isolate the population of cells that accumulate around the endometrial cups (Kydd and Allen, 1986 and personal communication). Investigations into the functional activities of the lymphocytes has shown that cells recovered between days 55 and 70 of gestation fail to proliferate in response to Con.A. The same cells have also been shown to abrogate the normal mitogenic response of peripheral blood lymphocytes from the donor of the cup lymphocytes and also the same response of lymphocytes from unrelated horses (Kydd and Allen, 1986; Allen et al., 1987). It thus appears that at least one population of cells attracted to the endometrial cups have suppressor rather than cytotoxic activity.

Studies of murine xenogeneic pregnancy failure (Mus caroli transferred to the Mus Musculus uterus) have shown that the activity of the decidua associated suppressor cells is deficient whereas suppressor cell activity was evident in a normal Mus caroli pregnancy (Clark et al., 1984). Similarly, it is suggested that in the donkey-in-horse model of pregnancy failure, the lack of endometrial cups may result in a lack of suppressor cell activity (Allen et al., 1987). The deficiency in both models of xenogeneic pregnancy failure has been related to a failure of trophoblast to recruit suppressor cells (Clark et al., 1984; Allen et al., 1987). This results in cytotoxic cell activation and infiltration into the embryo and subsequent susceptibility to anti-paternal transplantation immunity (Clark et al., 1986b).

A number of cell types with suppressor activity have been isolated from human and murine decidua. Human decidual cells (Parhar and Lala, 1986) and murine macrophages from the decidua (Tawfik et al., 1986) appear to suppress T cell reactivity by the release of prostaglandin E_2 (PGE_2)

which inhibits the production of IL-2 (Parhar and Lala, 1986). In 1986 (b), Clark et al. investigated the soluble suppressor activity released by decidual tissue in vitro. Activity was only obtained from non-T cells of pregnancy-associated decidua and not from hormone-stimulated endometrium or deciduoma cells. Clark et al. (1986c) found that indomethacin did not block production of suppressive supernatants showing that prostaglandin is not the mediator of suppression. HPLC studies of human decidual supernatant revealed peak immunosuppressive activity at two molecular weights, namely 43,000 and 21,000 daltons with a smaller peak of activity at 60,000 daltons (Daya et al., 1986). Clark et al. (unpublished) found suppressive activities in murine decidual supernatant with similar molecular weights. Indeed, PP-14 is similar in molecular weight to one decidual supernatant factor (43,000) and it also exhibits similar immunosuppressive activity (Bolton et al., 1986; Clark et al., 1987). The decidua derived inhibitor is not T cell dependent, is selective for IL-2 dependent cells and does not appear to be a ganglioside since the latter blocks the binding of IL-2 to its receptor whereas decidual derived inhibitor does not (Daya et al., 1986). The decidual inhibitor factor appears to inhibit the capping of IL-2 receptors but whether this explains the inhibition of T cell proliferation is unknown.

Other types of suppressive factor have been described. In 1983, Badet et al. obtained a 60,000 dalton factor from the decidua of syngeneically mated C3H mice and also from deciduomas in the absence of trophoblast. Hardt et al. (1981) identified a 50,000 dalton T cell-derived factor in the serum of mice which blocked the response to IL-2. To date, no data are available regarding the existence of similar factors in the horse.

The decidua associated anti IL-2 activity in the human and mouse has provided some insight into the mechanism of spontaneous abortion. Administration of a highly potent rat anti-mouse IL-2 receptor antibody to the xenogeneic pregnancy Mus caroli in Mus musculus was found to block totally the maternal mononuclear infiltration of Mus caroli foetuses resulting in the birth of large foetuses with apparently healthy placentae (Croy et al., 1987). This provides evidence that maternal killer cell infiltration of the foetus occurs due to a lack of IL-2 blocking and that maternal killer T cells accelerate embryo destruction. It is unknown at present whether a similar anti-IL-2 activity is associated with endometrial/placental tissue of species with epitheliochorial placentation.

Recent studies of spontaneous abortion suggest that a deficiency of local suppression in the decidua will lead to an increased susceptibility to para-immune effector cell systems in the uterus. This is reflected in the immunosuppressive properties found in murine pregnancy decidua supernatants. Jadus et al. (1986) reported the inhibition of IL-3 action preventing natural killer (NK) cell activation. Similarly, Clark et al. (1987) have observed an inhibition of macrophage activation to toxicity by lipo-polysaccharide (LPS) and interferon.

Finally, it is possible that suppressor cells may secrete growth factors as described in the immunostimulation hypothesis. The basis of the hypothesis is that maternal immune recognition of foetally derived cell surface antigens results in the secretion of factors that promote the growth of placental tissue, which in turn provides a fortified barrier to external damage from maternal immunity or microbes. Athanassakis et al. (1987) found that certain T cell lymphokines were capable of stimulating

placental growth and activation of foetally derived cells in vitro. It was found that the entire proliferative effect could be ascribed to lymphokines of the CSF family namely CSF-GM, CSF-1 and IL-3. IL-2 and B cell derived growth factor (BCDF) had no effect either alone or in conjunction with CSF-GM. Furthermore, the proliferating cells were found to be of the macrophage lineage (Guilbert, 1985). Anti-T cell antibody has been found to reduce trophoblast cell mass in vivo providing the specificity was anti-Lyt 2 and/or anti-Thy 1 (Athanassakis et al., 1987) It has been shown that decidua derived supernatants that contain suppressor activity can also stimulate placental cell growth in vitro (Athanassakis and Wegmann, 1986). Clark et al. (1987) suggest that the failure of xenogeneic trophoblast to recruit suppressor cells could also mean failure to recruit non-T cells that elaborate growth factors and this would lead to defective placental development and embryo death.

2.7.6.3 Placental Immunoregulatory Factors

Four placental immunoregulatory factors have been described in the literature and partially characterised. In 1977, Chaouat et al . reported that multiparity results in the emergence of suppressor T cells able to transfer hypo-responsiveness to a paternal stain allograft and to block maternal anti-paternal MLR and cell-mediated lympholysis (CML). The induction of these cells appeared to depend upon the activity of placental molecules and subsequently the term 'Suppressor Inducer Factor' (SIF) was introduced to describe the active moiety in the placental extracts or supernatants (Chaouat, 1987). This factor has been shown to induce suppressor cells of the GVH reaction (Chaouat and Chaffaux, 1984).

In 1984, Chaouat and Kolb reported that placental supernatants contain a product able specifically to block an MLR and CML. The supernatant can also enhance the survival of a murine foetal heart allograft (Chaouat, 1987). This factor has been termed the 'Afferent Suppressor Factor' (ASF).

Finally, placental resistance to NK and CTL-mediated lysis has been investigated. The anti-lytic property has been related to the production of CTL and NK inhibiting factors termed CTL-IF and NK-IF. The factors are different molecular entities as shown in Table 2.4.

Despite the above findings, there are problems in the interpretation of the data. Firstly, there is no real proof of their trophoblastic origin, i.e. a small percentage of decidual cells may be the producers. Decidual factors are different molecular entities but have the same properties (Clark and Chaouat, unpublished). Secondly, apart from some evidence for low NK activity in the peripheral blood of pregnant women, there is no conclusive evidence for any in vivo role during pregnancy (Chaouat, 1987). Thirdly, there is an intrinsic capacity of trophoblast populations to resist lysis and in some cases no CTL and NK-IF is detectable.

TABLE 2.4

Biochemistry of placental factors

Factor	Mol.wt (x1000)	pI	L. Lectin bind.	Tryps/Neuram Sens.	IgG bd
ASF	30-40	4.5-5	Yes(EL +)	+	- +
SIF	40-60	7	No	+	? +
CTL-IF	50-60	4.5-5	Yes(EL +)	+	+ ?
NK-IF	50-60	5.5-5	No	+	- ?

L. Lectin Bind., binding (and subsequent elution with alpha-methyl mannoside) to lentil lectin-coated Sepharose 4b; Tryps/Neuram.Sens: sensitivity (loss of activity) upon treatment with trypsin and neuraminidase; IgG bd, Binding to, and elution from (at acidic pH) mouse IgG-coated Sepharose 4b column (Chaouat, 1987).

CHAPTER -3-

PREGNANCY ASSOCIATED/SPECIFIC PROTEINS IN THE HORSE AND THEIR INTERACTION WITH THE ENDOCRINE SYSTEM

3.1 Introduction

Research into pregnancy associated proteins and hormones in the horse has been limited in comparisonto that into human pregnancy. The major focus of this chapter and of the research findings presented later in this thesis is on early equine pregnancy. In order to facilitate an understanding of the biological events that occur during early equine pregnancy, 'time-scale' figures (Figs.3.1, 3.2) have been drawn which summarize the events relevent to the content of this thesis.

3.2 The Establishment of Equine Pregnancy

During early equine pregnancy, stable placentation is not established until approximately thirty-seven days after ovulation compared to five to eight days in the human (Allen et al.,1973). The consequence of this is that throughout this period the conceptus is entirely dependent on uterine secretions for nourishment and protection. Indeed all animals with epitheliochorial placentation rely upon uterine secretions for development of the conceptus to a greater extent than do those animals with a haemochorial placenta. Many proteins are found in uterine secretions and these have been shown to serve as enzymes, carrier molecules for hormones, vitamins and minerals (Bazer et al., 1978), in fact these secretions have been likened to a complex embryo culture medium.

PRE-IMPLANTATION

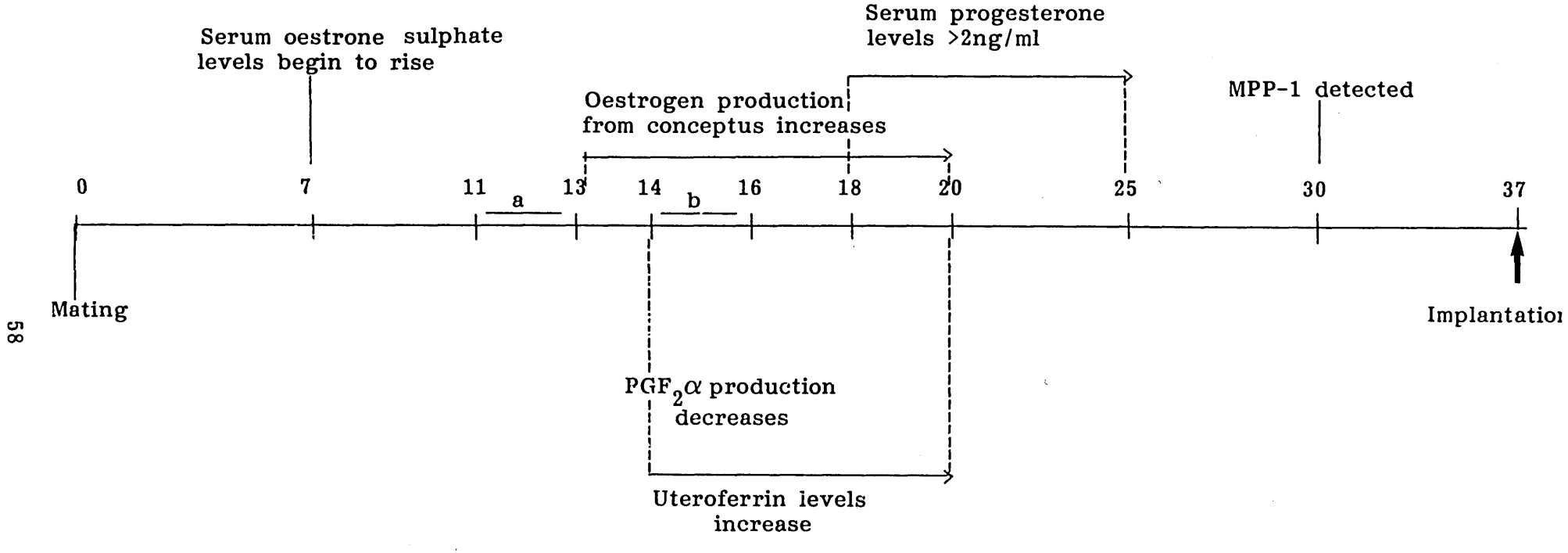

Fig.3.1 Biological Events in the Mare prior to Implantation

a,b: The suggested times for the maternal recognition of pregnancy.
(a):- Hershman and Douglas, 1979. (b):- Goff et al., 1987.

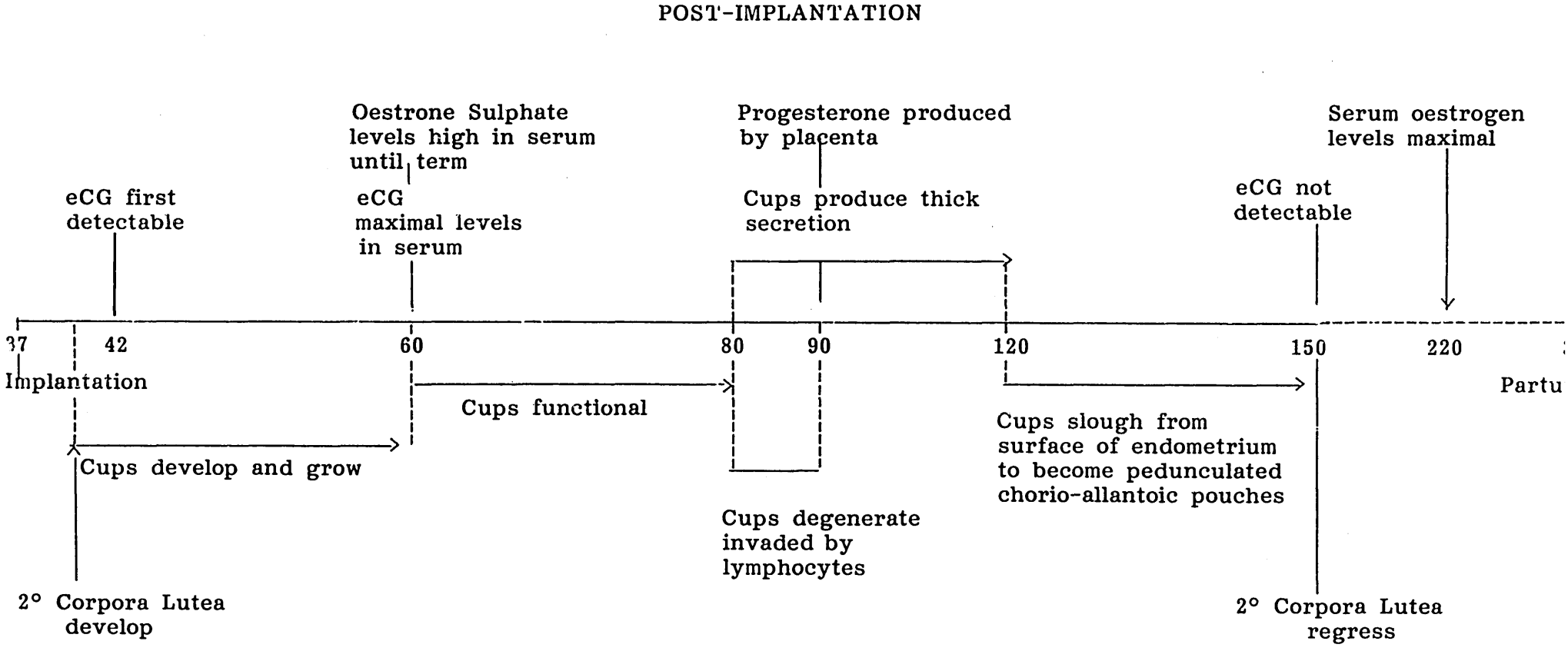

Fig.3.2 Biological Events in the Mare Post-Implantation

One area of equine reproduction receiving much attention in recent years is the study of the maternal recognition of pregnancy, those signals that inform the mare of the presence of an embryo, and the subsequent biological changes that occur. Knowledge of the way in which the embryo signals its presence could be of great benefit to the animal breeding industry in a variety of ways, the most obvious being the ability to diagnose pregnancy early, 14 to 16 days after ovulation. Furthermore, such information may clarify why a mare sometimes fails to recognize a viableembryo and also why pseudopregnancy occurs. As a consequence of this, much work has been done on the uterine fluid environment during the oestrous cycle and early pregnancy. It has been found that uterine secretory function progressively increases after ovulation and then decreases sharply after corpus luteum regression in non-pregnant mares (Zavy et al., 1978). It appears that the secretion of protein in the uterine lumen results primarily from the influence of progesterone, progesterone production and the protein content of the uterine lumen both increase markedly after ovulation and both decrease after luteolysis (Sharp, 1980). The increase in uterine protein content reflects uterine specific and non-uterine specific proteins which together produce an environment condusive to embryo survival and development. The overall message from such studies appears to be that the uterus secretes whatever components are necessary for the establishment of pregnancy every oestrous cycle and removes such secretory products if pregnancy does not occur. In the event of pregnancy, the corpus luteum is maintained (luteostasis) as are the protein components critical for embryo survival. The mechanism by which uterine secretory products are removed when pregnancy does not occur is uncertain. Uterine secretory activity is reported to decrease as progesterone decreases and thus the process appears to be progesterone dependent. Bazer and Thatcher (1977) proposed that uterine secretory

components exit the uterus by way of the venous drainage (endocrine secretion) at the time of luteolysis in the non-pregnant gilt but are maintained intraluminally (exocrine secretion) as part of the establishment of pregnancy. There is some evidence that this occurs in the mare based on a report by Douglas and Ginther (1976) who found that uterine venous plasma prostaglandin F_2 alpha (PGF) was reduced in pregnant pony mares compared with non-pregnant mares on day 14. PGF has been shown to have luteolytic properties (Section 3.4).

One interesting feature of the maternal recognition of pregnancy is the dual role of progesterone. Clearly, if a pregnancy is to be established and continue successfully, the corpus luteum must continue to secrete progesterone, but the irony is that progesterone also prepares the uterus for its role in luteolysis.

3.3 Equine Uteroferrin (acid phosphatase)

In 1972, Murray et al. demonstrated the presence of a basic glycoprotein with a purple coloration in porcine uterine secretions. Since then, the protein has become known as uteroferrin. In 1974, Schlosnagle et al. showed that uteroferrin had acid phosphatase activity and contained iron. Although uteroferrin has acid phosphatase activity, its main role is believed to be concerned with iron transport from mother to conceptus (Roberts and Bazer, 1980). Uteroferrin is in fact, one member of what may be a broad class of iron-containing phosphatases with unusual spectral properties which result from a novel type of di-iron active site (See Roberts and Bazer, 1984). As both the horse and pig have the same type of diffuse epitheliochorial placentation, it was suggested that both species may possess similar maternal-embryonic transfer systems for support of the conceptus.

In 1979, Zavy et al. chromatographed equine uterine luminal proteins and revealed 5 major fractions of approximate molecular weight 7,275,000 (I); 275,000 (II); 170,000 (III), 56,000 (IV) and 15,000 (V). It was found that peaks I, IV and V, when expressed as a percentage of the total protein, were significantly affected by day of the oestrous cycle whereas peaks II and III were not. Acid phospatase activity was found in fractions III and IV and was also significantly affected by day of the oestrous cycle. The enzyme had maximum activity in the late luteal phase which declined after luteolysis. In 1982, Zavy et al. further analysed the protein milieu of cyclic pony mares by two-dimensional polyacrylamide gel electrophoresis and found that a number of unique proteins, apparently of non-serum origin, appeared by the mid-luteal phase but disappeared by day 20 of the oestrous cycle. Furthermore, these proteins were maintained until at least day 20 of pregnancy. One of these pregnancy-associated proteins had a low molecular weight of 35,000 and was basic with an isoelectric point of 9.7. Characterisation of this polypeptide revealed that its properties were very similar to those of the porcine glycoprotein, uteroferrin, it had similar molecular weight, identical co-migration in two-dimensional electrophoresis systems (Zavy et al., 1982) and a similar Km (Michaelis-Menton constant) for acid phosphatase activity (McDowell et al., 1982). The proteins also cross-react immunologically and appear to be induced by progesterone (Zavy et al., 1978; Zavy et al., 1982). The equine uterine luminal protein in question has thus been called equine uteroferrin and it is believed to have the same physiological role as porcine uteroferrin, iron transport across the placenta to the developing embryo. The acid phosphatase enzymic activity of uteroferrin is easily monitored and has subsequently been used as an indicator of proteins synthesized and secreted by the progestational uterus. Equine uteroferrin has been found to increase in uterine

flushings during the luteal phase of the oestrous cycle, reaching a peak on day 14 in ponies and day 16 to 18 in horses (Sharp, 1980). After this pivotal stage in non-pregnant mares, uteroferrin decreases until acid phosphatase activity is no longer detected at day 20. In pregnant mares, however, the acid phosphatase activity of uteroferrin continues to increase until at least day 20, (Sharp, 1980). Experiments using explants of porcine endometrium have shown that porcine uteroferrin is synthesized by endometrial tissue (Basha et al., 1978). It was further reported that porcine uteroferrin was synthesized maximally in mid-pregnancy around day 60 [of a 115 day gestation] in the pig. Porcine uteroferrin production from late pregnancy endometrium (day 105) was, however, very low. The secretion of equine uteroferrin throughout gestation is thought to mirror the secretion pattern of porcine uteroferrin although this awaits clarification.

In 1987, McDowell et al. investigated the effects of progesterone and oestradiol on the secretion of equine uterine proteins. Exogenous progesterone was found to increase the total protein content of uterine secretions and also to increase the secretion of uterine specific proteins, as monitored by uteroferrin. Endometrial explants were used in this work suggesting that equine endometrial tissue secretes uteroferrin. In addition, it was demonstrated that oestrogen acts synergistically with progesterone to increase both total protein and uteroferrin in uterine secretions of mares.

3.4 Conceptus Secretory Products with Particular Reference to a Potential Antiluteolysin

Conceptuses produce a number of factors which may play a vital role in the establishment and maintenance of pregnancy, e.g steroids, prostaglandins and proteins. The continued secretion of progesterone is clearly

important in this context and thus its source of production, the corpus luteum, must be protected from any luteolytic activity. It has been noted that progesterone is particularly important in the induction of endometrial development and the secretion of endometrial histotroph (uterine secretion/milk) which appears essential in the nourishment of conceptuses having epitheliochorial fusion-type implantation (Schlafke and Enders, 1975).

The regression of corpus luteum function and the decrease in progesterone secretion have been related to an increase in uterine prostaglandin F_2alpha (PGF) synthesis and/or secretion (Zavy et al., 1978; Vernon et al., 1981), increased uterine venous PGF (Douglas and Ginther, 1976) and increased 15-keto-13,14-dihydro-PGF_2alpha otherwise known as prostaglandin F_2alpha metabolite (PGFM) (Neely et al., 1979). Furthermore, it seems that the maternal recognition of pregnancy involves primarily a mechanism that prevents luteolysis by preventing PGF synthesis or secretion. Non-pregnant mares during luteolysis (days 14 to 16) have been found to have higher concentrations of PGF in their uterine fluid and uterine venous plasma than did pregnant mares at the analogous stage of pregnancy.

In order to avert the luteolytic process, the embryo must be present during a critical period of the luteal phase. Hershman and Douglas (1979) demonstrated that flushing embryos from the uterus on day 14 resulted in a return to oestrous for the majority of mares, whereas flushing embryos from the uterus on day 16 resulted in corpus luteum maintenance. The requirement for the presence of the embryo coincides with the time of PGF secretion and expected luteolysis and it has been suggested that this time (day 14 to 16) should be referred to as the critical period for the maternal recognition of pregnancy. Further evidence for this was

presented by Vernon et al. (1979) who found that the time of maximal PGF secretion coincides with the time of maximum PGF binding by luteal cell membrane receptors. It was also reported that the ability of corpora lutea from pregnant mares to bind PGF was equivalent to that of corpora lutea from non-pregnant mares in oestrous. This suggest that corpus luteum maintenance in pregnancy is better explained by reduced PGF secretion than by altered PGF receptor function.

In 1981, Vernon et al. observed the apparent paradox of increased in vitro PGF production from endometrium of pregnant mares. In physiological terms, however, this was not surprising as the endometrium had been optimally primed for PGF secretion by virtue of its long exposure to progesterone. Berglund et al. (1982) investigated this phenomenon further and found that the co-culture of pregnant endometrium with conceptus membranes reduced PGF production. This inhibitory effect of conceptus membranes suggests the possibility that embryonic factors may be inhibiting PGF synthesis and/or release. These findings were corroborated by Sharp and McDowell (1985) who also reported that Banamine was able to block increased PGF production from endometrium in vitro proving that increased PGF concentrations reflected synthesis and release of the prostaglandin.

The nature of the signal from conceptus to the uterine endometrium inhibiting prostaglandin F_2alpha synthesis has not yet been elucidated. Zavy et al.(1979b) reported that the horse conceptus produces substantial amounts of oestrogens between days 8 and 20 pregnancy. Other workers have shown prolonged corpus luteum maintenance in mares treated with various oestrogens during dioestrus (Bery and Ginther, 1978). These findings are however controversial as others have found no such effects of oestrogen in mares. In 1983, Fazleabas and McDowell showed that day 12 to 14 equine

conceptuses incubated in vitro secrete a unique array of at least five proteins ranging in molecular weight from about 50,000 to greater than 400,000. Conceptus membranes incubated on day 15 or later, displayed entirely different pattern of proteins, and proteins of yolk sac origin such as transferrin and alpha-foetoprotein were dominant. In addition, after day 15 the chorioallantois produced an array of acidic proteins with the 2 dominant groups having estimated molecular weights of 28,000 and less than 15,000 and pIs of about 6.4 and less than 4, respectively. Day 14 conceptuses secreted either protein pattern or aspects of both, indicating that this particular day was the pivotal point As the critical period for the maternal recognition of pregnancy coincides with this pivotal day, it is suggested that the "early" proteins may be involved in the pregnancy recognition mechanisms (Sharp and McDowell, 1985).

In 1986, McDowell characterised proteins produced by cultured equine conceptuses and endometrium by 2 dimensional polyacrylamide gel electrophoresis (2-D PAGE) and fluorography of culture medium. Prior to day 14, there were two groups of isoelectric variants with molecular weights of 50,000 and 20,000 (approx.). After day 14, serum-like proteins were evident as reported by Fazleabas and McDowell (1983). Furthermore, it was found that the addition of high molecular weight conceptus secretory products (greater than 14,000) and low molecular weight secretory products (less than 14,000) decreased endometrial PGF production. In addition, endometrium from pregnant mares produced proteins similar to those produced by endometrium from diestrous mares. The maternal recognition of equine pregnancy has been suggested to be a process in which "the uterus is programmed to produce a given array of proteins that is not altered by the recognition and establishment of pregnancy. Rather, pregnancy permits the continued secretion of luteal

progesterone by a process of luteo-stasis which, in turn, allows the secretion of uterine specific proteins in a pattern that is pre-ordained" (Sharp and McDowell, 1985).

3.4.1 Oxytocin and the Maternal Recognition of Pregnancy

Oxytocin has been shown to have a role in luteolysis in both the cow (Fields et al., 1983) and the sheep (Wathes and Swann, 1982). As early as 1959, Arsmtrong and Hansel demonstrated that exogenous oxytocin could shorten the luteal phase of the oestrous cycle in the cow and that the luteolytic effect was mediated via the release of PGF. Very little work of this nature has been carried out in the mare.

In 1985, Betteridge et al. demonstrated that oxytocin stimulates the release of PGF around the time of luteolysis in the mare which suggests that oxytocin plays a role in luteolysis. It was suggested that the increasing response to oxytocin was due to the action of progesterone since progesterone priming of an ovariectomized mare for 14 days resulted in induction of responsiveness to oxytocin. It has recently been demonstrated, however, that the decline in the response to oxytocin seen after day 16 may not be due to the decrease in progesterone concentrations (Goff et al., 1987). It has been suggested that the increase in oestrogen concentration that occurs before ovulation may be involved in turning off the response to oxytocin, but this awaits clarification. Goff proposes that during the oestrous cycle decreasing progesterone and increasing oestradiol quickly inhibit the response to oxytocin. In the pregnant mare, it is thought that the release of PGF in response to oxytocin is reduced. Furthermore, Goff et al. (1987) found that in non-pregnant mares there was an increasing response to oxytocin between days 11 and 13 post-ovulation but during pregnancy this increase was abolished. On this basis, Goff suggests that the maternal recognition of pregnancy occurs

between days 11 and 13 and not between days 14 and 16 as suggested by Hershman and Douglas (1979). One theory regarding the mechanism involved, is that as increasing plasma oestrogen concentrations correspond to decreased responsiveness to oxytocin, then oestrogen produced by the embryo may maintain the corpus luteum in this fashion (Goff et al., 1987).

3.5 Oestrogen and Early Equine Pregnancy

Oestrogen production from the equine conceptus rises during the period between days 12 and 20 after ovulation, but may begin as early as day 6 (Paulo and Tischner, 1985). The possible luteostatic function of oestrogens has already been discussed but there are further features of these hormones worthy of mention in this context. Zavy et al. (1984) found that changes in concentration of oestrone and 17 beta-oestradiol occurring within the uterine environment were not discernable in plasma obtained from the peripheral circulation during this period. It was suggested that this may be due to the action of the sulfotransferase enzyme found in the endometrium. Mares have appreciable amounts of this enzyme between days 18 and 36 of pregnancy (Heap et al., 1982). Such an activation system could sulphate oestrogens before leaving the uterine environment so that they would appear in the peripheral circulation in their conjugated form. Perry et al. (1976) suggest that in the pig, oestrogens of conceptus origin are sulphated by the endometrium and transported to the ovary where it exerts luteostatic activity. It is possible that a similar mechanism exists in the horse. An increase in total oestrogens (conjugated plus unconjugated) has been shown to occur in the peripheral circulation of the mare by day 35 of pregnancy. Oestrone sulphate is one of these conjugated oestrogens, and its detection in mares is used as a pregnancy test (Parkes et al., 1977) [Section 3.11.1]. Both

oestrone and oestradiol are produced by the conceptus membranes (Heap et al., 1982; Zavy et al., 1984). Oestrone and oestradiol concentrations have been measured in the yolk sac during early pregnancy (Zavy et al., 1984). Both oestrogens increased between days 12 and 14 of pregnancy which mirrors a similar increase seen in uterine fluid and conceptus membrane incubations (Zavy et al., 1979). On days 12, 14 and 16, both oestrogens were comparable in concentration but by day 18, oestrone became predominant. A similar switch over also occurred in uterine fluid. Zavy suggests that oestradiol may be converted to oestrone and this may occur due to oestrogenic stimulation of the endometrium by the conceptus, resulting in increased 17-beta-hydroxysteroid dehydrogenase activity which catalyses the reaction. Such a mechanism does exist in the equine endometrium (Flood and Marrable, 1975). As oestrone sulphate increases during early equine pregnancy,it is suggested that the shift from oestradiol to oestrone may be the first step in preparation for the sulphation process.

In addition to having a potential role in the maternal recognition of pregnancy, hormones of conceptus origin may regulate secretion from the endometrium which have nutritional importance to the developing conceptus. This was illustrated by Zavy et al. (1982) who reported an increase in glucose, fructose, ascorbic acid and glucose phosphate isomerase activity within the uterine fluid of the mare during the early stages of pregnancy. Oestrogens secreted by the conceptus may be involved in regulating carbohydrate and amino acid flux from the maternal system into the uterine lumen and conceptus (Zavy et al., 1984). A mechanism such as this would assure nourishment of the conceptus during its 37 day pre-implantation period.

3.6 The Synthesis and Biological Functions of Equine Chorionic Gonadotrophin (eCG)

eCG (formerly known as pregnant mare serum gonadotrophin) is pregnancy specific and found in the mares bloodstream during the first half of pregnancy (Allen, 1969b). More precisely, it is first detectable in serum between days 35 and 41 of gestation, rises to a maximum concentration at about day 60 and this is followed by a steady decrease in concentration to become undetectable between days 100 and 150 (Cole and Hart, 1930; Allen 1969c).

eCG is produced by uterine structures, the endometrial cups. Endometrial cups were first described by Schauder (1912) and consist of a series of small ulcer-like, endometrial outgrowths in the pregnant horn of the uterus (Amoroso, 1952) forming in a circle around the developing foetal sac. The cups are initially present as small pale raised areas in the endometrium at day 38-40, then steadily grow for 30-40 days taking on their distinct saucer like shape due to continuing growth at the periphery and degeneration of the central regions of each structure. Allen (1970) has observed that after about day 80 they become increasingly pale and begin to release a sticky secretion which adheres to the surface of the overlying allantochorion. This exocrine phase-heralds the start of a steady degeneration which finally results in the necrotic cup tissue and admixed exocrine coagulum being sloughed from the surface of the endometrium between days 120 and 150. This sloughed material sits upon the surface of the foetal membranes where, as a consequence of gravity and other physical forces, it sinks towards the centre of the conceptus and so gives rise to the pedunculated chorioallantoic pouches described by Clegg et al. (1954).

eCG is a major component of endometrial cup secretions. This unique glycoprotein (mol.wt 70,000) consists of two non-identical subunits and its carbohydrate component is large, relative to that of other glycoprotein hormones, as it approaches 45% by weight (Moore and Ward, 1980). The molecule occurs in different forms depending on whether it is isolated from serum or from endometrial cups: the circulating form of the molecule appears to be larger, contain more carbohydrate and have different amino-terminal residues from that isolated from endometrial cups (Aggarwal et al., 1980). In addition, a number of authors have reported that the biological activity of eCG in the mare is similar to that of luteinizing hormone (LH) (Squires and Ginther, 1975; Moore and Ward, 1980). In 1979, Licht et al. reported that in other mammals, eCG has both LH and follicle-stimulating hormone (FSH) activity. Removal of sialic acid from eCG caused an increase in LH activity (Manning et al., 1987).

Other equids, including donkey (Bell et al., 1967) and zebra (King, 1965), also secrete a gonadotrophin during early pregnancy. In 1980, Aggarwal et al. isolated donkey chorionic gonadotrophin (dCG) from the serum of pregnant donkeys using the same methodology as that used for the isolation of eCG. The levels of dCG in pregnant donkeys are lower than the levels of eCG in pregnant mare sera (Allen, 1975b). Chemically, dCG was found to be similar to eCG in fractionation behaviour and glycoprotein nature. The donkey hormone was found, however, to have significantly less carbohydrate (31%) than had eCG (45%) and several differences were found in a comparison of amino-acid compositions. Unlike eCG, which has a considerable amount of FSH-like activity, dCG was found to have primarily LH-like biological activity, similar to hCG (Louvet et al., 1976). Stewart et al. (1977) found that hybrid mule and hinny conceptuses produce eCG

with an FSH:LH ratio approximately midway between those of the horse and donkey. It is a mixture of both eCG and dCG. Immunologically, dCG was found to significantly cross react in a homologous radioimmunoassay for eCG.

All CG values referred to in the following sections were calculated using a common immunoassay of eCG's regardless of the type of pregnancy under investigation.

3.7 The Endometrial Cup Reaction

The total lack of any direct physical connection between the cups and the overlying allantochorionic membrane initially led to the idea that the cups were maternal in origin representing some form of decidual response to the presence of the developing conceptus in the uterus (Clegg *et al.*, 1954; Allen, 1970).

In 1972, Allen and Moor established that specialised equine trophoblast cells recovered at a specific stage of gestation can produce large quantities of eCG *in vitro*. Only allantochorionic girdle cells were found to have this potential for eCG synthesis and these cells were found to exhibit the same distinctive morphological features characteristic of normal endometrial cup cells. Furthermore, Allen found that these trophoblastic cells secreted eCG over at least 180 days *in vitro*, whereas *in vivo*, eCG is secreted for only 80 days. The endometrial cups are therefore of foetal origin.

By day 36 after ovulation, the equine trophoblast is clearly divided into its invasive and non-invasive components. Where the enlarging allantoic and regressing yolk sac mambranes meet, a pale annulate band is formed that surrounds the conceptus (Allen, 1982). This chorionic girdle

separates from the foetal membranes and invades the underlying maternal endometrium between days 36 and 38. Once in the endometrial stroma, the cells quickly lose their pseudopodia, enlarge and start secreting eCG. The cells remain clumped together to form the endometrial cups (Allen et al., 1973). The cups can thus be described as highly specialized gonadotrophin-secreting foetal trophoblast cells which retain no physical connection with the remainder of the conceptus.

Throughout the entire lifespan of the cells, increasing numbers of lymphocytes and to a lesser extent, plasma cells and eosinophils accumulate in the endometrial stroma around the cups. Lymphocytes begin to aggregate immediately after the cups begins to develop and aggregation increases during the next 60-80 days resulting in a band of cells which appear to wall off the cup tissue from the surrounding normal endometrium. The numbers of lymphocytes in those aggregates have been shown to vary considerably between individual animals and to be related to the size of the endometrial cups, i.e. fewer lymphocytes are attracted to larger, broader cups than to smaller, more isolated structures. When the cups begin to degenerate, around day 80-90, the lymphocytes begin to invade the cup tissue and destroy the large cup cells (Allen, 1982).

3.7.1 Endometrial Cup Development and Foetal Survival in Intra-, Inter- and Extraspecific Equine Pregnancies

Female equids have the unique capacity to carry to term a variety of inter- and extraspecific pregnancies that differ markedly in genotypic and phenotypic characteristics from the surrogate mother (Allen, 1982; Kydd et al., 1985; Summers et al., 1987). The available data on endometrial cup development and foetal survival has recently been tabulated (Allen et al., 1987) and this is shown in Table 3.1.

Table 3.1

Endometrial cup development and fetal survival in intra-, inter- and extra-specific equine pregnancies

Fetal genotype	Mare genotype	Fetal:maternal karyotype (2n=)	Endometrial cup development and lifespan*	Outcome of pregnancy
Intraspecific mating				
Horse	Horse	64:64	+++ L	Term
Donkey	Donkey	62:62	+ L	Term
Grant's zebra	Grant's zebra	46:46	+ L	Term
Interspecific mating				
Mule	Horse	63:64	+ S	Term
Hinny	Donkey	63:62	++++ L	Term
Extraspecific embryo transfer				
Przewalski's horse	Horse	66:64	++ L	Term
Horse	Donkey	64:62	++++ L	Term
Horse	Mule	64:63	+++ L	Term
Donkey	Mule	62:63	+ L	Term
Grant's zebra	Donkey	46:62	+ S	Term
Grant's zebra	Horse	46:64	± VS	Term
Donkey	Horse	62:64	—	80% abort at Day 80–95

*Endometrial cups persist for: L = >60 days; S = 15–30 days; VS = <10 days.

(Allen et al., 1987)

A common feature of all successful intra-, inter- and extra-species pregnancies is invasion of foetal chorionic girdle cells into the endometrium at day 36 to 38. Endometrial cups exhibit two variables when the types of pregnancy are compared.

a) The amount of cup tissue that develops.

b) The lifespan of the cups:- this may reflect the intensity and success of the maternal leucocyte reaction in hastening the death of the cups (Allen et al., 1987).

It is evident from Table 3.1 that endometrial cup development and life span ranges from large and relatively long-lived cups in mares carrying horse conceptuses and donkeys carrying hinny and horse conceptuses to very small and transient cups in mares carrying zebra conceptuses. Allen et al. (1987) conclude that the paternal genome has a marked influence on the growth of the progenitor chorionic girdle and hence, on the amount of endometrial cup tissue that develops after invasion; regardless of changes in uterine environment resulting from embryo transfer, a much broader and more active chorionic girdle develops on the conceptus when a horse is the sire than when a donkey or Grant's zebra is the sire. It seems however that any conceptus that gives rise to any degree of endometrial cup development in the maternal uterus, has a chance of implanting normally and proceeding normally to term.

3.7.2 The Immunological Implications of the Endometrial Cup Reaction

Studies of the donkey-in-horse pregnancy as a model of foetal death has led to a greater understanding of the foeto-maternal immunological interactions in early gestation which protect the antigenically foreign foetus from potentially lethal maternal immune responses. It has been suggested that the invasion of chorionic girdle cells into the endometrium

to form endometrial cups provides a vital and temporally important antigenic stimulus to the mother that results in her becoming tolerant of any foreign foetal antigens that may subsequently be expressed at the foeto-maternal interface (Allen et al., 1987).

Antczak et al. (1987) have developed monoclonal antibodies which can distinguish between the invasive and non-invasive trophoblast. In this study, it was found that the expression of antigens by the invasive trophoblast was gradually lost during the development of the chorionic girdle. The modulation of these molecules may be indicative of a functional role related to cell-cell interactions (Antczak et al., 1987). In total, it appears that both paternally-derived alloantigens and trophoblast-specific molecules (Section 2.7.6) are presented for maternal immune recognition by the development of the endometrial cups and also that both types of antigens are concerned in eliciting the humoral and cell-mediated responses observed (Allen et al., 1987).

3.8 Equine Chorionic Gonadotrophin: A Pregnancy Specific Immunosuppressive Hormone

Another way in which endometrial cup development might suppress the maternal response to antigens expressed by the trophoblast during placentation is by the action of the principal secretory product of the cups: eCG. It is possible that the presence of eCG at high concentrations on the invasive trophoblast and in circulation may give a high negative charge to both trophoblast cells and maternal lymphocytes resulting in mutual repulsion between them. Such a mechanism has been described for hCG (Kirby, 1968).

Further support for this theory is the presence of the hormone at the time when the main foetal stimulus to the mother is greatest, i.e. during placentation it is secreted directly into the uterine lumen between the trophoblast and endometrial epithelium as an exocrine endometrial cup secretion.

eCG is also found in the lymphatic and blood circulation. Preliminary in vitro studies suggest that eCG may suppress lymphocyte proliferation in response to both the mitogen Con.A and the one way mixed lymphocyte reaction (Kydd and Allen - personal communication). It has been speculated, however, that this may be due to an immunosuppressive contaminant (cf EPF in human chorionic gonadotrophin preparations) (Rolfe et al., 1983). This is an area awaiting further clarification. Evidence of an immunosuppressive role of eCG in vivo is, however, not clear cut as eCG administration to a mare carrying a donkey conceptus offered no protection for the conceptus.

It is thus proposed that eCG alters the immunological activity of maternal lymphocytes in the blood and its presence in the uterine lumen contributes to the fibrinoid layer which coats the trophoblast cells thus masking the foetal antigens from maternal recognition. Preliminary in vitro studies on eCG suggest that it may have an immunosuppressive effect, particularly as a local effect at the maternal-foetal interface.

3.9 The Immunoprotection of Equine Pregnancy after Endometrial Cup Degeneration

The endometrial cup reaction lasts for less than 100 days, i.e. the cups have degenerated and eCG has disappeared from the circulation by mid-gestation. It is thought that other proteins take over the immuno-protective role of eCG during the second half of gestation. The most

likely source of these factors is the placenta which produces very large quantities of both phenolic and ring-B unsaturated oestrogens from day 90 onwards of gestation.

Progesterone has been shown to have immunomodulatory properties by both *in vivo* and *in vitro* techniques (Clemens *et al*, 1979; Stites and Siiteri, 1983) and thus this may play a significant role in the later stages of equine pregnancy. Progesterone and its 5 alpha-pregnane derivatives are secreted by the equine placenta from day 90 onwards (Raeside & Liptrap, 1975; Pashen & Allen, 1979). It is thus proposed that the immunoprotection of the equine foetus in the latter stages of pregnancy may be due to the local production of large quantities of progestogens and oestrogens.

3.10 Mare Pregnancy Protein 1 (MPP-1): a Pregnancy Specific Protein

MPP-1 was discovered by Gidley-Baird *et al*. in 1983. Allen (1979) had already shown by his work on luteal maintenance in the mare that by day 16 after ovulation the conceptus has exerted its first influence on maternal utero-ovarian function and this provided the impetus for an investigation into whether other pregnancy specific proteins existed in the horse and if one or more was measurable during pregnancy before the appearance of eCG.

Using an anti-pregnant mare antiserum adsorbed with stallion serum, Gidley-Baird *et al*. (1983) demonstrated two precipitates in two-dimensional crossed immunoelectrophoresis using serum from pregnant mares as the antigen. One of these precipitated in the beta-1 region andthe other in the alpha-2 region. By tandem-crossed immunoelectrophoresis, Gidley-Baird *et al*. (1983) showed identity between eCG and the alpha-2 mobile protein. In addition, it was found that the antiserum completely

inhibited the action of eCG in hypophysectomised mice. The beta-1 mobile protein was found in all of the pregnant mares tested and was detectable in the earliest samples taken at day 30. This protein designated mare pregnancy protein-1 (MPP-1) was not detected in stallions, geldings or non-pregnant mares and thus like eCG, may be pregnancy specific.

3.11 Pregnancy Testing in Mares

3.11.1 Serum Hormone Tests

a) Equine Chorionic Gonadotrophin (eCG)

eCG can be detected in the mare's blood by 42 days after conception. The use of eCG as a pregnancy test has not, however, been 100% successful, and more thoroughbred mares show detectable levels of eCG than actually give birth. The reason for this is that once the chorionic girdle cells have invaded the uterine stroma to form endometrial cups at around day 38, subsequent loss of the pregnancy, by whatever means, does not alter the normal sequence of development and regression of the endometrial cups (Allen, 1969b). High concentrations of eCG will thus appear in the blood for many weeks resulting in a pseudopregnancy. The other disadvantage of measuring serum eCG as an indicator of pregnancy is its late appearance. Serum eCG levels are, however, routinely measured in order to offer assurance that the mare has conceived and reached the stage when the endometrial cups have formed and begun producing gonadotrophin.

b) Progesterone

After ovulation, the corpus luteum produces progesterone and serum levels increase to greater than 2 ng/ml in two to three days. In non-pregnant mares in oestrous, the progesterone level drops sharply to less than 1 ng/ml when luteolysis occurs around day 16. In pregnant mares, the corpus luteum is maintained and levels remain elevated. Progesterone assays

can be used to detect elevated levels of the hormone during pregnancy that is from 18 to 25 days after ovulation. A proportion of oestrous cycles, however, are followed by a persistent corpus luteum (Stabenfeldt et al., 1974). Indeed, Allen et al. (1974) reported that during a breeding season, 93% of 362 non-pregnant, non-cycling thoroughbred mares had this condition. As a functional corpus luteum may keep serum progesterone levels high for up to 90 days a confirmatory pregnancy test is required.

The major information obtained from a progesterone assay is thus whether or not the corpus luteum has been maintained, which might be indicative of a pregnancy.

c) Oestrone Sulphate (ES)

Oestrone sulphate is a conjugated steroid hormone which, in pregnant animals, is secreted primarily by the conceptus membranes (Heap et al., 1982; Zavy et al., 1984; Kasman et al., 1987) [Section 3.5]. Serum ES concentrations have been shown to increase within a few weeks post-breeding in pregnant mares and to increase very sharply after 60 days to reach levels greater than 30,000 pg/ml at 80 days. Thereafter, these high levels were maintained throughout pregnancy (Sist et al., 1987). The mean ES serum concentrations in non-pregnant mares was approximately 750 pg/ml. Serum oestrone sulphate can thus be used as a reliable diagnosis of pregnancy and foetal viability from day 60 onwards. Concentrations of ES in milk can also be used for the diagnosis of pregnancy but the levels are lower than in serum and the test is only reliable after day 90 of gestation (Sist et al., 1987).

3.11.2 Pregnancy Diagnosis by Manual Examination and Other Techniques

Rectal palpation can be used to diagnose pregnancy, sometimes as early as day 19. A more reliable diagnosis by this technique, however, can be made at day 40 (Rossdale and Ricketts, 1974). In recent years, transrectal ultrasonography has made possible the detection of equine pregnancy by week two after ovulation with 98% accuracy (Chevalier and Palmer, 1982; Ginther, 1986; Woods et al., 1987).

3.12 Immunosuppression in Species with Epitheliochorial Placentation

Research to date into immunosuppressive mechanisms associated with the equine epitheliochorial placenta has been limited to a preliminary study on the cells which accumulate around the endometrial cups. As described in section 3.7.4, a population of cells with a suppressor function has indeed been identified (Kydd and Allen, 1986). Pregnancy associated immunosuppression has, however, been more widely investigated in other species exhibiting epitheliochorial placentation. A major motivation for such work has been due to the high rate of early embryonic mortality in domestic animals. In horses, early embryonic loss has recently been determined by ultrasound examinations of mated mares; it was found that 25.7% of conceptuses observed on day 11 were lost by day 25 (Ginther, 1985). In pigs, approximately 30% of the eggs fertilised at breeding are lost due to embryonic mortality and a high proportion of these are lost during the first 25 days of pregnancy (Pomeroy, 1960). In cattle, there is an embryonic mortality rate of 40% (Thatcher et al., 1985). Clearly, if these animals could be immunologically manipulated in early pregnancy so that the pregnancy success rate could be improved, then the financial input into farming and stud-breeding could be considerably curtailed.

This economic incentive has led to much research into the immunological maintenance of early bovine pregnancy. Bovine, ovine and porcine research in this area has generally been focussed on immunosuppression associated with uterine flushings in early pregnancy and with potentially immunosuppressive products of the conceptus in short term culture. In addition, preliminary work is in progress on uterine suppressor cells in the pig as well as in the horse (Croy et al., 1987).

3.12.1 Immunosuppression and Uterine Flushings

In 1977, Roberts demonstrated a dose-dependent inhibition of phytohaemagglutinin (PHA) stimulated blastogenesis in uterine flushings from a day 18 pregnant cow, whereas uterine flushings from a day 16 unmated cow and a day 13 pregnant cow were not inhibitory. More recent studies, however, report an inhibition of PHA stimulated lymphocyte blastogenesis by uterine flushings from both pregnant and non-pregnant cows (Segerson et al., 1984; Fisher et al., 1985). Similarly, uterine flushings from pregnant and non-pregnant ewes inhibited PHA stimulated blastogenesis and the MLR in a dose-dependent manner (Segerson, 1981). An immunosuppressive activity of porcine uterine flushings (pregnant and pseudo-pregnant) has been attributed to two progesterone-induced acidic proteins known as porcine acidic proteins (pAP) (Murray et al., 1978; Allen et al., 1981; Knight et al., 1973). Despite the above reports of immunosuppressive activity in uterine flushings, Fisher et al. (1985) were unable to show immunosuppression in bovine uterine vein serum and plasma. This suggests that the immunosuppressive substance(s) in the uterine lumen does not enter the uterine vein or, if present there, is diluted so that it cannot be detected under assay conditions. Similarly, serum taken from pregnant sheep has been shown not to inhibit the MLR or

PHA-stimulated blastogenesis (Miyasaka and McCullough, 1981; Segerson, 1981).

The report that the immunosuppressive porcine acidic proteins were induced by progesterone (Knight et al., 1973) led to speculation that the immunosuppressive activity in uterine flushings of other species may be associated with progesterone. Segerson (1981) found that the increase in ovine uterine flushing immunosuppression correlated with a rise in progesterone. Murray and Chenault (1982) demonstrated that progesterone itself is not immunosuppressive to bovine lymphoblastogenesis and consequently suggested that progesterone may influence immune function indirectly rather than directly by inducing the synthesis of immunomodulatory proteins or glycoproteins. In 1985, Sherblom et al. investigated the relationship between serum progesterone and immunosuppression in cows. Serum samples were taken from progesterone-oestrogen treated ovariectomised cows and from control ovariectomised cows. The average progesterone level in treated animals rose from 1 ng/ml (day 0) to plateau at 5 ng/ml (days 12 to 36). Immunosuppression was observed in serum samples in which the circulating progesterone concentration was in the normal physiological range (4 to 6 ng/ml). Lymphocyte proliferation appeared to be a function of rising progesterone concentrations because as the progesterone level reached a plateau, immunosuppressive activity returned to control levels. Furthermore, a significant difference in the degree of immunosuppression between the treated and non-treated animals occurred when PHA was used as the mitogen but not when pokeweed mitogen was used. This phenomenon suggests that the observed immunosuppression was directed against T rather than B lymphocytes. In the same report, sialic acid and sialyl transferase activity was measured during bovine pregnancy and a close correlation was found with progesterone levels, i.e.

all three factors reached peak levels at the same time (day 12). Immunosuppressive activity, however, was found to peak at day 8. Sherblom et al. (1985) suggest that progesterone may stimulate the synthesis of glycoproteins which are immunosuppressive and sialyl transferase may be a marker for a late phase in the process.

The identity of immunosuppressive substance(s) in sheep and cow uterine flushings has not been elucidated but available data indicates that they are >3,500 Mol.wt in the sheep (Segerson, 1981) and >1,000 Mol.wt in the cow (Fisher et al., 1985) determined by sample dialysis. Small molecules such as prostaglandin E and progesterone are thus eliminated as potential candidates for the immunosuppressive function. One possibility, however, is alpha-foetoprotein (68,000 Mol.wt). Roberts (1977) and Fisher et al. (1985) found that uterine flushings from pregnant cows inhibit lymphocyte stimulation in a manner similar to that reported for alpha-foetoprotein. In 1982, Butler et al. detected two 'pregnancy specific' proteins by immunoelectrophoresis using antisera developed to homogenates of bovine extraembryonic membranes. These proteins were present in extracts of bovine placental membrane from foetuses aged 25 to 270 days. One of the proteins had a similar molecular weight and isoelectric point to alpha-foetoprotein and also yielded a reaction of identity with bovine alpha-foetoprotein by immunodiffusion. The other protein, designated by Butler as 'protein B', has not been identified but appears to be pregnancy specific. Janzen et al. (1982) detected an alpha-foetoprotein in the bovine uterine lumen by RIA at days 14 to 22 of pregnancy. In 1984, Segerson et al. partially characterised uterine luminal secretions from pregnant and non-pregnant cows. Polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulphate-PAGE revealed that bovine day 17 samples consisted of three uterine specific cathode migrating bands that were

composed of several proteins/polypeptides, each with a molecular weight of less than 48,000. Segerson observed that the percentage composition of two of all three bands was greater in the pregnant than in the non-pregnant state. In addition, there have been reports of pregnancy specific proteins in the cow. Laster (1977) reported a 50,000 to 60,000 Mol.wt pregnancy-specific protein present by 15 days post-mating. Bartol et al. (1981) found four proteins present in uterine flushings by day 19 of pregnancy but absent on day 19 of the oestrous cycle. Segerson et al. (1984) conclude from these data that the greater immunosuppressive activity of uterine protein secretions for pregnant and for non-pregnant cows within day 17 uterine flushings may be due to a greater quantity of uterine specific protein(s), the appearance of pregnancy specific protein(s) or an activation, synergism etc. of these macromolecules. The suppressive factor(s) for non-pregnant cows is presumably of endometrial origin and of endometrial and(or) conceptus origin for pregnant cows.

3.12.2 Immunosuppression and Conceptus Products

In 1983, French and Northey demonstrated that media from cultured 18 day old bovine embryos inhibited PHA-induced incorporation of thymidine into bovine lymphocytes. They found that the substance had a molecular weight of between 500 and 10,000, it was only produced by live cells, it was not toxic to lymphocytes, acts in a partially reversible manner and shows some species specificity. This work was further developed by Fisher et al. (1985) indicating that the Mr was between 500 to 1,000. The embryo culture medium (ECM) decreased tritiated uridine incorporation by proliferating bovine lymphocytes suggesting that the ECM was inhibiting RNA synthesis. The effect on uridine incorporation was, however, less than the effect on tritiated thymidine incorporation indicating that RNA synthesis inhibition was secondary to decreased blastogenesis.

Furthermore, ECM added 20 to 30 hours after PHA was as suppressive as if added at the time of addition of PHA. This latter phenomenon suggested that ECM was not simply interfering with the binding of PHA to the cell surface or binding to PHA itself, making it unavailable for interaction with the cell surface. A similar immunosuppressive mechanism may also exist in the sheep. Extracts of day 25 sheep conceptus membranes have been shown to inhibit mitogen-stimulated blastogenesis and the activity was reported to reside in the <10,000 molecular weight fraction (Staples et al., 1983). In 1982, Masters et al. reported that preattachment ovine and porcine conceptuses in culture secrete a high molecular weight (>660,000) acidic glycoprotein (HMWGP) which is relatively resistent to proteolysis. In 1987, Murray et al. reported that HMWGP purified from incubation medium of preimplantation elongating ovine (day 16 and 17) and porcine (day 16) conceptuses was immunosuppressive. HMWGP inhibited tritiated thymidine incorporation into lymphocytes stimulated by PHA and also the two way MLR in a dose dependent manner. It is of interest that both the pig and sheep conceptuses secrete chemically-similar glycoproteins shown to be immunosuppressive. Murray et al. (unpublished) have also identified a similar glycoprotein in the incubation medium of day 17 bovine conceptuses which also has immunosuppressive activity. Murray et al. (1987) conclude that the secretion of a biochemically related glycoprotein may represent a common mechanism whereby the conceptuses of ungulates influence maternal immune reactivity within the uterus. In addition, mouse embryos and embryonal carcinoma appear to secrete similar HMWGP's (Muramatsu et al., 1979) to the one reported by Murray et al. (1987).

3.13 Platelets and Pregnancy

The rational behind the input of platelet studies into this project is twofold. Firstly, it has recently been suggested that the maternal recognition of pregnancy in humans and laboratory animals is associated with a transitory thrombocytopenia. Secondly, a number of pregnancy associated proteins play a role in the inhibition of platelet aggregation, particularly during the later stages of pregnancy.

3.13.1 Platelets and the Maternal Recognition of Pregnancy

The establishment of a successful pregnancy has been shown to be heavily dependent on a signal from the embryo which results in the prolonged maintenance of the corpus luteum. Changes in levels of pregnancy-associated proteins and hormones during this period are used to diagnose pregnancy. Prior to this, however, the embryo has been effecting changes in the maternal physiology for days. The earliest changes reported are a change in the properties of circulating maternal lymphocytes and a significant thrombocytopenia. The latter is defined as a significant reduction in the absolute numbers of platelets below the normal level. O'Neill (1985a) demonstrated that an early pregnancy-associated thrombocytopeniawas reported to occur as an initial maternal response to conception in humans and mice, both animals with haemochorial placentation. This thrombocytopenia occurs within 6 hours of fertilisation and is maintained for the first few weeks of pregnancy. Platelet numbers are reduced due to increased activation and consumption demonstrated by an enhanced platelet factor III activity during the thrombocytopenia period.

Platelet activation occurs only in the presence of a viable embryo and the extent of the activation can be correlated with the number of embryos present in the reproductive tract. The platelet activation is presumably

caused by a soluble factor produced by the embryo. O'Neill (1985a, b) further demonstrated that this thrombocytopenia was not an immunological reaction to antigenically alien embryos since syngeneic matings and transfer of parthenogenetically activated eggs produced the same result. The factor responsible for the thrombocytopenia has been partially purified and shown to have similar chemical, biochemical and physiological properties to the potent platelet activating factor 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphocholine (O'Neill, 1985c). Consequently, it has been called 'embryo derived platelet activating factor' (EDPAF).

O'Neill et al. (1985, 1987) looked at EDPAF and early pregnancy associated thrombocytopenia as a means of monitoring embryonic viability in women undergoing embryo transfer. Evidence was produced demonstrating that only those embryos producing EDPAF in vitro caused early pregnancy associated thrombocytopenia (EPAT) after embryo transfer and only patients displaying EPAT had a successful transfer. In addition, it has been shown in rodents that the inhibition of the activity of EDPAF by specific antagonists prevents implantation which further exemplifies the crucial role of this factor in the establishment of pregnancy (Spinks and O'Neill, 1987).

The only other very early embryonic signal so far described is an 'ovum factor' which induces the generation of early pregnancy factor (EPF) activity in maternal serum during the preimplantation stage of pregnancy (Morton et al., 1980; Cavanagh et al., 1982) (Section 2.7.6.1c). In 1985(c), O'Neill noted circumstantial evidence for similarities between 'ovum factor' and EDPAF. One example of this is that the partial characterisation of 'ovum factor' has shown it to be a low molecular weight molecule(s) with a strong tendency to associate with carrier proteins (Cavanagh et al., 1982) and in these physical characteristics, it is similar to PAF (Synder, 1985). Platelets play a central role in many

immune responses and thus may have a role in modulating the maternal immune response in pregnancy. This modulatory role has been demonstrated in that the removal of maternal platelets with anti-platelet antisera results in an inhibition of implantation (Gasic and Gasic, 1970) and that factors released by platelets promote blastocyst outgrowth (O'Neill et al., 1985). Cavanagh et al. (1982) demonstrated that the injection of oestrous mice with culture fluid from fertilised eggs resulted in EPF activity and that the factor responsible was the partly characterised 'ovum factor'. In 1986, Orozco et al. found that when synthetic PAF was injected into mature female mice during dioestrus, proestrus or oestrus, it also induced the expression of EPF activity detectable in the sera of the animals within one hour of injection. Neither ovum factor nor PAF are active in the rosette inhibition test (RIT) but is believed that they act as a primary signal, inducing and interacting with secondary factors which act to generate the RIT-active EPF species from preexisting inactive precursors (Clarke and Wilson, 1985). The above studies indicate that the ovum factor responsible for the induction of EPF-activity in the periimplantation stage of pregnancy may be the same as the EDPAF which is responsible for the altered maternal platelet physiology at this time. There has been no research into EPAT in animals with epitheliochorial placentation. The detection of EPF activity in this species indicates, however, that a similar mechanism may exist.

3.13.2 Pregnancy and Platelet Aggregation

In the placenta, particularly the haemochorial, there is much pooling of blood in the placental blood sinuses and thus coagulation may be expected to occur. As the occurrence of widespread placental thrombosis is rare, it seems likely that the placenta protects itself by a platelet anti-aggregatory mechanism. It is well known that the walls of many blood

vessels contain prostacyclin which is a very potent platelet anti-aggregatory agent and the structure of the placenta indicates that its platelet anti-aggregatory properties may be attributed to its vascularity.

In 1977, Myatt and Elder found that human placental extracts inhibited ADP-induced platelet aggregation. The inhibition was prostacyclin-like, as the inhibitor was labile at 37 ^{o}C and neutralised by the prostacyclin synthetase inhibitor, tranylcypromine. In 1982, however, Dembele-Duchesne et al. presented evidence that prostacyclin was not the active inhibitory agent in placental extracts when they failed to detect 6 keto-prostaglandin F_{1}alpha, the major stable metabolite of prostaglandin in either extracts or in the medium when placental microsomes were incubated with arachidonic acid. Hutton et al. (1980) added further weight to this claim when they reported that placental extracts specifically inhibited platelet aggregation induced by ADP, whereas umbilical artery-derived prostacyclin inhibited a number of proaggregatory agents. In addition, Hutton et al. (1980) found that the aggregatory activity of the ADP was removed after incubation of ADP with the placental extracts, i.e. the extracts were degrading the ADP. This degrading activity was found to be considerably higher in the placenta than in the myometrium, umbilical cord or foetal membranes. The effect of the extracts was mimicked by purified human placental alkaline phosphatase but the heat lability of the anti-aggregatory activity suggested another enzyme. In 1987, O'Brien et al. confirmed that human placental extracts are capable of inhibiting specifically ADP-induced platelet aggregation by approximately half. Furthermore, it was found that platelets fail to undergo complete aggregation but, in fact, disaggregate after an initial response. In this recent investigation, the ADP-ase activity of the placental extracts was

shown not to be affected by pH whereas heat stable alkaline phosphatase has maximum activity at pH 9.0; this effectively eliminated the enzyme as a potential anti-aggregatory candidate. O'Brien et al. (1987) also found that there was a deficiency of the inhibitor in some pregnancies with abruptio placentae and intrauterine growth retardation. In 1984, Maki et al. described an anti-aggregatory placental protein with different properties to those reported above. The inhibitor was high in molecular weight (450,000) and inhibited platelet aggregation induced by ADP, collagen and adrenalin but not ristocetin. Furthermore, the inhibitor was stable at room temperature for 5 days and devoid of phosphatase activity. It appears from the above reports that unimpeded retroplacental blood flow may depend upon the local inhibition of platelet aggregation.

Platelet aggregation inhibitors and anti-coagulants work together in protecting the foetus from a hazardous thrombosis. Uszynski (1979) reported a placental dialysable peptide inhibitor of both thrombin and urokinase. In 1984, Maki et al. described a coagulation inhibitor which was not dialysable, having a molecular weight of 45,000 and had no anti--urokinase activity. Recent work on human pregnancy associated proteins has revealed that at least 2 of them may have roles as anti-coagulants, namely PP5 [placental protein 5] (Soma et al., 1985) and PAPP-A [pregnancy associated plasma protein-A] (Bischof et al., 1982). Both of these proteins have an affinity for the heterogenous sulphated glycosaminoglycan heparin which is itself a potent anti-coagulant. This property has proved useful in the isolation and purification of the proteins. To date, there is no available data on the inhibition of equine and bovine platelet aggregation by placenta derived inhibitors and also on pregnancy associated anti-coagulants.

CHAPTER -4-

MATERIALS AND METHODS

4.1 Samples

Blood serum samples were taken from equids throughout pregnancy by Dr W R Allen at the Equine Fertility Unit, 307 Huntington Road, Cambridge. The types of pregnancy were as follows:

(a) Normal Horse Pregnancy

(b) Transferred Horse Pregnancy

(c) Normal Donkey Pregnancy

(d) Transferred Donkey Pregnancy

(e) Hybrid Donkey-in-Horse Pregnancy

The transferred pregnancies were established by the surgical implantation of a fertilised egg (washed from the uterus of a donor mare) into the uterus of a recipient mare. Equine placental samples, taken during the course of various operations on horses at various stages of pregnancy, and uterine flushings were also obtained from the Equine Fertility Unit.

Blood serum samples from 13 performance horses before and after mating were obtained from RIA UK (Sunderland). Samples were taken from day 0 to day 12 where day 1 referred to the day when the mare was sired by a stallion. Both successful and unsuccessful pregnancies were investigated blind, pregnancy being determined by an ultra-sonic scan at day 90.

Equine and bovine term and aborted placental material was obtained from two sources:

Dr L Wood at the Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, New Haw, Weybridge, Surrey

Dr C J Green, Veterinary Surgery, 129a Newbold Lane, Broomhill, Sheffield.

Fresh horse blood for lymphocyte and platelet studies was obtained from two sources:

Dr P Dixon, Churchfield Veterinary Centre, 29 Sackville Street, Barnsley

Dr C J Green, Veterinary Surgery, 129a Newbold Lane, Broomhill, Sheffield.

Fresh bovine blood for platelet studies was obtained from Woolley Brothers Abattoir, Mosborough, Sheffield, under the supervision of Mr P Hutchinson.

4.2 Protein assay

Protein was determined quantitatively using the method of Ohnishi and Barr (1978). This simplified method of protein determination combines the method of Lowry et al. (1951) with the Biuret method and results in a more stable colour complex. This enables many samples to be developed at the same time and enhances reproducibility. A further advantage of this method is that the reagent can be stored at room temperature for several months.

To 0.8 ml of the sample, 3.2 ml of BS7 reagent [Appendix II] was added. The solutions were mixed and left for 10 min at room temperature. After this period, 0.1 ml of Folin and Ciocalteau phenol reagent was added and quickly mixed. Samples were incubated for a further 20 min at room

temperature to allow the colour to stabilize and were then read at 600 nm against a blank made up from treating 0.8 ml of water in the same way as the sample.

A calibration curve was produced using bovine serum albumin (BSA, fraction V) dissolved in sodium acetate buffer (0.1 M; pH 4.8) [Appendix I]. 0.8 ml of each standard (0-1000 ug/ml) was treated in the same way as the sample. All standards and samples were determined in duplicate.

4.3 Development of Antisera against Proteins in Horse Pregnant and Non-Pregnant Serum

4.3.1 Immunization

Samples used were early pregnancy serum (6 weeks) obtained from Dr W R Allen and virgin mare serum obtained from Dr L Wood. Two New Zealand white rabbits were immunized, one with early pregnancy serum and the other with virgin mare serum. The immunization procedure was as follows:

Equine blood serum was emulsified in an equal volume of Freunds complete adjuvent and 1 ml of the emulsion was injected subcutaneously at four sites along the neck and back of the animal. Following this primary immunization, the first booster was given 3-4 weeks later. Further boosters were given routinely at 2-3 week intervals.

4.3.2 Bleeding

Approximately 10 days after the second and subsequent booster, 25-30 ml of blood were taken from a marginal ear vein. The rabbit was wrapped in a towel with its head protruding. The ear was wiped with xylene to induce vasodilation. The margin of the ear was streaked with Vaseline to prevent

the spreading of blood and a longitudinal cut (approx. 2 mm) was made in the vein with a sharp sterile scalpel blade. The blood was collected by gravity drip into a plastic universal container.

4.3.3 Preparation of Antiserum from Whole Blood

Blood was allowed to clot by standing at room temperature for approximately 2 h. The clot was then carefully separated from the vessel wall (ringed) and allowed to retract at 4°C overnight. Blood serum was then removed using a Pasteur pipette and centrifuged at 1000 x g for 15 min The remaining clear supernatant was the antiserum.

As repeated freezing and thawing is known to cause protein denaturation and eventual loss of antibody activity, the antiserum was frozen in 3.0 ml aliquots at -20°C.

4.4 Immunological Screening of Antisera

4.4.1 Two-Dimensional Crossed Immunoelectrophoresis

Two-dimensional crossed immunoelectrophoresis (Weeke, 1973) was used to screen antisera for the presence of antibodies against known and unknown serum proteins.

1% agarose (Sigma - Type II medium EEO) was made up in tris-barbitone buffer (pH 8.6) [Appendix II]. The 1% agarose solution was heated to 90°C in a steamer to give a clear molten agarose gel. The gel was then placed in a water bath regulated 60°C to prevent it solidifying (agarose gel becomes solid at temperatures < 56°C). Molten agarose was poured on to 8 x 8 cm glass plates (10 ml agarose per plate). Once the gel had set, sample wells were punched along one side of the plate (3 wells per plate). One of the samples (serum antigens) was mixed with tracker dye (bromophenol blue) and 1 µl of each sample was applied to each well. The

samples were electrophoresed usingan LKB 2217 Multiphor, until the bromophenol blue spot had traversed the plate. The current used for 3 plates was 20 mA (constant current) and the time taken to complete the first dimension was about 2.5 h. After this period, the first dimension gel was divided into three strips so that each strip contained a sample well and the separated components of the sample. Each strip of gel (approx. 2.5 x 8.0 cm) was transferred to the cathodic end of a clean glass plate by sliding the strip of gel carefully off the plate onto another using a clean razor blade. The glass plates were placed on a horizontal surface and 7 ml of antibody-containing gel was poured onto the remainder of the plate. Volumes of antisera used were generally 100 µl or 150 µl per 7 ml of agarose. Once the gel had set, the plates were transferred to the Multiphor tanks and the gel was connected with the buffer by means of filter paper wicks (5 pieces thick). The separated components of the initial sample were electrophoresed at 20 mA constant current (3 plates) into the antibody containing gel. This second dimension was allowed to run overnight (16-20 h). Throughout the entire process, the gels were kept cool by means of a water circulator (LKB 2209 Multi Temp) at 10-20°C. Once the second dimension was complete, the peaks could be visualised by lateral illumination but in most cases the plates were stained. On completion of the second dimension, the plates were pressed, soaked in 0.1M NaCl for 2 x 1 h, stained with 0.05% Coomassie in acetic acid: methanol:water (50:40:10) for 10 min and then destained in acetic acid:methanol:water (10:20:70) for 30 min. The plates were then dried in a stream of warm air.

Samples of blood serum taken from ponies and mares at various stages of pregnancy including before and after mating were electrophoresed against rabbit antisera raised against pregnant horse serum (anti-pregnant horse

serum) and compared with virgin serum samples run against the same antiserum. In addition, the same samples of equine serum were run against rabbit antiserum raised against virgin horse serum (anti-virgin horse serum) and compared with the above. Pregnant donkey serum was also electrophoresed against the anti-pregnant horse antiserum to investigate the degree of between species cross-reactivity. Serum samples taken from a donkey-in-horse pregnancy was also electrophoresed against the same antisera. Placental extracts were electrophoresed against the anti-pregnant horse antiserum in order to investigate the origin of observed pregnancy-associated proteins.

4.4.2 Tandem Crossed Immunoelectrophoresis

Tandem crossed immunoelectrophoresis (Kroll, 1973) was used to investigate the identity of proteins in different samples as visualised by two-dimensional crossed immunoelectrophoresis. Two circular application wells were punched out in the same track in the first dimensional agarose electrophoresis gel with an intercentre distance of 8 mm. The samples to be compared were each placed in one of the application wells. Two-dimensional crossed immunoelectrophoresis was then performed as previously described. The fusion of precipitin lines in the final pattern is indicative of protein identity between the two samples.

4.5 Adsorbed Antisera

Antiserum raised against pregnant horse serum was adsorbed with virgin horse serum to remove these specificities not related to equine pregnancy. This was achieved by treating the antiserum with virgin horse serum covalently linked to Sephacryl S-300 prepared by the borohydride reduction method as follows:

(a) Oxidation of the Gel

15 ml (settled volume) of Sephacryl S-300 in a 50 ml plastic centrifuge tube (volume measured after allowing to settle overnight) was washed thoroughly with 2 l of distilled water. Washing was achieved by topping up the centrifuge tube with distilled water and thus suspending the gel followed by gentle centrifugation. The supernatant was discarded and the process repeated. The washed gel was resuspended in 50 ml of 5mM/l sodium m-periodate in 0.1 M sodium acetate buffer pH 5.0. The reaction was carried out with end-over-end mixing for 1 h at room temperature. The excess periodate was reacted by the addition of 5 ml 10% (v/v) glycerol with further end-over-end mixing for 1 h. The gel was washed thouroughly with 0.1 M sodium bicarbonate as described above. The oxidised Sephacryl S-300 was using for coupling to protein.

(b) Coupling of Virgin Horse Serum to the Oxidised Gel

The protein content of virgin horse serum was determined by the Ohnishi-Barr method. The serum sample was diluted in 0.1 M sodium bicarbonate to a protein concentration of 5-8 mg per ml of gel (approx. 1 in 4 to 1 in 5 dilution). 15 ml (settled gel volume) of oxidised Sephacryl S-300 was resuspended in 20 ml of the dilute serum in 0.1 M $NaHCO_3$ pH 9.0. This suspension was mixed by end-over-end rotation overnight at room temperature. After the reaction, the uncoupled protein solution was recovered from the gel by thoroughly washing with 2 l of phosphate buffer pH 7.4. The volume of each wash was recorded and samples taken of each for protein determination. The amount of protein bound to the gel could then be calculated.

(c) Borohydride Reduction

The reacted Sephacryl S-300 was resuspended in 0.5 M phosphate-buffered saline pH 7.4 and allowed to settle for 30 min. The supernatant was removed and kept for protein determination. The gel was then washed with an equal volume of phosphate-buffered saline to remove the majority of unbound protein and thus this supernatant was also kept for a protein determination. The solid phase was then resuspended in 500 mg of sodium borohydride in a final volume of 100 ml and reacted with occasional stirring for 30 min at room temperature. After this period, the solid phase was washed alternately in 0.25 M citrate buffer (pH 4.0) and 0.25 M Tris-HCl (pH 8.5), approximately four times with each buffer. All washes were kept for a check on the removal of further unbound protein. Finally the gel was resuspended in phosphate-buffered saline.

4.6 Specificity Test of Adsorbed Antisera

Adsorbtion was considered complete if no precipitin lines were formed when virgin horse serum was used as the antigen and electrophoresed against the adsorbed antisera.

A more rapid test of specificity was also adopted utilising the technique of counter-immunoelectrophoresis on glass plates or microscope slides. 1% agarose gel (High EEO) was poured onto the glass surface and allowed to set. Pairs of 4 µl wells were punched in the gel 1.5 cm apart, one well being situated towards the cathodic end of the plate and one towards the anodic end. Antigen was placed in the cathodic wells and adsorbed antisera was placed in the anodic wells. The plates were transferred to

Multiphor tanks and the gel was connected with the buffer by means of filter paper wicks (5 pieces thick). The samples were electrophoresed at 20 mA constant current for 2.5 h using barbitone buffer (pH 8.6).

During electrophoresis the antigens move towards the anode in the normal fashion. The immunoglobulins, however, move towards the cathode because IgG has a slight positive charge at pH 8.6. and also due to the electro-endosmotic effect of the agarose on electrophoresis (Agarose- High EEO). Electro-endosmosis in agarose occurs due to the presence of ionisable groups in the gel that become negatively charged on electrophoresis. Agarose contains sulphonic acid groups which are responsible for this negative charge. The natural tendency of the negative charges is to move towards the anode, but this is impossible since the medium is stationary. To counter the force generated, a movement of positively protonated water molecules (H_3O^+) occurs towards the cathode. Subsequently, the electrically neutral gamma-globulin fraction of serum moves towards the cathode.

4.7 Precipitin Line Immunization

This is a technique of producing monospecific or oligospecific antisera against protein(s) visualised as a precipitin line in an agarose gel after two-dimensional crossed imunoelectrophoresis, i.e. specific antigen-antibody complexes in the gel are used as immunogen (Kroll, 1981).

A two-dimensional crossed immunoelectrophoretic run was completed and the peaks were viewed by lateral illumination. After identification of a peak of interest, the agarose gel was blotted with filter paper under a slight pressure to remove non-precipitated antigens and to reduce the agarose gel to a thin but not completely dry sheet. The specific precipitin line was cut out of the gel by means of a Linocutter. Strips of agarose gel

containing the precipitin line of interest were washed in 5 l of 0.1 M NaCl overnight to remove unprecipitated protein (antigen). The precipitin lines were then placed in a centrifuge tube with fresh 0.1 M NaCl and centrifuged at 15,000 g for 10 min to ensure that all non-precipitated antigen and weakly associated antigens had been removed from the agarose. The washed agarose gel strips containing the immunocomplex were solubilised by suspension in 1 ml of 6 M KI. The solubilisation process was aided by sonication of the sample in an ice bath. After complete solubilisation, 0.5 ml of the solution was mixed with an equal volume of Freunds complete adjuvent and emulsified. The initial 1 ml suspension contained approximately 2.5 cm of the initial precipitin line. To ensure enough immunogen was immunized, 1.0 ml of the emulsion was injected subcutaneously. The immunization protocol was as previously described (Section 4.3.1).

4.8 Heparin Affinity Chromatography

Heparin is a naturally occurring sulphated heterogenous glycosaminoglycan and since its discovery by McLean in 1916, much evidence has accumulated which demonstrates that heparin functions as a powerful anticoagulant. Twenty percent of the total number of circulating proteins in human plasma have an affinity for heparin, which can be demonstrated by investigating those plasma proteins which bind to immobilised heparin in heparin affinity chromatography (Teisner et al., 1983). Furthermore, two human pregnancy associated proteins appear to have an affinity for heparin, namely PAPP-A (pregnancy associated plasma protein-A) and PP-5 (placental protein-5) [Section 3.15].

4.8.1 Chromatographic Procedure

Two types of affinity media were used, namely heparin-sepharose and heparin-ultrogel. A 10 cm column was packed with the heparin solid phase. The affinity media was washed with 10 column volumes of distilled water. Distilled water was used both to wash and to elute unbound protein from the column so that fractions collected from the column could subsequently be concentrated by lyophilization and the resulting concentrate investigated by immunoelectrophoresis. High salt concentrations would otherwise interfere with subsequent immunoelectrophoresis. The flow rate of the column was adjusted to 12 ml/h and 3 ml fractions were collected. The eluate level on the column was lowered to the surface of the affinity medium, the pump switched off and 1 ml of pregnant equine serum was carefully applied on to the affinity medium using a Pasteur pipette. The pump was switched on until the sample had just entered the affinity media when 2 ml of distilled water was applied to the column. The column was then filled with distilled water and the pump and fraction collector switched on.

The absorbance of each fraction eluting from the column was measured at 256 nm against a distilled water blank to test for protein. All the fractions with measurable optical densities were collected and numbered. The column was washed until no further protein was detected.

Proteins bound to the heparin-Sepharose and heparin Ultrogel were eluted at the same flow rate with a linear salt gradient ranging from 0.2 M NaCl to 1.0 M NaCl in distilled water. As before, elution was monitored at each salt concentration at 256 nm against the appropriate blank. All absorbance measurements were taken using the LKB Ultrospec spectrophotometer. Once all the originally bound protein had been eluted from the column, all the samples with an absorbance reading above 0.600

were freeze dried after the pooling and dialysis of homogenous protein peaks. The samples were redissolved in one tenth of the original volume and run as the antigen source in two-dimensional crossed immuno-electrophoresis. The affinity column was regenerated by thoroughly washing with distilled water.

4.9 Lymphocyte Blastogenesis

4.9.1 Preparation of Placental/Uterine Extracts

All placental and uterine extracts were homogenised in phosphate buffered saline (PBS) containing a small amount of the protease inhibitor-phenylmethylsulphonyl fluoride [PMSF]. 10 mg of PMSF was dissolved in 1 ml of ethanol to give a saturated solution of PMSF. 100 µl of this PMSF solution was added to 100 ml of PBS.

Samples of placental and uterine tissue were taken from fresh equine and bovine placentae as a consequence of the termination of a pregnancy, post-mortem or after normal delivery. The tissue was chopped up as finely as possible using sharp surgical scissors and a razor blade and added to the PBS with PMSF to give about 100 mg of wet tissue per ml of solution. The solutions were then homogenised using an electric homogeniser while maintaining the samples at low temperature in an ice bath. After homogenisation the samples were centrifuged at 2,000 g for 30 min to remove all debris. For use in lymphocyte blastogenesis studies, these samples were still found to contain too much sediment for filter sterilisation and were thus centrifuged at 18,000 g for 30 min. The resulting supernatant was dialysed against 0.05 M PBS for 24 h. After dialysis the samples were centrifuged again at 18,000 g to remove any precipitated protein. The protein content of each sample was then determined by the method of Ohnishi-Barr (Section 4.2) and all samples

were diluted to the same protein concentration using sterile PBS. All samples used in lymphocyte cultures were filter sterilised through 0.45 micron Millipore membranes before use. Doubling dilutions were prepared in sterile PBS.

4.9.2 The Two-Way Allogeneic Mixed Lymphocyte Reaction (MLR)

(a) Isolation of peripheral blood lymphocytes from the blood of a horse

Whole blood was obtained by venepuncture from two non-inbred horses. Blood was taken from the jugular vein of each horse into 10 ml Vacutainers each containing 200 units of lithium heparin as anticoagulant. Lymphocytes and mononuclear cells were then separated from the whole blood by the method of Boyum (1968). Each blood sample was diluted with two volumes of Dulbecco's modification of Eagles medium containing 20 mM/l HEPES (DMEM). Alternatively, the blood was allowed to sediment under gravity for 40 min at room temperature and the lymphocyte rich plasma drawn off and diluted with two volumes of DMEM. 9 ml of lymphopaque, specific gravity 1.084 [Appendix I] was aseptically dispensed into sterile 25 ml universal containers (8 per horse). 15 ml of diluted blood or plasma was carefully layered on to the lymphopaque in each universal using a 5 ml adjustable pipette, previously cleaned with ethanol fitted with a sterile tip. The samples were then centrifuged at 300 x g (calculated at the interface of the serum and lymphopaque) for 30 min at room temperature. Within the sterile surroundings of a laminar air flow cabinet the clear supernatant was removed (using a sterile plastic tip) down to the opaque band of white cells at the lymphopaque interface. The band of white cells was removed from every gradient and placed into separate universal containers for each horse.

Approximately 25 ml of DMEM was added to each cell sample and the cells were resuspended by gentle inversion. The cells were then centrifuged at 150 x g for 15 min and the supernatants discarded. The cells, visualised as a small pellet at the bottom of the universal, were resuspended in 1 ml of DMEM by slowly circulating the DMEM above the pellet using a sterile disposable pipette tip attached to a 1 ml adjustable pipette. The whole washing procedure was repeated a further twice and the cells were finally suspended in 1 ml of DMEM containing 0.04 M $NaHCO_3$, 10% controlled process serum replacement-2, 2 mM/l glutamine, 200 ml of 5000 iu/ml penicillin and 5000 µg/ml streptomycin. This supplemented DMEM was referred to as $DMEM^+$.

(b) Estimation of viable cell numbers

An estimation of the number of viable cells isolated from each horse was determined using the trypan blue exclusion method. 25 ul of cell suspension was transferred into a disposable plastic tube with 25 ul of trypan blue (0.2% in physiological saline). The cells were left at room temperature for a 10 min incubation period and then small aliquots of the suspension were introduced into a double chamber improved Neubauer haemocytometer. Using a light microscope and a x40 objective, the number of viable cells were counted. The plasma membrane of a viable cell does not permit the entry of non-electrolyte dye substances and thus the unstained cells were counted as viable, i.e. non-viable cells stain blue. Cell numbers were estimated utilizing the following formulae:

$$\text{No of cells/ml} = \frac{\text{No of cells counted x 25 x } 10^4}{\text{No of triple ruled squares used in count}}$$

The samples used for the cell count were discarded and the remaining cells diluted to a final concentration of 1×10^6 cells/ml in $DMEM^+$ or 2×10^6 cells/ml in $DMEM^+$ depending on the number of viable cells.

(c) Setting up mixed lymphocyte cultures

Cell cultures were prepared from a stock cell suspension of 1 x 10^6 cells/ml and were incubated in a 96 well microtitre plates (Falcon Laboratories). 200 µl of a cell suspension from one donor horse was added to 6 microtitre plate wells (2 x 10^5 cells per well). Each of the next 6 wells contained 2 x 10^5 cells from the second donor horse. These represented control cultures. Equal volumes of the cell suspensions obtained for the 2 individual donor horses were then mixed together and 200 µl of this lymphocyte culture (2 x 10^5 lymphocytes per well) was added to the remaining wells. Control cultures contained 50 µl of DMEM and experimental cultures contained 50 µl of sample. All samples were incorporated into the assay in triplicate wells.

The peripheral wells of the plate were not used as sample wells but were filled with 200 µl of DMEM with bicarbonate in order to maintain the humidity around the cultures. The plates were then incubated for 5 days at 100% humidity at 37°C in 5% CO_2 in air in a Hereaus CO_2 incubator.

(d) Pulsing of cells with tritiated thymidine (^{3}H-Tdr)

After 5 days of culture, 1 µCi of ^{3}H-Tdr (90 Ci/mM) in a total volume of 25 µl (diluted in DMEM) was added to each well except three zero hour control wells containing mixed lymphocyte cultures. The cultures were pulsed under sterile conditions and than incubated for a further 24 h. The zero hour controls were pulsed 24 h later just prior to harvesting.

(e) Termination and harvesting of cultures

After this final 24 h incubation period, the cells were harvested onto glass fibre filter paper, either manually using 5% trichloroacetic acid (TCA) containing 30 mM/l pyrophosphate, or by the use of a Skatron semi-automated cell harvester.

For the manual termination of the cell cultures, the contents of each well were aspirated into plastic disposable tubes on ice. A 200 µl volume of trypsinising solution (0.05% trypsin, 0.02% EDTA in physiological saline) was then added to each well in order to free the adherent cells. After a 30 min incubation period at room temperature, the wells were once again aspirated into the appropriate cell suspensions on ice. 1 ml of ice cold 5% TCA containing 30 mM pyrophosphate was added to each cell suspension and incubated at either room temperature for 1 h or 4°C overnight, in order to precipitate the DNA. The DNA precipitate was isolated by filtration of the cell suspension through glass fibre filter paper using a Millipore sintered glass filter assembly attached to a vacuum line. Prior to filtration, the filters were soaked in TCA, placed into the vacuum filter holder after which 5 ml of ice cold TCA was dispensed onto it. Each sample in turn was vortex mixed and tipped into the filter holder containing the TCA. The tube was washed with 2 x 1 ml volumes of TCA, the contents being dispensed into the filter holder each time. The vacuum was applied and the filter allowed to drain after which 2 x 5 ml volumes of TCA were added to the filter holder followed by 5 ml of ice cold ethanol. Once the filter had drained, the vacuum pump was switched off and the filter papers transferred to scintillation vials and allowed to dry for a minimum of 30 min. The extent of ^{3}H-Tdr incorporation into the DNA of each culture was assessed by liquid scintillation counting using an LKB Rackbeta 1212 for a 10 min period. The scintillation fluid was either cocktail 'O' scintillant (0.3% PPO, 0.01% POPOP in toluene) or 'Optiphase Hisafe'.

A majority of the cell harvesting was performed using the Skatron semi-automatic cell harvester. All samples were harvested onto glass fibre filter paper. The filter paper was allowed to dry overnight and then

punched out into plastic disposable vials. 3 ml of scintillant was added to each vial and the vials were counted for 10 min in a LKB Beta-Counter as described above.

4.9.3 The Mitogenic Stimulation Assay (MSA)

Peripheral blood lymphocytes (PBL's) were isolated from whole blood as previously described (Section 5.9.2a). The cell suspension was diluted with $DMEM^+$ to a concentration of 1 x 10^6 cells/ml. 100 µl of cell suspension was added to each well (1 x 10^5 cells/well) in addition to 50 µl of mitogen, 50 µl of sample and 50 µl of $DMEM^+$. The control cultures contained DMEM in place of the sample and the background stimulation was assessed by incubating cells in the absence of mitogen, i.e. 100 µl cell suspension, 100 µl DMEM and 50 µl $DMEM^+$. Zero hour controls were also set up by pulsing 3 wells just prior to harvesting. These control wells contained cells and mitogen but no sample. This procedure also serves as an assessment of contamination due to unincorporated ^3H-Tdr. The mitogen used in this work was concanavalin A (Con.A) at a final concentration of 2 µg/ml.

The plates were incubated for 72 h at 100% humidity, 37°C in 5% CO_2 in air. 6 h prior to termination, the cultures were pulsed with ^3H-Tdr (1 uCi) and on termination were harvested using a Skatron semi-automatic cell harvester and counted as described above.

4.9.4 Cytotoxicity Test

The cytotoxicity test is used as a safeguard against sample toxicity being mistaken for immunosuppression in the MLR and the MSA. Cultures of lymphocytes from one donor (2 x 10^5 cells/well) were incubated with each sample over a 6 day period. Each culture was set up in triplicate. The cells were counted every 24 h using the Trypan Blue exclusion method

(Section 4.9.2b). No more than 2 extracts were taken from any one culture in order to avoid a reduction in cell numbers obscuring the results. The number of viable cells was recorded as a percentage of the total numbers of cells in each well and a significant decrease in this percentage was indicative of sample toxicity.

4.9.5 Conditioned Medium

Conditioned medium is the supernatant from an MLR and contains lymphokines and other factors produced by stimulated lymphocytes. MLR cultures were incubated over a 6 day period in a 96 well microtitre plate as previously described. At the end of the incubation period, the cultures were aspirated, pooled and centrifuged to sediment the cells. The cell free supernatant was removed and hence referred to as conditioned medium. In both the MLR and MSA, 50 µl of conditioned medium was added to each culture containing immunosuppressive samples in order to investigate whether the immunosuppression could be reversed by the secreted lymphocyte factors. The number of cells per well had to remain the same and thus the original dilution of the cells was adjusted to account for this. In the MLR, the cell suspension was diluted to a concentration of 2×10^5 cells/150 µl (1.34×10^6 cells/ml) and consequently 150 µl of this suspension was added to each well. In the MSA, the cell suspension was diluted to 1×10^5 cells/150 µl (2×10^6 cells/ml) and 50 µl of this suspension was added to each well. The volumes of the other additives were therefore as previously described.

4.9.6 Delayed Addition of Samples

This is a means of finding out whether a sample with a known immunosuppressive activity inhibits the activation stage of lymphocyte proliferation or a later stage. Samples were added to MLR and MSA cultures after various incubation times, i.e. 24, 48, 72, 96 h for the MLR and after 24, 36, 48 and 72 h for the MSA.

4.10 Equine Hormone Assays

A number of samples used in the lymphocyte culture work were assayed for equine chorionic gonadotrophin (eCG) and progesterone. These assays were carried out by members of the research team at the Equine Fertility Unit, in Cambridge. A double antibody (sandwich) type of enzyme linked immunosorbent assay (ELISA) was used for both hormones. Progesterone was assayed using a kit obtained from Hoechst U.K. Limited. The eCG ELISA was developed at the Equine Fertility Unit and the details of this assay are as follows.

4.10.1 ELISA Assay for eCG (PMSG)

All buffers used in this assay are listed in Appendix III. The basic components of the assay were an unlabelled antibody (anti-eCG), a labelled antibody (anti-eCG conjugated to alkaline phosphatase via a lysine link) and a standard, the 2nd International Standard-serum gonadotrophin.

A Nunc immuno-plate was coated with purified polyclonal anti-eCG at a concentration of 5 μg/ml; 120 μl was added to each well. The plate was incubated at 37°C for 16 h in a closed box. After this period, the plate was washed with 4 x 200 μl of wash buffer and glaze. A range of standards was prepared from the original standard serum gonadotrophin in standard buffer (0; 0.5; 1.0; 5.0; 10.0; 50.0; 100.0; 200.0 iu/ml). All standards and samples were diluted 1:50 with dilution buffer. 20 μl of sample or

standard were added to appropriate wells and 100 µl of dilution buffer was added to every well after which the plate was incubated for 1 h at 37°C. The wells were washed with 4 x 200 µl of assay wash buffer to remove unbound eCG. After draining, 100 µl of labelled anti-eCG (polyclonal) was added to each well and this was followed by a further 1 h incubation period. The wells were washed and drained as before, this time removing any unbound labelled anti-eCG, and 100 µl of substrate was added to each well. After a further 20 min incubation period, 100 µl of amplifier was added to each well and the plate was left until the colour development in the 200 iu/ml standard gave an optical density reading between 1.5 and 2.0. The colour development was stopped with 100 ml of 0.4 M sulphuric acid and the plate was read and calibrated using a Titertek uniskan spectrophotometer with a computer link. The lowest detectable amount using this assay was 0.5 iu/ml and its working range was from 3 to 200 iu/ml.

4.11 Platelet Aggregation Studies

4.11.1 Preparation of Placental Extracts

As described previously for lymphocyte work (Section 4.9.1).

4.11.2 Equine Blood Sample Collection and Preparation

Whole blood was obtained by jugular venepuncture into 4.5 ml vacutainers containing 0.105 M sodium citrate as anticoagulant. The ratio of blood to anticoagulant was 9:1. The vacuntainer was gently inverted to prevent separation of the blood, after which it was centrifuged at 50 x g for 10 min at room temperature. After centrifugation, there was approximately 1 ml of plasma per 10 ml of cell suspension. This plasma has a high

platelet content and is thus referred to as platelet-rich plasma (PRP). The PRP was carefully removed using a plastic transfer pipette and expelled into a plastic tube.

A second method for preparation of PRP was investigated. Blood was stood at room temperature for approximately 10 min. When the erythrocytes had sedimented leaving about 1 ml of plasma at the top of the tube, the plasma was removed as PRP. Once the PRP had been removed, the remaining blood was centrifuged at 1,500 x g for 10 min. The plasma left had no or very small number of platelets and was retained as platelet poor plasma (PPP). PRP and PPP preparation was carried out as soon as possible after sample collection (within 30 min).

A marked feature of horse blood is the natural clumping of erythrocytes leading to the separation of cells and plasma within minutes. Since the sedimentation rate of horse blood is very rapid and resuspension by inversion does not always provide an even distribution of platelets because of spontaneous aggregation, the platelet number in the PRP can vary (White et al., 1975). Furthermore, the number of platelets in horse PRP is, on the average, lower than the number in human PRP and thus the magnitude of platelet responses is lower (White et al., 1975).

4.11.3 Human and Bovine Blood Sample Collection and Preparation

Human and bovine erythrocytes do not sediment as quickly as equine erythrocytes therefore a higher centrifugation speed is required for the preparation of PRP. Whole blood was centrifuged at 250 x g for 10 min.

4.11.4 Platelet Aggregation Studies

After preparation, PRP was equilibrated at 37°C for 15 min. Platelet aggregation studies were carried out using collagen and ADP as agonists. Aggregation was assessed using a platelet aggregometer (ADG Instruments

Ltd - Model 1002). The aggregometer measures the turbidity of the PRP which is continually stirred in a cuvette. Changes in optical density are constantly assessed as a means of detecting the effects of aggregating agents. When platelets aggregate the optical density decreases and when the aggregates disperse the optical density increases.

300 µl of PRP was incubated with 60 µl of 0.85% saline in an aggregation cuvette for 3 min. 40 µl of agonist was added and the aggregation recorded by means of a chart recorder (Kip-Zonen) connected to the platelet aggregometer. Maximum deflection of the recording pen was pre-set with platelet-rich and platelet-poor plasma. Thus, as platelets aggregated and fell out of the light path, transmission increased and was recorded as an upward swing of the pen. To assess the inhibitory effect of the placental extracts, the saline was replaced by a sample. The platelet aggregation response was recorded for approximately 5 min.

4.11.5 Collagenase Assay

One agonist used to induce platelet aggregation was collagen. The inhibition of collagen induced platelet aggregation by a sample may be due to the breakdown of collagen by collagenase.

The substrate for collagenase in the assay was p-phenylazo-benzyloxycarbonyl-Pro-Leu-Gly-Pro-Arg. Collagenase splits the hydrophilic substrate specifically between leucine and glycine resulting in a coloured lipophilic fragment and a non-coloured tripeptide. The change in optical density of the fragment at 320 nm per unit time is a measure of the enzyme activity.

$$\text{PZ-Pro-Leu-Gly-Pro-Arg} \xrightarrow{\text{collagenase}} \text{PZ-Pro-Leu} + \text{Gly-Pro-Arg}$$

2.0 ml of substrate solution (Appendix II) was added to 0.4 ml of calcium chloride solution (0.1 M) in a test tube and allowed to stand for 5 min at 25°C. After this period, 0.1 ml of sample (placental extract) or standard (collagenase) was added, mixed and incubated for exactly 15 min at 25°C. 0.5 ml of incubation mixture was then quickly transferred to prepared test tubes filled with 1.0 ml citric acid solution (25 mM; pH 3.5) and 5.0 ml ethylacetate. The test tubes were shaken for 15 sec and the ethylacetate phase was transferred by pipette into test tubes containing 300 mg of anhydrous Na_2SO_4. These test tubes were shaken and the contents transferred into plastic cuvettes, after which the change in absorbance was measured. The 'volume activity' and 'specific activity' of the enzyme can be calculated using the following formulae:-

$$\text{Volume activity} = \frac{2.5 \times 5 \times E}{21 \times 0.1 \times 0.5 \times 15} \quad \text{(U/ml sample)}$$

$$\text{Specific activity} = \frac{\text{Volume activity}}{\text{Concentration}} \quad \text{(U/mg protein)}$$

(E= E sample - E blank; E= Extension (absorbance))

CHAPTER -5-

IMMUNOCHEMICAL STUDIES OF EQUINE PREGNANCY

5.1 Introduction

Published data on the presence of serum pregnancy specific/associated proteins in the horse is limited. The most widely reported protein is equine chorionic gonadotrophin (eCG), first detectable in the maternal circulation at day 42 of gestation (Allen and Moor, 1972). In addition, a single report describes another pregnancy specific protein designated mare pregnancy protein-1 (MPP-1), detectable in the earliest sample investigated which was day 30 of gestation (Gidley-Baird et al., 1983). Furthermore, serum based pregnancy tests have been shown only to be reliable after day 60 of gestation when elevated levels of the conceptus derived steroid, oestrone sulphate, can be detected (Sist et al., 1987). Prior to this time, serum pregnancy tests are only tentative indicators of pregnancy - elevated progesterone levels are used to confirm a continued activity of the corpus luteum (days 18-25) and elevated eCG levels are used to confirm conception (days 42-90).

The rationale behind this section of work was to investigate the presence of other pregnancy specific or pregnancy-associated serum proteins which may be detectable in the peripheral circulation during early gestation. Subsequently, it was hoped to evaluate the use of one or more of these factors in the detection of equine pregnancy prior to day 60 of gestation. This Chapter describes the detection of a new pregnancy specific serum protein which is present prior to day 30 of gestation. The first half of the Chapter deals primarily with the detection of the new protein, its

quantitation and investigates the potential use of this protein as an indicator of pregnancy. The source and purification of the protein are dealt with in the latter half of the Chapter.

5.2 The Detection of a New Pregnancy-Specific Protein in the Horse by Two-Dimensional Crossed Immunoelectrophoresis

Rabbits immunized with both pregnant and non-pregnant horse serum developed antibody titres measurable in immunoprecipitation techniques. Two-dimensional crossed immunoelectrophoresis of equine serum obtained from pregnant or non-pregnant animals, run against rabbit anti-pregnant horse antisera resulted in the formation of many precipitin lines.

Serum samples were taken from two mated horses (H1, H2) at different times from day 2 to day 12 after mating where day 0 represented the day that the mare was sired by a stallion. Pregnancy was investigated in both animals by an ultrasound scan carried out at day 90 after mating. Pregnancy was confirmed in one whereas the second horse (H2) failed to become pregnant. Serum samples were also taken from a third horse (H3), which was subsequently revealed to be pregnant by succesful outcome of the pregnancy, at later gestational ages ranging from day 21 to day 239. Serum samples from all the above animals were electrophoresed against rabbit anti-pregnant horse antisera. Examination of the immunoelectrophoretic plates (Figs 5.1, 5.2, 5.3) revealed a precipitin line representing a protein which appeared to be associated with the pregnant state of the horse (Compare H2 Fig.5.2 with H1 and H3 Fig.5.1 and 5.3). The protein had beta$_2$-electrophoretic mobility and was first detected in the peripheral blood serum at day 6 in a pregnant horse (Fig.5.1B). This protein was not present in serum from the horse that failed to become pregnant after mating (H2) (Fig.5.2), in serum from a gelding (H4) (Fig.5.4A), in serum from a virgin mare (H5) (Fig.5.4B) or in serum from a mare that

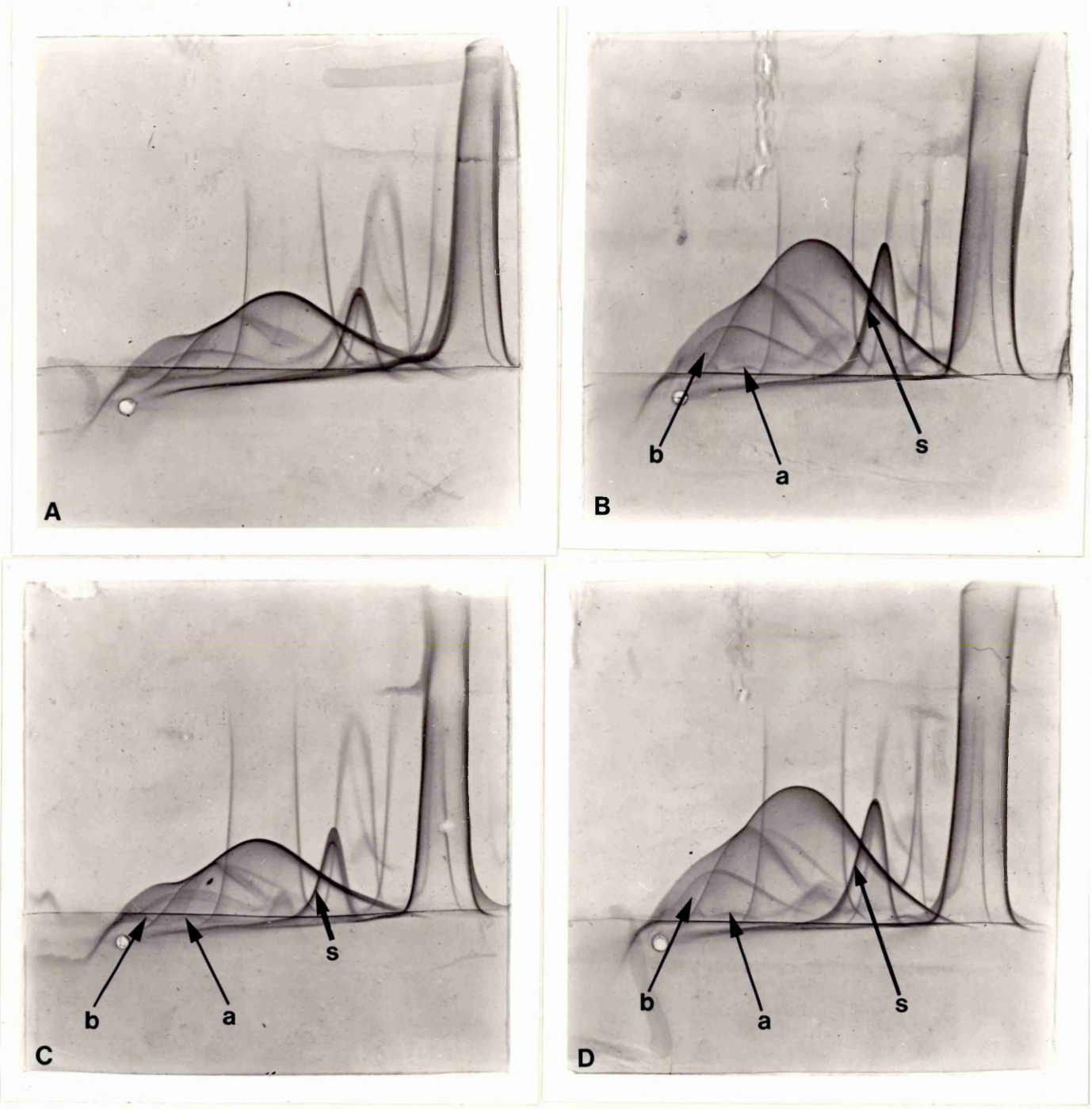

Fig.5.1 Two-dimensional crossed immunoelectrophoresis of serum taken from a pregnant mare (H1) at intervals during the first 10 days after mating. [a= β_2-hors.PP, b= γ_2-hors.P, antisera= rabbit anti-pregnant horse] A (day 3), B (day 6), C (day 9), D (day 10).
Beta$_2$-hors.PP is first detectable at day 6. Pregnancy was confirmed by ultrasound scan at 90 days.

s= α_2-macroglobulin

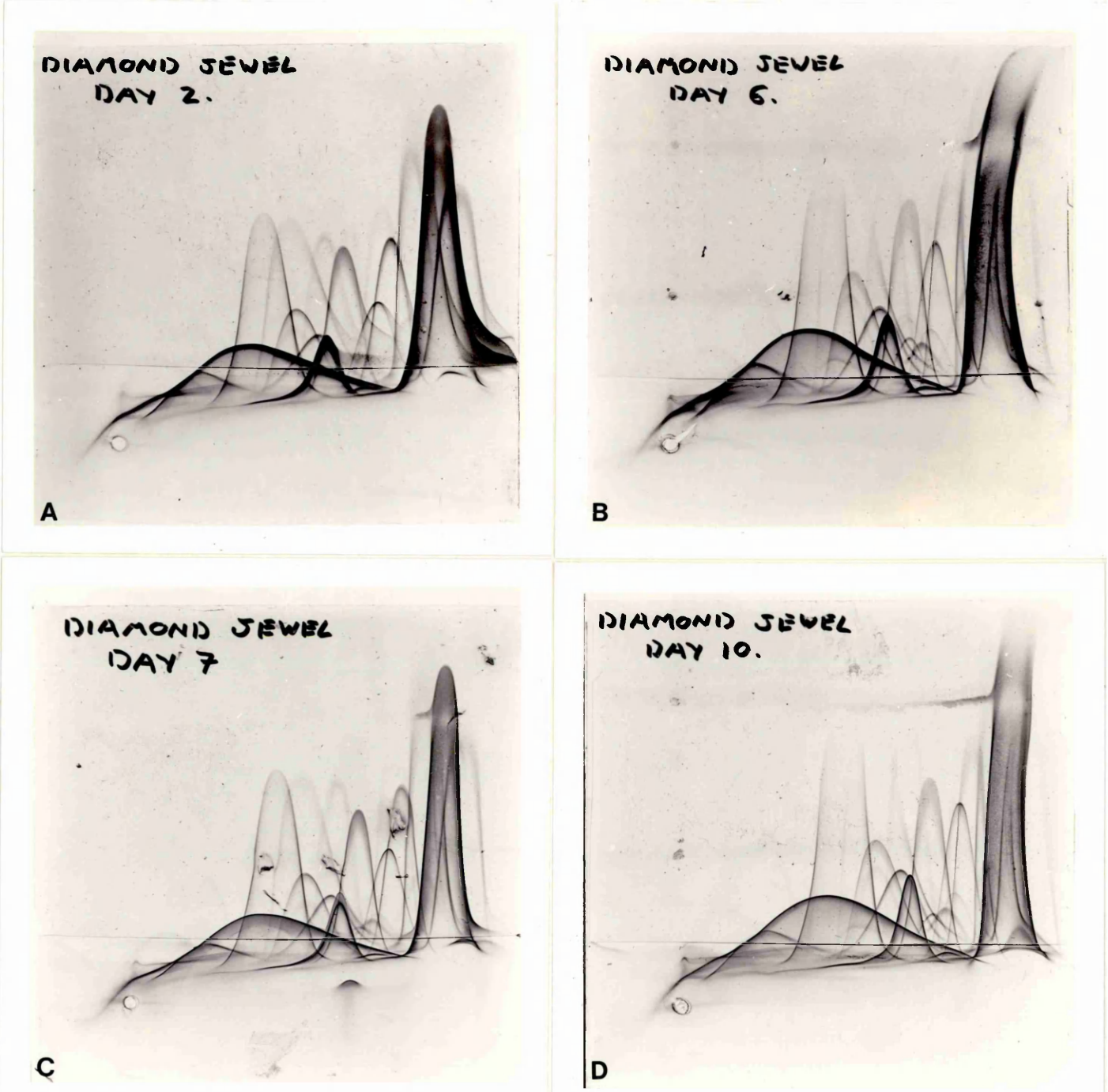

Fig.5.2 Two-dimensional crossed immunoelectrophoresis of serum taken from a mare (H2) (after mating) which failed to become pregnant; serum samples were taken at intervals during the first 10 days.
[antisera= rabbit anti-pregnant horse]
A (day 2), B (day 6), C (day 7), D (day 10).

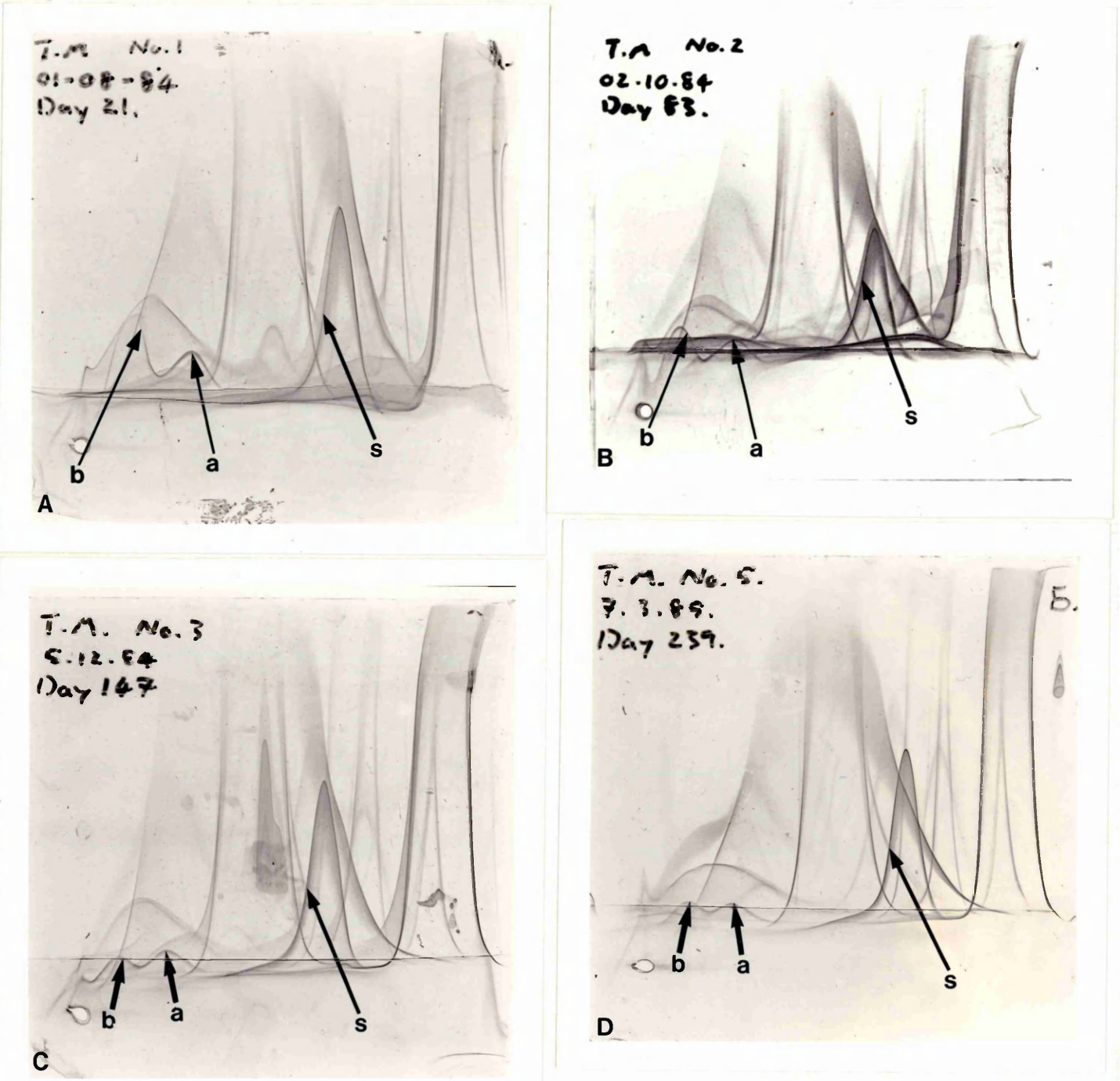

Fig.5.3 Two-dimensional crossed immunoelectrophoresis of serum from a pregnant mare (H3) at day 21 at intervals during gestation. [a= β_2-hors.PP, b= γ_2-hors.P, antisera= rabbit anti-pregnant horse] A (day 21), B (day 83), C (day 147), D (day 239). Pregnancy was confirmed by successful outcome.

s= α_2-macroglobulin

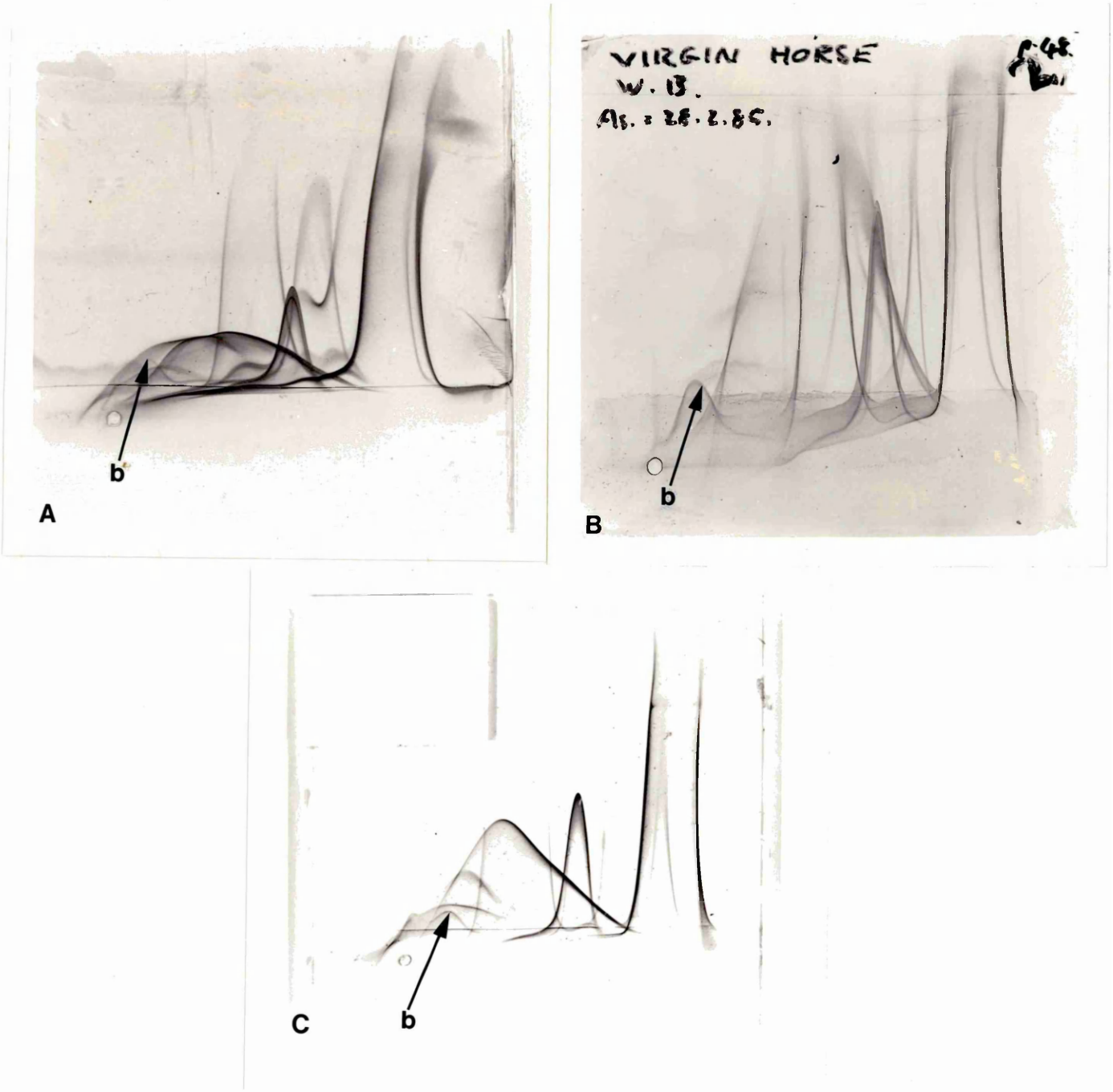

Fig.5.4 Two-dimensional crossed immunoelectrophoresis of gelding (H4) (A), virgin horse serum (H5) (B) and non-pregnant horse serum from a previously pregnant mare (H6) (C) against rabbit anti-pregnant horse antiserum.
Only gamma$_2$-hors.P (b) is present.

hadpreviously been pregnant (H6) (Fig.5.4C). A second protein with gamma$_2$-electrophoretic mobility that was present in gelding (H4) serum and in serum from both of the non-pregnant mares (H5, H6) (Fig.5.4A, B,C) was also present in serum taken from pregnant mares (H1, H3) (Figs 5.1, 5.3). This protein appeared to be immunologically related to the pregnancy specific protein of beta$_2$-electrophoretic mobility in that the precipitin lines of the proteins showed continuity (See eg, Fig 5.3). In addition, serum from a number of other pregnant mares was pooled and electrophoresed against rabbit anti-pregnant horse antiserum. The two precipitates were continuous indicating the presence of identical antigenic determinents in the compared samples (Fig.5.5).

The two proteins were termed beta$_2$-hors. pregnancy protein (β_2-hors.PP)and gamma$_2$-hors. protein (γ_2-hors.P) respectively. Two-dimensional crossed immunoelectrophoresis of pregnant mare serum against rabbit anti-virgin horse antiserum revealed the presence only of the β_2-hors.PP recipitate (Fig.5.6). Tandem crossed immunoelectrophoresis of virgin horse serum and pregnant horse serum against rabbit anti-pregnant horse antisera, revealed the fusion of the γ_2-hors.P precipitin lines from the two samples indicating an identity between the postulated γ_2-hors.P in pregnant and non-pregnant horses (Fig.5.7).

To summarise, a protein with beta$_2$-electrophoretic mobility is described, which appears in the serum of mares specifically during pregnancy. The protein is first detected at day 6 post-covering and is immunologically related to another protein present in the non-pregnant mare.

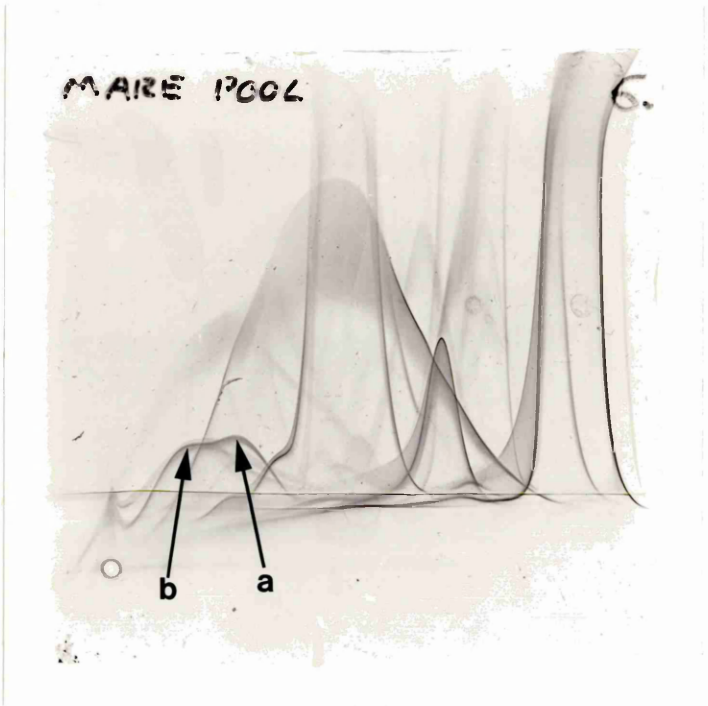

Fig.5.5 Two-dimensional crossed immunoelectrophoresis of pooled serum from a number of pregnant mares. The equine pregnancy protein precipitates have fused together.
[antiserum= rabbit anti-pregnant horse]

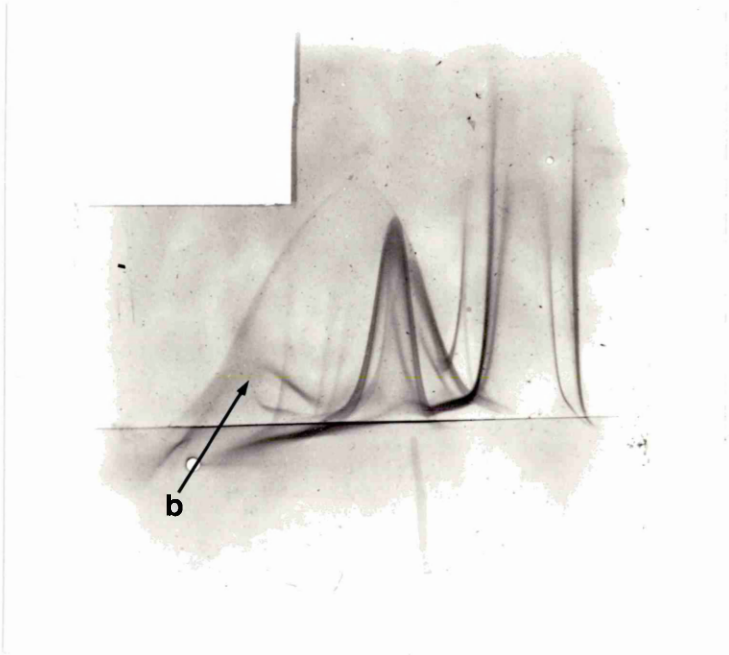

Fig.5.6 Two-dimensional crossed immunoelectrophoresis of pregnant horse serum (H3) against rabbit anti-virgin horse serum.
Only gamma$_2$-hors.P (b) is present.

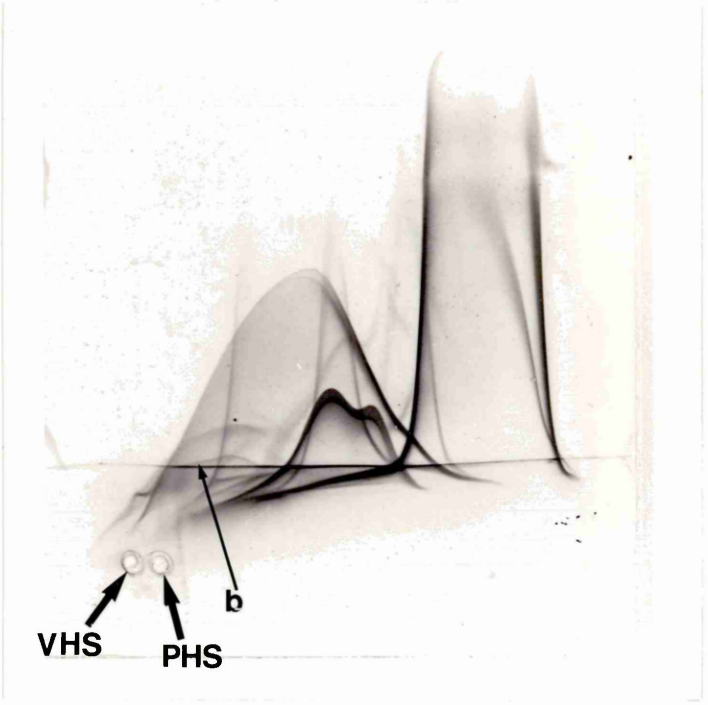

Fig.5.7 Tandem crossed immunoelectrophoresis of virgin horse serum and pregnant horse serum against rabbit anti-pregnant horse antisera. The γ_2-hors.P precipitates have fused together.
(VHS= virgin horse serum; PHS= pregnant horse serum)

5.3 Identification of Equine Chorionic Gonadotrophin (eCG)

As eCG is the most widely reported pregnancy specific protein, a possible identity between eCG and β_2-hors.PP was investigated, although this seemed unlikely in view of the source of eCG and the early appearance of β_2-hors.PP.

eCG was obtained from two commercial sources, Sigma and Intervet. The hormone was electrophoresed against rabbit anti-pregnant horse antiserum. Both hormone preparations revealed a peak in the alpha$_2$ area of the gel (Fig.5.8A, B) as described by Gidley-Baird et al. (1983). A number of contaminating proteins were, however, also present particularly in the Sigma preparation (Fig.5.8A). Pregnant mare serum was also electrophoresed against rabbit anti-eCG (Equine Fertility Unit, Cambridge) and a similar precipitin line was observed (Fig.5.8C). Neither β_2-hors.PP or γ_2-hors.P was revealed in any of the above immunoelectrophoretic runs. Therefore, since neither eCG preparations contain material identifiable immunologically as either β_2-hors.PP or γ_2-hors.P and as anti-eCG failed to precipitate either of these 2 proteins in pregnant mare serum known to contain β_2-hors.PP (H3), it was concluded that β_2-hors.PP and γ_2-hors.P are proteins distinct from eCG.

5.4 Semi-Quantitation of β_2-hors.PP Levels during Pregnancy

In order to determine the relative changes in concentration of β_2-hors.PP throughout pregnancy the area under the protein precipitin line was compared to the area under a common protein precipitate present on each plate. The common protein selected was the protease inhibitor alpha$_2$-macroglobulin, the concentration of which does not vary during pregnancy (Carlson et al., 1986). This protein also produced a clearly identifiable precipitate on all immunoelectrophoresis plates. When fresh serum is used in two-dimensional crossed immunoelectrophoresis, alpha$_2$-macroglobulin

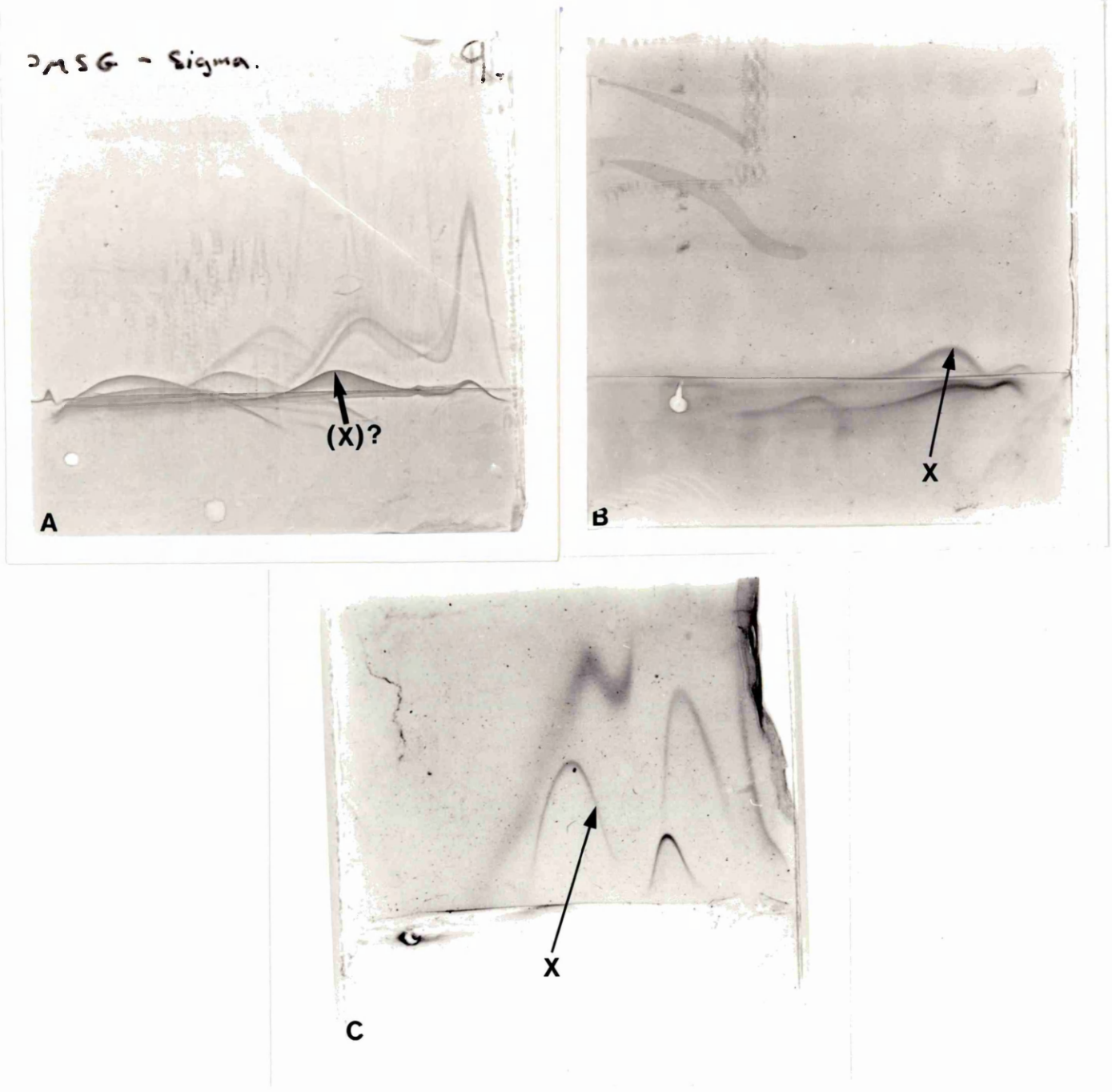

Fig.5.8 Two-dimensional crossed immunoelectrophoresis of two preparations of eCG, from the suppliers Sigma (A) and Intervet (B), against rabbit anti-pregnant horse antiserum and also of pregnant mare (H3) serum (eCG phase) against rabbit anti-eCG (C). [X=eCG]

sometimes forms two precipitates in the second dimension, each with identical electrophoretic mobility in the first dimension (Dott et al., 1985). In such cases, the upper peak was used as the standard as only the lower peak varies in height.

The orientation of specific precipitin peaks on an immunoelectrophoretic plate is variable. Consequently, the positioning of a base line for the determination and comparison of areas on the same plate is difficult. To overcome this problem, the base line was drawn in the two most plausible positions and the peak areas calculated for each position. The two chosen base line positions were firstly, each peak with its own base line and secondly, a common base line for all protein precipitates. Once the peak areas and ratios were determined for both base line positions, the average areas were calculated. This procedure was repeated with different anti-pregnant mare antisera and the results compared (Table 5.1). This experiment was carried out with serum samples from two pregnant horses, namely H1 (day 3-10) and H3 (day 21-239). The peak areas shown in Table 5.1 suggest that there is an increase in secretion of β_2-hors.PP for at least the first 21 days of gestation. From day 83 onwards, however, β_2-hors.PP levels decline suggesting that peak levels of the protein are produced at some stage between days 21 and 83. Such a secretion pattern suggests that the function of such a protein is of prime importance during early pregnancy, for example in human pregnancy certain immunomodulatory proteins are present at a higher concentration during early pregnancy than later in gestation. Furthermore, when implantation occurs in the horse at day 37, β_2-hors.PP levels are elevated, which suggests that the protein may have a biological function during this process.

Table 5.1

Relative Changes in Concentration of β_2-hors.PP during Equine Pregnancy as Assessed by the Use of 3 different antisera in two-dimensional crossed immunoelectrophoresis

	Day of Gestation	Antisera 1 (β2 ratio)	Antisera 2 (β2 ratio)	Antisera 3 (β2 ratio)
		$\bar{x} \pm \sigma n-1$ of 2 positions of base line		
H1	3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	5	0.00 ± 0.00	3.20 ± 2.47	6.68 ± 0.42
	6	6.65 ± 2.52	4.71 ± 3.13	5.91 ± 0.29
	9	5.96 ± 4.76	9.12 ± 8.62	7.20 ± 4.96
	10	8.54 ± 8.11	5.65 ± 3.79	5.77 ± 1.16
H3	21	15.82 ± 2.69	6.28 ± 0.55	19.00 ± 20.26
	83	13.96 ± 1.05	4.38 ± 0.76	12.88 ± 11.29
	147	11.46 ± 6.36	5.30 ± 3.82	12.43 ± 11.73
	175	14.89 ± 10.18	6.22 ± 4.58	3.12 ± 0.00
	239	8.87 ± 3.26	5.27 ± 4.32	6.00 ± 0.00

* β_2 ratio calculated by comparing the area under the β_2-hors .PP precipitate to that under standard protein precipitate (α2-macroglobulin): $\beta 2 \text{ ratio} = \frac{\beta 2\text{-hors.PP area}}{\text{standard protein area}} \times 100$

5.5 β_2-hors.PP as an Indicator of Pregnancy

Sequential serum samples were taken from 16 horses for up to 3 weeks after mating with a stallion. Pregnancy was determined either at 90 days by ultrasonic scan (N=13) or by successful outcome (N=3). Eleven horses were found to be pregnant by these criteria. Serum samples from all horses were run in two-dimensional crossed immunoelectrophoresis against rabbit anti- pregnant horse antiserum. At this stage, it was unknown which horses were pregnant and which were not, the immunoelectrophoresis was performed blindly. In addition, serum samples from 14 mares known not to be pregnant were also run against the same antiserum in the immuno-electrophoretic system described. β_2-hors.PP was detected in 10 of the 11 mares which were later confirmed to be pregnant. Two of the five mares that were covered by a stallion and did not develop a successful pregnancy transiently showed the presence of the protein. In one of these two horses (H7), the protein was detectable at day 5 only but not in the samples taken on the following four days. In the other horse (H8) β_2-hors.PP was detected on day 10, the last sample obtained. In horses where pregnancy was confirmed, β_2-hors.PP appeared in all subsequent samples investigated. The protein was not detected in any of the samples taken from the 14 mares known not to be pregnant. When the significance of these results was assessed by the Chi-squared test using a 2 x 2 contingency table, it was found that the use of β_2-hors.PP as an indicator of pregnancy was clinically significant at the 10% level (Table 5.1a).

It is possible that the transient appearance of β_2-hors.PP in the circulation of those mated horses which subsequently proved not to be pregnant may represent a situation analogous to the state of biochemical pregnancy described in the human (Miller et al., 1980). Indeed, it has been reported that similar fertilization rates occur in both normal and

Table 5.1a

Test	Actual: Pregnant	Actual: Non-pregnant
+	10 / 8.25	2 / 3.75
-	1 / 2.75	3 / 1.25

$\chi^2 = 4.75$ with 1 degree of freedom

This is significant at the 10% level. $P<0.10$

Key

Obs / Exp

Contigency table for Chi^2 test in order to determine the significance of β_2-hors.PP detection as a pregnancy test.

barren mares but significantly different day 14 pregnancy rates (Ball et al., 1985).

5.6 The Detection of Serum β_2-hors.PP during a Donkey and Donkey-in-Horse Pregnancy

Once the presence of β_2-hors.PP in the circulation of pregnant horses had been confirmed, it was of interest to investigate whether or not the protein was present during pregnancy in closely related species within the same Genus.

A series of 10 serum samples were taken from one pregnant donkey at different stages of gestation from day 32 to day 252. Two-dimensional crossed immunoelectrophoresis of each serum sample against anti-pregnant horse antiserum resulted in the formation of many precipitin line peaks demonstrating a high degree of cross-reactivity between horse and donkey serum proteins. Only on one occasion did a precipitate appear in the beta-2 area of the gel, which resembled β_2-hors.PP (Fig.5.9A). That this could not be repeated on subsequent runs with the same sample suggests that the precipitate could have been an artifact. β_2-hors.PP did not, therefore, appear to be present in the serum from that particular pregnant donkey or it was not detectable within the limits of sensitivity imposed by the detection technique.

10 serum samples were taken from a donkey-in-horse pregnancy, and clearly showed the existence of β_2-hors.PP after running each sample in the immunoelectrophoretic system described against rabbit anti-pregnant horse antiserum (Fig.5.9B).

These preliminary findings suggest that β_2-hors.PP may be species specific. If this is the case then its presence in a donkey-in-horse pregnancy may be indicative of a maternal origin of the protein.

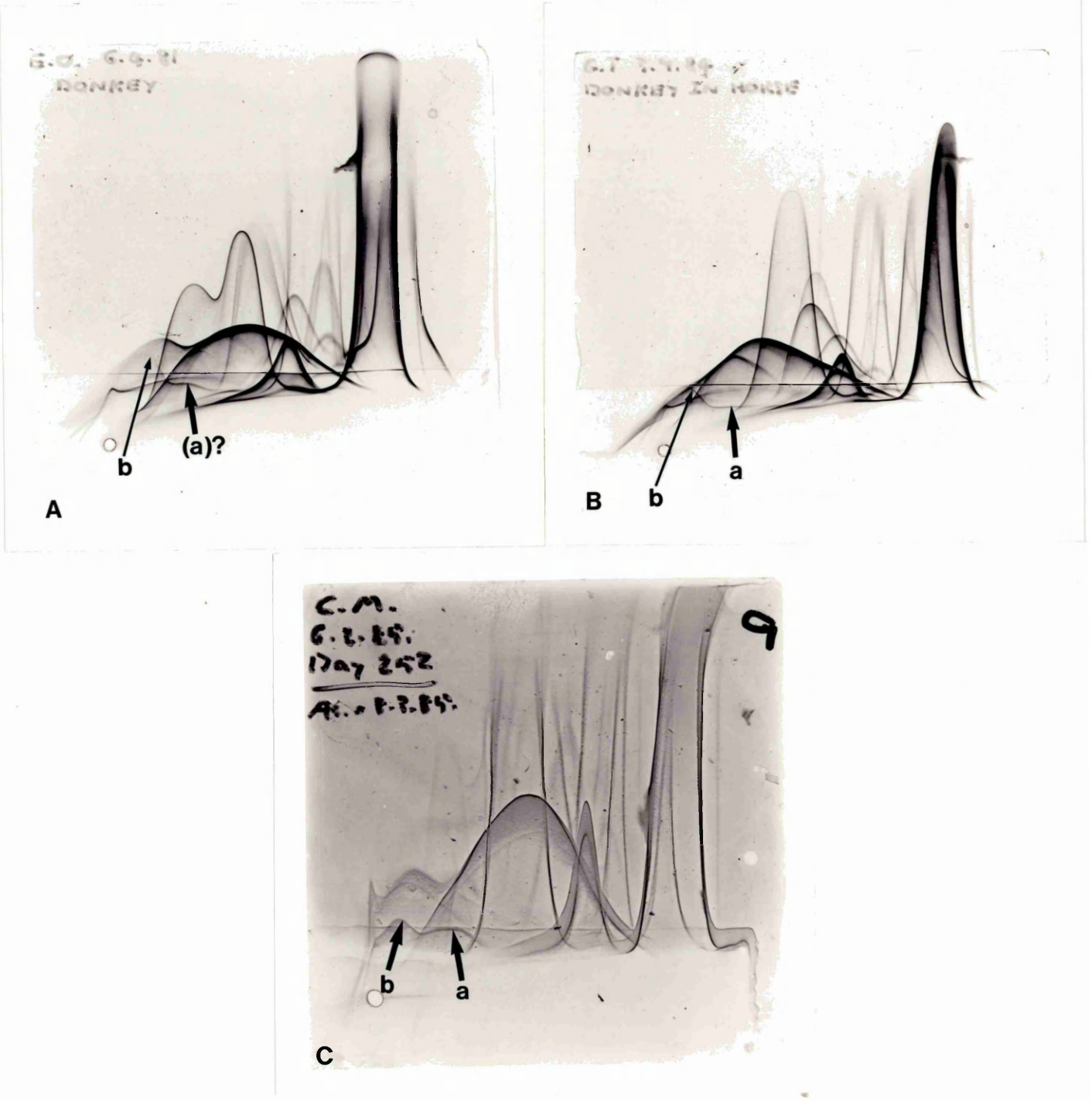

Fig.5.9 Two-dimensional crossed immunoelectrophoresis of serum from a donkey pregnancy [A], a donkey-in-horse pregnancy [B] and a normal horse pregnancy (H10) [C].
[a= β_2-hors.PP, b= γ_2-hors.P, antiserum= rabbit anti-pregnant horse].

5.7 The Source of β_2-hors.PP

It is widely accepted that before day 14 after ovulation the equine conceptus produces some signal enabling the continued survival of the corpus luteum (Hershman and Douglas, 1979). Evidence suggests that oestrogens produced from the conceptus reduce the sensitivity of the uterus to oxytocin resulting in a decrease in the secretion of the luteolytic agent prostaglandin F_2alpha which enables the continued functioning of the corpus luteum (Goff et al., 1987). Conceptus derived factors may, however, have other localised effects on the uterus possibly resulting in the secretion of further early pregnancy factors, such as β_2-hors.PP and MPP-1 from maternal tissue. The presence of the protein in a donkey-in-horse pregnancy and its apparent absence in a donkey pregnancy may be taken as preliminary evidence for a maternal source of β_2-hors.PP. More concrete evidence for this theory was required and this led to an investigation of the factors present in uterine flushings and endometrial extracts.

5.7.1 Immunoelectrophoresis of Horse Uterine Flushings

Approximately 4 litres of Dulbecco's modification of Eagles medium (DMEM) was flushed through the uterus of a horse (H9) that had been sired by a stallion 6 days previous. The flushings were collected in measuring cylinders (2 litre volume) and allowed to stand for approximately 15 min. to allow any fertilised eggs or tissue present, to sediment. The DMEM was aspirated from the measuring cylinders leaving approximately 100 ml of liquid at the bottom of the cylinders. The fertilised egg was then located by the microscopical examination of the uterine flushings after which it was used in embryo transfer work at the Equine Fertility Unit in Cambridge.

The remaining uterine flushings were pooled, extensively dialysed against PBS, concentrated by a factor of 10 by freeze drying and dialysed again. The resulting uterine flushing concentrate was electrophoresed against rabbit anti-pregnant horse antiserum in immunoelectrophoresis. After staining, a precipitin line was visible in the beta$_2$ area of the gel, indicating that β_2-hors.PP was present. The peak was, however, very faint and could not be seen by photography. It is likely that the proteins were present in very low concentration in the flushings but as these were reconstituted in a small volume (approx. 200 ul), it was not possible to concentrate them further. It is also possible that the freeze drying process decreased the antigenicity of the proteins. The flushings used in this study were obtained from one horse (H9). Serum samples were not taken from this individual around the time of flushing and thus the possible presence of β_2-hors.PP in the serum could not be investigated. For future investigations of this nature, flushings from a number of pregnant horses should be pooled and concentrated to give an enriched flushing concentrate. The above results indicate that β_2-hors.PP may be of uterine origin although this requires further clarification.

5.7.2 The Immunoelectrophoresis of Horse Placental/Uterine Extracts from Tissues Taken during the eCG Phase of Pregnancy (day 60 and day 80)

In order to try and identify the tissue responsible for β_2-hors.PP secretion, a number of placental/endometrial extracts were electrophoresed in the system described.

Implantation and the establishment of the equine placenta are characterised by the invasion of the maternal endometrium by trophoblast cells which develop into the endometrial cups. In order to investigate a

possible physiological role of β_2-hors.PP during the establishment of placentation, the endometrial/placental extracts to be immuno-electrophoresed were taken during the eCG phase of pregnancy.

Placental and uterine tissues were obtained from a 60 day (after mating) pregnant mare (H11) post-mortem. Additional placental tissue samples were taken from a second horse (H12) whose pregnancy was terminated at day 80 (after mating). After preparation of the homogenates, all samples were diluted to a protein concentrations of 0.6 mg/ml. A summary of the tissue source and day of gestation of these homogenates is shown in Table 5.2. All samples were electrophoresed in two-dimensional crossed immuno-electrophoresis against rabbit anti-pregnant horse antiserum. Serum taken from the eCG phase of gestation of a pregnant mare (day 83) was electrophoresed simultaneously as a positive control.

Table 5.2

Source of Homogenate (0.6 mg/ml)	Day of Gestation (after mating)
Pregnant horn endometrium	Day 60
Endometrial cup homogenate	Day 60
Non-pregnant horn endometrium	Day 60
Pregnant horn allantochorion with endometrial cup secretion	Day 80
Pregnant horn allantochorion with no visible cup secretion	Day 80
Non-pregnant horn allantochorion	Day 80

Placental/uterine extracts taken from two pregnant mares (H11: day 60, H12: day 80)and used in two-dimensional crossed immunoelectrophoresis.

Using this methodology, β_2-hors.PP could not be identified in any of the samples taken either at day 60 or day 80. At day 60, no eCG was detected in the non-pregnant horn endometrium (Fig.5.10D). Both pregnant horn endometrium (Fig.5.10B) and endometrial cup homogenate (Fig.5.10C) produced virtually identical patterns after immunoeletrophoresis, both showing the presence of eCG. All three samples revealed a second predominant peak in the $alpha_1$ area of the gel in the position of albumin.

At day 80, no eCG was detected in the non-pregnant horn allantochorion (Fig.5.12D). Two different anti-pregnant horse antisera were used in the immunoelectrophoresis of the day 80 samples. Both pregnant horn allantochorion with endometrial cup secretion and the same tissue without visible cup secretion, showed the presence of eCG particularly with antiserum 1 (Fig.5.11B, C). The electrophoretic mobility of eCG did show some variation which may be attributed to a variable glycosylation of the molecule. The carbohydrate component of eCG is large, approaching 45% by weight (Moore et al., 1980). In addition, it is reported that the molecule occurs in different forms, indeed the circulating form of the molecule is more highly glycosylated than that isolated from endometrial cups (Aggarwal et al., 1980). In addition to eCG and albumin, day 80 pregnant horn allantochorion with cup secretion produced a number of other precipitin line peaks post-immunoelectrophoresis with both antisera. Antiserum 1 resulted in a further 4 peaks in addition to eCG and albumin (Fig.5.11B). Antiserum 2 revealed a total of 10 precipitin line peaks, six of which were very sharp and clear (Fig.5.12B). However, none could be identified as β_2-hors.PP. These results contrast sharply with pregnant horn allantochorion with no endometrial cup secretion which gave one clear precipitin line identified as albumin and perhaps another peak of

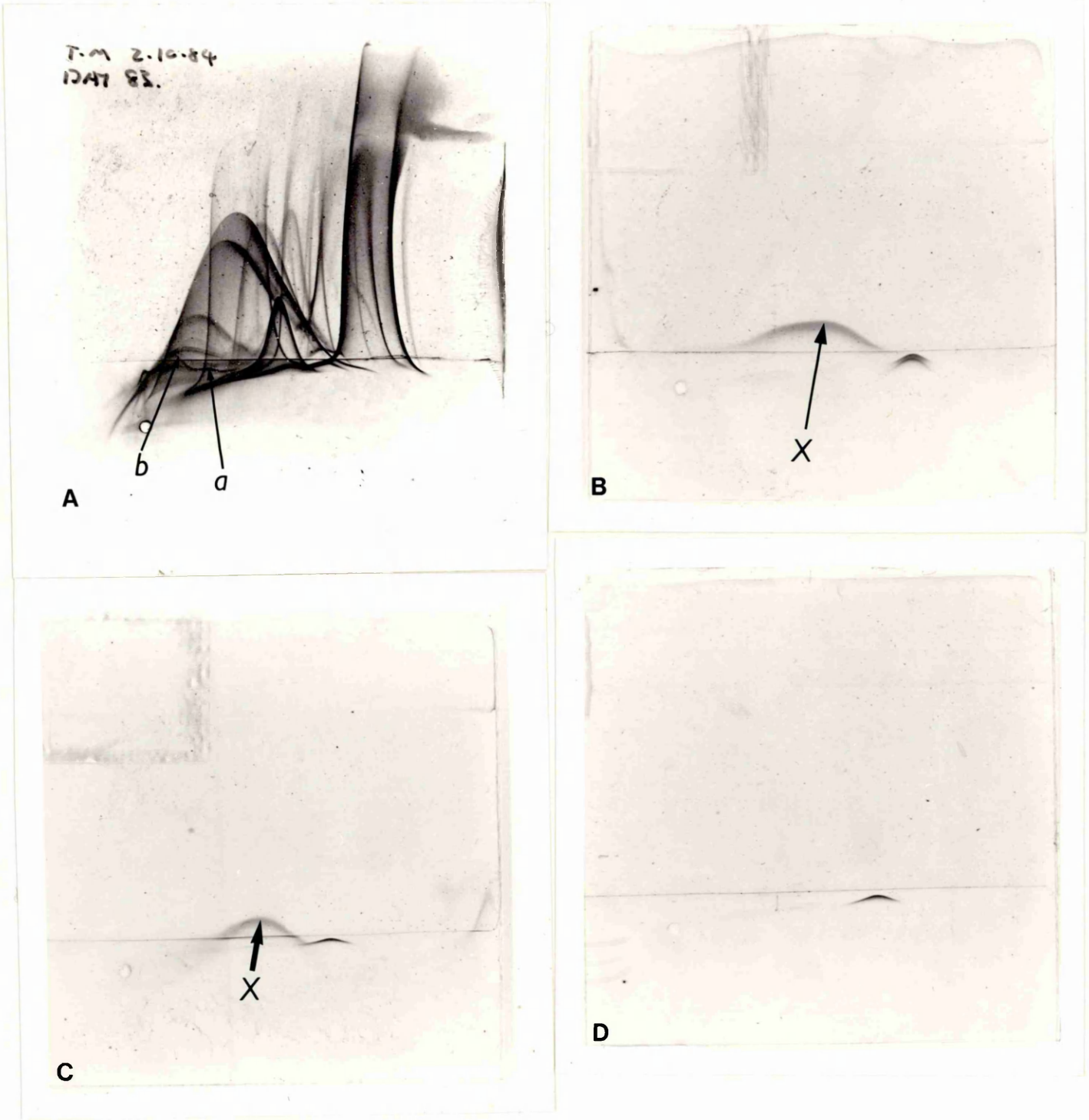

Fig.5.10 Two-dimensional crossed immunoelectrophoresis of horse placental and uterine extracts taken at day 60 of gestation.
[a= β_2-hors.PP, b= γ_2-hors.P, X= eCG, antiserum= rabbit anti--pregnant horse]

A: Day 83 pregnant horse serum (Control)

B: Pregnant horn endometrium

C: Endometrial cup homogenate

D: Non-pregnant horn endometrium

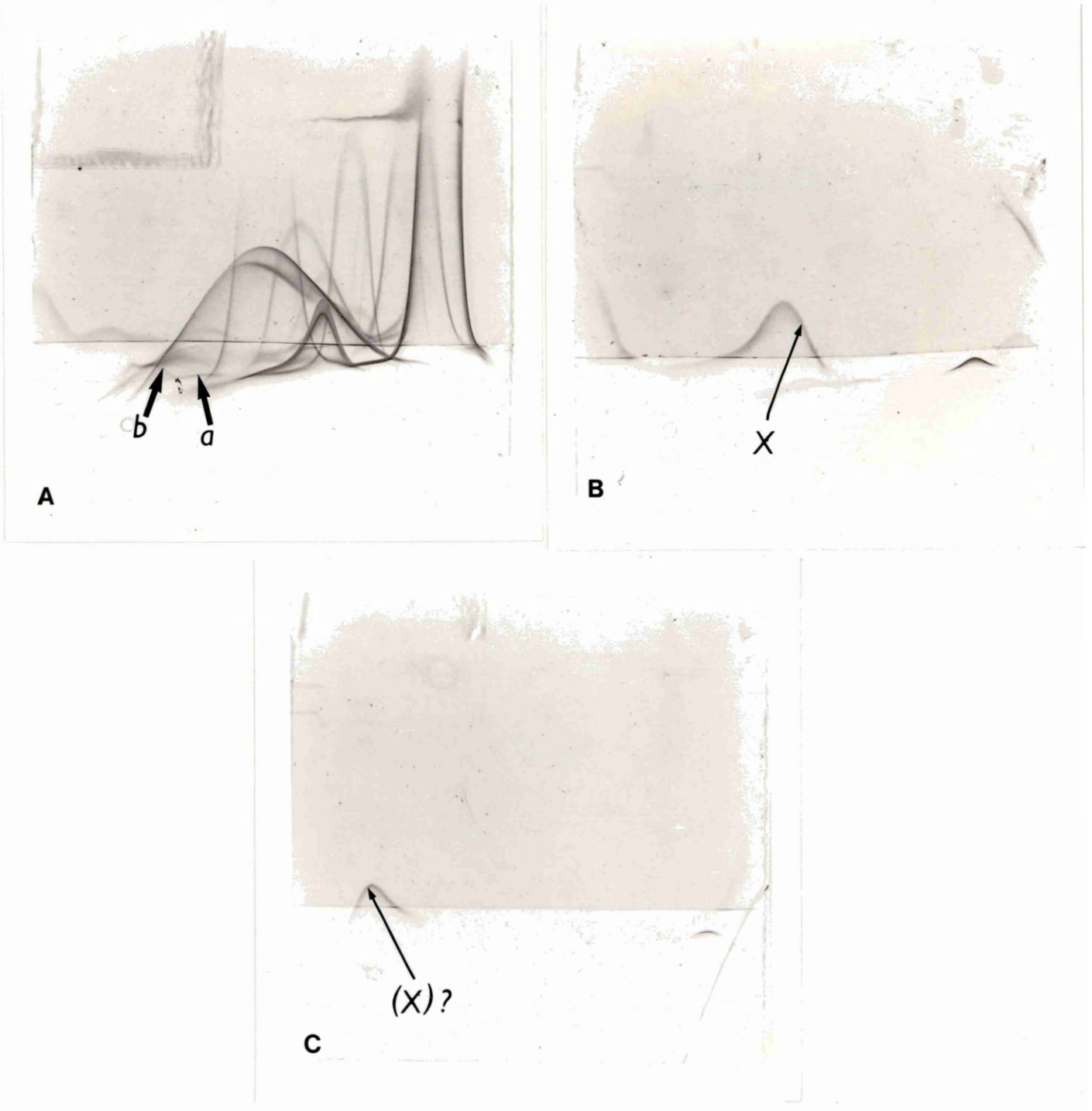

Fig.5.11 Two-dimensional crossed immunoelectrophoresis of horse allantochorion extracts taken at day 80 of gestation - rabbit anti-pregnant horse antiserum 1.
[a= β_2-hors.PP, b= γ_2-hors.P, X= eCG]
A: Day 114 pregnant horse serum (Control)
B: Pregnant horn rich with endometrial cup secretion
C: Pregnant horn - little endometrial cup secretion.

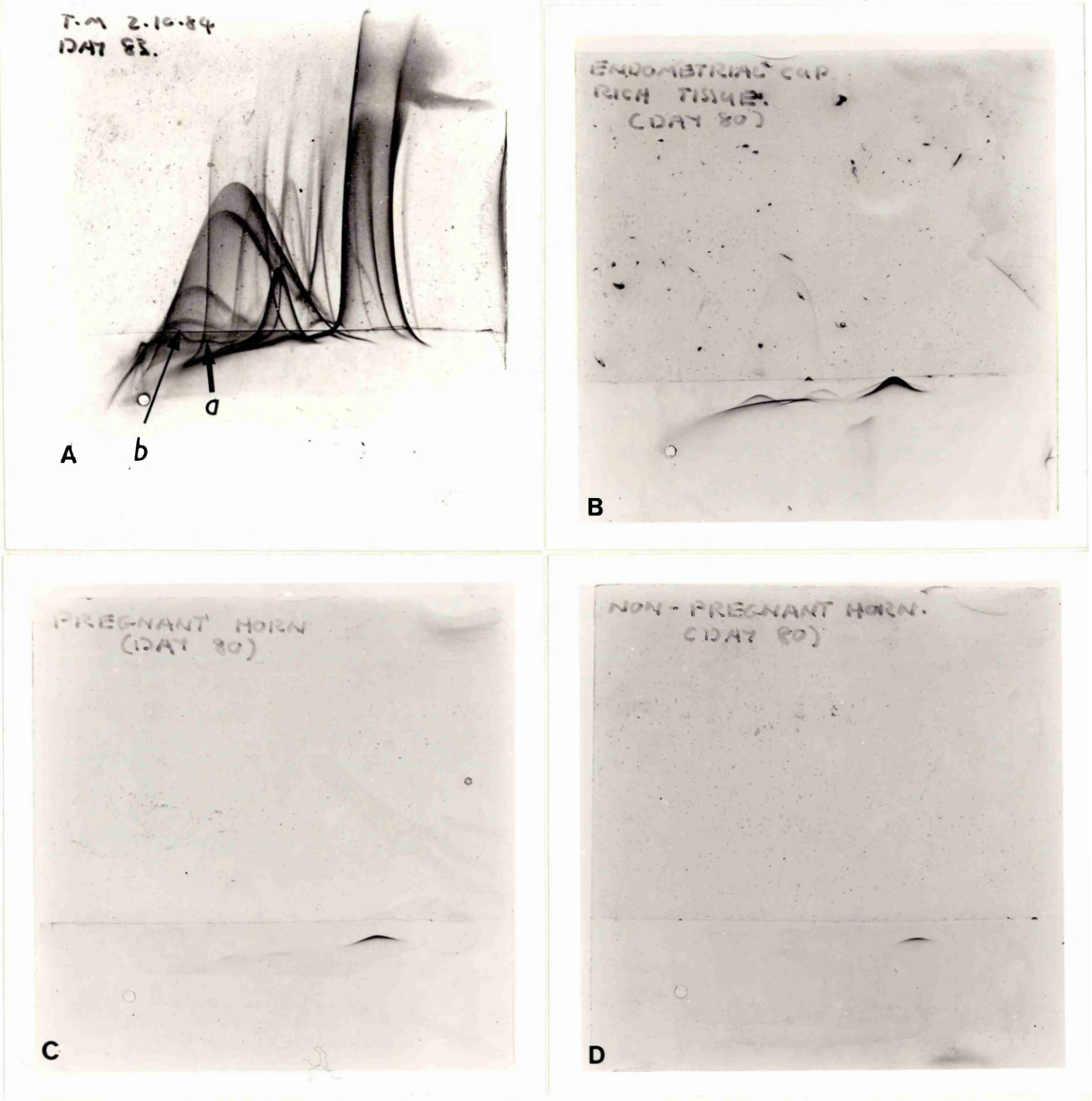

Fig.5.12 Two-dimensional crossed immunoelectrophoresis of horse allantochorion extracts taken at day 80 of gestation - rabbit anti-pregnant horse antiserum 2.
[a= β_2-hors.PP, b= γ_2-hors.P, X= eCG]
A: Day 83 pregnant horse serum (Control)
B: Pregnant horn rich with endometrial cup secretion
C: Pregnant horn - little endometrial cup secretion
D: Non-pregnant horn allantochorion

unknown identity (Figs.5.11C, 5.12C). Similarly, only one peak could be seen in the position of albumin when non-pregnant horn allantochorion was immunoelectrophoresed (Fig.5.12D).

These results suggest that eCG may be one of a number of factors produced by the endometrial cups. That many of these factors are detected by an antiserum raised against the serum of a pregnant mare may, however indicate that the sample was contaminated with serum protein. Indeed, one of the peaks corresponds to the position of α_2-macroglobulin and another to that of IgM.

With respect to β_2-hors.PP, it was not possible to investigate the presence of the protein in the serum of the animals from which the tissue was taken as the appropriate serum samples could not be obtained from the Equine Fertility Unit.

In conclusion, it appears that β_2-hors.PP is either not present or is not produced in high enough concentrations to be detected in any of the eCG phase tissue extracts investigated. Furthermore, the tissue homogenates were prepared without any extensive washing of the tissue (Section 4.9.1) and thus it is unlikely that the protein was leached out of the tissue during extract preparation. Two lines of evidence indicate, however, that β_2-hors.PP is of uterine origin. Firstly, the protein is present in maternal serum taken from a donkey-in-horse pregnancy and does not appear to be present during a donkey pregnancy. Secondly, β_2-hors.PP has been detected in uterine flushings taken from a horse (H9) 6 days after mating with a stallion. The uterine origin of β_2-hors.PP, however, still requires furtherinvestigation. Additional evidence may be obtained by immunoeletrophoresing uterine extracts taken earlier during pregnancy,

i.e. before the eCG phase. Such endometrial tissue can, however, only be taken as a consequence of a post-mortem examination and is thus difficult to obtain.

5.8 The Partial Purification of β_2-hors.PP

Approximately 20% of total circulating proteins in human plasma have been observed to bind to a immobilised heparin, the majority of which can be eluted by increasing the salt concentration to 0.45 M NaCl (Teisner et al., 1983). These authors suggest that heparin affinity chromatography may be an effective procedure for the isolation and purification of each interacting protein. Heparin affinity has already been used in the isolation and purification of two human pregnancy associated proteins (PAPP-A and PP-5) (Teisner et al., 1983; Salem et al., 1980) and thus may be useful in the purification of equine pregnancy associated proteins. In the same report, Teisner et al. showed that the interaction of proteins with heparin in two dimensional crossed immuno-electrophoresis can be used to predict the outcome of heparin affinity chromatography. Consequently, in order to investigate whether β_2-hors.PP interacted with heparin, pregnant mare serum was immunoelectrophoresed using heparin in the first dimension gel.

5.8.1 Investigation of an Interaction between Heparin and the Horse Proteins (β_2-hors.PP and γ_2-hors.P)

A stock solution of 300 U/ml heparin was prepared. Different amounts of this were mixed with molten agarose prior to pouring and gels containing 21, 30 and 60 units of heparin per ml gel were poured in the usual way. Pregnant mare serum was electrophoresed in the first dimension in each gel and subsequently run against rabbit anti-pregnant horse antisera in the second dimension. In addition, a control immunoeletrophoresis plate was run with no heparin in the first dimension gel.

β_2-hors.PP and γ_2-hors.P were identified in all gels. The electrophoretic mobility of the proteins was unaltered in the first dimension but the peaks appeared to increase in height in the second dimension as the concentration of heparin increased in the first dimension gel (Fig.5.13). This was confirmed by measuring the area under β_2-hors.PP and γ_2-hors.P and comparing them to the area under the α_2-macroglobulin peak as described in Section 5.4 (Table 5.3). α_2-macroglobulin has been shown not to interact with heparin (Teisner et al., 1983). The peak areas outlined in Table 5.3 suggest that there is an increase in the relative peak areas for both β_2-hors.PP and γ_2-hors.P as the concentration of heparin in the first dimension increases.

In conclusion, heparin has no effect on the electrophoretic mobility of β_2-hors.PP and γ_2-hors.P in the first dimension. The presence of heparin in the first dimension gel, however, increased the size of the β_2-hors.PP and γ_2-hors.P second dimension peaks indicating that there was an interaction between heparin and the equine proteins. As peak height is directly proportional to antigen concentration, it is possible that the binding of heparin to the proteins results in an increase in the number of available epitopes.

5.8.2 Partial Purification of β_2-hors.PP by Heparin Affinity Chromatography

The above data illustrates that there is some interaction between heparin and β_2-hors.PP. In order to partially purify β_2-hors.PP, 1.0 ml of pooled pregnant mare serum was passed through 5 ml of washed heparin Ultrogel. Once all of the unbound protein had been washed from the column by washing with distilled water (Fig.5.14a), the bound protein was eluted from the column using a stepped salt gradient of 0.2 M, 0.4 M, 0.6 M, 1.0 M NaCl (Fig.5.14b). The most concentrated fractions from each peak were pooled,

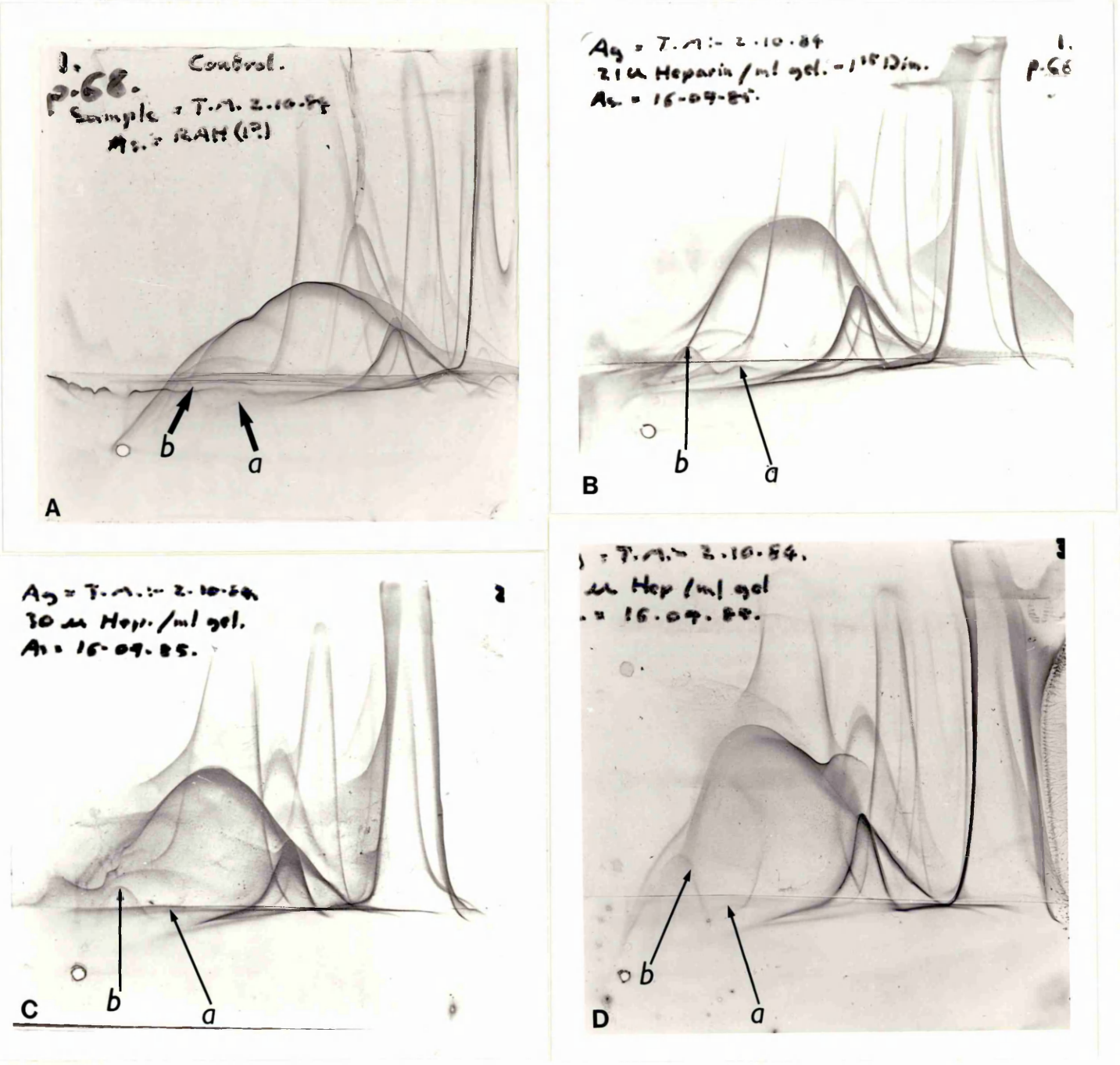

Fig.5.13 Two-dimensional crossed immunoelectrophoresis of pregnant mare serum with heparin incorporated into the first dimension gel.
A (Control), B (21 U/ml gel), C (30 U/ml gel), D (60 U/ml gel)
[a= β_2-hors.PP, b= γ_2-hors.P]

Table 5.3

Heparin in First Dimension Gel	β_2 Ratio $\bar{x} \pm \delta n-1$ of 2 positions of base line	γ_2 Ratio
0 (Control)	7.23±0.47	36.34±9.13
21 u/ml	6.94±1.97	40.97±0.98
30 u/ml	7.82±1.63	45.74±0.58
60 u/ml	11.00±5.97	106.72±1.77

The effect of heparin in the first dimension gel on the size of the β_2-hors.PP and γ_2-hors.P second dimension peaks.

dialysed and concentrated by freeze drying. Each pooled peak, the wash and the original sample were then electrophoresed in two-dimensional crossed immunoelectrophoresis against rabbit anti-pregnant horse antiserum. The results showed that β_2-hors.PP and γ_2-hors.P appeared to be eluted from the column with 0.2 M NaCl (Fig.5.15). However, when this experiment was repeated and 2 ml of pooled mare serum was passed through a 10 ml column of heparin Ultrogel these two pregnancy proteins were not retained in the column but were slightly retarded in the wash.

One cause of the apparent variable heparin affinity of the proteins could be the salt concentration of the original sample - physiological saline is 0.15 M with respect to NaCl and this approaches closely the molarity at which the proteins were first eluted. To investigate whether reduction of the salt concentration of serum enhanced the heparin binding capacity of the proteins, a 2 ml sample of serum from a pregnant mare was dialysed against 5 l of distilled water. 1 ml of this dialysed sample was passed through 5 ml of heparin Ultrogel and 3 ml fractions collected. The wash and fractions eluted with 0.2 M NaCl were dialysed, freeze dried and redissolved in 300 ul of distilled water and immunoelectrophoresed as before. β_2-hors.PP and γ_2-hors.P appeared to be present in the 0.2 M eluent. Assuming that the observed precipitin lines were β_2-hors.PP and γ_2-hors.P, this experiment indicates that the salt concentration has some effect on the heparin binding capacity of the two proteins. Furthermore, the close biochemical relationship of β_2-hors.PP and γ_2-hors.P is emphasized by the fact that they are always eluted together in an affinity experiment such as this.

The use of a weak heparin affinity for the partial purification of proteins has proved problematic for other researchers (Teisner et al., 1983). It has been reported that if a protein comes through a heparin

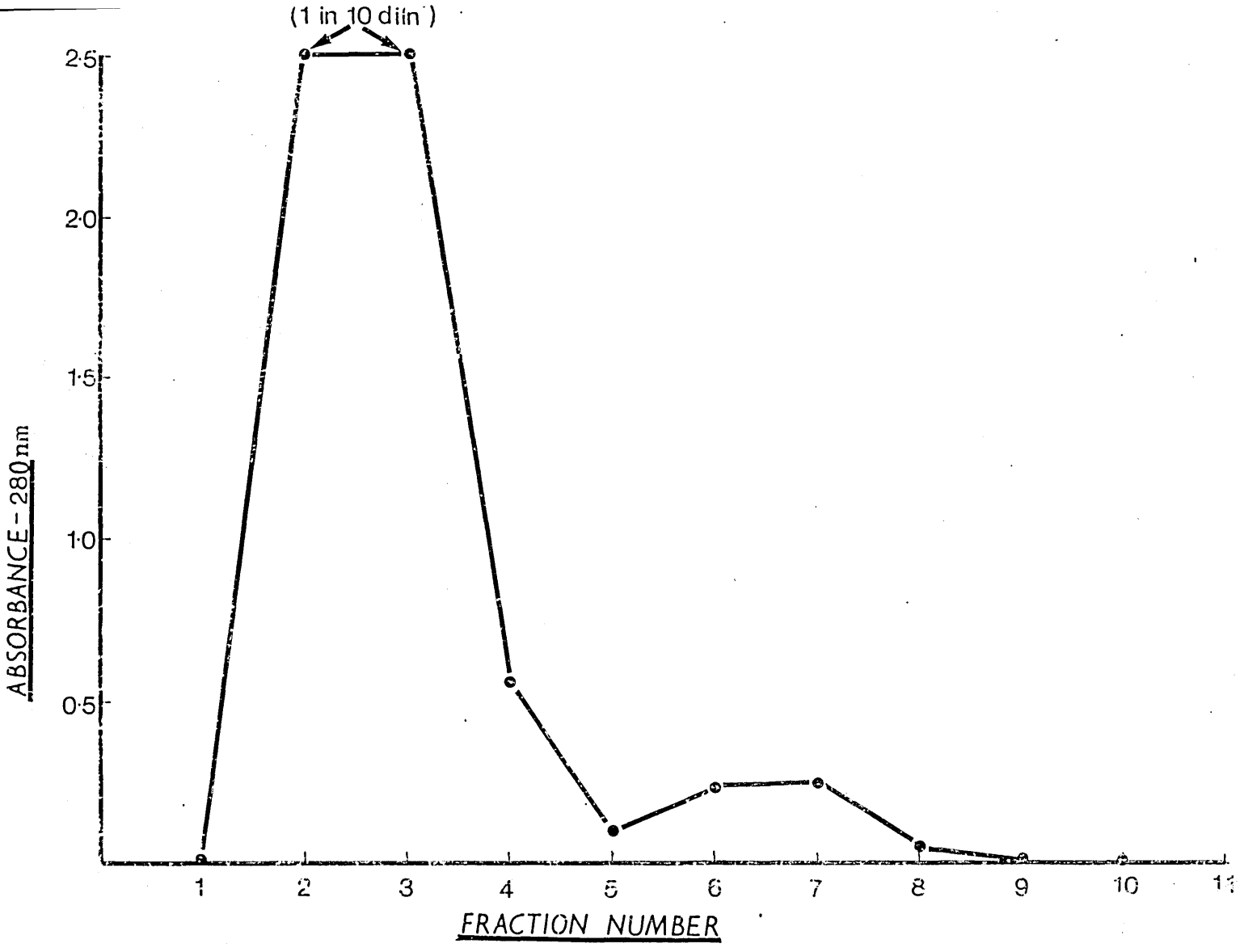

Fig.5.14(a) Elution profile - serum sample from pregnant mare passed through heparin ultrogel column (peaks represent non-bound protein).

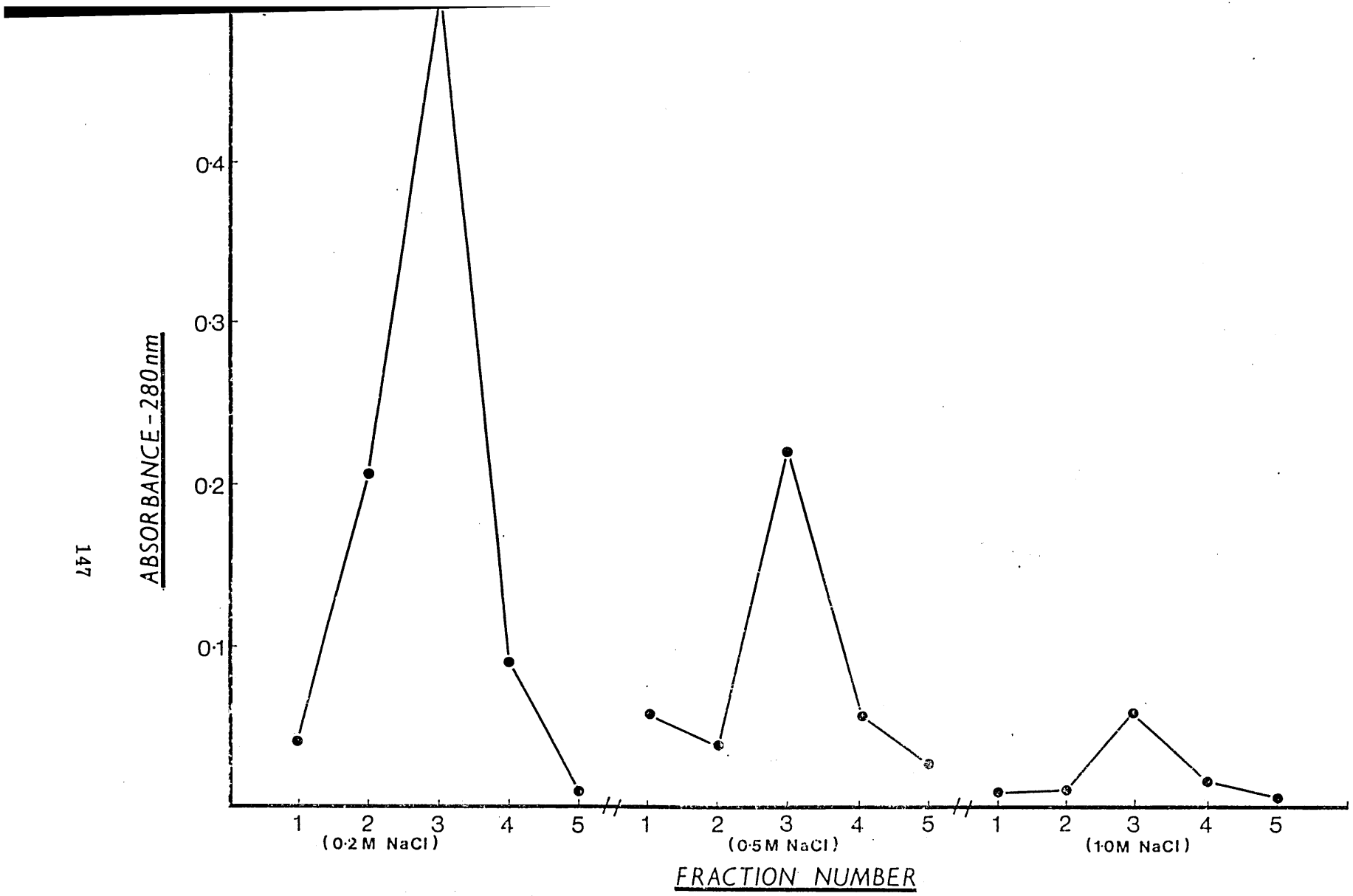

Fig.5.14(b) Elution profile - serum sample from a pregnant mare passed through heparin ultrogel column (peaks represent bound protein).

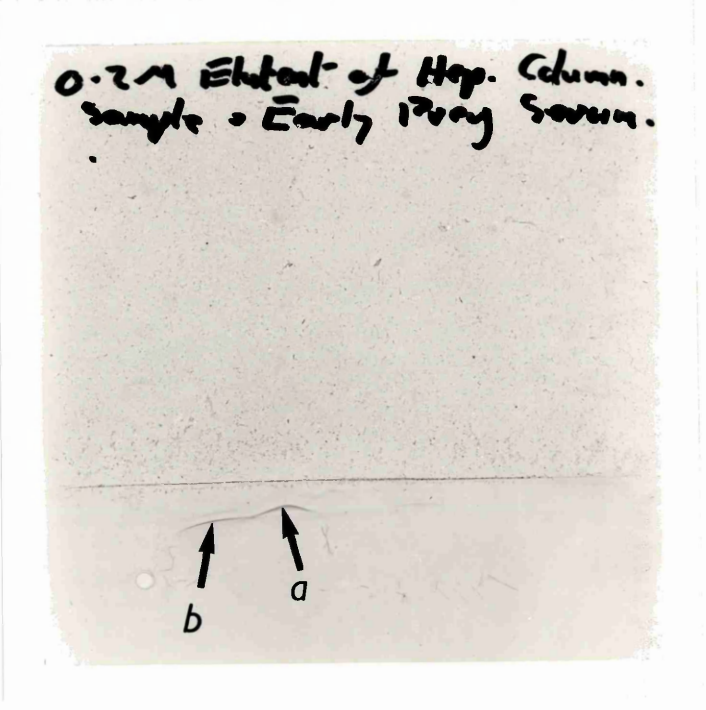

Fig.5.15 Two-dimensional crossed immunoelectrophoresis of 0.2 M eluent obtained from the heparin affinity chromatography of a pooled serum sample from a number of pregnant mares. [a= β_2-hors.PP, b= γ_2-hors.P]

affinity column in the void volume, then an interaction with heparin is by no means ruled out (Teisner et al., 1983). In the same report, Teisner postulates that affinity may be affected by column dimensions and some proteins may only be slightly retarded in the void volume, e.g. complement factors.

It appears from the above that heparin affinity chromatography could be used to partially purify β_2-hors.PP. The variable heparin affinity of the protein, however, requires further investigation so that the purification conditions can be optimised.

5.8.3 Development of an Oligospecific Antiserum for β_2-hors.PP

An oligospecific antiserum for β_2-hors.PP and γ_2-hors.P would be useful in the identification of the proteins in various tissues and also in their immuno-purification. Precipitin line immunization and the adsorbing of antisera raised against serum from a pregnant mare with virgin horse serum were two techniques used to try and obtain such an antiserum.

5.8.3.1 Precipitin Line Immunization

This technique requires the removal of precipitin lines from an agarose gel after two-dimensional crossed immunoelectrophoresis. Consequently, attempts were made to increase the clarity of the peaks by using different quality agarose. Special precautions concerning the choice of gel quality are necessary when analyzing proteins with known heparin interactions because the sulphate groups of agarose have protein binding properties similar to those in commercial heparins (Laurell and McKay, 1981). Such proteins can only be analysed reliably by precipitation techniques if highly purified agarose with a sulphate content of less than 0.01% is used. Pregnant mare serum was electrophoresed in two-dimensional crossed immunoelectrophoresis using a low sulphate containing agarose (Sigma-Type

VIII) for both dimensions. The resulting peaks, however, were very large and diffuse which suggests that the sulphate groups in the agarose play an important role in the precipitation process (Fig.5.16). When the experiment was repeated using low sulphate containing agarose for the first dimension and normal agarose for the second, to aid precipitation, the peaks were of a better size but the equine pregnancy proteins could not be identified. Subsequently, normal agarose (Type II) was used for the identification and removal of precipitin lines for immunization.

Pregnant mare serum was immunoelectrophoresed against rabbit anti-pregnant horse antiserum. The β_2-hors.PP and γ_2-hors.P precipitin lines were removed from the gel and solubilized as described in Section 4.7. A New Zealand white rabbit was immunized with the β_2-hors.PP and γ_2-hors.P antigen-antibody complex following the protocol outlined in Section 4.3.1. A total of 6 precipitin lines were solubilized in 1 ml of KI and thus 3 precipitin line equivalents (β_2-hors.PP and γ_2-hors.P) were used for the primary immunization and subsequent booster immunizations. After the second booster immunization and after each subsequent booster, the rabbit was bled and antiserum obtained (Section 4.3.2). Pregnant mare serum known to contain β_2-hors.PP andγ_2-hors.P was immunoelectrophoresed against various concentrations of each preparation of the antiserum. Despite repeated immunoelectrophoretic runs, no precipitin lines were observed, and there was no evidence of an oligospecific antiserum against β_2-hors.PP and γ_2-hors.P.

The most likely reason for this is that the amount of protein immunized may have been too low to induce antibody production. Another possibility is that as antigen-antibody complex was immunized instead of antigen, the

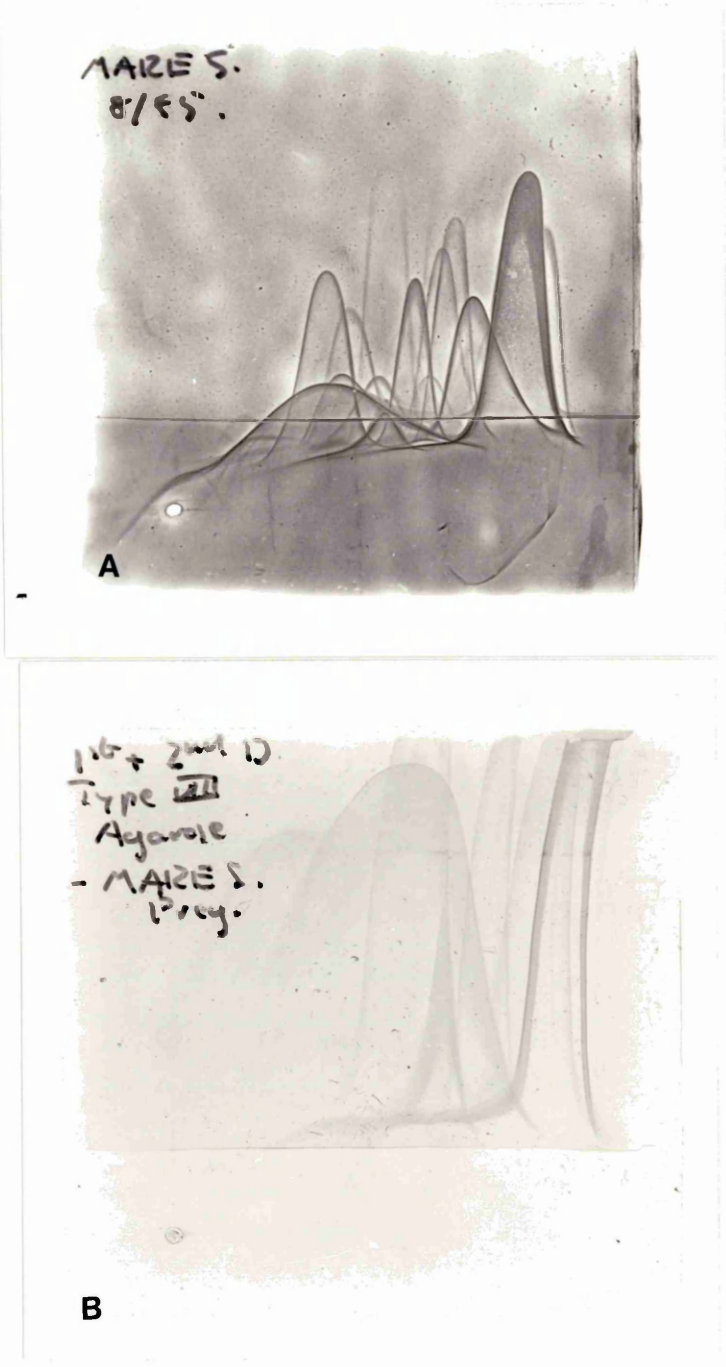

Fig.5.16 Two-dimensional crossed immunoelectrophoresis of pregnant mare (H3) serum in two types of agarose gel.

A: Type II

B: Type VIII [low sulphate content]

epitopes of the antigen may have been masked by the antibody component of the complex.

5.8.3.2 Adsorbing Antisera with Virgin Horse Serum

Complete adsorbtion of rabbit anti-pregnant horse antiserum was unsuccessful. Antisera passed through Sephacryl S-300 linked to virgin horse serum was used in two dimensional crossed immunoelectrophoresis using virgin and pregnant serum as the antigen source. Precipitates in the alpha$_1$ region of the plate were, however, formed in both cases. However, in view of the close immunological relationship of β_2-hors.PP and γ_2-hors.P, the removal of specificities to one of the proteins by adsorption of the antisera would probably remove specificities to the other. Consequently, the above results were not unexpected.

CHAPTER -6-

INVESTIGATIONS INTO THE BIOLOGICAL ACTIVITIES ASSOCIATED WITH PLACENTAL AND ENDOMETRIAL TISSUES OF THE HORSE

6.1 Introduction

Local immunosuppressive mechanisms are thought to play a critical role in the immunoprotection of the mammalian foetal allograft (Clark et al., 1987; Chaouat, 1987). Research in this area has, however, largely focussed on mammals with haemochorial placentation particularly the human and mouse. Conversely, analogous research in mammals with epitheliochorial placentation has been very limited. The establishment of the equine epitheliochorial placenta is of particular interest in view of the endometrial cup reaction unique to the genus Equus. During this period, the foetal stimulus to the mother is maximal (Allen et al., 1986) and it is thought that local immunosuppressive mechanisms may play an important role in the protection of the equine foetus (Antczak and Allen, 1984). There is evidence to show that at least one population of T cells attracted to the endometrial cups have suppressor rather than cytotoxic activity (Kydd and Allen, 1986). Furthermore, preliminary in vitro studies suggest that the principal secretory component of the cups, equine chorionic gonadotrophin (eCG), may suppress lymphocyte proliferation (Kydd and Allen, unpublished). The effects of extracts of equine placental and uterine tissues on lymphocyte proliferation and the inhibitory mechanisms of the factors on this proliferation have not previously been reported. A major objective of this section of work was to evaluate the inhibitory effects of specific maternal and foetal tissues on lymphocyte proliferation.

The *in vitro* tests used to assess lymphocyte proliferation were the allogeneic two way mixed lymphocyte reaction (MLR) and the mitogen stimulation assay (MSA) using Concanavalin A (Con.A) as the mitogen. In both assays, lymphoproliferation was assessed by measuring the uptake of ^{3}H-Tdr into the DNA of the proliferating lymphocytes (Section 6.3). The inhibitory effect of a sample on lymphocyte proliferation was taken as evidence of an immunosuppressive effect. The MLR is considered to be a better test for immunosuppressive activity in that the assay conditions approximate those that occur during *in vivo* lymphocyte stimulation and proliferation. In this assay, two populations of cells are stimulated to proliferate by foreign MHC class II antigens on the cells surface of the opposite population. In the context of pregnancy, the MLR can be used as a model of maternal cell mediated immunity. The suppression of the MLR by placental extracts can be described as an *in vitro* model of the graft -versus-host reaction.

The first part of this Chapter deals with the effect of the lectin Con.A on equine lymphocytes. This is followed by the major section dealing with the effects of eCG phase maternal and foetal tissues on lymphocyte proliferation. Finally the inhibitory activity of term placental tissue is investigated and the results compared to activities of analogous tissues taken earlier in gestation (eCG phase).

6.2 The Mitogenic Stimulation of Equine Lymphocytes with Concanavalin A (Con.A)

All mitogen stimulation assays (MSA's) used in this work were set up using the lectin Con.A as the response of equine lymphocytes to phytohaemaglutinin (PHA) has been reported to be very variable (Lazary *et al.*, 1973a).

Con.A is a mitogenic lectin with a specificity for the sugars αD-Mannose, α-D-Glucose and D-Fructose. As a consequence of their carbohydrate specificity, a number of lectins can activate from 30 to 60% of cells and are thus often referred to as polyclonal activators. No information is currently available regarding the mechanism of action of Con.A. It is reported, however, that antibodies against the sheep red blood cell receptor (CD2 antigen) block cell proliferation in response to PHA but not in response to Con.A (O'Flynn et al., 1985). This demonstrates that the two lectins mediate their effects on the cell via different cell surface receptors.

Equine lymphocyte cultures at a concentration of 1 x 10^6 cells/ml were incubated with different concentrations of Con.A ranging from 0 to 16 ug/ml culture fluid. To determine the optimum mitogenic concentration of Con.A, tritiated thymidine incorporation by triplicate cultures (expressed as mean counts per minute) was plotted against the final concentration of Con.A in each culture. This procedure was carried out with lymphocytes from two different horses and both sets of results are shown in Fig.6.1. The maximum response was achieved with a mitogen (Con.A) concentration of 2 ug/ml culture fluid and this concentration was selected for further mitogen stimulation studies. In both horses, the sensitivity of the lymphocytes appeared to be the same although the magnitude of response varied. Increasing concentrations of Con.A from 0 to 2 ug/ml resulted in an increase in lymphocyte proliferation as measured by tritiated thymidine uptake. As the concentration of Con.A was increased beyond 2 ug/ml, the degree of lymphocyte proliferation decreased until no proliferation occurred at concentrations of 8-12 ug/ml.

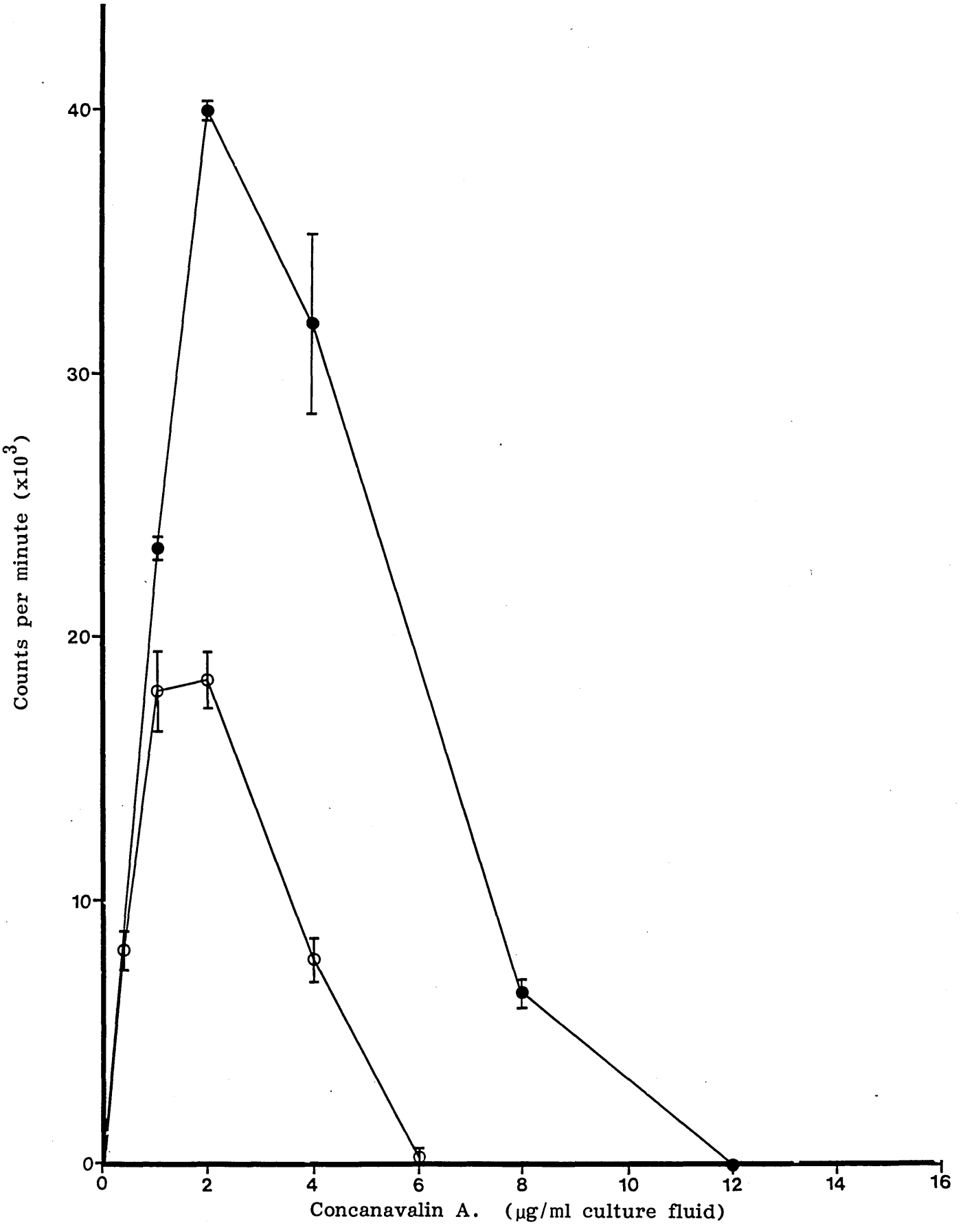

Fig.6.1 The effect of different concentrations of Con.A on equine lymphocyte proliferation; determination of maximum mitogenic concentration. Results are expressed as means ± standard error of each calibration. (n=2)

There are no published data on the dose-response relationships of equine lymphocytes to Con.A. However, this appears to be similar to the observed effect of mitogens on human lymphocyte proliferation, only in the later case, the decline in the proliferative response at high mitogen concentrations is more gradual and tends to tail off so that there is always some response (Pockley and Bolton, personal communication). High concentrations of the mitogen were shown not to be cytotoxic. It is thought that as more mitogen is added to a cell culture, the more mitogen will become bound to the cell surface. Consequently, it is possible that this will interfere with the production and/or interaction of the cytokines with the cell surface.

The sensitivity of mammalian lymphocytes to Con.A under optimal conditions has been reported to vary between 10,000 and 200,000 CPM/culture (Kristensen et al., 1982).

6.3 Assessing the Inhibitory Activity of Biological Samples on Lymphocyte Proliferation: Control Cultures

The incorporation of ^{3}H-Tdr into the DNA of the control cultures (those containing no tissue extract) of mitogenically and allogeneically stimulated lymphocytes was taken as 100%. Incorporation into cultures incubated with tissue samples was compared with these values. Each such experiment was carried out in triplicate. Non-specific (background) stimulation of the lymphocytes, as assessed by the uptake of ^{3}H-Tdr into unstimulated lymphocytes either in the absence of Con.A (MSA) or in lymphocytes from one donor (MLR), was generally less than 20% of this control value for the mixed lymphocyte reaction and less than 5% for the Con.A stimulated lymphocytes when the final concentration of Con.A was 2.0 µg/ml. In a few instances, non-specific stimulation was as high as 60%, particularly in the MLR, where the cell numbers in each culture were twice

that of the MSA cultures. It has been reported, however, that freshly prepared lymphocytes are not necessarily in the G_0 phase of the cell cycle (resting) but may already have reached a later phase already committed to proliferation due to an unknown endogenous stimulus (Kristensen et al., 1982). Consequently these cells will have a high thymidine uptake until the cycle has passed, or might maintain a high activity as a consequence of further cell proliferation (Bodeker et al., 1980; Kristensen et al., 1982).

Significance levels referred to in the text were calculated using Student's t test.

6.3.1 Assessing the Cytotoxic Effects of Samples Incorporated into Lymphocyte Cultures

All placental and endometrial extracts used in this research were incubated with 2×10^5 cell for 6 days. After this period, the viability of the lymphocytes was assessed by using the Trypan Blue exclusion method. The percentage of viable cells in all treated cultures was not significantly different to that in control cultures suggesting that the samples were not cytotoxic to the lymphocytes.

6.4 The Inhibitory Effect of eCG on Lymphocyte Proliferation as Assessed by the MLR and MSA

A majority of placental samples used in this study were taken during the eCG phase of equine pregnancy and thus some samples were expected to contain measurable amounts of this hormone. As preliminary in vitro studies suggested that eCG may have an immunosuppressive effect (Kydd and Allen, unpublished), it was deemed appropriate to confirm and further investigate these findings.

eCG obtained from 'Intervet' was extensively dialysed to remove the sugar stabiliser. Con.A-stimulated equine lymphocytes and mixed lymphocyte cultures were incubated with concentrations of 0-40 iu/ml eCG throughout the entire culture period.

In the MLR, a dose-dependent inhibition of lymphocyte proliferation was observed (Fig.6.2). A significant inhibition occurred between 5 and 10 iu/ml ($P<0.10$). Increasing eCG concentrations beyond 10 iu/ml resulted in a plateau with no further increase in inhibitory activity and this maximal inhibition was less than 100%.

In the MSA, a dose-dependent inhibition of lymphocyte proliferation occurred between eCG concentrations of 10 and 40 iu/ml (Fig.6.3). The difference in the inhibitory effect of eCG at each of these concentrations was found to be significant ($P<0.02$). Furthermore, maximal eCG concentrations tested were found not to induce 100% inhibition.

eCG is documented as containing 45% carbohydrate consisting of 14.3% hexose, 20.6% hexosamine and 10.2% sialic acid (Schams and Papkoff, 1972). Moreover, Con.A has a specificity for the hexoses α-D-mannose and α-D-glucose as well as D-fructose. It is possible therefore that eCG may inhibit the MSA by binding to the Con.A and thus preventing an interaction between the mitogen and the cell surface.

The inhibitory effect of eCG in the two way MLR is indicative of an immunosuppressive effect of the hormone preparation. That eCG did not induce 100% inhibition in either the MLR or MSA suggests that the hormone does not inhibit the proliferation of all cell populations in culture. Furthermore, the inhibitory effects of eCG at 40 iu/ml in both the MLR and

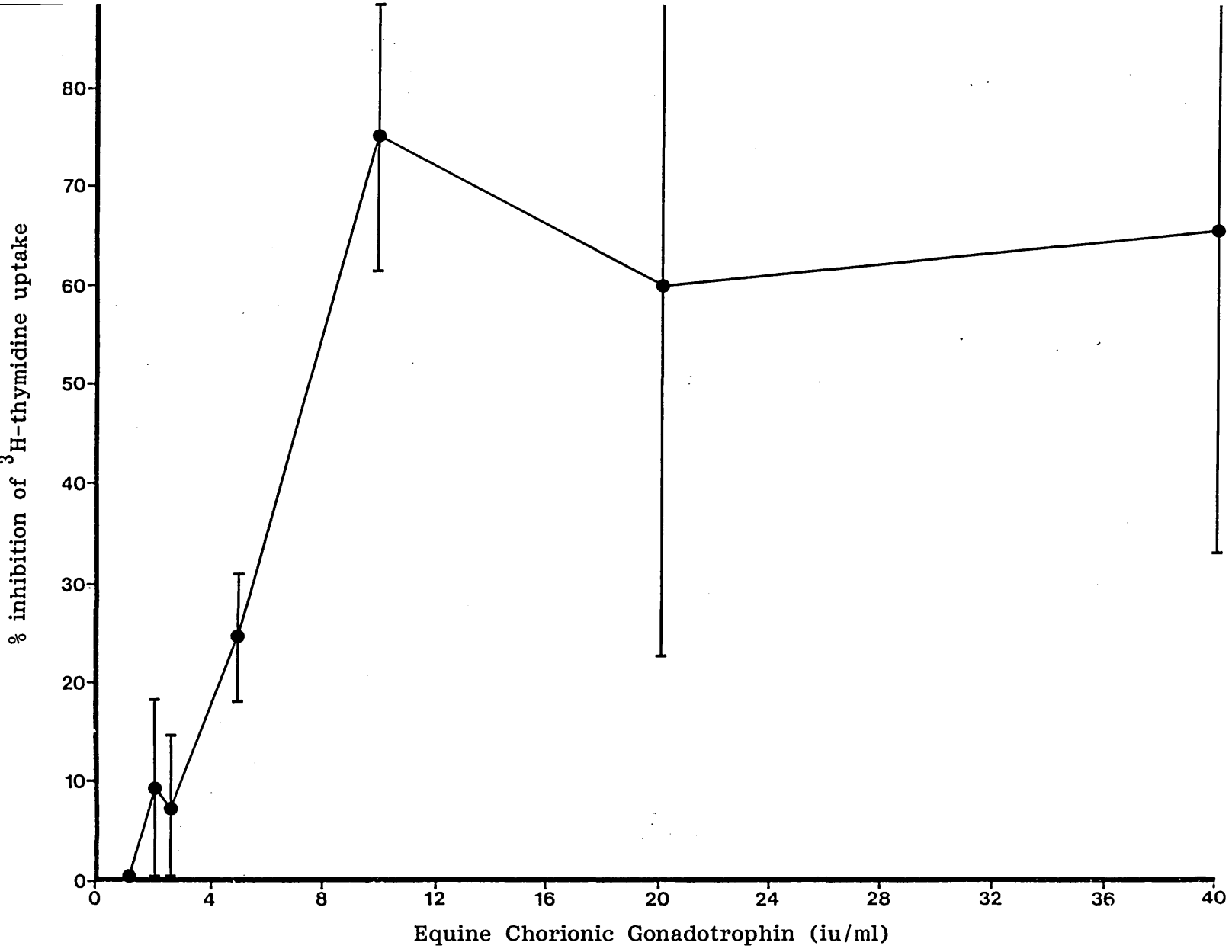

Fig.6.2 The effect of eCG, related to hormone activity, on the inhibition of ^{3}H-thymidine uptake by mixed lymphocyte cultures. C.P.M. in control cultures = 56,500. Results are expressed as means ± standard error, (n=2).

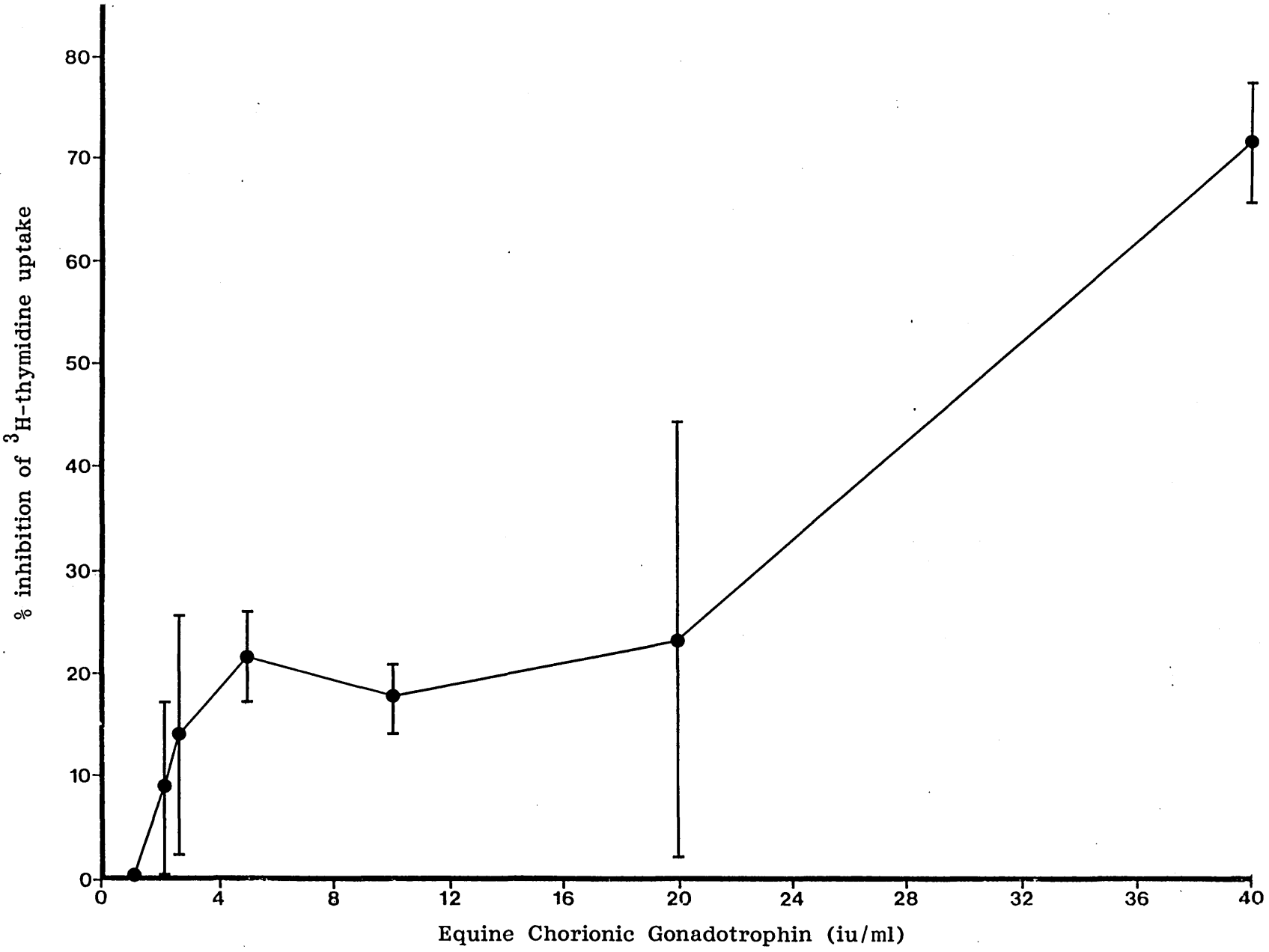

Fig.6.3 The effect of eCG, related to hormone activity, on the inhibition of ^{3}H-thymidine uptake by Con.A stimulated lymphocytes. C.P.M. in control cultures = 170,000. Results are expressed as means ± standard error, (n=2).

MSA were not significantly different (P>0.10). This suggests that eCG was not binding to the Con.A in the MSA and could be inhibiting lymphoproliferation in both assays via similar mechanisms.

The above data confirms the earlier findings of Kydd and Allen (unpublished) and may be indicative of an immunosuppressive role of the hormone. During placentation, eCG is secreted directly into the uterine lumen between the trophoblast and endometrial epithelium as an exocrine endometrial cup secretion suggesting that any immunomodulatory role would be local.

6.5 The Effect of Maternal/Foetal Tissues, Taken during the eCG Phase of Pregnancy, on Lymphocyte Proliferation

Samples of tissue were obtained from two pregnant mares at days 60 (H11)and 80 (H12) of gestation. These particular days represent key stages of the endometrial cup reaction, i.e. at day 60, the endometrial cups are healthy, active and maximal amounts of eCG are found in the maternal circulation. At day 80, serum eCG levels have fallen and the cups are starting to degenerate. The ability of all tissue extracts to inhibit lymphocyte proliferation was assessed and the apparent immunomodulatory activity of the tissues and their importance in local immunosuppression during the endometrial cup reaction was evaluated.

6.5.1 eCG and Progesterone Concentrations of Endometrial and Placental Tissue Extracts Taken at 60 Days of Gestation

A horse, 60 days pregnant, was sacrificed and the uterus removed. Maternal and foetal tissue extracts, prepared as described in Section 4.9.1, were diluted to 0.6 mg protein per ml and assayed for eCG and progesterone by ELISA (Table 6.1). eCG is produced by the endometrial cups and consequently the endometrial cup homogenate contained more eCG than the

Table 6.1

SAMPLE (0.6 mg/ml)	eCG (iu/ml)	Progesterone (ng/ml)
PREGNANT HORN ENDOMETRIUM	n/d	0.1
NON-PREGNANT HORN ENDOMETRIUM	n/d	0.5
PREGNANT HORN ALLANTOCHORION	15.0	30.0
NON-PREGNANT HORN ALLANTOCHORION	n/d	16.6
ENDOMETRIAL CUP HOMOGENATE	70.5	0.8
AMNION	4.6	0.7

eCG and progesterone concentrations of day 60 placental and endometrial extracts as determined by ELISA. Values are means of duplicates, working range of eCG assay: 3-200 iu/ml, recommended range of standards for progesterone assay: 1-10 ng/ml. (n/d = not detectable).

other samples, in this case 70.5 iu/ml. Pregnant horn allantochorion also contained detectable levels of eCG at 15.0 iu/ml. No eCG was detected in the pregnant horn endometrium as the cups are only beginning to secrete into the surrounding tissues at day 60 and the tissue itself was not taken in the endometrial cup region. eCG could not be detected in non-pregnant horn endometrium and allantochorion as placentation and thus endometrial cup formation is localised in the pregnant horn. The presence of 4.6 iu/ml of eCG in the amnion extract can be attributed to eCG contamination which occurred during the dissection of the uterus within the confinement of a pyrex dish.

The incorporation of the above samples into an MLR or MSA would result in a 1 in 5 dilution of the extracts. Consequently, the incorporation of undiluted endometrial cup homogenate into such an assay would result in a final eCG concentration of 14.1 iu/ml. As discussed in Section 6.4, this level of eCG would result in approximately 70-75% inhibition in the MLR and around 20% inhibition in the MSA. The incorporation of undiluted pregnant horn allantochorion extract into a similar assay would result in a final eCG concentration of 3.0 iu/ml which would not inhibit lymphocyte proliferation in either assay.

The highest level of progesterone was found in the pregnant horn allantochorion extract (30.0 ng/ml) and a measurable level of the hormone was found in the non-pregnant horn allantochorion extract (16.6 ng/ml). Levels of the hormone in the other tissues were negligeable. Progesterone is produced by the equine placenta from the third month of gestation onwards (Allen, 1975). Furthermore, it has been demonstrated that foetal membranes can secrete the hormone before this time (Marsan et al., 1987).

This may explain why the hormone was detectable in the allantochorion extracts but not detectable in the maternal tissue or endometrial cup extracts.

Although the effect of progesterone on equine lymphocytes has not been published, Murray and Chenault (1982) found that bovine lymphocyte proliferation was not affected by progesterone. However, in the human, progesterone has been reported to inhibit human lymphocyte proliferation in both the MSA and MLR (Clemens et al., 1979), although at concentrations of between 1 and 20 μg/ml. It therefore seems unlikely that the 30 ng/ml of progesterone measured in the pregnant horn allantochorion would have a significant inhibitory effect on lymphocyte proliferation. Furthermore, the incorporation of the tissue extract into the lymphocyte cultures further diluted the progesterone concentration to 6 ng/ml. Progesterone levels are in fact lower in epitheliochorial placentae than in haemochorial placentae (Stites and Siiteri, 1983) which suggests that any direct immunomodulatory role progesterone might have is less important in epitheliochorial placentae.

6.5.2 The Inhibition of Lymphocyte Proliferation by Endometrial Extracts and Endometrial Cup Homogenate Taken from a Day 60 Pregnancy, as Assessed by the MSA and MLR

Studies of pregnancy associated immunosuppression in the horse has been limited to an investigation of suppressive activity associated with the lymphocytes that accumulate around the cups throughout their 80-100 day lifespan. When the cells were recovered between days 55 and 70 of gestation, they were found to suppress the normal mitogenic response of peripheral blood lymphocytes from the dam and unrelated horses (Kydd and Allen, 1986). In the human and mouse, however, a number of factors with immunomodulatory activity have been described in decidual/endometrial

tissue (Clark et al., 1984; Parhar and Lala, 1986; Bolton et al., 1986). As pregnancy in these species is typified by the establishment of a haemochorial placenta, it was of interest to investigate the presence of analogous or similar factors in a species exhibiting epitheliochorial placentation (Chapter -1-). Consequently the immunomodulatory activity of the equine endometrium was investigated further.

In the study, three samples were incorporated into the MSA and MLR, namely pregnant and non-pregnant horn endometrial extract and endometrial cup homogenate. Each sample induced a dose-dependent inhibition of lymphocyte proliferation in both assays (Figs.6.4, 6.5). In the MSA, at protein concentrations higher than 15 ug/ml, the non-pregnant horn endometrium was found to have significantly more inhibitory activity than the pregnant horn endometrium ($P<0.05$). Furthermore, no significant difference was found between the non-pregnant and the endometrial cup homogenate in terms of inhibitory activity ($P>0.10$).

In conclusion, the inhibitory activity of each sample in the MLR is indicative of immunosuppressive activity. It was uncertain, however, whether the inhibitory activity observed in the MSA reflected immunosuppressive activity or a sequestering of the mitogen by glycoproteins in the crude extracts. The inhibitory activity of the endometrial extracts was not related to eCG content suggesting that inhibitory/immunosuppressive activity associated with this tissue can be attributed to other factors.

In the MSA, only about 20% of the inhibitory activity of the undiluted endometrial cup homogenate may be due to the eCG concentration of 14.1 iu/ml (Fig.6.3). The majority of the inhibitory activity in the undiluted sample (approx 80%) and all of the inhibitory activity in the 1 in 4

dilution of the same sample is therefore due to other unidentified factors (Table 6.2). It can therefore be concluded that in the MSA, the inhibitory activity of the endometrial cup homogenate is not related to its eCG content.

6.5.3 The Inhibition of Lymphocyte Proliferation by Allantochorion Extracts and Endometrial Cup Homogenate Taken from a Day 60 Pregnancy, as Assessed by the MSA and MLR

Extracts of allantochorion from the pregnant and non-pregnant horns induced a dose-dependent inhibition of lymphocyte proliferation in both the MLR and MSA (Figs.6.5, 6.6). The effects of the endometrial cup homogenate on the MLR and MSA were discussed in Section 6.5.2. The undiluted allantochorion extracts were more inhibitory in the MLR than in the MSA. Furthermore, in the MSA the pregnant horn allantochorion extract was not significantly different in its inhibitory activity to the non-pregnant horn allantochorion extract ($P>0.10$). In addition, the dose-dependent inhibition of the endometrial cup homogenate was virtually identical to that of the pregnant horn allantochorion.

These data suggest that the allantochorion samples contain immunosuppressive factor(s). Furthermore, the inhibitory activity was not related to eCG content (Table 6.2), suggesting the presence of another

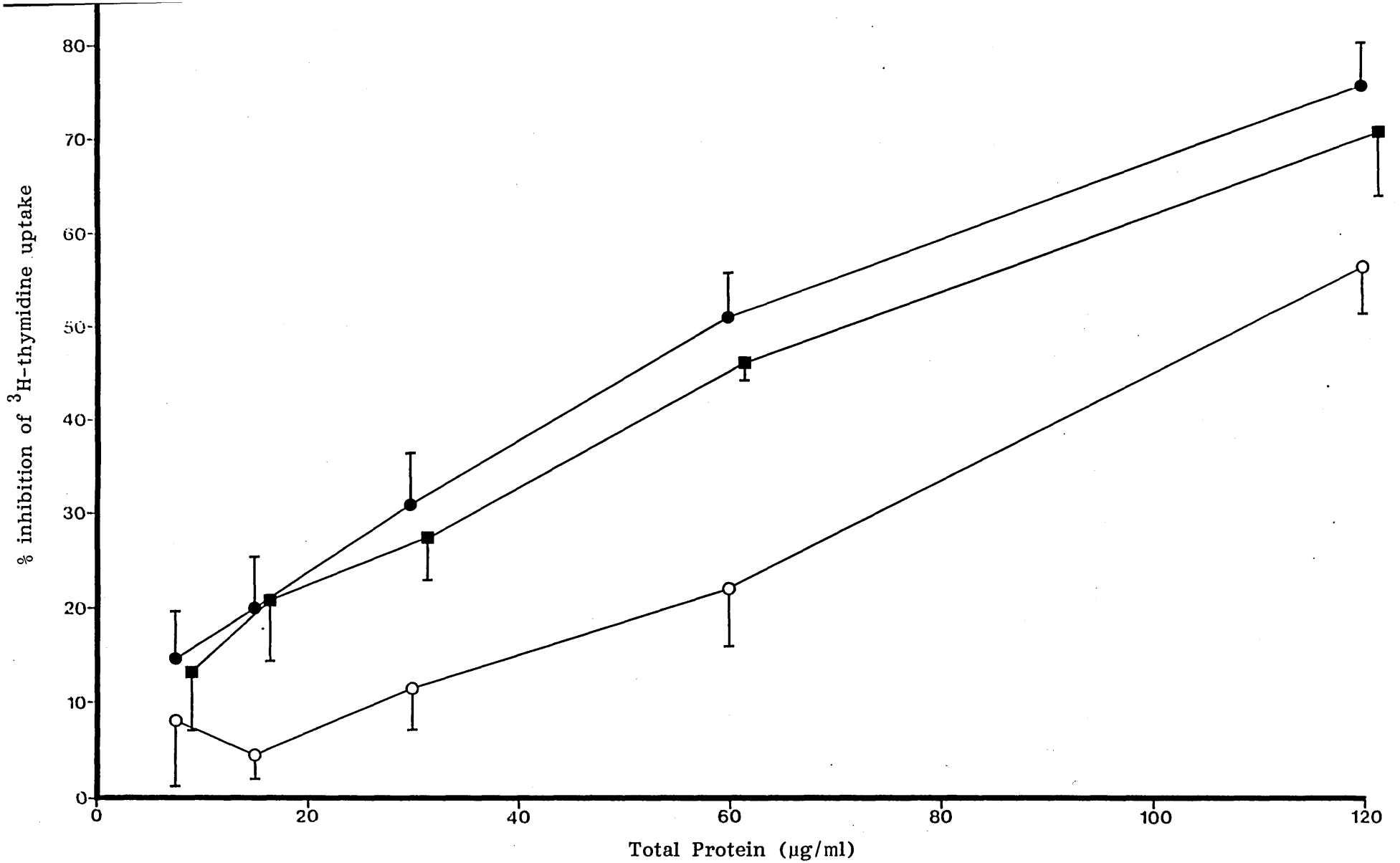

Fig.6.4 The effect of day 60 endometrial extracts, related to protein content, on the inhibition of ^{3}H-thymidine uptake by Con.A stimulated lymphocytes. Samples used were endometrial cup homogenate (■), pregnant horn endometrium (O) and non-pregnant horn endometrium (●). C.P.M. in control cultures = 87,300. Results are expressed as means ± standard error, (n=4).

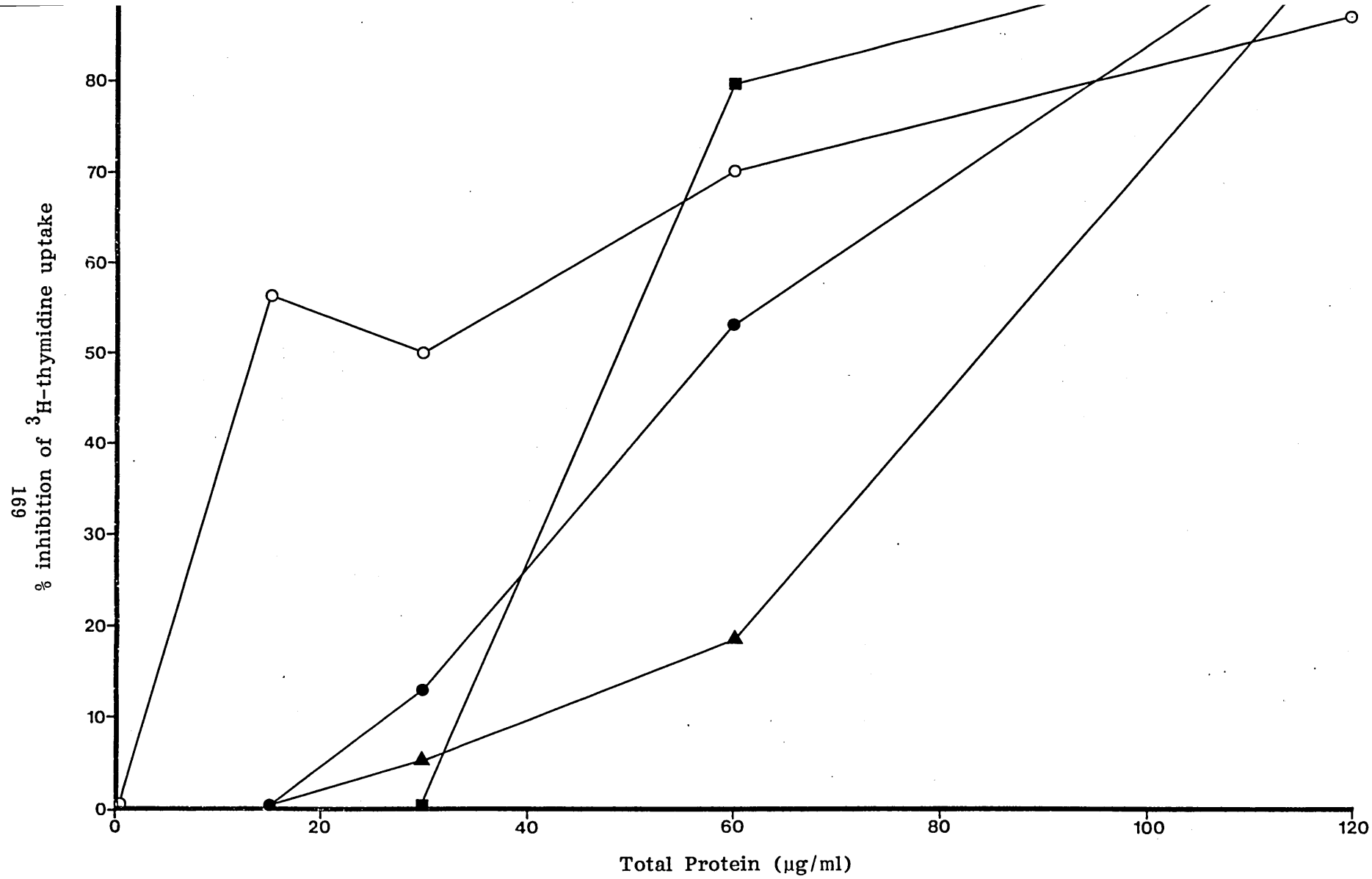

Fig.6.5 The effect of day 60 placental and uterine extracts, related to protein content, on the inhibition of ^{3}H-thymidine uptake by mixed lymphocyte cultures. Samples used were endometrial cup homogenate (●), pregnant horn endometrium (O), pregnant horn (▲) and non-pregnant allantochorion (■). C.P.M. in control cultures = 28,600. Results are expressed as mean incorporation into triplicate cultures (n=1).

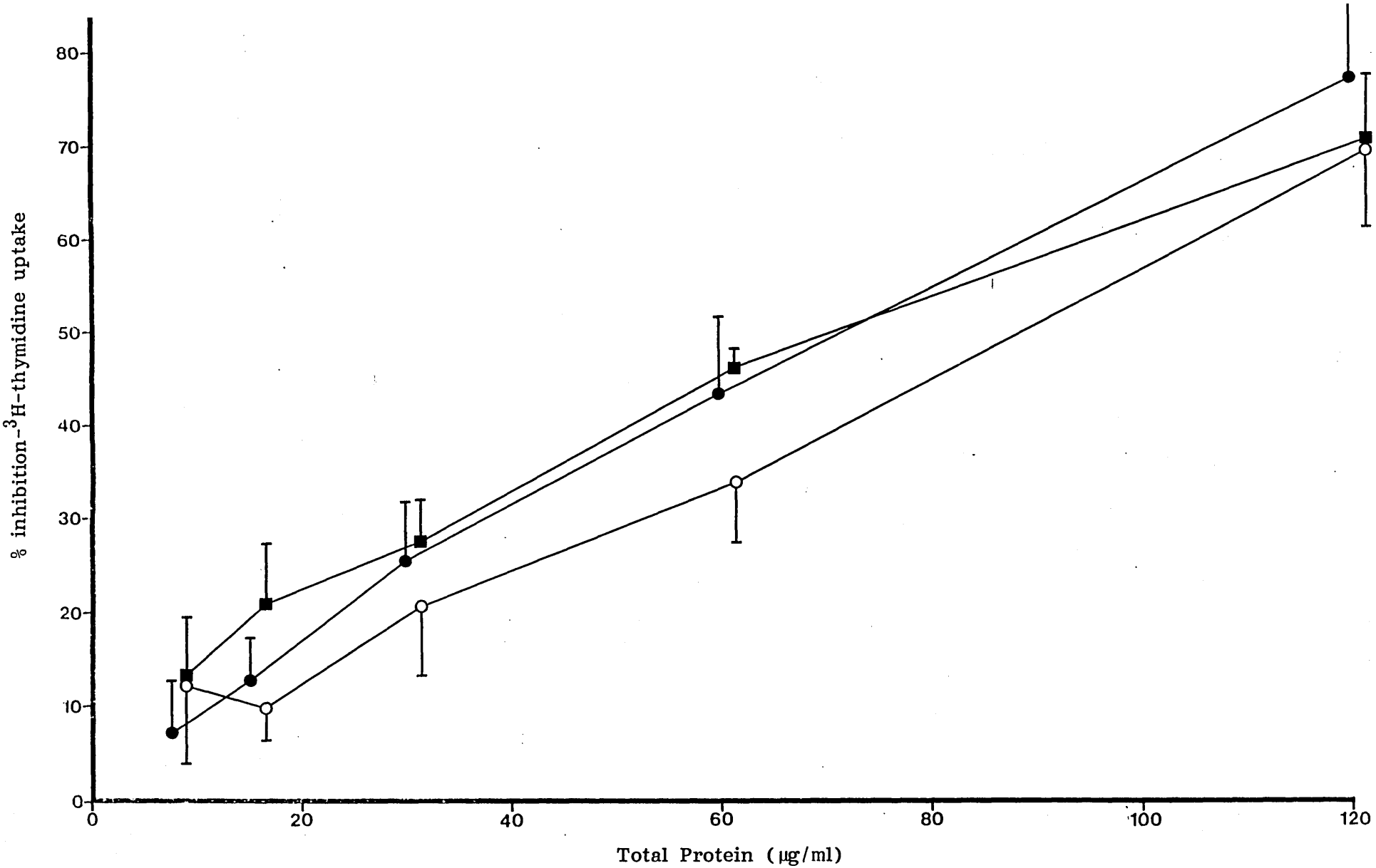

Fig.6.6 The effect of 3 day 60 allantochorion and endometrial cup homogenate, related to protein content, on the inhibition of ^{3}H-thymidine uptake by Con.A stimulated lymphocytes. Samples used were pregnant horn allantochorion (●), non-pregnant horn allantochorion (○) and endometrial cup homogenate (■). C.P.M. in control cultures = 87,300. Results are expressed as means ± standard error, (n=4).

Table 6.2

Sample	Protein ug/ml	eCG iu/ml	Progesterone ng/ml	% inhibition MSA (n=4)	MLR (n=1)
PREGNANT HORN ENDOMETRIUM	15	n/d	n/d	4.1 ± 2.5	55.7
	30	n/d	n/d	11.1 ± 4.3	49.4
	60	n/d	n/d	21.7 ± 5.9	69.6
	120	n/d	n/d	55.8 ± 5.1	86.6
ENDOMETRIAL CUP HOMOGENATE	15	1.762	0.020	20.5 ± 6.4	0.0
	30	3.525	0.040	27.2 ± 4.4	12.5
	60	7.050	0.080	45.9 ± 1.9	52.6
	120	14.100	0.160	70.1 ± 6.5	95.7
PREGNANT HORN ALLANTOCHORION	15	0.375	0.750	12.3 ± 4.7	0.0
	30	0.750	1.500	25.2 ± 6.1	4.8
	60	1.500	3.000	42.5 ± 8.4	18.5
	120	3.000	6.000	76.5 ±10.8	99.4
NON-PREGNANT HORN ALLANTOCHORION	15	n/d	0.415	9.4 ± 3.3	0.0
	30	n/d	0.830	20.4 ± 7.2	0.0
	60	n/d	1.660	33.7 ± 6.4	78.8
	120	n/d	3.320	68.7 ± 8.0	99.6

Relative inhibitory potencies of day 60 samples in the MLR and MSA as related to protein content and including eCG and progesterone levels.

inhibitory/immunosuppressive factor(s) associated with the allantochorion. At day 60, the allantochorion will not extend very far into the non-pregnant horn of the uterus and thus there should be little difference in the secretory activity of the tissues (Allen, personal communication). This appears to be reflected in the above data as no significant difference was found between the inhibitory activities of the pregnant horn and non-pregnant horn allantochorion extracts. The similarity between the inhibitory activity of the pregnant horn allantochorion and the endometrial cup homogenate may reflect the close biological relationship of the tissues, that is the endometrial cups are derived from modified allantochorionic cells (Allen et al., 1973). The reason for differing levels of inhibition between the MLR and MSA with undiluted samples is uncertain. This may reflect different immunosuppressive effects of the same factor(s) in different assays as appears to be the case with eCG. Alternatively, the inhibition observed in the MSA may reflect a sequestering of the mitogen by glycoproteins in the allantochorion extracts.

6.5.4 eCG and Progesterone Concentrations of Placental Tissue Extracts Taken at Day 80 of Gestation

The pregnancy of a horse was terminated at 80 days gestation and specific tissue samples were obtained. All samples were diluted to 0.6 mg protein per ml and assayed for eCG and progesterone by ELISA (Table 6.3). eCG is a major product of the endometrial cups and thus the endometrial cup secretion observed in one of the allantochorion extracts produced a high sample eCG concentration of 251 iu/ml. The other allantochorion sample contained little of this secretion and this was reflected in its lower eCG content of 8.6 iu/ml. eCG was not present in the non-pregnant horn allantochorion as placentation and endometrial cup formation is localised

Table 6.3

SAMPLE (0.6 mg/ml)	eCG (iu/ml)	Progesterone (ng/ml)
PREGNANT HORN ALLANTOCHORION WITH ENDOMETRIAL CUP SECRETION (day 80)	251.0	15.7
PREGNANT HORN ALLANTOCHORION - no visible secretion (day 80)	8.6	30.0
NON-PREGNANT HORN ALLANTOCHORION (day 80)	n/d	26.6
AMNION (day 80)	n/d	1.9

eCG and progesterone concentrations of day 80 placental extracts as determined by ELISA. Values are means of duplicates, working range of eCG assay: 3-200 iu/ml, recommended range of standards for progesterone assay: 1-10 ng/ml. (n/d = not detectable).

in the pregnant horn. The lack of eCG in the amnion confirms earlier observations by Allen et al. (personal communication). The incorporation of the undiluted pregnant horn allantochorion extract, with endometrial cup secretion, into a lymphoproliferation assay would result in a final eCG concentration of 50.2 iu/ml. This concentration of eCG induces approximately 70-75% inhibition in both the MLR and MSA (Figs.6.2, 6.3). Furthermore, doubling dilutions of the sample down to a 1 in 4 dilution would still leave sufficient eCG (12.5 iu/ml) to cause the same degree of inhibition in the MLR (Table 6.4).

The progesterone concentrations of the day 80 samples were similar to those of the day 60 samples. The pregnant horn allantochorion was found to have the highest levels of the hormone at 30 ng/ml at both stages of gestation. At these concentrations of the hormone, it is unlikely that they would exert an effect on lymphocyte proliferation (See Section 6.5.1).

6.5.5 The Inhibition of Lymphocyte Proliferation by Day 80 Pregnant Horn Allantochorion as Assessed by the MSA and MLR

Pregnant horn allantochorion induced a dose dependent inhibition of ^{3}H-Tdr uptake in both the MLR and MSA (Figs.6.7, 6.8 and Table 6.4). The inhibitory effects of allantochorion both with and without endometrial cup secretion were not significantly different ($P>0.10$) in either the MSA or MLR. Furthermore, the percentage inhibition values at each protein concentration in the MLR were found not to be significantly different from the analogous values in the MSA ($P>0.10$).

As outlined above, the extract of pregnant horn allantochorion with cup secretion contained enough eCG to account for all the inhibition observed in the MLR at each sample dilution (Table 6.4). The undiluted pregnant

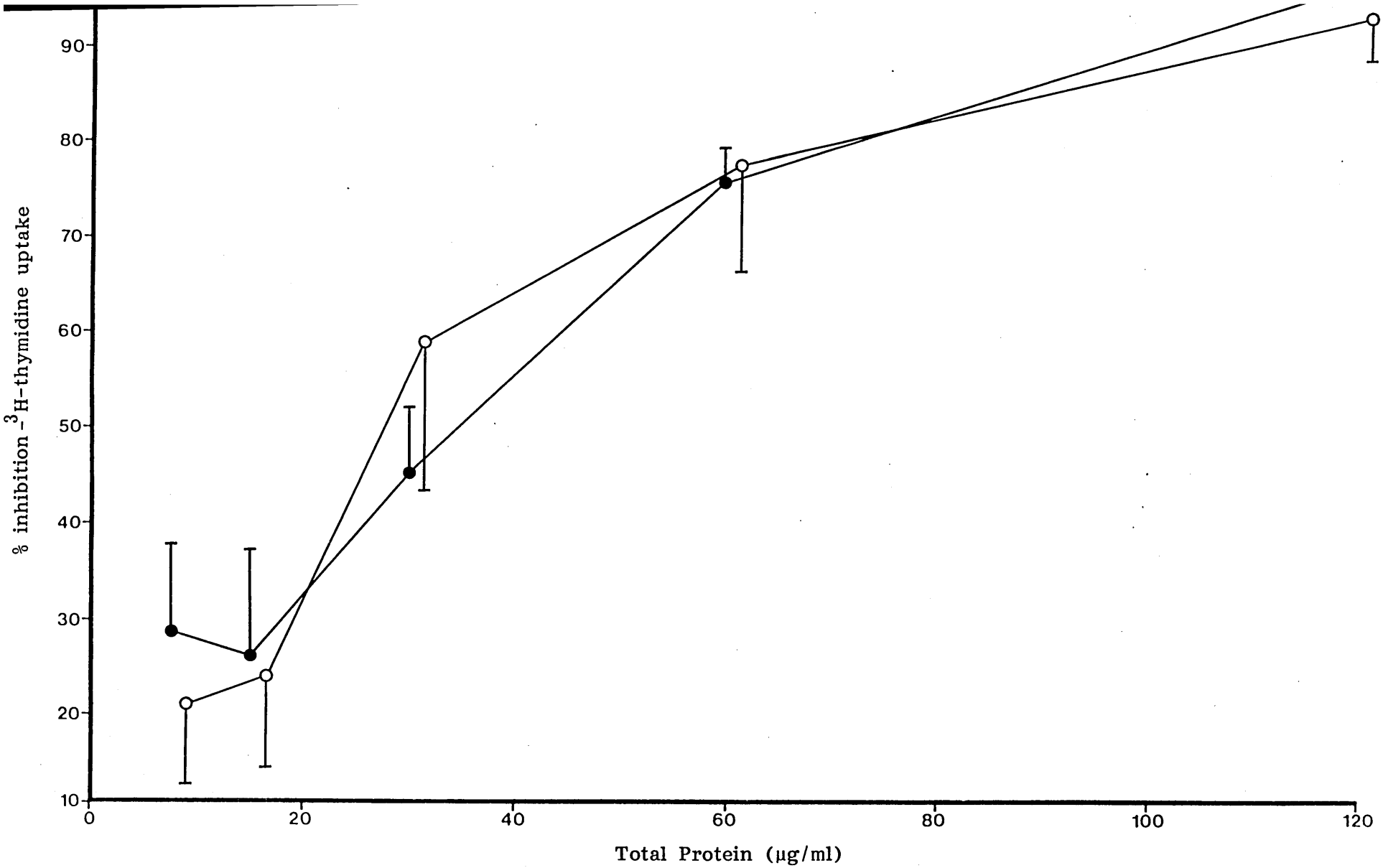

Fig.6.7 The effect of day 80 pregnant horn allantochorion, related to protein content, on the inhibition of ^{3}H-thymidine uptake by Con.A stimulated lymphocytes. Samples used were allantochorion with visible endometrial cup secretion (●) and allantochorion with no obvious secretion (O). C.P.M. in control cultures = 112,600. Results are expressed as means ± standard error, (n=4).

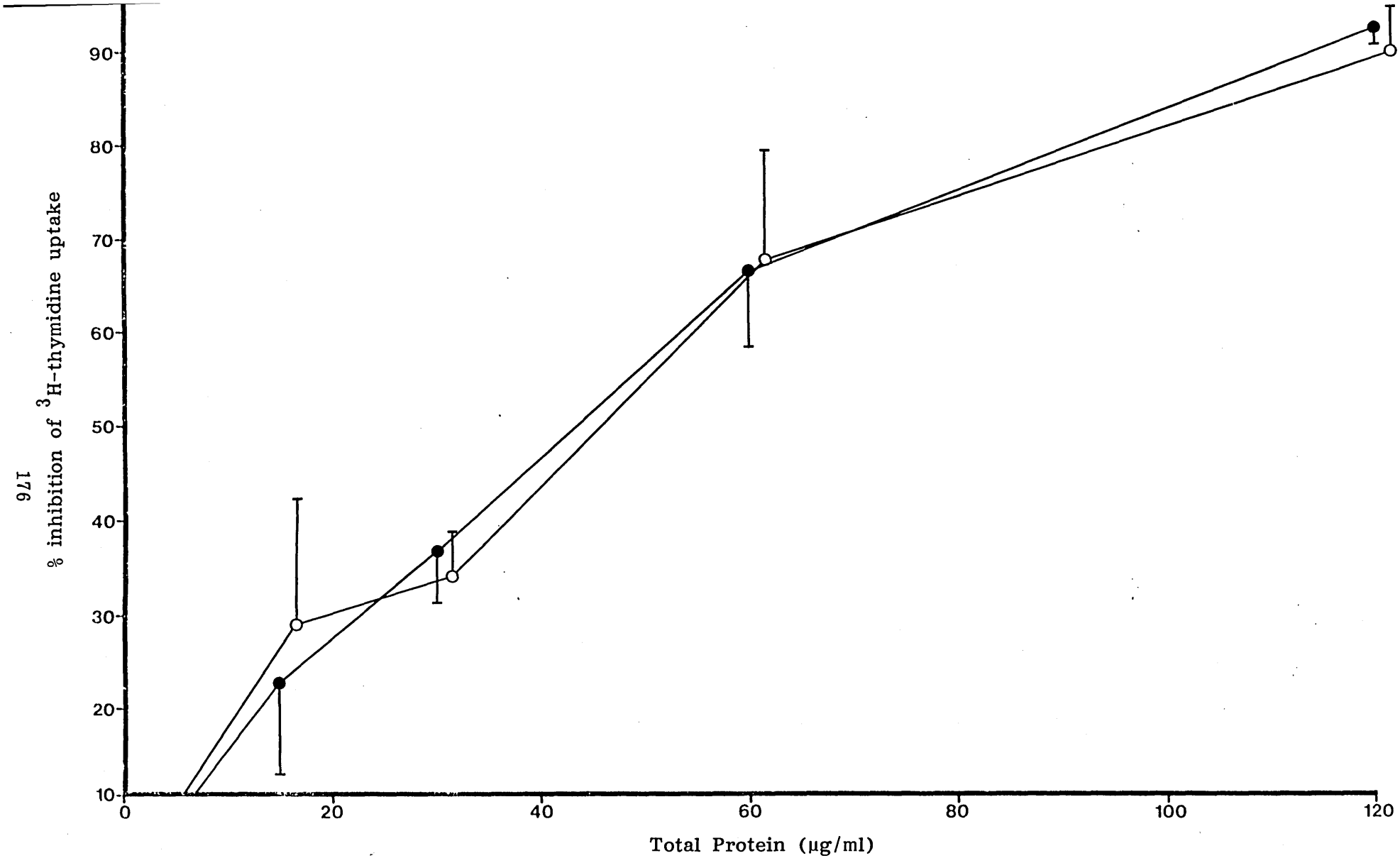

Fig.6.8 The effect of day 80 pregnant horn allantochorion, related to protein content, on the inhibition of ^{3}H-thymidine uptake by mixed lymphocyte cultures. Samples were allantochorion with visible endometrial cup secretion (●) and allantochorion with no obvious secretion (O). C.P.M. in control cultures = 156,700. Results are expressed as means ± standard error, (n=3).

TABLE 6.4

Sample	Protein ug/ml	eCG iu/ml	Progesterone ng/ml	% inhibition MSA (n=4)	MLR (n=3)
PREGNANT HORN	15	6.2750	0.3925	26.4±11.1	23.0±10.0
ALLANTOCHORION WITH	30	12.5500	0.7850	45.7± 7.0	36.9± 5.8
ENDOMETRIAL CUP	60	25.1000	1.5700	75.8± 3.6	66.6± 8.3
SECRETION	120	50.2000	3.1400	96.8± 0.9	92.7± 1.8
PREGNANT HORN	15	0.2150	0.7500	23.8± 9.4	28.9±13.5
ALLANTOCHORION - no	30	0.4300	1.5000	58.7±15.7	34.2± 4.7
visible secretion	60	0.8600	3.0000	77.6±11.2	67.6±11.5
	120	1.7200	6.0000	92.6± 4.8	89.8± 4.9
NON PREGNANT	15	n/d	0.6650	33.3± 3.5	54.2±12.2
HORN	30	n/d	1.3330	23.7± 2.4	45.6± 9.2
ALLANTOCHORION	60	n/d	2.6600	81.2± 5.7	84.8± 4.0
	120	n/d	5.3200	94.5± 2.2	87.6± 5.6
AMNION	15	n/d	0.0475	17.4± 7.5	9.8± 9.7
	30	n/d	0.0950	53.4± 9.0	51.8±14.3
	60	n/d	0.1900	83.1± 3.9	68.7±10.7
	120	n/d	0.3800	97.7± 1.5	97.4± 0.6

Relative inhibitory potencies of day 80 samples in the MLR and MSA as related to protein content and including eCG and progesterone concentrations (n/d= not detectable).

horn allantochorion extract without cup secretion, however, contained too little eCG (1.72 iu/ml final concentration) to account for any of the observed inhibition. This indicates that the ability of the pregnant horn allantochorion extract to suppress lymphocyte proliferation is independent of eCG content. The presence of additional inhibitory factors to eCG would be expected to result in an additive inhibitory effect. That this does not occur suggests that the inhibitory factors in both samples are the same and that eCG is not the factor responsible. It appears therefore that the commercial preparations of eCG tested in the MLR and MSA (Section 6.4) may contain an inhibitory/immunosuppressive contaminant and that the hormone itself may not inhibit lymphocyte proliferation.

The MSA data further confirms a non-inhibitory effect of eCG. In the MSA, the undiluted allantochorion extract with cup secretion contained enough eCG (50.2 iu/ml) to account for most of the observed inhibition (Fig.6.3, Table 6.4). The undiluted sample of pregnant horn allantochorion without cup secretion, however, contained too little eCG (1.7 iu/ml) to account for any observed inhibition. That the two samples were equivalent in suppressive activity illustrates that no additive effect was occurring between eCG and any other inhibitory factor(s), which suggests that eCG may not inhibit lymphocyte proliferation. Furthermore, as the inhibitory effects of the samples in the MLR and MSA were not significantly different, it would appear that similar inhibitory mechanisms were occurring in both lymphoproliferation assays. This latter point argues against a sequestering of the mitogen by the samples in the MSA and thus the inhibitory activity in this assay can be taken as evidence for immunosuppressive activity.

In conclusion, evidence presented above suggests that pregnant horn allantochorion extract contains an immunosuppressive factor(s) which is independent of the presence of endometrial cup homogenate and eCG. Furthermore, evidence is presented which indicates the eCG does not inhibit lymphocyte proliferation in the MSA or MLR and is therefore not immunosuppressive. Indeed, it appears that the inhibitory activity of a commercial preparation of eCG, in the MSA and MLR (Section 6.4), may be due to the presence of an unknown contaminant.

6.5.6 The Inhibition of Lymphocyte Proliferation by Day 80 Non-Pregnant Horn Allantochorion and Amnion as Assessed by the MSA and MLR

Non-pregnant horn allantochorion and amnion induced a dose dependent inhibition of lymphocyte proliferation in both the MSA and MLR (Figs.6.9, 6.10 and Table 6.4). The level of inhibition induced by non-pregnant horn allantochorion was comparable to that induced by pregnant horn allantochorion in both assays. Amnion, however, inhibited ^{3}H-Tdr incorporation to a greater extent than the other tissues indicating a higher level of inhibitory activity as assessed by the MLR. These findings confirm those of Kydd and Allen (personal communication) who also found that amnion had a greater inhibitory effect on lymphocyte proliferation than other tissues. Whether the later observation was due to higher levels of a common immunosuppressive factor, or due to the activity of a different factor, is unknown. In the human and mouse, alpha foeto-protein (AFP) present in amniotic fluid has been reported to suppress lymphocyte proliferation at physiological concentrations (Tomasi, 1978; Yachin and Lester, 1979). In addition, PP14 is present in amniotic fluid at levels high enough to suppress lymphocyte proliferation (Julkunen et al., 1985;

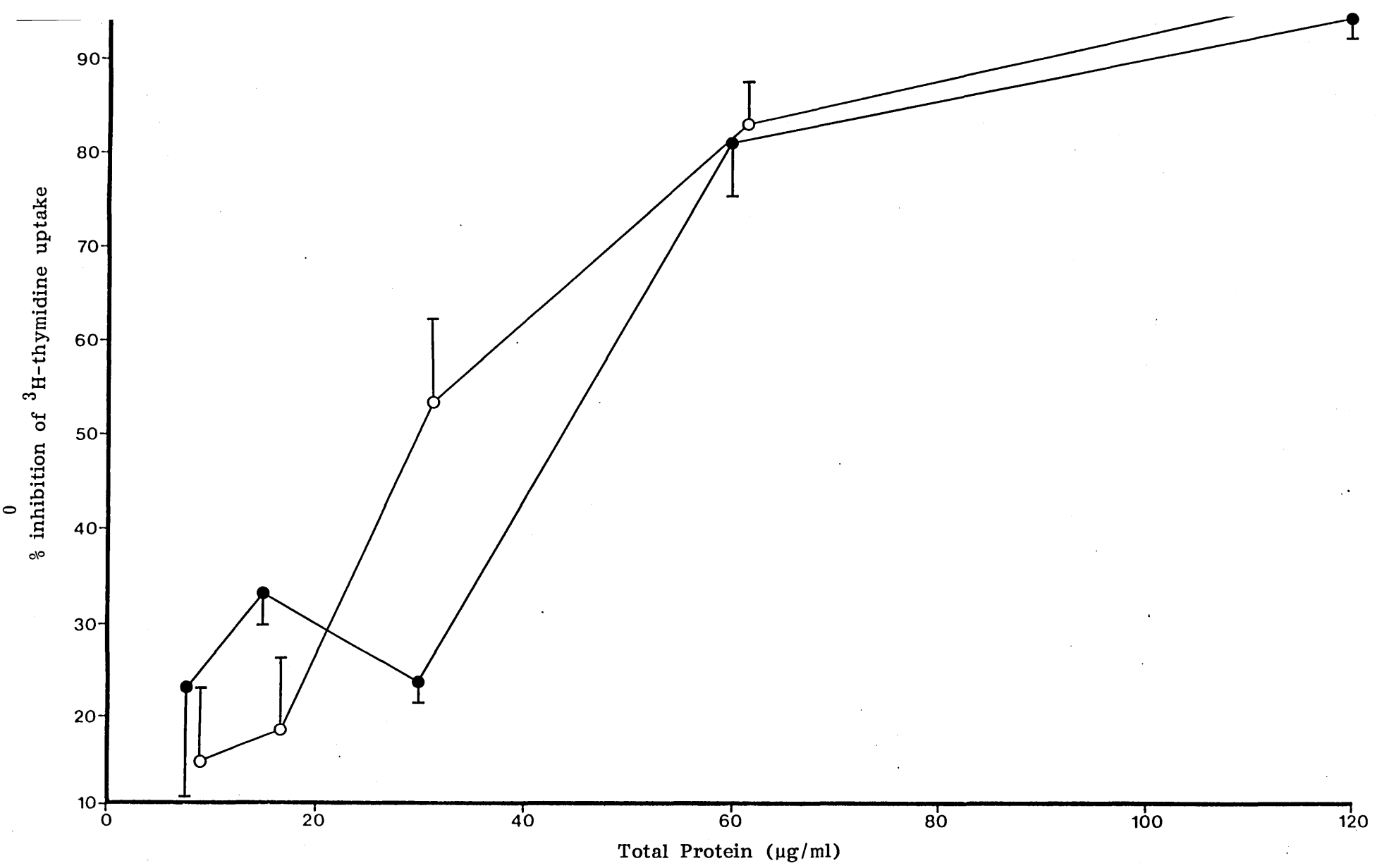

Fig.6.9 The effect of day 80 placental extracts, related to protein content, on the inhibition of ^{3}H-thymidine uptake by Con.A stimulated lymphocytes. Samples were non-pregnant horn allantochorion (●) and amnion (○). C.P.M. in control cultures = 112,600. Results are expressed as means ± standard error, (n=4).

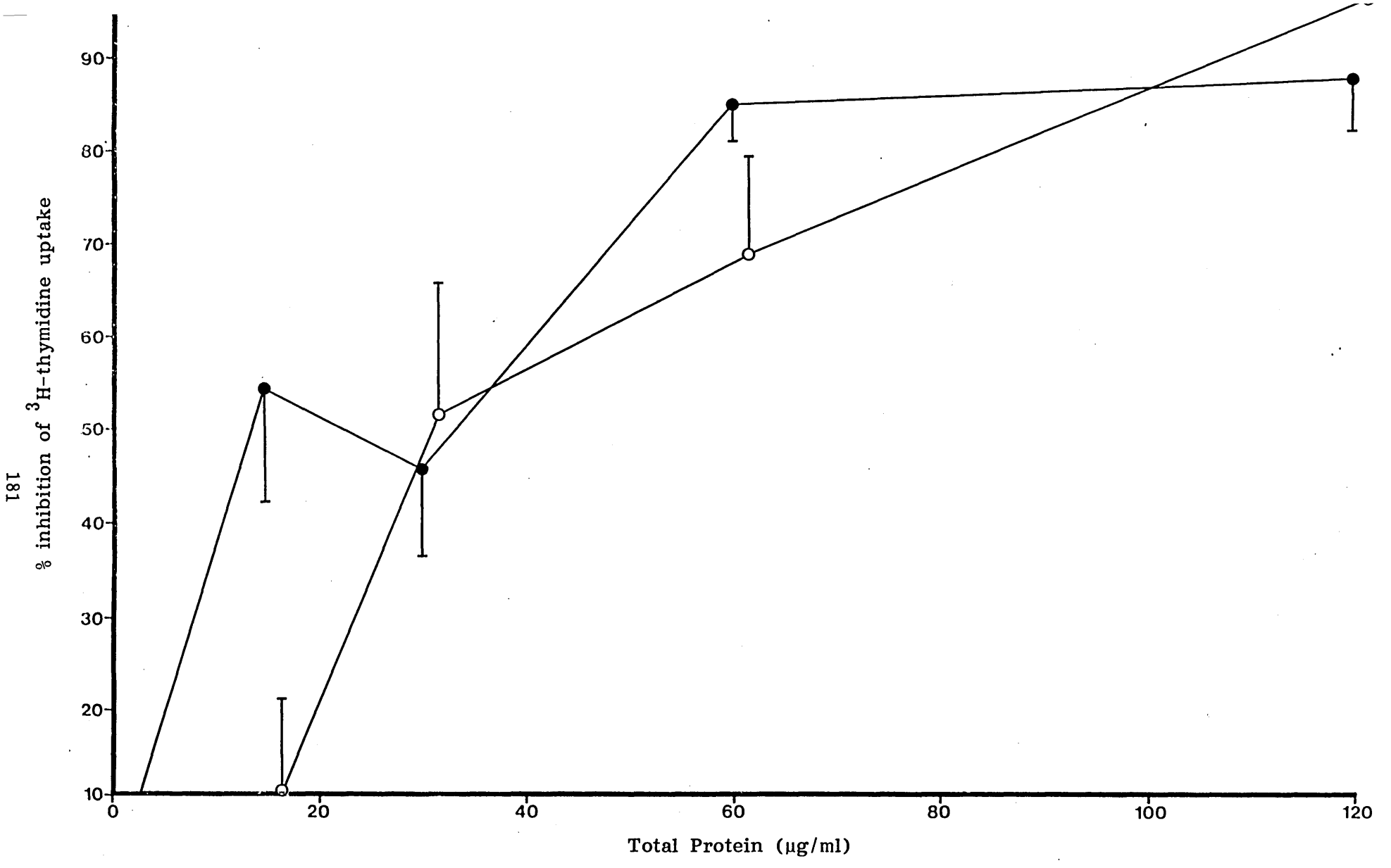

Fig.6.10 The effect of day 80 placental extracts, related to protein content, on the inhibition of ^{3}H-thymidine uptake by mixed lymphocyte cultures. Samples were non-pregnant horn allantochorion (○) and amnion (●). C.P.M. in control cultures = 156,700. Results are expressed as means ± standard error, (n=3).

Bolton et al., 1986). It is possible therefore that an equine analogue to either human AFP or PP14 may have contributed to the observed inhibition.

6.5.7 The Relative Inhibitory Effects of Pregnant/Non-Pregnant Horn Allantochorion and Amnion Taken from a Day 60 and Day 80 Pregnancy, on Lymphocyte Proliferation as Assessed by the MSA and MLR

Each of the above tissues has been shown to induce a dose-dependent inhibition of lymphocyte proliferation in both the MSA and MLR. When the % inhibition induced by each day 60 tissue extract in the MSA was compared to the % inhibition induced by the corresponding day 80 tissue extracts in the same assay, it was found that the latter day 80 extracts contained more inhibitory/immunosuppressive activity, on an activity per mass of total protein basis, than the corresponding day 60 extracts (Figs.6.11, 6.12, 6.13). The differences in the inhibitory activity of the pregnant horn allantochorion samples (day 60 and 80) were not found to be significantly different ($P>0.10$). A significant difference was, however, found when the non-pregnant horn allantochorion samples were compared at protein concentrations of 60 µg/ml and 120 µg/ml ($P<0.05$). In addition, the inhibitory activities of the amnion samples were found to be significantly different at sample protein concentrations of 30 µg/ml and above ($P<0.02$). Available data in the MLR also indicate that the inhibitory/immunosuppressive activity of day 80 extracts is higher than that of day 60 extracts (Table 6.5). As all samples were prepared in an identical manner and diluted to the same total protein concentration of 0.6 mg/ml, it was concluded that the factors responsible for the inhibitory activity were present in greater quantity or more active at day 80 of gestation than at day 60.

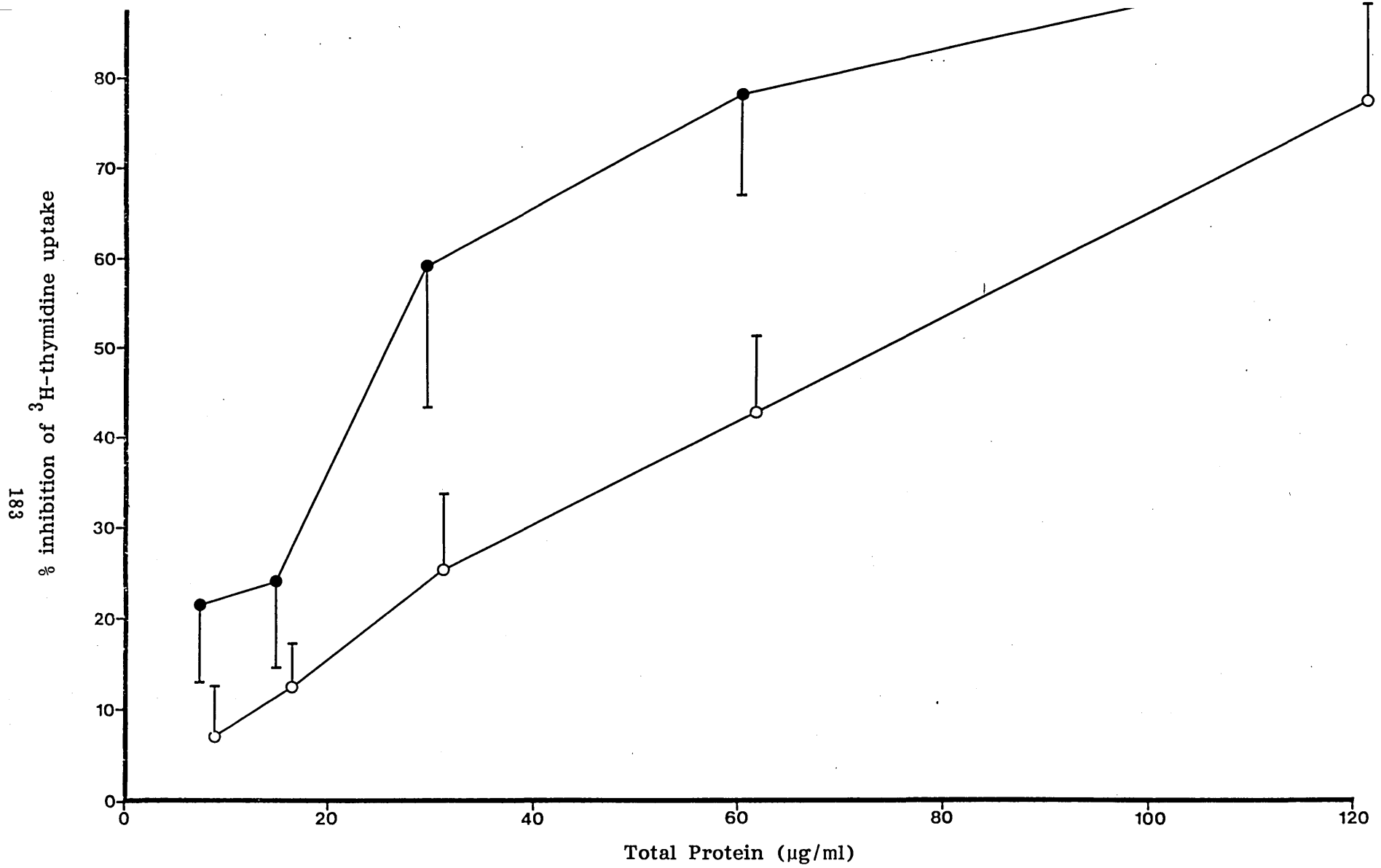

Fig.6.11 The comparative effects of day 60 (○) and day 80 (●) pregnant horn allantochorion, related to protein content, on the inhibition of ^{3}H-thymidine uptake by Con.A stimulated lymphocytes. C.P.M. in control cultures = 87,300 and 112,000 respectively. Results are expressed as means ± standard error, (n=4).

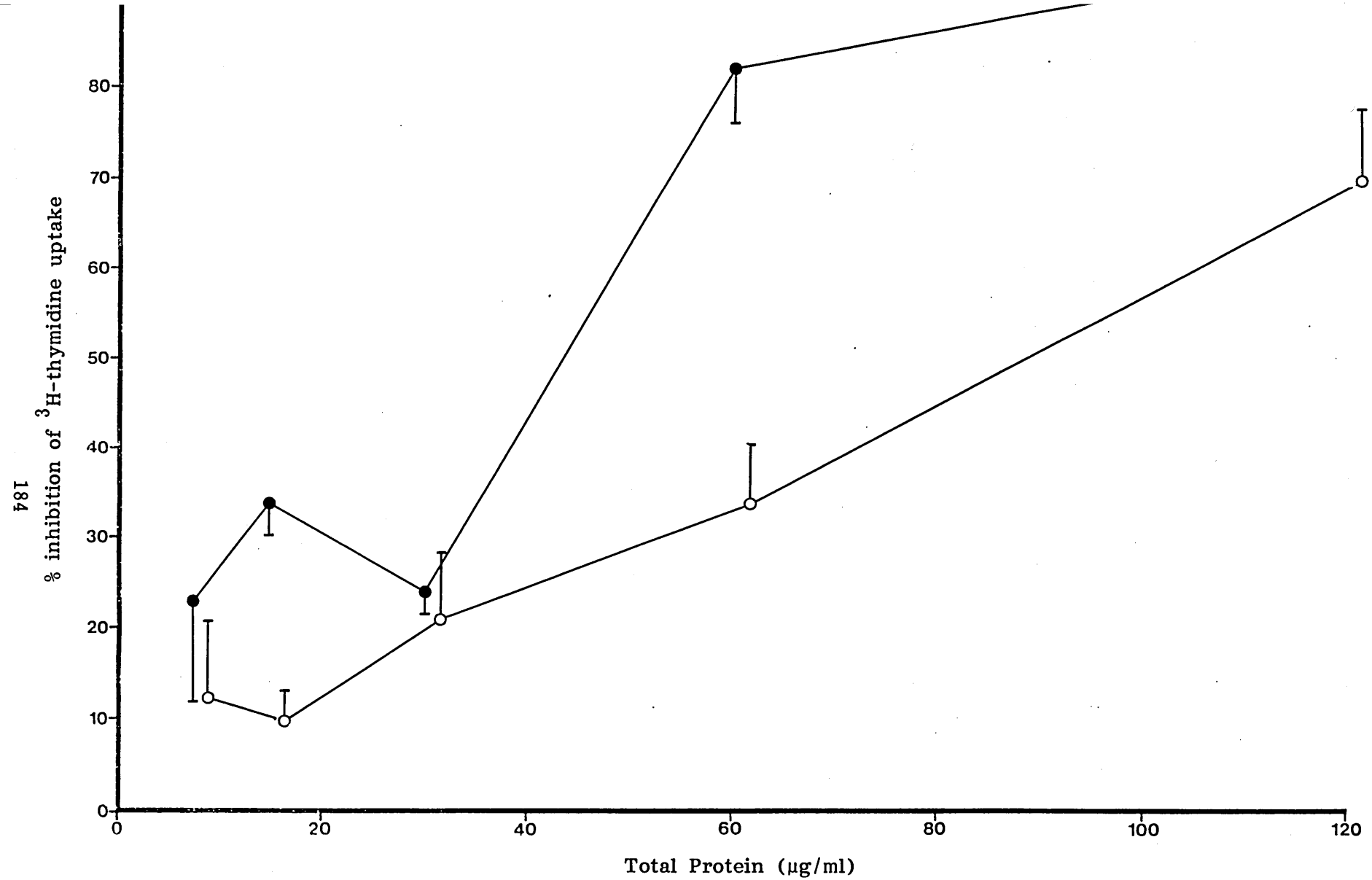

Fig.6.12 The comparative effects of day 60 (O)$_3$ and day 80 (●) non-pregnant horn allantochorion, related to protein content, on the inhibition of ^{3}H-thymidine uptake by Con.A stimulated lymphocytes. C.P.M. in control cultures = 87,300 and 112,000 respectively. Results are expressed as means ± standard error, (n=4).

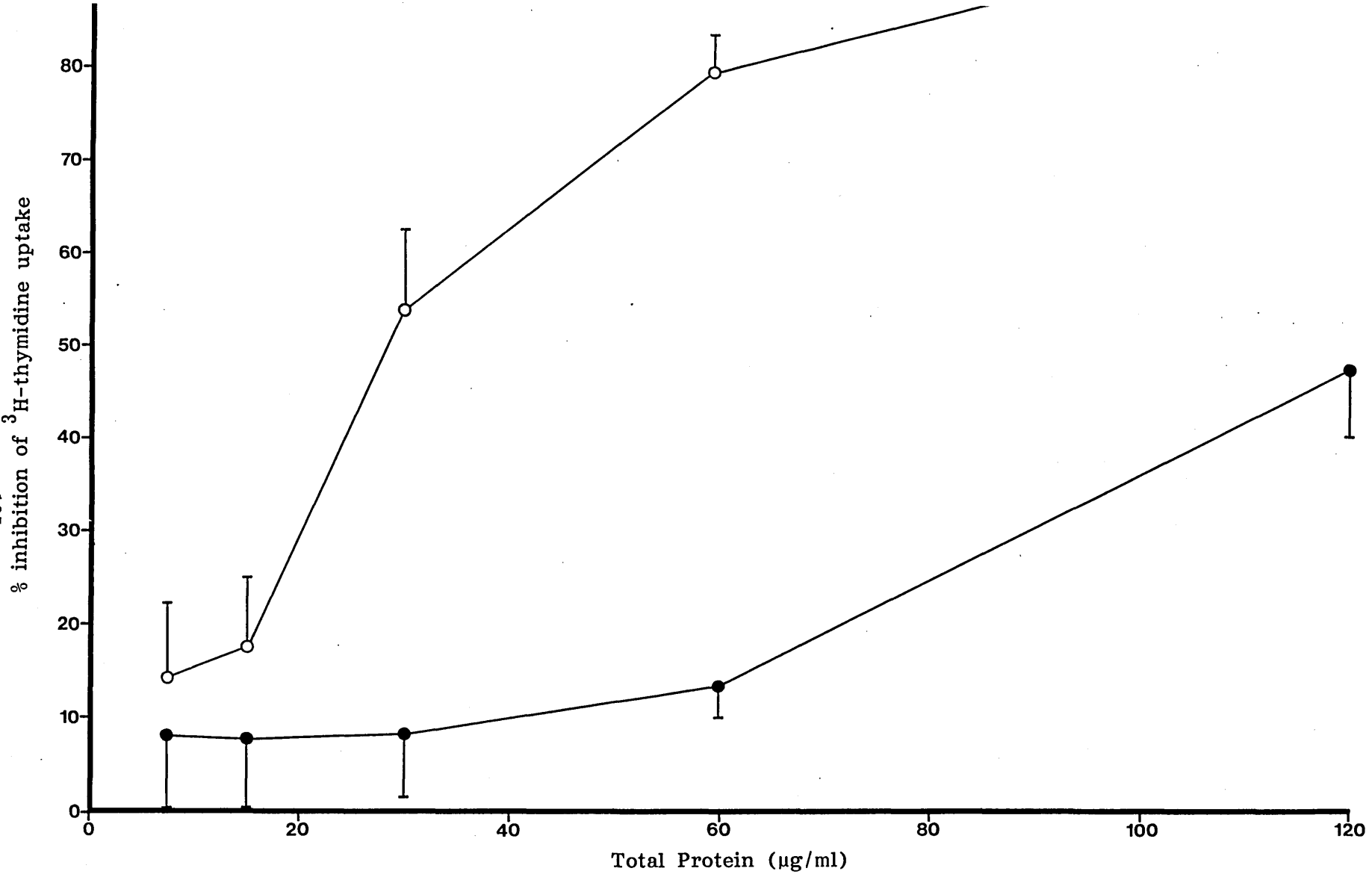

Fig.6.13 The comparative effects of day 60 (●) and day 80 (O) amnion, related to protein content, on the inhibition of ^{3}H-thymidine uptake by Con.A stimulated lymphocytes. C.P.M. in control cultures = 87,300 and 112,000 respectively.
Results are expressed as means ± standard error, (n=4).

Table 6.5

Sample	Protein ug/ml	% inhibition MLR (day 60) n=1	MLR (day 80) n=3
PREGNANT HORN ALLANTOCHORION	15	0.0	28.9 ± 13.5
	30	4.8	34.2 ± 4.7
	60	18.5	67.6 + 11.5
	120	99.4	89.8 ± 4.9
NON-PREGNANT HORN ALLANTOCHORION	15	0.0	54.2 ± 12.2
	30	0.0	45.6 ± 9.2
	60	78.8	84.8 ± 4.0
	120	99.6	87.6 ± 5.6

Relative inhibitory potencies of allantochorion extracts in the MLR at day 60 and 80 of gestation as related to protein content.

The difference between day 60 and day 80 amnion may be attributable to increasing levels of an equine factor similar to alpha-foeto protein (AFP) in the human. AFP has been reported to suppress human and murine lymphocyte proliferation (Tomasi, 1978; Yachin and Lester, 1979) and is present in the amnion in increasing concentrations during gestation (Hau, 1986). Further support for this theory came from the finding that term equine amnion contained a greater level of inhibitory activity than day 80 amnion (Section 6.7).

As it appears that eCG does not suppress lymphocyte proliferation (Section 6.5.5.), the increase in inhibitory activity of the pregnant horn allantochorion cannot be explained by a change in the eCG levels of this hormone. Whether or not this rise in inhibitory activity bears any relationship to the endometrial cup reaction is, at present, uncertain.

6.6 The Inhibition of Lymphocyte Proliferation: Investigations into the Mode of Action of the Placental/Endometrial Factors Involved

Data discussed so far in this chapter indicate that one or more factors, that appear to be immunosuppressive, are associated with equine foetal and maternal tissues during the eCG phase of pregnancy. The identity of the factor(s) and their mechanism of action are, however, unknown. In the human and mouse, a number of immunoregulatory factors have been investigated, which are produced by endometrial/decidual tissue and which appear to block the IL-2 dependent stage of the immune response (Clark et al., 1985; Tawfik et al., 1986).

Consequently, the comparative basis of this project was developed further by investigating the mode of action and properties of the equine placental/endometrial derived factors responsible for the inhibition of lymphocyte proliferation.

6.6.1 The Effect of Delayed Addition of Day 80 Allantochorion and Amnion to Mitogen Stimulated Cultures on the Inhibition of the Lymphocyte Proliferation

In an investigation of the mechanism by which extracts of allantochorion and amnion inhibit the proliferation of Con.A stimulated lymphocytes, the extracts were added at timed intervals after the initiation of the cultures. In the case of the amnion and non-pregnant horn allantochorion, the observed suppression of lymphoproliferation was independent of the time of addition up to 24 h after initiation of the cultures. The pregnant horn allantochorion, however, continued to suppress lymphocyte proliferation at a consistent level up to 36 h which was the latest addition of sample (Fig.6.14).

As Con.A can be removed from target lymphocytes any time after 20 h with no diminution of mitogenic response (Gunther et al., 1974), the results presented here suggest that inhibition of previously activated lymphocytes occurs. Furthermore, these observations suggest that the samples were not inhibiting lymphoproliferation simply by binding to Con.A. That the pregnant horn allantochorion extract had a different suppressive pattern in this experiment suggests that the inhibitory activity of the sample may occur via a different mechanism in comparison to the other samples and may therefore be due to a different molecule. In order to interpret these findings, the time scale of cellular interactions that occur during lymphocyte stimulation was considered. During the first 24 h of human mitogen stimulated lymphocyte proliferation, IL-2 is produced by some T cell subsets and approximately 30% of the cells express IL-2 receptors (Cantrell and Smith, 1983; Kaye et al., 1984). In the absence of cell cycle progression, the IL-2 receptor density per cell gradually increases until a critical threshold is reached necessary for

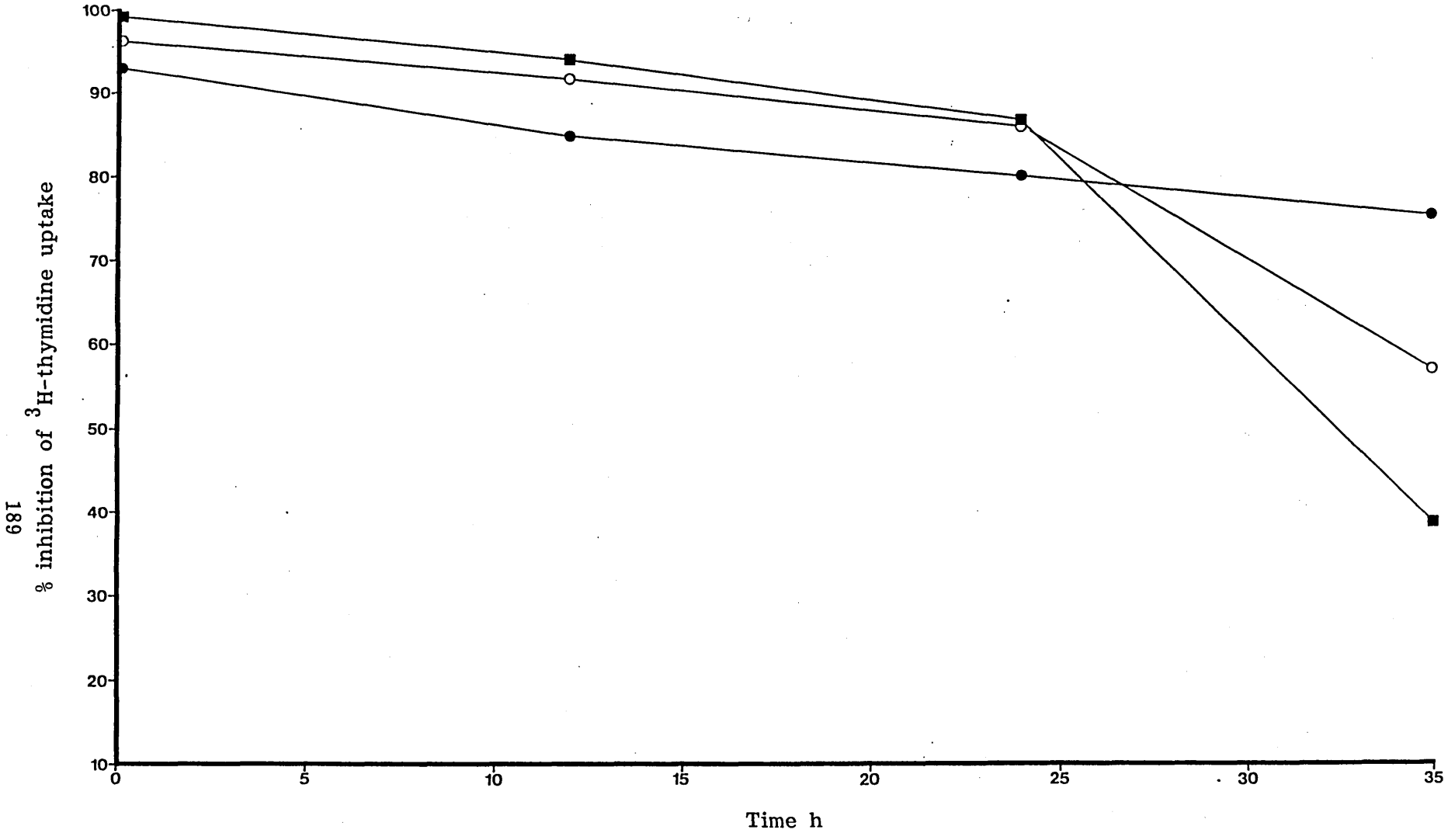

Fig.6.14 The effect of delayed sample addition (day 80) to Con.A stimulated lymphocytes. Samples were pregnant horn allantochorion (●), non-pregnant horn allantochorion (O) and amnion (■) at a concentration of 0.6 mg/ml.
Results are expressed as mean incorporation into triplicate cultures, (n=1).

cell proliferation (Cantrell and Smith, 1983). This critical threshold, however, is related to the number of occupied receptors and thus the free IL-2 concentrations is also a limiting factor in clonal expansion (Kaye et al., 1984). Indeed, the rate and magnitude of clonal expansion is dependent on 3 variables: the free IL-2 concentration, the expression of IL-2 receptors and the affinity of thereceptor for IL-2 (Smith, 1984). Because the inhibition caused by tissue extracts occurs if added within 24 h of initiation of the culture, it is possible that the tissue extracts are blocking IL-2 receptor expression, the binding of IL-2 to its receptor or they may be inhibiting IL-2 production. After this 24 h period, however, the receptor density and IL-2 concentration may be too high for the same concentration of sample to inhibit lymphocyte proliferation. The inhibition of IL-2 production has in fact been reported to occur in prostaglandin E2 induced immunosuppression where no immunosuppressive effect was observed if the sample was added 18 h after culturing T cells with mitogen (Chouaib et al., 1985).

6.6.2 The Effect of Conditioned Medium on the Inhibition of Lymphocyte Proliferation by Endometrial and Placental Samples (day 60 and day 80) as Assessed by the MSA and MLR

One mechanism by which tissue extracts might inhibit lymphocyte proliferation could be by inhibiting the generation of cytokines from the interacting cells. In order to test this possibility, MLR supernatant (conditioned medium), generated as described in Section 4.9.5, was tested for its ability to reverse the inhibitory effects of the samples in both the MLR and MSA.

The incorporation of conditioned medium into the MSA with selected placental samples at 30 μg/ml final protein concentration or with a commercial preparation of eCG (100, 50, 25 iu/ml) was found to partially or in most cases totally reverse the observed level of inhibition (Table 6.6). When the experiment was repeated using more concentrated placental/endometrial samples (120 μg/ml final protein concentration), the inhibition was in most instances not reversed (Table 6.7). However, the addition of conditioned medium to mixed lymphocyte cultures in the presence of placental/endometrial samples at a final concentration of 120 μg/ml was found only in a limited number of cases to reverse the observed level of inhibition. This occurred with allantochorion extract which reversed the inhibition by 46% and 13% in separate MLR's.

In conclusion, data presented above suggest that the inhibition of lymphocyte proliferation by allantochorion extract can be reversed by conditioned medium in both the MSA and MLR. It appears therefore that factor(s) present in the allantochorion block the generation and/or activity of cytokines necessary for lymphocyte proliferation. The MSA data (Table 6.6) suggest that the amnion, endometrial cup homogenate and the commercial preparation of eCG also contain factor(s) with similar cytokine blocking activity, that is the mechanisms by which each sample inhibits lymphocyte proliferation appear to be similar. That the addition of cytokines to cells inhibited by more concentrated samples (120 ug/ml) had little effect on the level of inhibition, suggest that at this greater concentration the samples may be non-specifically binding to the lymphocytes and blocking cytokine-cell surface interactions.

A number of local immunosuppressive mechanisms investigated in the human and mouse are reported to block the IL-2 dependent stage of the immune response (Clark et al., 1985; Parhar and Lala, 1986). Preliminary

Table 6.6

SAMPLE	% inhibition of ^{3}H-Tdr uptake		CONTROL CULTURE COUNTS
	- conditioned medium	+ conditioned medium	
ENDOMETRIAL CUP HOMOGENATE (day 60)	48.6	34.6	12,796
PREGNANT HORN ALLANTOCHORION (day 60)	13.4	0.0	12,796
NON-PREGNANT HORN ALLANTOCHORION (day 80)	27.1	1.1	12,796
AMNION (day 80)	3.9	0.0	12,796
eCG (100 iu/ml)	40.6 83.9	12.3 65.8	17,500 12,796
eCG (50 iu/ml)	18.0 65.0	0.0 84.1	17,500 12,796
eCG (25 iu/ml)	45.0 75.0	0.0 70.0	17,500 12,796

The effect of conditioned medium on the inhibition of Con.A stimulated horse lymphocyte proliferation induced by equine chorionic gonadotrophin and placental extracts taken from a day 60 and day 80 pregnancy. Final protein concentration in cultures = 30 µg/ml.

Table 6.7

SAMPLE	% inhibition of ^{3}H-Tdr Incorporation		CONTROL CULTURE COUNTS
	- conditioned medium	+ conditioned medium	
PREGNANT HORN ALLANTOCHORION WITH ENDOMETRIAL CUP SECRETION (day 80)	97.7	99.0	58,000
	98.7	97.6	17,500
PREGNANT HORN ALLANTOCHORION (day 80)	69.2	68.3	58,000
	99.8	99.6	17,500
NON-PREGNANT HORN ALLANTOCHORION (day 80)	96.3	91.9	58,000
	50.0	5.7	17,500
AMNION (day 80)	99.8	99.7	58,000
	78.6	86.5	17,500
PREGNANT HORN ENDOMETRIUM (day 60)	39.5	28.4	25,000
NON-PREGNANT HORN ENDOMETRIUM (day 60)	66.9	61.2	25,000
ENDOMETRIAL CUP HOMOGENATE (day 60)	57.4	64.3	25,000
PREGNANT HORN ALLANTOCHORION (day 60)	66.3	46.4	25,000
NON-PREGNANT HORN ALLANTOCHORION (day 60)	37.2	27.7	25,000

The effect of conditioned medium on the inhibition of Con.A stimulated horse lymphocyte proliferation induced by placental and endometrial extracts taken from a day 60 and day 80 pregnancy. Final protein concentration in cultures = 120 µg/ml.

evidence presented here in both the delayed addition and conditioned medium experiments suggest that a similar mode of action may be associated with local immunosuppressive mechanisms occurring in the horse. Until this can be clarified, however, the potential inhibition of other cytokines must be considered, the most likely being IL-1 and interferon.

6.7 The Inhibition of Lymphocyte Proliferation by Pregnant Horn Allantochorion and Amnion, Taken from a Pregnancy Aborted near to Term, as Assessed by the MLR

Parturition has been likened to the rejection of a previously tolerated graft (Siiteri and Stites, 1982). Indeed, these authors suggest that the endocrine changes which occur prior to parturition remove immunoregulatory influences which have maintained the graft. In support of this, a number of immunosuppressive factors are reported to be low in the circulation at term, e.g. progesterone and PP14 (Siiteri and Stites, 1982; Bolton et al., 1983).

In order to investigate levels of immunosuppressive factors approaching term in equine pregnancy, pregnant horn allantochorion and amnion extracts taken from a placenta aborted spontaneously near to term, were incorporated into an MLR. Both original samples were diluted to a protein concentration of 0.6 mg/ml. The allantochorion only inhibited ^{3}H-Tdr incorporation (48.6%) when undiluted and thus appeared to be low in immunosuppressive activity (Fig.6.15). The amnion, however, had an inhibitory effect on ^{3}H-Tdr incorporation when present at concentrations greater than 20 µg/ml culture fluid. Furthermore, the degree of observed inhibition was greater than that associated with day 80 amnion.

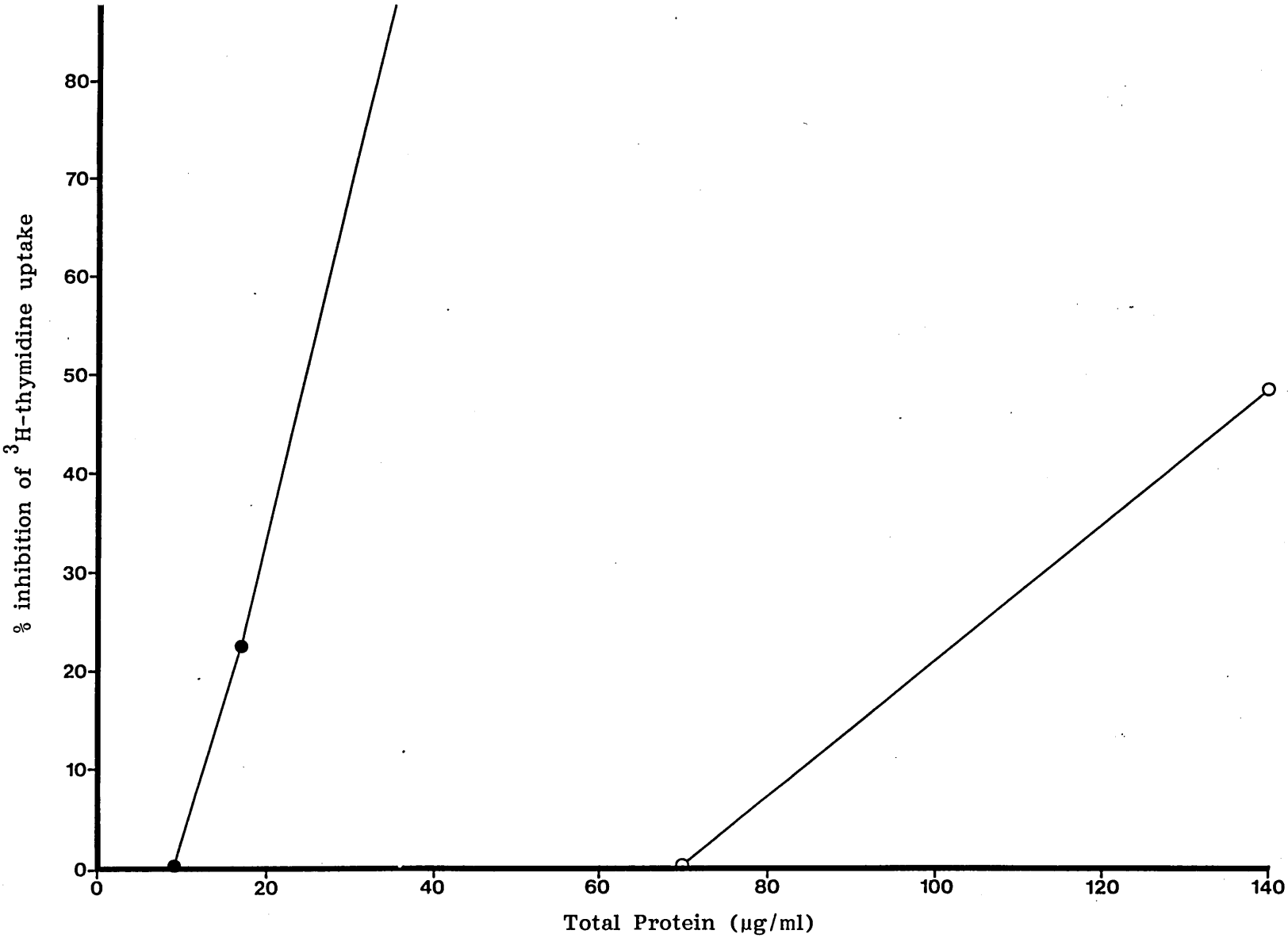

Fig.6.15 The effect of term (aborted) allantochorion (○) and amnion (●), related to protein content, on the inhibition of ^{3}H-thymidine uptake by mixed lymphocyte cultures.
C.P.M. in control culutres = 156,700. Results are expressed as mean incorporation into triplicate cultures, (n=1).

The low inhibitory activity of the allantochorion may be taken as evidence for a reduction in allantochorion associated immunoprotective mechanisms in preparation for parturition. As the allantochorion is the outermost layer of the placenta in direct contact with the uterine wall, a reduction in immunoprotective mechanisms associated with this tissue would lead to the rejection of the foeto-placental unit. As this particular pregnancy was spontaneously aborted, it is possible, however, that the low level of inhibitory activity reflected the poor clinical state of the tissue.

The amnion is the inner most membrane of the placenta that envelopes the foetus directly. It would appear therefore that a reduction in immunosuppressive activity associated with this tissue may be less important at parturition. Indeed, it can be argued that such a reduction in amnion associated immunoprotective mechanisms would put the foetus itself at risk during the later stages of pregnancy. This appears to be supported by the high level of inhibitory activity in term amnion reported above.

CHAPTER -7-

STUDIES OF ANTI-PLATELET AGGREGATORY MECHANISMS IN THE EPITHELIOCHORIAL PLACENTA

7.1 Introduction

In the human haemochorial placenta, the foetal villi are in direct contact with the maternal blood and consequently much pooling of blood occurs in the placental blood sinuses. In addition, it is reported that during pregnancy there is an increased activation of the maternal clotting system (Stirling et al., 1984). Despite these observations, the occurrence of a placental thrombosis is rare. This suggests that the placenta may protect itself by inhibiting platelet aggregation and/or coagulation. Indeed, a number of investigations have revealed that extracts of human placental tissue inhibit ADP induced platelet aggregation (Myatt and Elder, 1977; Hutton et al., 1980; O'Brien et al., 1987). In addition, a single report by Maki et al. (1984) described an anti-aggregatory placental protein which inhibited platelet aggregation induced by ADP, collagen and adrenalin. A number of placental associated anti-coagulants have also been described (Section 3.14).

In contrast to the above, there is no data available concerning analogous studies in species with epitheliochorial placentation. In such placentae, there are six layers of tissue between the maternal and foetal circulations. However, despite the preservation of these layers, the passage of nutrients across the placenta is facilitated by the penetration of both maternal and foetal capillaries into the epithelium and trophoblast where they assume adjacent positions (Ramsey, 1982).

Furthermore, the vascularisation of the epitheliochorial placenta is typified by the occurrence of microvilli whose interdigitation increases the area of maternal-foetal contact many fold.

In the equine epitheliochorial placenta, there is no pooling of maternal blood. The outer surface of the chorion is, however, highly vascularised, that is the chorion becomes studded with thousands of tufts of short branched villi which enter into corresponding invaginations in the endometrium to form small globular structures known as microcotyledons (Steven and Morris, 1975).

The bovine epitheliochorial placenta is typified by its cotyledonary configuration, that is the villi are scattered in large clusters over the foetal sac. The bovine uterine mucosa exhibits localized thickenings independent of pregnancy known as caruncles. During pregnancy, proliferations of chorionic villi (cotyledons) become attached to the caruncles and it is through these connections that placental exchange occurs. The combination of caruncle and cotyledon is known as a placentome. The maternal caruncles become enlarged and highly vascularised during pregnancy in a similar way to the endometrial component of the equine microcotyledon. In contrast to the horse, however, small haematomas appear in some of the bovine placentomes. Hematomas are stagnant accumulations of maternal blood which lie in direct contact with trophoblastic villi. The trophoblast cells are reported to actively phagocytose the red blood cells and degrade the products of haemoglobin (Ramsey, 1982).

The presence of anti-aggregatory activity in the human haemochorial placenta has been largely associated with the pooling of maternal blood in the placental sinuses where it is in direct contact with trophoblast.

However, in view of the different vascular features of the equine and bovine placentae, the importance of analogous anti-aggregatory mechanisms in the epitheliochorial placenta is uncertain. Consequently, the following investigations were carried out to determine whether or not anti-aggregatory mechanisms are associated with term equine and bovine epitheliochorial placentae.

7.2 The Inhibition of Platelet Aggregation by Equine Term Placental Extracts

Extracts of amnion and allantochorion, taken from a normal term placenta, were added to equine platelets in the presence of collagen and ADP. Both undiluted extracts were used at the same final protein concentration of 52.5 µg/ml. It was found that equine allantochorion inhibited platelet aggregation induced by collagen but not platelet aggregation induced by ADP. Doubling dilutions of the sample showed the same anti-aggregatory effect down to a 1 in 16 dilution (Fig.7.1). Term amnion had no effect on platelet aggregation at even the highest concentration investigated.

High concentrations of collagenase in a sample will prevent collagen induced platelet aggregation by degeneration of the collagen. The allantochorion extract was assayed for collagenase and levels of the enzyme were found to be below the working range of the assay. The sample did not therefore contain enough collagenase to degrade sufficient collagen under the conditions of the experiment.

In conclusion, it appears that equine term allantochorion contains a factor(s) capable of inhibiting platelet aggregation induced by collagen but not aggregation induced by ADP. This contrasts with reports of human placental extracts, most of which inhibit ADP induced aggregation but not

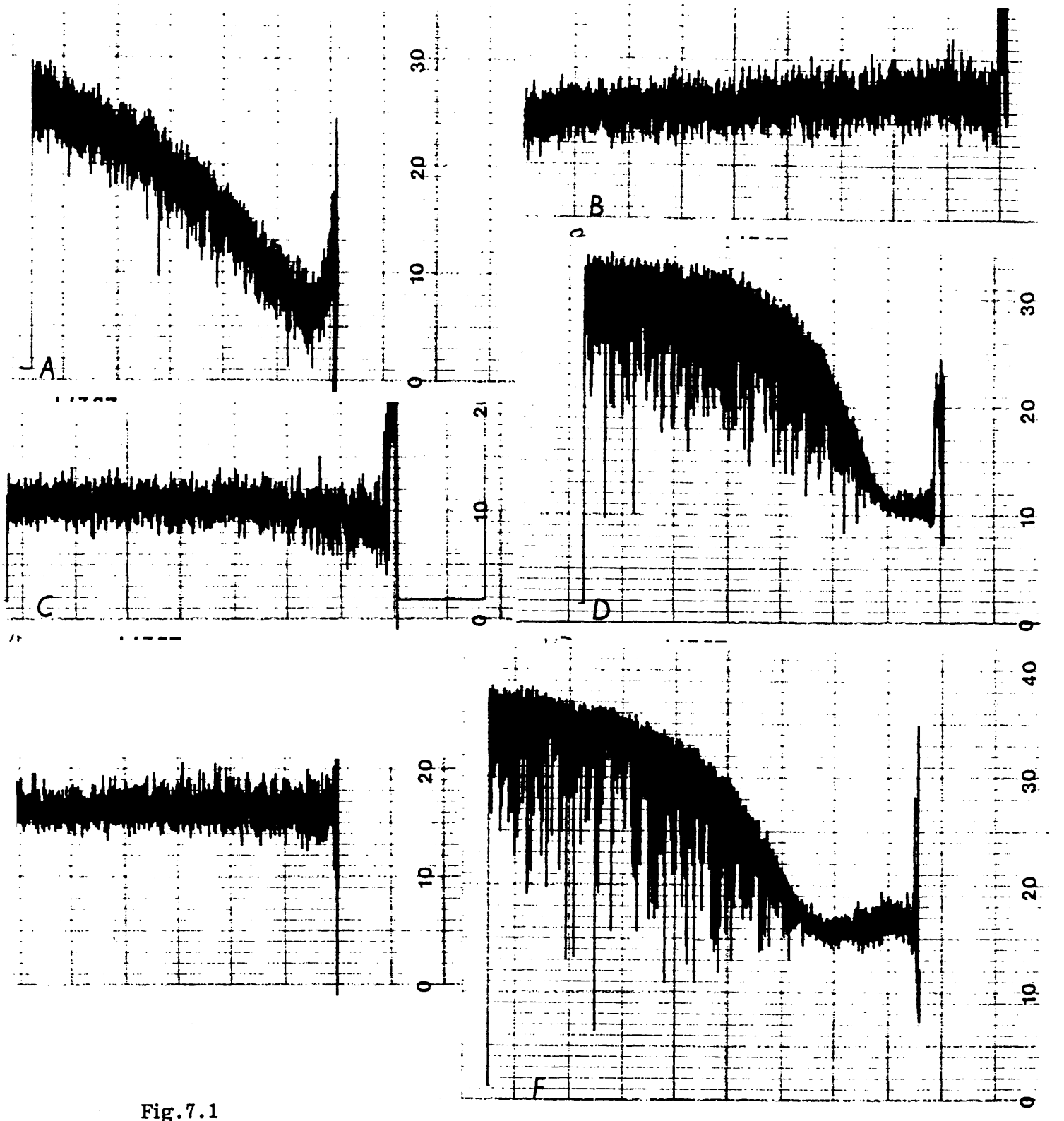

Fig.7.1
Inhibition of collagen induced equine platelet aggregation by term placental allantochorian:-
A(Sample+ADP), B(Sample+Collagen), C(Sample[1:2 diln']+Collagen), D(Control for C), E(Sample[1:8 diln']+Collagen), F(Control for E).

collagen induced aggregation. Only in one case was a factor isolated from the human placenta which inhibited both ADP and collagen induced platelet aggregation (Maki et al., 1984).

On the addition of collagen to a suspension of platelets, there is a short delay followed by the secretion of ADP which induces the platelets to aggregate. As the equine sample had no effect on ADP induced platelet aggregation, it must be either blocking the activity or binding of collagen to the platelets or it may be interfering with one of the reactions occurring inside the platelet leading to ADP secretion. In brief, this series of reactions leading to secretion involves the release of arachinodic acid from the platelet membrane which is quickly converted into endoperoxides. These ,in turn, are converted into thromboxane A_2 which is the stimulus for ADP secretion. It is interesting that most factors isolated from the human placenta do not interfere with these early events of platelet aggregation but appear to degrade the ADP (O'Brien et al., 1987). With respect to the equine placenta, it is possible, however, that the highly vascular nature of the allantochorion may necessitate an inhibitor of collagen induced platelet aggregation due to high levels of collagen in the membrane.

7.3 The inhibition of Platelet Aggregation by Bovine Placentome Extract

An extract of bovine placentome taken from a normal term placenta was added to bovine platelets in the presence of collagen and ADP. The final protein concentration of the extract when added to the platelets was 52.5 µg/ml. On addition of the extract to platelet rich plasma, there was a small amount of aggregation followed by an apparent dissociation. When ADP was added to this mixture, there was no response and thus the extract had inhibited ADP induced platelet aggregation (Fig.7.2). It is possible that this activity may be due to a phosphatase activity, indeed such a

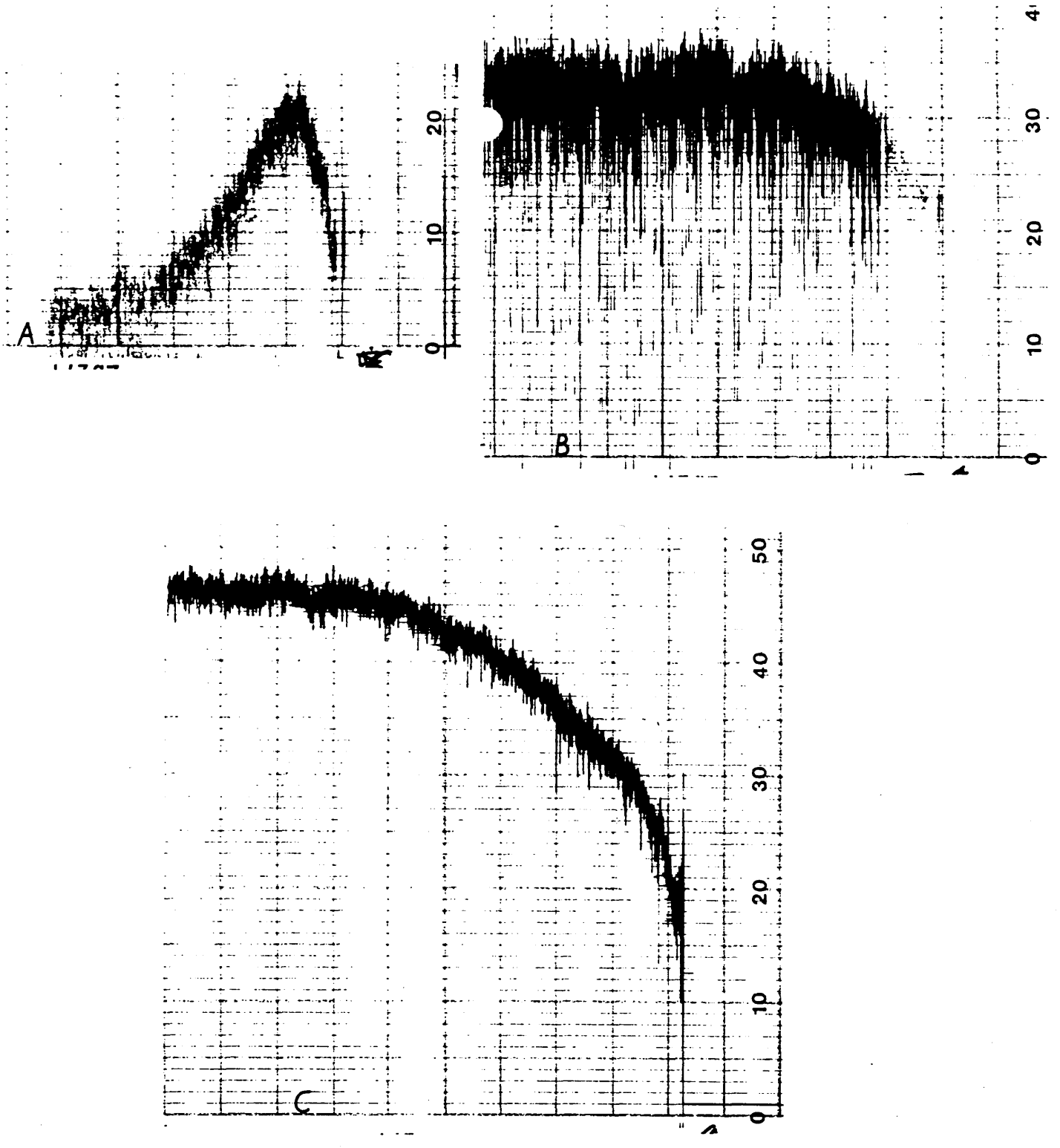

Fig.7.2
Inhibition of ADP induced bovine platelet aggregation by bovine placentome homogenate:-
A(Sample+Platelet Rich Plasma), B(A.+ADP), C(Control for A. and B.).

mechanism has been described in the human placenta (Hutton et al., 1980). The placentome extract (2 ml) was, however, dialysed against 5 l of distilled water (overnight) before incorporation into the experiment. As it is reported that extensive dialysis results in the loss of zinc atoms from alkaline phosphatase necessary for enzymatic activity (McComb et al., 1979), it is unlikely that this enzyme is responsible for the anti-aggregatory activity associated with the sample. This awaits further clarification.

When collagen was added to the bovine platelets incubated with placentome extract, aggregation occurred as normal. This contrasts with the effect of equine allantochorion which inhibited collagen induced aggregation but had no effect on ADP induced aggregation.

An additional study was carried out on the effect of equine and bovine placental extracts on human platelet aggregation induced by ADP and collagen. Platelet aggregation was found to occur as normal suggesting that the anti- aggregatory factors described are species specific. Moreover, the lack of inhibition when ADP stimulated human platelets were incubated with bovine placentome extract indicates that it is even less likely that a phosphatase activity in the sample was responsible for the inhibition of bovine lymphocyte aggregation.

In conclusion, it appears that both the equine and bovine epitheliochorial placentae exhibit anti-platelet aggregatory activity. The bovine activity is more akin to that described in the human placenta. It appears, however, that the bovine factor(s) responsible may not be degrading the ADP as is the case with the human inhibitory factors. Whether or not the presence of haematomas in bovine placentomes and placental sinuses in the human placenta have any bearing on the inhibition of the ADP agonist is at

present uncertain. The equine anti-aggregatory factor is unique in that it specifically inhibits collagen induced platelet aggregation. That platelet anti-aggregatory activity is found in placentae as diverse as the haemochorial and epitheliochorial types suggest that placenta anti-aggregatory factors are of critical importance in the prevention of a hazardous thrombosis in mammals.

CHAPTER -8-

FINAL DISCUSSION

Early embryonic and foetal loss in the mare, most of which occurs before day 12 after ovulation (Forde et al., 1987), has proved to be a serious economic loss to the horse breeding industry. Moreover, the serum tests used for the actual detection of pregnancy are not reliable until the detection of oestrone sulphate produced by the foeto-placental unit from day 60 onwards. From days 18-25, maternal progesterone levels are elevated during pregnancy due to the continued activity of the corpus luteum. In the non-pregnant mare, luteolysis occurs at day 16 and consequently levels drop. A proportion of oestrous cycles are, however, followed by a persistent corpus luteum which continues to secrete progesterone, resulting in a pseudo-pregnant state (Stabenfeldt et al., 1984). Indeed, in one study, 93% of 362 non-pregnant mares during the breeding season were reported to have elevated plasma progesterone levels (Allen et al., 1974). Consequently, elevated progesterone levels can only be used as a tentative indicator of pregnancy. Similarly, eCG detectable in the maternal plasma at 42 days post-conception is only indicative of the presence of functional endometrial cups. This was demonstrated by Allen and Moor (1972) who found that once the chorionic girdle cells invade the uterine stroma to form endometrial cups at around day 37 (implantation), the subsequent loss of the pregnancy did not alter the normal sequence of development and regression of the endometrial cups. Although other techniques are available for pregnancy diagnosis such as rectal palpation and ultrasound echography, little information is obtained regarding the

physical state of the foetus. Consequently, it would appear that there is a need for a non-invasive method of detecting pregnancy before day 60 which also allows foetal well being to be monitored.

Evidence is presented in this thesis for the presence of a protein with β_2-electrophoretic mobility which appears in the peripheral circulation of the horse specifically during pregnancy. The pregnancy specific protein designated β_2-hors.PP was first detectable at day 6 after mating and than increased for at least the first 21 days of pregnancy. Levels of the protein appeared to be elevated between days 21 and 83, that is during implantation, after which it decreased throughout gestation. The assay system used, two-dimensional crossed immunoelectrophoresis, lacks the high sensitivity of some immunoassay systems and the failure to demonstrate this protein in the serum of non-pregnant mares may be a consequence of the lower limit of sensitivity imposed by the assay. Consequently, it is possible that β_2-hors.PP may be present in the non-pregnant state at low levels and elevated during pregnancy.

That β_2-hors.PP was transiently present in the serum of 2 pregnant mares that failed to become pregnant, suggests that the protein may be produced in response to a biochemical pregnancy as described in the human (Miller et al., 1980). Indeed, it has been reported that there are similar fertilization rates in normal and barren mares but significantly different day 14 pregnancy rates (Ball et al., 1985). The problem of early foetal loss in the mare throws into question the utility of β_2-hors.PP as a pregnancy test, particularly during the first month of gestation. The possible link of β_2-hors.PP with biochemical pregnancy, however, indicates that monitoring of the protein may be useful in assessing foetal well being. It is conceivable that the monitoring of β_2-hors.PP levels in this way may enable the early detection of pseudopregnancy as defined by

elevated levels of progesterone and/or eCG in the absence of $_2$-hors.PP. Consequently the mares could be artificially induced to return to oestrous and appropriate arrangements made for another mating.

It has been reported that much foetal loss occurs around the time of implantation at day 37 (Whyte and Allen, 1985). In addition, Woods et al. (1987) reported that the pregnancy loss rate is significantly less after implantation than before. The presence of elevated levels of β_2-hors.PP after implantation suggests that the risk of foetal loss may be sufficiently low to justify the development of a routine non-invasive pregnancy test earlier in gestation than day 60 when elevated levels of oestrone sulphate are detectable.

Data presented in this thesis suggests that β_2-hors.PP is detectable in a serum of a donkey-in-horse pregnancy but not detectable in a normal donkey pregnancy. These findings coupled with the detection of β_2-hors.PP in uterine flushings suggest that the protein has a maternal origin. It is possible therefore that the reported early equine conceptus derived factors (Hershman and Douglas, 1979) may have a localised effect on the uterus resulting in the secretion of further early pregnancy factors, one of which may be β_2-hors.PP.

To date, only two other equine pregnancy specific serum proteins have been reported in the literature: equine chorionic gonadotrophin (Cole and Hart, 1930) and mare pregnancy protein-1 (Gidley-Baird et al., 1983).

An identity between β_2-hors.PP and equine chorionic gonadotrophin (eCG) was excluded as antisera to eCG did not precipitate β_2-hors.PP and purified eCG did not contain material which was immunologically similar to β_2-hors.PP. Furthermore, β_2-hors.PP was detected in the serum before the development of the endometrial cups which secrete eCG.

A comparison of β_2-hors.PP with published data about mare pregnancy protein-1 (MPP-1) revealed some common factors between the proteins. Firstly, MPP-1 is reported to have $beta_1$-electrophoretic mobility and was detected using a similar immunoelectrophoretic system to that described here. Secondly, MPP-1 was present in the earliest serum sample investigated taken at day 30 of gestation and thus may be detectable at an earlier stage of pregnancy. It is possible that MPP-1 and β_2-hors.PP are identical proteins, although the slightly differing electrophoretic mobilities may suggest either different proteins or differing forms such as degree of glycosylation of the same protein.

There are, however, two factors which mitigate against such an identity. Firstly, β_2-hors.PP is immunologically related to a protein found in the serum of non-pregnant mares. This protein was found to have $gamma_2$ electrophoretic mobility and has been designated as $gamma_2$ horse protein (γ_2-hors.P). The relationship of the two proteins was apparent from a continuity of the protein precipitin lines formed in two-dimensional crossed immunoelectrophoresis. No similar protein related to MPP-1 was described by Gidley-Baird et al. (1983). Secondly, the antisera developed by Gidley-Baird et al.(1983) against pregnant mare serum was adsorbed with stallion serum. Data presented in this thesis demonstrate that such an adsorption process removes antibody specificity to β_2-hors.PP and this has been attributed to the close immunological relationship between β_2-hors.PP and a protein present in the non-pregnant horse (γ_2-hors.P). The similarities or differences of these two proteins, however, require further investigation.

Other pregnancy associated factors have been described in uterine fluid, the most widely reported one being uteroferrin (Sharp, 1980). The presence of uteroferrin in non-pregnant mare uterine flushingsand its absence from pregnant mare serum mitigate against an identity between β_2-hors.PP and the uterine protein.

There is little evidence to suggest that β_2-hors.PP is an equine analogue of a specific human pregnancy associated protein although the increase in concentration of β_2-hors.PP in the serum for the first few weeks, followed by a decline for the remainder of gestation is similar to that reported for PP-14 (Julkunen et al., 1985a). Furthermore, both proteins are secreted in the uterus.

Recent research into bovine pregnancy has revealed a pregnancy specific protein that is first detectable in the serum 1-8 days after artificial insemination (Klima et al., 1987). This protein, designated early pregnancy associated protein (EPAP) was detected using a similar immunoelectrophoretic method to that used in the detection of β_2-hors.PP. EPAP was detected in the γ-globulin area of the immunoelectrophoretic gel and appeared to be related to a protein present in non-pregnant serum. As assessed by the detection of EPAP, 91.5% of 71 sera samples were classified correctly to be pregnant or non-pregnant. In comparison, data presented in this thesis show that 81% of 16 equine sera samples investigated blind were correctly diagnosed to be pregnant or non-pregnant. It is possible that EPAP in the cow and β_2-hors.PP in the horse are the same or equivalent proteins and this is based on the following common factors.

1/ Both proteins are pregnancy specific and appear within 8 days after mating.

2/ Both proteins appear to be biologically related to a protein present in the non-pregnant state.

3/ The differing electrophoretic mobilities of EPAP and β_2-hors.PP may be due to different degrees of glycosylation.

4/ Both EPAP and β_2-hors.PP have been shown to be highly accurate in the diagnosis of early pregnancy.

The early appearance of β_2-hors.PP during pregnancy may be indicative of a role associated with implantation, particularly as levels of the protein appear to be elevated during this period. Furthermore, with respect to bovine pregnancy, EPAP has been shown by the 'adoptive transfer of contact sensitivity' to behave like EPF from sheep in suppressing the delayed cutaneous hypersensitivity in mice (Klima, 1985; Pitra et al., 1985). The similarities between β_2-hors.PP and EPAP may therefore be indicative of a biochemical/immunological relationship between β_2-hors.PP and EPF. It is therefore possible that β_2-hors.PP may have an immunomodulatory role.

In recent years, the role of pregnancy associated proteins and steroids as potential immunomodulatory agents during pregnancy has been much investigated. Reproductive immunologists generally agree that the survival of the mammalian foetal allograft within an immunocompetent mother may be partly explained by local intra-uterine immunosuppressive mechanisms. Furthermore, such mechanisms are considered to be particularly important during implantation and early pregnancy. There are, however, a number of other theories explaining the survival of the foetal allograft which are not necessarily mutually exclusive. Indeed, it has been suggested that a number of mechanisms interplay or even synergize, resulting in a sophisticated immunological equilibrium that is not fully

understood (Chaouat, 1984). Other theories include a poor immunogenic status of the trophoblast due to a lack of MHC antigens and the production of blocking antibodies as a humoral response to pregnancy.

The former theory may be more pertinent to placentae exhibiting gross invasive activity such as the haemochorial placenta although the invasive trophoblast of the equine placenta does not appear to express class I MHC antigens (Crump et al., 1987).

With regard to blocking antibody production, there is evidence to suggest that a humoral response to pregnancy is important if not critical for the continued survival of the foetal allograft in both the human and the horse. Indeed, 90% of primiparous mares develop hightitre anti-paternal alloantibody compared to 15-25% of primiparous women and 40-60% of multiparous women (Antczak et al., 1984; Doughty and Gelthorpe, 1974). The stimulus for this antibody production in the mare is believed to come from the invasive activity of the endometrial cups and may be due to the expression of non-MHC antigens such as the TLX antigen (Allen et al., 1987).

With respect to local immunosuppressive mechanisms, research has almost entirely concentrated on species exhibiting haemochorial placentation. Indeed, reservations have been expressed regarding the importance of such a mechanism in species with epitheliochorial placentation (Enders, 1983). Local immunosuppressive mechanisms are considered to be of particular importance in haemochorial placentae as there is direct contact between maternal blood and foetal trophoblast. However, the events occurring during implantation in the horse suggest that local immunosuppressive mechanisms may be important in species with more superficial placentation. Of particularly importance in this respect is the endometrial cup reaction

unique to the genus Equus. During this, specialised gonadotrophin secreting trophoblast cells of chorionic girdle membrane origin invade the maternal endometrium at days 36-38 of gestation and develop into endometrial cups. The cups have a life span 60-100 days throughout which increasing numbers of lymphocytes accumulate in the surrounding stroma (endometrial cup lymphocytes). When the cups begin to degenerate, around day 80-90, the lymphocytes begin to invade the cup tissue and destroy the large cup cells (Allen, 1982). As a consequence of the invasive activity of the trophoblast cup cells, the endometrial cup reaction can be described as the time when the main foetal stimulus to the mother is greatest and consequently it is during this period that local immunosuppressive mechanisms may be stimulated, resulting in elevated levels of immunosuppressive factors. That the cups are not invaded by lymphocytes until day 80-90 suggests that a local immunomodulatory factor may be active possibly produced by the endometrial cups. Evidence has already been presented to show that at least one population of cells attracted to the endometrial cups has suppressor rather than cytotoxic activity (Kydd and Allen, 1986 and personal communication). In addition, preliminary in vitro studies suggest that the principal secretory component of the cups, equine chorionic gonadotrophin (eCG), may suppress lymphocyte proliferation (Kydd and Allen, unpublished). These studies were carried out using a commercial preparation of eCG.

Data presented in this thesis confirms that commercially available eCG (Intervet) inhibits lymphocyte proliferation in a dose dependent manner. The sample did not induce 100% inhibition in either the MLR or MSA which suggests that one or more cell populations in culture were proliferating despite the presence of the eCG preparation. However, evidence is also

presented in this thesis which suggests that the inhibitory activity observed in the MLR and MSA may not be due to eCG but due to a contaminant in the hormone preparation.

This became evident when two extracts of pregnant horn allantochorion taken from a pregnant mare 80 days after mating were compared in terms of their inhibitory effects on lymphocyte stimulation. Only one of the extracts contained endometrial cup secretion and this was reflected in its high eCG content (251 iu/ml). The other sample contained very little endometrial cup secretion which was reflected in its low eCG content (8.6 iu/ml). As assessed using the commercially available eCG, the concentration of the hormone in the extract with cup secretion was found to be high enough for all of the observed inhibition in the MLR. The eCG level in the other sample was, however, too low to have any effect. No significant difference was found in the inhibitory effects of the two samples on lymphocyte proliferation and thus it appeared that the inhibitory activity of the allantochorion extract was independent of eCG content. Furthermore, as the hormone was present in culture with other inhibitory factor(s), an enhanced inhibitory effect was expected. That no additive effect was observed when the two allantochorion samples were compared suggests that the hormone itself does not inhibit lymphocyte proliferation and that the commercially available eCG contains a contaminant with inhibitory activity.

Similar results have been obtained in a series of experiments using commercially available human chorionic gonadotrophin (hCG). It was reported that hCG inhibits PHA-induced lymphocyte proliferation, mixed lymphocyte reactions and antigen induced blastogenesis (Siiteri and Stites, 1982). In 1983, however, a more highly purified form of hCG was reported not to be immunosuppressive and thus the original results were

attributed to an unknown contaminant in the more crude hormone preparation (Rolfe et al., 1983). The crude hCG preparations have been further reported to contain EPF activity and it has been suggested that this may be entirely responsible for the immunosuppression observed. Similarly, it is possible that the inhibitory effects of the crude eCG preparation may similarly be due to contamination.

Although it appears that eCG does not inhibit lymphocyte proliferation, the hormone may have other immunomodulatory effects during pregnancy. Indeed, it has been suggested that eCG may have a role as a physical barrier which covers and therefore prevents the maternal immune recognition of the foetal antigens (Allen, 1982). Alternatively or in conjunction with the above, the high carbohydrate and sialic acid content of eCG may give a high negative charge to both trophoblast cells and maternal lymphocytes leading to mutual repulsion (Allen, 1982).

The importance of the endometrial cup reaction to the successful maintenance of early equine pregnancy is evident from the work carried out on various experimental equine pregnancies. It has been shown that any conceptus that gives rise to any degree of endometrial cup development in the maternal uterus has a chance of implanting normally and proceeding to term (Allen et al., 1987). As eCG is the major secretory product of the endometrial cups, it is possible that the endometrium and/or endometrial cups may secrete an immunosuppressive agent to protect them against rejection. This would enable the continued production of eCG facilitating the survival of the pregnancy because of its endocrine action. Indeed, Kydd and Allen (1986) have found that endometrial cup lymphocytes, recovered between days 55 and 70, exhibit suppressor activity.

Data presented in this thesis have shown that extracts of endometrial tissue and endometrial cup homogenate taken from a pregnant mare 60 days after mating are able to inhibit lymphocyte proliferation in the MLR and MSA, indicative of the presence of immunosuppressive activity. A similar inhibitory effect has also been observed with extracts of foetal membranes (allantochorion and amnion) taken from two pregnant mares 60 days and 80 days after mating.

The inhibitory activity of these samples was reversed by the addition of conditioned medium (MLR supernatant). This suggests that factor(s) present in the equine endometrium and in foetal tissues may have a local immunosuppressive effect by inhibiting the action and/or generation of specific lymphokines such as IL-1 and IL-2. Furthermore, the placental samples were not required at the initiation of an MSA in order to inhibit lymphoproliferation. This demonstrates that the inhibitory factors were effective against previously activated lymphocytes. Indeed, as assessed by the MSA, the inhibitory activity of the placental samples was independent of the time of addition up to 24-36 h. In view of the events that occur during lymphocyte stimulation, it is possible that the placental factors were blocking the IL-2 dependent stage of the immune response. Such an effect may be due to blocking IL-2 receptor expression, the binding of IL-2 to its receptor or by the inhibition of IL-2 production. In total, it appears that intra-uterine immunomodulatory factors are active during early pregnancy in the horse and that some of these factors may suppress at the IL-2 level of the immune response.

Investigations into endometrial immunoregulatory activity in species exhibiting haemochorial placentation have generally focussed on human and murine pregnancy. In these species, a number of factors produced from a variety of cell types contribute to endometrial/decidual associated

immunosuppression. Moreover, it appears that a common but not exclusive mechanism of immunosuppression associated with these factors is a blockade of the IL-2 dependent stage of immune response. A population of trophoblast dependent non-T lymphocytes present in murine decidua have been shown to release a factor which blocks the activity of IL-2 (Clark et al., 1985). Moreover, the decidual supernatant factor is similar in molecular weight to the human endometrial protein PP-14 which has recently been shown to suppress lymphocyte proliferation (Bolton et al., 1987). Decidual cells (Parhar and Lala, 1986) and macrophages (Tawfic et al., 1986) have been shown to suppress T cell reactivity by the release of prostaglandin E_2 (PGE_2) which in turn inhibits the production of IL-2. In the light of results presented in this thesis, it appears that local immunoregulatory mechanisms, such as those described above, are important for the survival of mammalian pregnancy irrespective of the type of placenta (haemochorial compared with epitheliochorial).

It has been postulated that birth is essentially the rejection of an allograft no longer protected by local immunosuppressive mechanisms and consequently, levels of proteins with immunomodulatory activity are low just prior to term (Siiteri and Stites, 1982). This has been demonstrated in human and murine pregnancy and data presented here suggests that this is also the case in the horse. As assessed by the MLR, an allantochorion extract from a placenta spontaneously aborted near to term had little effect on lymphocyte proliferation. The allantochorion is the outermost layer of the placenta and consequently a reduction in the immunosuppressive activity of the tissue may lead to spontaneous abortion or natural birth. In contrast, the suppressive activity of day 80 allantochorion extracts were found to be greater than those of day 60 allantochorion extracts. This suggests that the suppressive activity of

the allantochorion may reach maximum levels early in pregnancy and then decrease throughout gestation in a similar manner to human immunoregulatory proteins, e.g. PP-14. These results suggest that local immunoregulatory mechanisms may be particularly important during the endometrial cup reaction when the foetal stimulus to the mother is maximal. Indeed, Allen et al. (1987) have suggested that a major function of the endometrial cup reaction during a normal pregnancy is to stimulate suppressor cell activity.

An amnion extract from the same placenta was found to have a higher level of inhibitory activity than similar extracts taken at day 60 and day 80. The amnion is the innermost membrane of the placenta and consequently, immunosuppressive activity associated with this tissue may not effect the onset of parturition but will be important in the protection of the foetus. The increase in suppressive activity of the amnion may be due to the secretion of a factor similar to AFP in the human. AFP has been shown to suppress human and murine lymphocyte proliferation and to increase in concentration during gestation (Tomasi, 1978; Yachin and Lester, 1979; Hau, 1986).

It has recently been stated that 10% of anything will suppress lymphoproliferation (Antczak and Allen, personal communication). In answer to this, it has been observed that term allantochorion will not suppress lymphocyte proliferation despite the high protein concentration in the lymphocyte cultures and thus this may be taken as evidence that the suppressive effect induced by the other samples was not solely due to the presence of protein.

During human pregnancy, there is an increased activity of the maternal clotting system (Stirling et al., 1984). Furthermore, in the haemochorial placenta, the pooling of maternal blood in the blood sinuses suggests that a thrombosis is likely to occur. The rare occurrence of such a thrombosis has been attributed to the presence of a number of factors in the placenta which function as anti-coagulants or have anti-platelet aggregatory activity. It has been reported that extracts of human term placenta specifically inhibit ADP induced platelet aggregation (Myatt and Elder, 1977; Hutton et al., 1980; O'Brien et al., 1987) except in one report where the same factor inhibited ADP, collagen and adrenalin induced platelet aggregation.

Although it has been reported that there is an increase in levels of clotting factors during bovine pregnancy (Gentry et al., 1979), there is no data available concerning anti-platelet aggregatory activity in the epitheliochorial placenta.

Data is presented in this thesis illustrating the presence of a factor in equine term allantochorion inhibiting the aggregation of platelets in response to collagen. Preliminary data are also presented suggesting the presence of a factor in the bovine placentome which inhibits the aggregation of platelets in response to ADP.

The bovine activity is similar to that described in the human placenta although no degrading of ADP seems to occur as described in the human placenta. It is interesting that some bovine placentomes contain haematomas where maternal blood accumulates in contact with trophoblast, in a similar way to the pooling of maternal blood in the human placental sinuses. The significance of this in terms of platelet aggregation is, however, uncertain.

In contrast to the human and bovine anti-aggregatory factors, the equine activity was specific in its inhibition of collagen induced platelet aggregation. This may be due to the high degree of vascularisation and consequently, a high collagen content of the equine term allantochorion.

In total, it appears that the presence of placental factors capable of inhibiting platelet aggregation is necessary in both haemochorial and epitheliochorial placentae in order to counteract the otherwise high risk of a placental thrombosis.

In conclusion, data discussed in this thesis have led to a greater understanding of some of the biological events that occur during equine pregnancy. The important findings of these studies are outlined in Figs 8.1 and 8.2. A protein specific to the pregnant state of the horse has been described, which is detectable in the maternal serum at day 6 after mating and is detectable throughout gestation. The protein (β_2-hors.PP) may be of considerable value to the horse breeding industry in terms of the diagnosis of pregnancy and possibly in the monitoring of foetal well being. As assessed during the first 21 days after mating, β_2-hors.PP detection as an indication of pregnancy was clinically significant at the 10% level. Further developments of this work could include the development of a more sensitive assay for β_2-hors.PP and also an assessment of β_2-hors.PP detection as an indication of pregnancy at later stages of gestation. Such investigations could lead to the development of a reliable non-invasive pregnancy test before day 60 when oestrone sulphate levels can be monitored. The pattern of secretion of β_2-hors.PP suggests a possible biological activity during implantation such as immunoregulation. Consequently, further investigations could also include an evaluation of the possible immunomodulatory properties of the protein. It has also been demonstrated that equine epitheliochorial placentation exhibits both

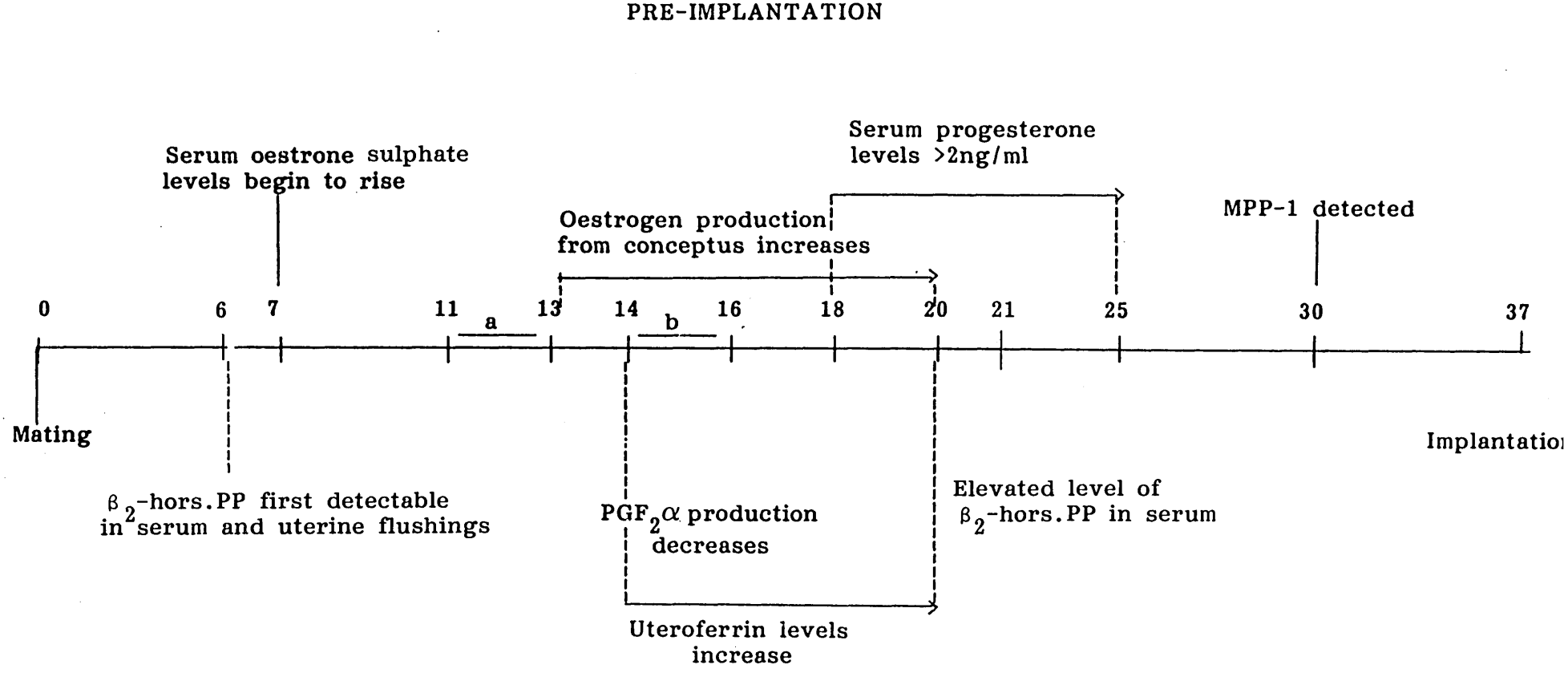

Fig.8.1 Biological Events in the Mare prior to Implantation
(incorporating results presented in this thesis)

a,b: The suggested times for the maternal recognition of pregnancy.
(a):- Hershman and Douglas, 1979. (b):- Goff et al., 1987.

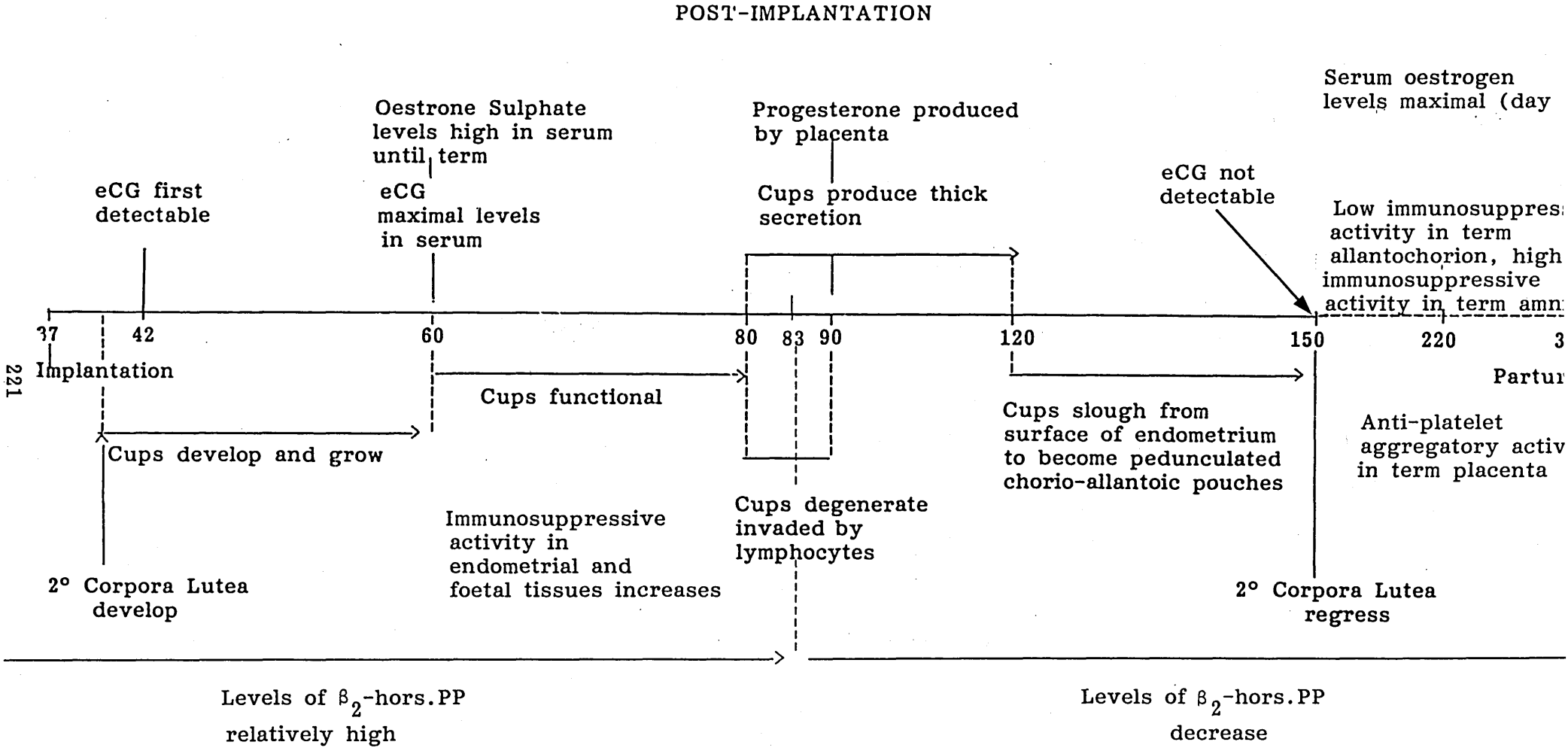

Fig.8.2 Biological Events in the Mare Post-Implantation
(incorporating results presented in this thesis)

immunomodulatory and anti-aggregatory activity with the former predominant during early gestation (eCG phase) and the latter predominant near to term as described in the human haemochorial placenta. It appears that eCG may not be immunosuppressive but that the commercially available eCG preparation may contain an immunosuppressive contaminant. Equine intra-uterine immunosuppressive mechanisms appear to be effective at the IL-2 level of the immune response which is comparable to existing data in the human and murine systems. Term equine placental allantochorion inhibited collagen induced aggregation but had no effect on ADP induced aggregation. This may be due to the high degree of vascularisation in the tissue. Term bovine placentome extract inhibited ADP induced platelet aggregation but not collagen induced aggregation in a similar fashion to many human anti-aggregatory factors. This work could be developed further in the characterisation of the immunoregulatory and anti-aggregatory factors present in the tissue extracts. In addition, further investigations could be carried out into their mode of action. It would also be of interest to investigate the immunoregulatory activity associated with non-pregnant endometrium for comparison to the effects of pregnant endometrium.

In total, this preliminary work has shown that there are many similarities in the intra-uterine immunoregulatory and anti-aggregatory activities between mammals exhibiting haemochorial or epitheliochorial placentation. The development of this work leading to the identification of some of the factors described may lead to the further understanding of mammalian placental function. It is hoped, however, that the data presented here will further help reproductive immunologists to solve 'the riddle of mammalian foetal allograft'.

REFERENCES

AGGARWAL A A, FARMER S W , PAPKOFF H, STEWART F, ALLEN W R (1980). Purification and characterisation of donkey chorionic gonadotrophin. J Endocr 85, 449-455

ALLEN R D, MURRAY F A, WIETSMAS H T (1981). Immunosuppression in the mouse by porcine uterine secretory protein. Am J Reprod Immunol 1, 193-198

ALLEN W R (1969a). A quantitative immunological assay for pregnant mare serum gonadotrophin. J Endocr 43, 581-591

ALLEN W R (1969b). The immunological measurement of pregnant mare serum gonadotrophin. J Endocr 43, 593-598

ALLEN W R (1969c). Factors influencing pregnant mare serum gonadotrophin production. Nature, 223, 64-66

ALLEN W R (1970). Endocrinology of early pregnancy in the mare. Equ. Vet J 2, 64-70

ALLEN W R (1975). Endocrine functions of the placenta. Monographs for students in medicine. In 'Comparative Placentation' (Essays in Structure and Function). Editor:Steven D H, Academic Press, pp.214-267

ALLEN W R (1975a). Immunological aspects of the equine endometrial cup reaction [Clinical and Experimental Immunoreproduction 1]. Immunobiology of trophoblast. Editor: Edwards R G, Howe C W S, Johnson M H. Cambridge University Press, pp.217-253

ALLEN W R (1975b). The influence of foetal genotype upon endometrial cup development and PMSG and progestagen production in equids. J Reprod Fert, Suppl. 23, 405-413

ALLEN W R (1979). Maternal recognition of pregnancy and immunological implications of trophoblast-endometrium interactions in equids. In 'Maternal Recognition of Pregnancy'. Ciba Foundation Symposium 64, Excerpta Media, pp.323-352

ALLEN W R (1982). Immunological aspects of the endometrial cup reaction and the effect of xenogeneic pregnancy in horses and donkeys. J Reprod Fert, Suppl. 31, 57-94

ALLEN W R, MOOR R M (1972). The origin of the equine endometrial cups. I. Production of PMSG by foetal trophoblast cells. J Reprod Fert 29, 313-316

ALLEN W R, HAMILTON D W, MOOR R M (1973). The origin of equine endometrial cups. II. Invasion of the endometrium by trophoblast. Anat Rec 177, 485-502

ALLEN W R, STEWART F, COOPER M J, CROWHURST R C, SIMPSON D J, McENERY R J, GREENWOOD R E S, ROSSDALE P D, RICKETTS S W (1974). Further studies on the use of synthetic prostaglandin analogues for inducing luteolysis in mares. Equine Vet J 6, 31-35

ALLEN W R, KYDD J, MILLER J, ANTCZAK D F (1984). Immunological studies on foeto-maternal relationships in equine pregnancy. 'Immunological Aspects of Reproduction in Mammals'. Editor:- Crighton D B, Publ. Butterworths, pp.183-193

ALLEN W R, KYDD J H, ANTCZAK D F (1986). Successful application of immunotherapy to a model of pregnancy failure in equids. Reproductive Immunology, 253-260

ALLEN W R, KYDD J H, BOYLE M S, ANTCZAK D F (1987). Extraspecific donkey-in-horse pregnancy as a model of early fetal death. J Reprod Fert, Suppl.35, 197-209

AMOROSO E C, HANCOCK J L, ROWLANDS I W (1948). Ovarian activity in the pregnant mare. Nature, Lond.161, 355-356

AMOROSO E C (1952). "Placentation". Marshalls Physiology of Reproduction, 3rd edn, Ed A S Parkes, Longman Green & Co, London, Vol.11, pp.127-311

ANTCZAK D F, BRIGHT S M, REMICK L H, BAUMAN B E (1982). Lymphocyte alloantigens of the horse. I. Serologic and genetic studies. Tissue Antigens 20, 172-187

ANTCZAK D F, MILLER J M, REMICK L H (1984). Lymphocyte alloantigens of the horse. II. Antibodies to ELA antigens produced during equine pregnancy. J Reprod Immunol 6, 283-297

ANTCZAK D F, ALLEN W R (1984). Invasive trophoblast in the genue Equus. Ann Immunol (Inst Pasteur), 325-331

ANTCZAK D F, ORIOL J G, DONALDSON W L, POLEMAN C, STENZLER L, VOLSEN S G, ALLEN W R (1987). Differentiation molecules of the equine trophoblast. J Reprod Fert, Suppl.35, 371-378

ARMSTRONG D T, HANSEL W (1959). Alteration of the bovine estrous cycle with oxytocin. J Dairy Sci 42, 533-542

ATHANASSAKIS I, WEGMANN T G (1986). The immunotrophic interaction between maternal T cells and fetal trophoblast/macrophages during gestation. "Reproductive Immunology 1986" (Clark D A and Croy B A Eds), pp.99-105, Elsevier Amsterdam

ATHANASSAKIS I, BLEACKLEY R C, PAETKAN V, GUILBERT L, BARR P J, WEGMANN T G (1987). The immunostimulatory effect of T cells and T cell lymphokines on murine fetally derived placental cells. J Immunol 138, 37-44

BADET M T, BELL S C, BILLINGTON W D (1983). Partial characterization of immunosuppressive factors from short term cultures of uterine decidual tissues. Ann Immunol (Inst Pasteur) 134C, 321-329

BALL B A, LITTLE T V, HILLMAN R B, WOODS G L (1985). Embryonic loss in normal and barren mares. Proc Am Assoc Equine Pract, 535-543

BARDAWIL W A, TOY B L (1959). The natural history of choriocarcinoma: problems of immunity and spontaneous regression. Ann N.Y Acad Sci 80, 197

BARKHAN P, TOMLIN S C, ARCHER R K (1957). Comparative coagulation studies on horse and human blood. J Comp Path 67, 358-368

BARTOL F F, THATCHER W W, BAZER F W, KIMBALL F A, CHENAULT J R, WILCOX C J, ROBERTS R M (1981). Effects of the oestrous cycle and early pregnancy in bovine uterine, luteal and follicular responses. Biol Reprod 25, 759-776

BASHA M M S, HORST M N, BAZER F W, ROBERTS R M (1978). Peptidases from pig reproductive tract: purification and properties of aminopeptidases from uterine secretions, allantoic fluid and amniotic fluid. Arch Biochem Biophys 185, 174-183

BAZER F W, THATCHER W W (1977). Theory of maternal recognition of pregnancy in swine based on oestrogen controlled endocrine versus exocrine secretion of PGF_2 by the uterine endometrium. Prostaglandins 14, 397-406

BAZER F W, ROBERTS R M, THATCHER W W (1978). Action of hormones on the uterus and effect on conceptus development. J Anim Sci 49, Suppl.2, 35-45

BEER A E, SIO J O (1982). Placenta as an immunological barrier. Biol Reprod 26, 15-27

BELL E T, LORAINE J A, JENNINGS S, WEAVER A D (1967). Serum and urinary gonadotrophin levels in pregnant ponies and donkeys. J Exp Physiol 52, 68-75

BELL S C, BILLINGTON W D (1981). Humoral immune responses in murine pregnancy. I. Anti-paternal alloantibody levels in maternal serum. J Reprod Immunol 3, 3-13

BERGLUND L A, SHARP D C, VERNON M W, THATCHER W W (1982). Effect of pregnancy and collection technique on prostaglandin F in the uterine lumen of Pony mares. J Reprod Fert, Suppl.32, 335-341

BERY S L, GINTHER O J (1978). Effect of oestrogens on uterine tone and life span of the corpus luteum in mares. J Anim Sci 47, 203-208

BETTERIDGE K J, RENARD A, GOFF A K (1985). Uterine prostaglandin release relative to embryo collection, transfer procedures and maintenance of the corpus luteum. Equine Vet J Suppl.32, 25-33

BISCHOF P, DUBERG S, SCHINDLER A M, HERMANN W L, SIZONENKO P C (1982). A hyphothesis on the biological function of pregnancy-associated plasma protein-A. Pregnancy Proteins - Biology, Chemistry and Clinical Application. Ed. Grudzinskas J G, Teisner B, Seppala M (Academic Press, Sydney) pp.321-332

BJORKMAN N, DANTZER V (1987). Placentation. "Textbook of Veterinary Histology". Dellman and Brown (Eds). Lea and Febiger Publishers, pp.340-360

BODEKER B G D, VAN EIJK R V W, MUHLRADT P F (1980). Mitogenic effects of partially purified interleukin 2 on thymocyte subpopulations and spleen T cells of the mouse. Eur J Immunol 10, 702-707

BODMER J G (1978). Ia antigens-definition of the HLA-DRw specificities. British Med Bull 34, 233-240

BOHN H, JOHANNSEN R, KRAUS W (1980). Neues Plazentaprotein (PP15) mit immunosuppressiver Wirkung. Arch Gynecol 230, 167-172

BOHN H, KRAUS W, WINCKLER W (1982). New soluble placental tissue proteins: their isolation, characterization, localization and quantification. "Immunology of Human Placental Proteins: Placenta". Klopper A (Ed), Praeger Publishers, Eastbourne, England, Suppl.4, pp.67-81

BOLTON A E, CHAPMAN M G, STOKER R J, ANDREW C EM WASS D, BOHN H (1983). The radioimmunoassay of human placental protein 14 (PP14). Clin Chim Acta 135, 282-291

BOLTON A E, CLOUGH K J, BOHN H, CHAPMAN M G (1986). Placental protein 14 (PP14) in the human reproductive tract. "Pregnancy Proteins in Animals". Walter de Gruyter & Co, Berlin, New York - Ed. Hau J, pp.165-171

BOLTON A E, POCKLEY A G, CLOUGH K J, MOWLES E A, STOKER R J, WESTWOOD O M R, CHAPMAN M G (1987). The identification of placental protein 14 (PP14) as an immunosuppressant factor involved in human reproduction. Lancet 1, 593-595

BORLAND R (1975). Placenta as an allograft. "Comparative Placentation". Eds Steven D H, Academic Press, pp.268-281

BOYUM A (1964). Separation of white blood cells. Nature 204, 793

BOYUM A (1968). Separation of leucocytes from blood and bone marrow. Scand J Clin Lab Invst 21, Suppl.97

BRADBURY S, BILLINGTON W D, KIRBY D R, WILLIAMS F A (1969). Surface mucin of human trophoblast. Am J Obstet Gynecol 104, 426

BRAUNSTEIN G D et al. (1976). Serum human chorionic gonadotrophin levels throughout normal pregnancy. Am J Obstet Gynecol 126, 178-181

BULMER J N, BILLINGTON W D, JOHNSON P M (1984). Immunohistologic identification of trophoblast populations in early human pregnancy with the use of monoclonal antibodies. Am J Obstet Gynecol 148, 19-26

BULMER J N, JOHNSON P M (1984). Macrophage populations in the human placenta and amniochorion. Clin Exp Immunol 57, 393-403

BULMER J N, SUNDERLAND C A (1984). Immunohistological characterization of lymphoid cell populations in the early human placental bed. Immunology 52, 349-357

BULMER J N, JOHNSON P M (1985). Antigen expression by trophoblast populations in the human placenta and their possible immunobiological relevance. Placenta 6, 127-140

BUTLER J E, HAMILTON W C, SASSER R G, RUDER C A, HASS G M, WILLIAMS R J (1982). Detection and partial characterisation of two bovine pregnancy-specific proteins. Biology of Reproduction 26, 925-933

CANTRELL D A, SMITH K A (1983). Transient expression of interleukin 2 receptors: consequences for T cell growth. J Exp Med 158, 1895-1911

CARLSON L, STIGBRAND T, FOLKERSEN J (1986). Immunochemical relationship between pregnancy-associated α_2-glycoprotein and α_2-macroglobulin as defined by poly- and monoclonal antibodies. "Pregnancy Proteins in Animals", Ed. Hau J, Walter de Gruyter, Berlin, pp.113-121

CAVANAGH A C, MORTON H, ROLFE B E, GIDLEY-BAIRD A A (1982). Ovum factor: a first signal of pregnancy. Am J Reprod Immunol 2, 97-101

CAWSTON T E, MURPHY G (1981). Mammalian collagenases. Methods in Enzymol 80, 711-722

CHAO P, FRANCIS L, ATKINS F (1977). The release of an endogenous pyrogen from guinea pig leucocytes in vitro: a new model for investigating the role of lymphocytes in fevers induced by antigen in hosts with delayed hypersensitivity. J Exp Med 145, 1288-1298

CHAOUAT G (1984). The riddle of the foetal allograft, introduction. Ann Immunol (Inst Pasteur) 135D, 301

CHAOUAT G (1987). Placental immunoregulatory factors. J Reprod Immunol 10, 179-188

CHAOUAT G, VOISIN G A, DAERON M, KANELLOPOULOS J (1977). Anticorps facilitants at cellules suppressives dans la reaction maternelle antifetale. Ann Immunol (Inst Pasteur) 129C, 685-687

CHAOUAT G, VOISIN G A (1979). Regulatory T cell subpopulations in pregnancy. I. Evidence for suppressive activity of the early phase of MLR. The Journal of Immunology 122, 1383-1388

CHAOUAT G, KOLB J P (1984). Immunoactive products of the placenta. II. Afferent suppression of maternal cell mediated immunity. Ann Immunol (Inst Pasteur) 135C, 205-218

CHAOUAT G, CHAFFAUX S (1984). Placental products induce suppressor cells of graft-versus-host reaction. Am J Reprod Immunol 6, 107

CHAOUAT G, KOLB J (1985). Immunoactive products of placenta. IV. Impairment by placental cells and their products of CTL function at effector stage. The Journal of Immunology 135, 215-222

CHAOUAT G, KOLB J P, KIGER N, STANISLAWSKI M, WEGMANN T G (1985). Immunologic consequence of vaccination against abortion in mice. J Immunol 134, 1594-1598

CHATTERJEE-HASROUNI S, LALA P K (1981). MHC antigens on mouse trophoblast cells: paucity of Ia antigens despite the presence of H-2K and D. J Immunol 127, 2070-2073

CHEVALIER F, PALMER E (1982). Ultrasonic echography in the mare. J Reprod Fert, Suppl.32, 423-430

CHOUAIB S, WELTE K, MERTELSMANN R, DUPONT B (1985). Prostaglandin E_2 acts at two distinct pathways of T lymphocyte activation: inhibition of interleukin 2 production and down-regulation of transferrin receptor expression. J Immunol 135, 1172-1179

CLARK D A (1984). Local suppressor cells and the success or failure of the "foetal allograft?" Ann Immunol (Inst Pateur) 135D, 321-323

CLARK D A, McDERMOTT M R, SZEWCZUK M R (1980). Impairment of host-versus-graft reaction in pregnant mice. II. Selective suppression of cytotoxic T cell generation correlates with soluble activity and with successful allogeneic pregnancy. Cell Immunol 52, 106-118

CLARK D A, SLAPSYS R, CROY B A, ROSSANT J (1983). Suppressor cell activity in uterine decidua correlates with success or failure of murine pregnancies. The Journal of Immunology 131, 540-542

CLARK D A, SLAPSYS R, CROY B A, KRECEK J, ROSSANT J (1984). Local active suppression by suppressor cells in the decidua: a review. Am J Reprod Immunol 5, 78-83

CLARK D A, SLAPSYS R M, CROY B A, ROSSANT J (1984). Immunoregulation of host-versus-graft responses in the uterus. Immunology Today 5, 111-115

CLARK D A, CHAPUT A, WALKER C, ROSENTHAL K L (1985). Active suppression of host-vs-graft reaction in pregnant mice. IV. Soluble suppressor activity obtained from decidua of allopregnant mice blocks the response to IL-2. The Journal of Immunology 134, 1659-1664.

CLARK D A, CHAOUAT G (1986). Characterization of the cellular basis for the inhibition of cytolitic effector cells by murine placenta. Cell Immunol 102, 43-51

CLARK D A, BRIERLEY J, SLAPSYS R, DAYA S, DAMJI N, CHAPUT A, ROSENTHAL K (1986a). Trophoblast-dependent and trophoblast-independent suppressor cells of maternal origin in murine and human decidua. "Reproductive Immunology 1986" (Clark D A and Croy B A Eds), pp.219-226, Elsevier, Amsterdam

CLARK D A, BRIERLEY J, SLAPSYS R, DAYA S, DAMJI N, CHAPUT A, ROSENTHAL K (1986b). Local intrauterine suppressor cells and suppressor factors in survival of the fetal allograft. "Reproductive Immunology: Materno-fetal Relationship" (Chaouat G, Ed), 154, pp.77-88, Colloque INSERM, Paris, France

CLARK D A, CHAPUT A, TUTTON D (1986c). Active suppression of host-versus-graft reaction in pregnant mice. VII. Spontaneous abortion of allogeneic CBA/J x DBA/2 fetuses in the uterus of CBA/J mice correlates with deficient non-T suppressor cell activity. The Journal of Immunology 136, 1668-1675

CLARK D A, CROY B A, ROSSANT J, CHAOUAT G (1986d). Immune presensitization and local intrauterine defenses as determinants of success of murine interspecies pregnancies. J Reprod Fert 72, 633-643

CLARK D A, DAMJI N, CHAPUT A, DAYA S, ROSENTHAL K L, BRIERLEY J (1986e). Decidua-associated suppressor cells and suppressor factors reculating interleukin-2: Their role in the survival of the "foetal allograft". Progress in Immunology VI, Ed: Cinader B and Miller R G, pp.1089-1099

CLARK D A, CROY B A, WEGMANN I G, CHAOUAT G (1987). Immunological and para-immunological mechanisms in spontaneous abortion: recent insights and future directions. J Reprod Immunol 12, 1-12

CLARK D A, MOWBRAY J, UNDERWOOD J, LIDELL H (1987). Histopathologic alterations in the decidua in human spontaneous abortion: loss of cells with large cytoplasmic granules. Am J Reprod Immunol and Microbiol 13, 19-22

CLARKE F, WILSON S (1985). In search of EPF. "Early Pregnancy Factors" (Ellendorff F, Koch E Eds). Perinatology Press, Ithaca, New York, pp.165-177

CLARKE A G (1969). Factors affecting the growth of trophoblast transplanted to the testis. J Reprod Fert 18, 539-541

CLEGG M T, BODA J M, COLE H H (1954). The endometrial cups and allantochorionic pouches in the mare with emphasis on the source of equine gonadotrophin. J Endocr 54, 448-463

CLEGG M T, COLE H H, HOWARD C B, PIGON H (1962). The influence of foetal genotype on equine gonadotrophin secretion. J Endocr 25, 245-248

CLEMENS L E, SIITERI P K, STITES D P (1979). Mechanism of immunosuppression of progesterone on maternal lymphocyte activation during pregnancy. J Immunol 122, 1978-1985

COLE H H, HART G H (1930). The potency of blood serum of mares in progressive stages of pregnancy in effecting the sexual maturity of the immature rat. Am J Physiol 93, 57-68

COOK R G, JENKINS R N, FLAHERTY L, RICH R R (1983). The Qa-1 alloantigens II. Evidence for the expression of two Qa-1 molecules by the Qa-1^{d} genotype and for cross-reactivity between Qa-1 and H-$2K^{f1}$. J Immunol 130, 1293-1299

COOPER D W, AITZEN R J (1981). Failure to detect altered rosette inhibition titres in human pregnancy serum. J Reprod Fert 61, 241-244

CROY B A, WOOD W, KING G (1987). Evaluation of intrauterine immune suppression during pregnancy in a species with epitheliochorial placentation. J Immunol 139, 1088-1095

CRUMP A, DONALDSON W L, MILLER J, KYDD J H, ALLEN W R, ANTCZAK D F (1987) Expression of major histocompatibility complex (MHC) antigens on horse trophoblast. J Reprod Fert, Suppl.35, 379-388

CURRIE G A, BAGSHAWE K D (1967). The masking of antigens on trophoblast and cancer cells. Lancet 1, 708-710

DAYA S, ROSENTHAL K L, CLARK D A (1986). Immunosuppressor factor(s) produced by decidua-associated suppressor cells: a proposed mechanism for foetal allograft survival. Am J Obstet Gynecol 156, 344-350

DEMBELE-DUCHESNE M J, THALER-DAO H, DE PAULET A (1982). The human placental anti-aggregating factor is neither prostacyclin nor a prostacyclin metabolite. Prostaglandins 24, 701

DEPPER J M, LEONARD W J, KRONKE M, NOGUCHI P D, CANNINGHAM R E, WALDMANN T A, GREENE W C (1984b). Regulation of interleukin-2 receptor expression: effects of phorbol diester, phosphalipase C, and reexposure to lectin and antigen. J Immunol 133, 3045-3061

DE WECK A L, LAZARY S, BULLEN S, GERBER H, MEISTER U (1978). Determination of leukocyte histocompatibility antigens in horses by serological tecnhiques. The Journal of Equine Medicine and Surgery, Suppl.1, 221-227

DOTT C S, HOWARD A, ANSELL B M (1985). The visualization and characterization of two immunological forms of alpha$_2$ macroglobulin by immunophoretic methods. Clin Chem Acta 146, 157-166

DOUGHTY R W, GELSTHORPE K (1976). Some parameters of lymphocyte antibody activity through pregnancy and further eluates of placental material. Tissue Antigens 8, 43-46

DOUGLAS R H, GINTHER O J (1976). Concentration of prostaglandin F in uterine venous plasma of anesthetized mares during the oestrous cycle and early pregnancy. Prostaglandins 11, 251-260

DOWER S K, URDAL D L (1987). The interleukin-1 receptor. Immunology Today 8, 46-51

DURUM S K, SCHMIDT J A, OPPENHEIM J J (1985). Interleukin-1: an immunological perspective. Ann Rev Immunol 3, 263,287

ENDERS A C (1983). Varying structural relationships between implantation and placentation of possible significance to maternal fetal immunology. "Immunology of Reproduction" (Eds: T G Wegmann and T Gill), Oxford University Press, London, pp.161-177

FARR A G, DORF M E, UNANUE E R (1977). Secretion of mediators following T lymphocyte macrophage interaction is regulated by the major histocompatibility complex. Proc Natl Acad Sci USA 74, 3542-3546

FAULK W P, JEANNET M, CREIGHTON W D, CARBONARA A (1974). Immunological studies of the human placenta: characterisation of immunoglobulins on trophoblast basement membrane. J Clin Invest 54, 1011-1019

FAULK W P, TEMPLE A (1976). Distribution of beta $_2$ microglobulin and HLA in chorionic villi of human placentae. Nature (London) 262, 799

FAULK W P, MCINTYRE J A (1986). Role of anti-TLX antibody in human pregnancy. "Reproductive Immunology". Clark D A and Croy B A (Eds), Elsevier Publ, New York, pp.106-114

FAZLEABAS A T, McDOWELL K J (1983). Synthesis and release of polypeptides by horse conceptus tissue and extra-embryonic membranes during early and mid-gestation. Biol Reprod 28, Suppl.1, (Abstr.) 138

FIELDS P A, ELRIDGE R K, FUCHS A R, ROBERTS R F, FIELDS M J (1983). Human placental and bovine corpora luteal oxytocin. J Endocr 113,1544-1546

FISHER S J, GIMENEZ T, HENRICKS D M (1985). Immunosuppressive activity associated with early pregnancy in the bovine. Biol Reprod 32, 894-906

FLOOD P F, MARRABLE A W (1975). A histological study of steroid metabolism in the equine fetus and placenta. J Reprod Fert, Suppl.23, 569

FORDE D, KEENAN L, WADE J, O'CONNOR M, ROCHE J F (1987). Reproductive wastage in the mare and its relationship to progesterone in early pregnancy. J Reprod Fert, Suppl.35, 493-495

FRENCH L R, NORTHEY D L (1983). Inhibitory effect of the bovine conceptus on lymphocyte stimulation. J Anim Sci 57, 456-465

GASIC G J, GASIC T B (1970). Total suppression of pregnancy in mice by postcoital administration of neuramidase. Proc Natn Acad Sci USA 67, 793-798

GENTRY P A, LIPTRAP R M, BLACK W D (1979). Changes in blood coagulation profiles of dairy cows during pregnancy and in heifer calves after hormone treatment. Can J An Sci 59, 503-510

GIDLEY-BAIRD A A, TEISNER B, HAU J, GRUDZINSKAS J G (1983). Immunochemical demonstration of a new pregnancy protein in the mare. J Reprod Fert 67, 129-132

GILLIS S, CRABTREE G R, SMITH K A (1979). Glucocoticoid-induced inhibition of T cell growth factor production. I. The effect on mitogen-induced lymphocyte proliferation. J Immunol 123, 1624-1631

GINTHER O J (1985). Embryonic loss in mares: incidence, time of occurrence and hormonal involvement. Therionology 23, 77-89

GINTHER O J (1986). "Ultrasonic Imaging and Reproductive Events in the Mare". Equiservices, Cross Plains, Wisconsin, pp.259-272

GOFF A K, PONTBRIAND D, SIROIS J (1987). Oxytocin stimulation of plasma 15-keto-13, 14-dihydro prostaglandin F2α during the oestrous cycle and early pregnancy in the mare. J Reprod Fert, Suppl.35, 253-260

GREENE W C, LEONARD W J (1986). The human interleukin-2 receptor. Ann Rev Immunol 4, 69-95

GROSSER O (1927). Fruhentwicklungen, Eihautbildung und Placentation des Menschen und der Saugetiere. "Deutsche Frauenheilkunde, Geburtshilfe, Gynakologie und Nachbergebiete in Einzeldarstellungen". Von Jaschke R T Ed., V Munich: Bergman

GUILBERT J (1985). Mononuclear phagocyte progenitors and growth factors. "Mononuclear Phagocytes and Inflamation" (Cohn Z, Van Furin R, Gordon S Eds), Martinus Niphof, the Hague, Boston, p.233

GUNTHER G R, WANG J L, EDELMAN G M (1974). The kinetics of cellular commitment during stimulation of lymphocytes by lectins. J Cell Biol 62, 366-377

HAMANN A, JABLONSKI-WESTRICH D, RAEDLER A, THIELE H (1984). Lymphocytes express specific antigen-independent contact interaction sites upon activation. Cell Immunol 86, 14-32

HARDT C, ROLLINGHOFF M, PFIZENMAIER K, MOSMANN H, WAGNER H (1981). Lyt-23+ cyclophosphamide-sensitive T cells regulate the activity of an interleukin-2 inhibitor in vivo. J Exp Med 154, 262-274

HART G H, COLE H H (1934). The source of oestrin in the pregnant mare. Am J Physiol 109, 320-323

HAU J (1986). Murine models to human pregnancy specific 1-glycoprotein and -fetoprotein and their application in teratogenic studies. "Pregnancy Proteins in Animals" (Ed Hau J), Walter de Gruyter, Berlin-New York, pp.465-479

HEAP R B, HAMON M, ALLEN W R (1982). Studies on oestrogen synthesis by the preimplantation equine conceptus. J Reprod Fert, Suppl.32, 343-352

HERSHMAN L, DOUGLAS R H (1979). The critical period for the maternal recognition of pregnancy in pony mares. J Reprod Fert, Suppl.27, 395-401

HEYNER S, HUNZIKER R D, ZSINK G L (1980). Differential expression of minor histocompatibility antigens on the surface of the mouse oocyte and preimplantation developmental stages. J Reprod Immunol 2, 269-280

HOSKIN D W, MURGITA S, HAMEL S, GRONVIK K O (1985). Pregnancy interruption by a monoclonal antibody that recognises non-T suppressor cells in maternal lymphoid tissue. "Pregnancy Proteins in Animals" (Hau J Ed), Walter de Gruyter and Co, Berlin-New York, pp.351-359

HUNZIGER R D, WEGMANN T G (1986). Placental immunoregulation. CRC Critical Reviews in Immunology 6, 245-285

HUTTON R A, DANDONA P, CHOW F P R, CRAFT I L (1980). Inhibition of platelet aggregation by placental extracts. Thromb Res 17, 465-471

IKLE F A (1964). Dissemination von Syncitiotrophoblast-zellen im mutterlichen Blut wahrend der Graviditat. Bull Schweiz Akad Med Wiss 20, 62

JADUS R, SCHMUNK G, DJEU J Y, PARKMAN R (1986). Morphology and mechanisms of interleukin 3-dependent natural cytotoxic cells: tumour necrosis factor as a possible mediator. J Immunol 137, 2774-2783

JANZEN R G, MABLY E R, TAMACKI T, CHURCH R B, LORSCHEIDER F L (1982). Syntesis of alpha-fetoportien by the pre-implantation and post-implantation bovine embryo. J Reprod Fert 65, 105-110

JULKUNEN M, WAHLSTROM T, SEPPLALA M, KOISTINEN R, KOSKIMIES A, STENMAN U H, BOHN H (1984). Detection and localisation of placental protein 14-like protein in human seminal plasma and in the male genital tract. Arch Androl 12, 59-67

JULKUNEN M, KOISTINEN R, SJOBERG J, RUTANEN E, WAHLSTROM T, SEPPALA M (1985a). Secretory endometrium synthesizes placental protein 14. J Endocr 118, 1782-1786

JULKUNEN M, RUTANEN E M, KOSKIMIES A, RANTA T, BOHN H, SEPPALA M (1985b) Distribution of placental protein 14 in tissue and body fluids during pregnancy. Br J Obstet Gynaecol 92, 1145-1151

JULKUNEN M, WAHLSTROM T, SEPPALA M (1986). Human fallopian tube contains placental protein 14. Am J Obstet Gynecol 154, 1076-1079

JULKUNEN M, RAIKER R S, JOSHI S G, BOHN H, SEPPLALA M (1986a). Placental protein 14 and progestagen-dependent endometrial protein are immunologically indistinguishable. Hum Reprod 1, 7-8

JULKUNEN M, APTER D, SEPPALA M, STENMAN U H, BOHN H (1986b). Serum levels of placental protein 14 reflect ovulation in non-conceptual menstrual cycles. Fertility and Sterility 45, 47-50

JULKUNEN M, KOISTINEN R, SJOBERG J, RUTANEN E M, WAHLSTROM T, SEPPALA (1986c). Secretory endometrium synthesizes placental protein 14. J Endocr 118, 1782-1786

KASAHARA T, HOOKS J J, DOUGHERTY S F, OPPENHEIM J J (1983). Interleukin 2 mediated immune interferon production by human T cells and T cell subsets. J Immunol 130, 1784-1789

KASMAN L H, STABENFELDT G H, HUGHES J P, CONTO M A, LASLEY B L (1987). Circulating oestrone sulphate concentrations in pregnant mares undergoing ovariectomy. J Reprod Fert, Suppl.35, 721-723

KAY J E (1969). The role of the stimulant in the activation of lymphocytes by PHA. Exptl Cell Res 58, 185-187

KAYE M D, JONES W R, ING R M Y (1974). An investigation of immunological privilege in the pregnant rat uterus. J Reprod Fert 36, 467

KAYE J, GILLIS S, MIZEL S B, SHEVACH E M, MALEK T R, DINARELLO C A, LACHMAN L B, JANEWAY C A (1984). Growth of a cloned helper T cell line induced by a monoclonal antibody specific for the antigen receptor: interleukin-1 is required fot the expression of receptors for interleukin-2. J Immunol 133, 1339

KING J M (1965). Comparative aspects of reproduction in Equidae. PhD thesis, University of Cambridge

KIRBY D R S (1968). Immunological aspects of pregnancy. "Advances in Reproductive Physiology", Ed. McLaren A, Logos Press, Vol.III, pp.33-79

KLIMA F (1985). Fetomaternale Weckselbeziehungen in der fruhe Trachtigkeit. Milu 6, 336-342

KLIMA F, TIEMANN U, PITRA C, KAUFFOLD P (1987). Serological detection of early pregnancy in cattle and partial characterisation of a serum glycoprotein associated with early pregnancy. J Reprod Immunol 11, 31-39

KNIGHT J W, BAZER F W, WALLACE H D (1973). Hormonal regulation of porcine uterine protein secretion. J Anim Sci 36, 546-553

KOWER S K, URDAL D L (1987). The interleukin-1 receptor. Immunology Today 8, 46-51

KRISTENSEN F, KRISTENSEN B, LAZARY S (1982). The lymphocyte stimulation test in veterinary immunology. Vet Immunol and Immunopathol 3, 203-277

KROLL J (1973). Tandem Crossed Immunoelectrophoresis. Scand J Immunol, Suppl.1, 57-59

KROLL J (1981). Production of specific antisera by immunisation with precipitin lines. Methods in Enzymology 73, 52-57

KUMAR D, AZOURY R S, BARNES A C (1963). Studies on human premature births. I. Placental progesterone concentrations. Am J Obstet Gynecol 87, 126-129

KYDD J, MILLER J, ANTCZACK D F, ALLEN W R (1982). Maternal anti-foetal cytotoxic antibody responses of equids during pregnancy. J Reprod Fert, Suppl.32, 361-369

KYDD J, ALLEN W R, BOYLE M S, SHEPARD A, SUMMERS P M (1985). Transfer of exotic equine embryos to domestic horses and donkeys. Equine Vet J, Suppl.3, 80-84

KYDD J, ALLEN W R (1986). Suppressor activity in the equine endometrial cup reaction. J Reprod Immunol (abstr), Suppl.119, 125

LASTER D B (1977). A pregnancy-specific protein in the bovine uterus. Biol Reprod 16, 682-690

LAURELL C B, McKAY E J (1981). Electroimmunoassay Methods in Enzymology 73, 339-369

LAWRENCE R, CHURCH J A, RICHARDS W, BORZY M (1980). Immunological mechanisms in the maintenance of pregnancy. Annals of Allergy 44, 166-173

LAZARY S, DE WECK A L, GERBER H, SCHATZMANN U, STRAUB R (1973a). Characteristics of the in vitro stimulation of horse leucocytes by phytohaemagglutinin and antigen. Z Immun-Forsch 145, 364-375

LAZARY S, DE WECK A L, BULLEN S, STRAUB R, GERBER H (1980a). Equine leukocyte antigen system. I. Serological studies. Transplantation 30, 203-209

LAZARY S, BULLEN S, MULLER J, KOVACS G, BODO I, HOCKENJOS O, DE WECK A L (1980b). Equine leukocyte antigen system. II. Serological and mixed lymphocyte reactivity studies in families. Transplantation 30, 210-215

LAZARY S, DUBATH M L, LUDER C, GERBER H (1986). Equine leukocyte antigen system. IV. Recombination within the major histocompatibility complex (MHC). Journal of Immunogenetics 13, 315-325

LICHT P, BON GALLO A, AGGARWAL B B, FARMER S W, CASTELINO J B, PAPKOFF H (1979). Biological and binding activities of equine pituitary gonadotrophins and pregnant mare serum gonadotrophin. J Endocrin 83, 311-322

LOUVET J P, HARMAN S M, NISULA B C, ROSS G T, BIRKEN S, CANFIELD R (1976) Follicle stimulating activity of human chorionic gonadotrophin; effect of dissociation and recombination of subunits. J Endocr 99, 1126-1128

LOWRY D H, ROSEBROUGH N J, FARR A L, RANDALL R J (1951). Protein measurement with the folin phenol reagent. J Biol Chem 193, 265-275

LU C Y, CHANGELIAN P S, UNANUE E R (1984). α-foeto protein inhibits macrophage expression of Ia antigens. J Immunol 132, 1722

LUDER C, LAZARY S, GERBER H (1985). Study on the contribution of Class I and II ELA antigens. Animal Blood Groups and Biochemical Genetics 16, 120-121

McCOMB R B, BOWERS G N, POSEN S (1979). "Alkaline Phosphatase". Plenum Press New York and London

McDOWELL K J, SHARP D C, FAZLEABAS A, ROBERTS R M, BAZER F W (1982). Partial characterisation of the equine uteroferrin-like protein. J Reprod Fert, Suppl.32, 329-334

McDOWELL K J (1986). Establishment of pregnancy in mares: inter-relationships among uterine proteins, prostaglandin $F_2\alpha$ and conceptus secretory protein. Dissertation Abstracts International 47, 4441-B

McDOWELL K J, SHARP D C, GRUBAUGH W (1987). Comparison of progesterone and progesterone + oestrogen on total and specific uterine proteins in pony mares. J Reprod Fert, Suppl.35, 335-342

McKAY E J, LAURELL C B (1980). The interaction of heparin with plasma proteins. Demonstration of different binding sites for antithrombin III complexes and antithrombin III. J Lab Clin Med 95, 69-80

MALE D, CHAMPOIN B, COOKE A (1987). Advanced Immunology, Gower Medical Publishing, London

MAKI M, MURATA M, SHIDARA Y (1984). Inhibitors of platelet aggregation and blood coagulation isolated from the human placenta. Europ J Obstet Gynec Reprod Biol 17, 149-154

MANNING A W, RAJKUMAR K, BRISTOL F, FLOOD P F, MURPHY B D (1987). Genetic and temporal variation in serum concentrations and biological activity of horse chorionic gonadotrophin. J Reprod Fert, Suppl.35, 389-397

MARCH C J, MOSLEY B, LARSEN A (1985). Cloning sequence and expression of two distinct human interleukin-1 complementary DNAs. Nature 315, 641-647

MARSAN C, GOFF A K, SIROIS J, BETTERIDGE K J (1987). Steroid secretion by different cell types of the horse conceptus. J Reprod Fert, Suppl.35, 363-369

MASTERS R A, ROBERTS R M, LEWIS G S, THATCHER W W, BAZER F W, GODKIN J D (1982). High molecular weight glycoproteins released by expanding preattachment sheep, pig and cow blastocysts in culture. J Reprod Fert 66, 571-583

MATTERI R L, PAPKOFF H, MURTHY H M S, ROSER J F, CHANG Y S (1986). Comparison of the properties of highly purified equine chorionic gonadotrophin isolated from commercial concentrates of pregnant mare serum and endometrial cups. Dom Anim Endocr 3, 39-48

MENCIO G F, MANNS J, MURPHY B D (1978). FHS and LH activity of PMSG from mares at different stages of gestation. Anim Reprod Sci (Netherlands), 137-144

MILLER J F, WILLIAMS E, GLUE J, GORDON Y B, GRUDZINSKAS J G, SYKES A (1980) Fetal loss after implantation. The Lancet, 554-556

MIYASAKA N M, McCULLOUGH P (1981). Immunological responsiveness of maternal and foetal lymphocytes during normal pregnancy in the ewe. J Reprod Immunol 3, 15-27

MOORE W T (Jr), WARD D N (1980). Pregnant mare serum gonadotrophin: an in vitro biological characterisation of the lutropin-follitropin dual activity. J Biol Chem 255, 6930-6936

MORTON H (1985). EPF as a pregnancy protein. "Early Pregnancy Factors". Eds: Ellendorf F and Koch E, Perinatology Press, Ithaca, N.Y, pp.53-64

MORTON H, HEGH V, CLUNIE G J A (1976). Studies of the rosette inibition test in pregnant mice: evidence of immunosuppression? Proc R Soc Biol 193, 413-419

MORTON H, CLUNIE G J A, SHAW F D (1979). A test for early pregnancy in sheep. Res Vet Sci 26, 261-262

MORTON H, ROLFE B E, McNEILL L, CLARKE P, CLARKE F M, CLUNIE G J A (1980) Early pregnancy factor: Tissues involved in its production in the mouse. J Reprod Immunol 2, 73-82

MORTON H, TINNEBERG H R, ROLFE B, WOLF M, METTLER L (1982). Rosette inhibition test: a multicentre investigation of early pregnancy factor in humans. J Reprod Immunol 4, 251-261

MORTON H, MORTON D J, ELLENDORF F (1983). The appearance and characteristics of early pregnancy factor in the pig. J Reprod Fert 68, 437-446

MOSSMAN H W (1937). Comparative morphogenesis of the fetal membranes and accessory uterine structures. Contrib Embryol Carnegie Inst 26, 129-246

MOWBRAY J F, LIDELL H, UNDERWOOD J L, GIBBINGS C, REGINALD P W, BEARD R W (1985). I. Controlled trial of treatment of recurrent spontaneous abortion by immunization with paternal cells. Lancet 1, 941-943

MURAMATSU T, GACHELIN G, DAMONNEVILLE M, DELARBRE C, JACOB F (1979). Cell surface carbohydrates of embryonal carcinoma cells: polysaccaridic side chains of F9 antigens and of receptors to two lectins, FBP and PNA. Cell 18, 183-191

MURGITA R A, TOMASI T B (1975). Suppression of the immune response by α-feto protein. II. The effect of α-fetoprotein on mixed lymphocyte reactivity and mitogen induced lymphocyte transformation. J Exp Med 141, 440-452

MURGITA R A, GOIDL E A, KONTIAINEN S, WIGZELL H (1977). Alpha-foeto protein induces suppressor T cells in vitro. Nature (London) 267, 257-259

MURGITA R A, HOOPER D C, STEGAGNO M, DELOVITCH T L, WIGZELL H (1981) Characterization of murine newborn inhibitor T lymphocytes: functional and phenotypic comparison with an adult T cell subset activated in vitro by alpha-foeto protein. Eur J Immunol 11, 957-964

MURRAY F A, BAZER F W, WALLACE H D, WARNICK A C (1972). Quantitative and qualitative variation in the secretion of protein by the porcine uterus during the oestrous cycle. Biol Reprod 7, 314-320

MURRAY F A, SEGERSON E C, BROWN F T (1978). Suppression of lymphocytes in vitro by porcine uterine secretory protein. Biol Reprod 19, 15-25

MURRAY F A, CHENAULT J R (1982). Effects of steroids on bovine T-lymphocyte blastogenesis in vitro. J Anim Sci 55, 1132-1138

MURRAY M K, SEGERSON E C, HANSEN P J, BAZER F W, ROBERTS R M (1987). Suppression of lymphocyte activation by a high-molecular-weight glycoprotein released from preimplantation ovine and porcine conceptuses. Am J of Reprod Immunol 14, 38-44

MYATT L, ELDER M (1977). Inhibition of platelet aggregation by a placental substance with prostacyclin-like activity. Nature 268, 159-160

NANCARROW C D, WALLACE A L L, GREEWAL A S (1981). The early pregnancy factor of sheep and cattle. J Reprod Fert, Suppl.30, 191-197

NEELY D P, KINDAHL H, STABENFELDT G H, EDQUIST L E, HUGHES J P (1979). Prostaglandin release patterns in the mare: physiological, pathophysiological and therapeutic response. J Reprod Fert, Suppl 27, 181-189

NOONAN F P, HALLIDAY W J, MORTON H, CLUNIE G J A (1979). Early pregnancy factor is immunosuppressive. Nature 278, 649-650

NUSSENZWAIG M C, STEINMAN R M (1980). Contribution of dendritic cells to stimulation of the murine syngeneic mixed leukocyte reaction. J Exp Med 151, 1196-1212

O'BRIEN W F, KNUPPEL R A, SABA H I, ANGEL J L, BENOIT R, BRUCE A (1987). Platelet inhibitory activity in placentas from normal and abnormal pregnancies. Obstet and Gynec 70, 597-600

O'FLYNN K, KRENSKY A M, BEVERLY P C L, BURAKOFF J, LINCH D (1985). Phytohaemagglutinin activation of T cells through the sheep red blood cell receptor. Nature 313, 686-687

O'HEARN M, STITES D P (1983). Inhibition of murine suppressor cell function by progesterone. Cell Immunol 77, 340-350

OHNISHI S T, BARR J K (1978)
A simplified method of quantitating protein using the biuret and phenol reagents. Anal Biochem 86, 193-200

O'NEILL C (1985a). Thrombocytopenia is an initial maternal response to fertilization in mice. J Reprod Fert 73, 559-566

O'NEILL C (1985b). Examination of the causes of early pregnancy-associated thrombocytopenia in mice. J Reprod Fert 73, 567-577

O'NEILL C (1985c). Embryo derived platelet activating factor. "Early Pregnancy Factors". Eds: Ellendorf F and Koch E, Perinatology Press, Ithaca, pp.261-266

O'NEILL C, GIDLEY-BAIRD A A, PIKE I L, PORTER R N, SINOSICH M J, SAUNDERS D M (1985). Maternal blood platelet physiology and luteal-phase endocrinology as a means of monitoring pre- and post-implantation embryo viability following in vitro fertilization. Journal of in Vitro Fertilization and Embryo Transfer 2, 59-65

O'NEILL C, PIKE I L, PORTER R N, GIDLEY-BAIRD A A, SINOSICH M J, SAUNDERS D M (1985). Maternal recognition of pregnancy prior to implantation: methods for monitoring embryonic viability in vitro and in vivo. Annals New York Academy of Sciences, pp.429-439

O'NEILL C, GIDLEY-BAIRD A A, PIKE I L, SAUNDERS D M (1987). Use of a bioassay for embryo-derived platelet-activating factor as a means of assessing quality and pregnancy potential of human embryos. Fertility and Sterility 47, 969-975

OPPENHEIM J J, KOVACS E J, MATSUSHIMA K, DURUM S K (1986). There is more than one interleukin-1. Immunology Today 7, 45-56

OROZCO C, PERKINS T, CLARKE F M (1986). Platelet-activating factor induces the expression of early pregnancy factor activity in female mice. J Reprod Fert 78, 549-555

PARHAR R S, LALA P K (1986). Mechanisms of PGE_2-mediated T cell inactivation by the human decidua. J Reprod Immunol (Abstract) 122

PARKES R O, BLACKMORE D J, RANCE T A, PARK B K, DEAN P D G (1977). Plasma concentrations of equilin and oestrone in the assessment of fetoplacental function in the amre. Vet Record 100, 511-512

PASHEN R L, ALLEN W R (1979). The role of the fetal gonads and placenta in steroid production, maintenance of pregnancy and parturition in the mare. J Reprod Fert, Suppl.27, 499-509

PAULO E, TISCHNER M (1985). Activity of 5 -hydroxy-steroid dehydrogenase and steroid hormones content in early preimplantation horse embryos. Folia Histoch Cytobiol 23, 81-84

PERRY J S, HEAP R B, BURTON R D, GADSBY J E (1976). Endocrinology of the blastocyst and its role in the establishment of pregnancy. J Reprod Fert, Suppl 25, 80

PITRA C, KLIMA F, KAUFFOLD P (1985). Detection of early pregnancy in cattle and pigs by the adoptive transfer of contact sensitivity. Am J Reprod Immunol Microbiol 7, 56

POCKLEY A G, BOLTON A E, MOWLES E A, STOKER R J, WESTWOOD O M R, CHAPMAN M (1987). The suppression of the mitogenic response to phytohaemagglutinin by placental protein 14. B J Obstet Gynaecol 94, 918 (Abstract)

POCKLEY A G, MOWLES E A, STOKER R J, WESTWOOD O M R, CHAPMAN M G, BOLTON A E(1988). The suppression of in vitro lymphocyte reactivity to phytohaemagglutinin (PHA) by placental protein 14 (PP 14). J Reprod Immunol (In press)

POMEROY R W (1960). Infertility and neonatal mortality in the sow.IV. Further observations and conclusions. J Agric Sci 54, 57-66

POWER D A, CATTO G R D, MASON R J, MACLEOD A M, Stewart K N, SHEWEN W G (1983). The fetus as an allograft: evidence for protective antibodies to HLA-linked paternal antigens. Lancet ii, 701-704

RAMSEY E M (1982). The placenta: human and animal. New York, Praeger Publishers

REASIDE J I, LIPTRAP R M (1975). Pattern of urinary oestrogen excretion in individual pregnant mares. J Reprod Fert, Suppl.23, 469-475

REDMAN C W G, McMICHAEL A J, STIRRAT G M (1984). Class I major histocompatibility complex antigens on human extravillous trophoblast. Immunology 52, 457-468

ROBB R J, MUNCK A, SMITH K A (1981). T cell growth factor receptors: quantitation, specificity and biological relevance. J Exp Med 154, 1455-1474

ROBB R J, SMITH K A (1981). Heterogeneity of human T cell growth factor(s) due to variable glycosylation. Mol Immunol 18, 1087-1094

ROBB R J, GREENE W C, RUSK C M (1984). Low and high affinity cellular receptors for interleukin-2. Implications for the level of Tac antigen. J Exp Med 160, 1126-1146

ROBERTS G P (1977). Inhibition of lymphocyte stimulation by bovine uterine proteins. J Reprod Fert 50, 337-339

ROBERTS R M, BAZER F W (1980). The properites, function and hormonal control of uteroferrin, the purple protein of the pig uterus. "Steroid Induced Proteins" (Ed M Beato), Elsevier-North Holland, Amsterdam, pp.133-149

ROBERTS R M, BAZER F W (1984). Uteroferrin: a protein in search of a function. Bioessays 1, 8-11

ROCKLIN R E, KITZMILLER J L, CARPENTER C B, GARAVOY M R, DAVID J R (1976). Maternal-fetal relation: absence of an immunologic blocking factor from the serum of women with chronic abortions. New Eng J Med 295, 1209-1213.

ROCKLIN R E, KITZMILLER J L, KAYE M D (1979). Immunobiology of the maternal-fetal relationship. Ann Rev Med 30, 375-404

ROITT I, BROSTOFF J, MALE D (1986). Immunology, Gower Medical Publishing, London

ROLFE B E, MORTON H, CLARKE F M (1983). Early pregnancy factor is an immunosuppressive contaminant of commercial preparations of human chorionic gonadotrophin. Clin Exp Immunol 51, 45-52

ROSSDALE P D, RICKETTS S W (1974). The Practice of Equine Stud Medicine (McMillan Publishing Company Inc, New York)

SALEM H T, OBIEKWE B C, AL-ANI A T M, SEPALLA M, CHARD T (1980). Molecular heterogeneity of placental protein 5 (PP5) in late pregnancy serum and plasma: evidence for a heparin-PP5 polymer. Clin Chim Acta 107, 211-215

SCHAMS D, PAPKOFF H (1972). Chemical and immunochemical studies on pregnant mare serum gonadotrophin. Biochim et Biophys Acta 263, 139-148

SCHAUDER W (1912). Untersuchungen uber die Eihaute und Embryotrophe des Pferdes. Arch Anat Physiol, 259-302

SCHLAFKE S, ENDERS A C (1975). Cellular basis of interaction between trophoblast and uterus at implantation. Biol Reprod 12, 41-65

SCHLOSNAGLE D C, BAZER F W, TSIBRIS J C M, ROBERTS R M (1974). An iron-containing phosphatase induced by progesterone in the uterine fluids of pigs. J Biol Chem 249, 7574-7479

SCOTT J R, ROTE N S, BRANCH D W (1987). Immunologic aspects of recurrent abortion and fetal death. Obst and Gynecol Review 70, 645-656

SEGERSON E C (JR) (1981). Immunosuppressive effect of ovine uterine secretory protein upon lymphocytes in vitro. Biol Reprod 25, 77-84

SEGERSON E C, LIBBY D W, GETZ W R, RANDEL R D (1984). Immunosuppressive effect of uterine secretory protein from Angus and Brahman cows upon lymphocytes in vitro. J Anim Sci 59, 1047-1059

SELLENS M H, JENKINSON E J, BILLINGTON W D (1978). Major histocompatibility complex and non-major histocompatibility complex antigens on mouse ectoplacental cone and placental trophoblastic cells. Transplantation 25, 173-179

SHARP D C (1980). Factors associated with the maternal recognition of pregnancy in mares. Symposium on equine reproduction. Veterinary Clinics of North America 2, pp.277-290

SHARP D C, McDOWELL K J (1985). Critical events surrounding the maternal recognition of pregnancy in mares. Equine Vet J, Suppl.3, 19-22

SHERBLOM A P, SMAGULA R M, MOODY C E, ANDERSON G W (1985). Immunosuppression, sialic acid and sialyltransferase of bovine serum as a function of progesterone concentration. J Reprod Fert 74, 509-517

SHORT R V (1959). Progesterone in blood. IV. Progesterone in the blood of mares. J Endocr 19, 207-210

SIITERI P K, FEBRES F, CLEMENS L E, CHANG R J, GONDOS B, STITES D P (1977) Progesterone and the maintenance of pregnancy: Is progesterone natures immunosuppressant? Ann N.Y. Acad Sci 284, 384-397

SIITERI P K, STITES D P (1982). Immunologic and endocrine interrelationships in pregnancy. Biol Reprod 26, 1-14

SIST M D, WILLIAMS J F, GEARY A M (1987). Pregnancy diagnosis in the mare by immunoassay of oestrone sulfate in serum and milk. J Equine Vet Sci 7, 20-23

SMART Y C, ROBERTS T K, CLANCY R L, CRIPPS A W (1981). Early pregnancy factor: its role in mammalian reproduction - research review. Fertility and Sterility 35, 397-402

SMITH K A (1984). Interleukin 2. Ann Rev immunol 2, 319-333

SMITH R N, STERNLICHT M, BUTCHER G W (1982). The alloantibody response in the allogeneically pregnant rat. I. The primary and secondary responses and detection of Ir gene control. J Immunol 129, 771-776

SOMA H, SAYAMA S, OKUDERA K, ISAKA K, TAKAYAMA (1985). Biological function of placental protein PP_5 on coagulation and fibrinolytic activities during placental separation. "Proteins of the Placenta", Bischof, Klopper (Eds). 5th Int Congr on Placental Proteins, Annecy 1984 (Karger, Basel), pp.129-141

SPINKS N R, O'NEILL C (1987). Embryo-derived platelet activating factor is essential for establishment of pregnancy in the mouse. Lancet 1, 106-107

SQUIRES E L, GINTHER O J (1975). Follicular and luteal development in pregnant mares. J Reprod Fert, Suppl.23, 429-433

STABENFELDT G H, HUGHES J P, EVANS J W, NEELY D P (1974). Spontaneous prolongation of luteal activity in the mare. Equine Vet J 6, 158-163

STAPLES L D, BROWN D, BINNS R M, HEAP R B (1983). The influence of protein hormones and conceptus extracts on sheep lymphocyte transformation induced in vitro. Placenta 4, 125-131

STEVEN D (1975). Anatomy of the placental barrier. Monographs for students of medicine. "Comparative Placentation" (Esssays in Structure and Function). Editor: Steven D.H. (Academic Press), pp.25-57

STEVEN D, MORRIS G (1975). Development of the Foetal Membranes. Monographs for students of medicine. "Comparative Placentation" (Essays in Structure and Function). Editor: Steven D.H. (Academic Press), pp.58-85

STEWART F, ALLEN W R, MOOR R M (1977). Influence of foetal genotype on the follicle-stimulating hormone: luteinizing hormone ratio of pregnant mare serum gonadotrophin. J Endocr 73, 419-425

STEWART G M, MASON R J, THOMSON M A R, McLEOD A M. CATTO G R D (1984). Noncytotoxic antibodies to paternal antigens in maternal sera and placental eluates. Transplantation 38, 111-115

STIMSON W H, STRACHAN A F, SHEPERD A (1979). Studies on the maternal immune response to placental antigens: absence of a blocking factor from the blood of abortion-prone women. Br J Obstet Gynaecol 86, 41-45

STIRLING Y, WOOLF L, NORTH W R S, SEGHATCHIAN M J, MEADE T W (1984). Haemostasis in normal pregnancy. Thromb Haemostas (Stuttgart) 52, 176-182

STITES D P, SIITERI P K (1983). Steroids as Immunosuppressants in pregnancy. Immunol Rev 75, 117-137

STREILEIN J W, WEGMANN T G (1987). Immunologic privilege in the eye and the fetus. Immunology Today 8, 362-366

SUMMERS P M, SHEPARD A M, HODGES J K, KYDD J, BOYLE M S, ALLEN W R (1987). Successful transfer of the embryos of Przewalski's horses (Equus przewalskii) and Grant's zebra (Equus burchelli) to domestic mares (Equus caballus). J Reprod Fert 80, 13-20

SUNDERLAND C A, REDMAN C W G, STIRRAT G M (1981). HLA A, B, C antigens are expressed on non-villous trophoblast of the early human placenta. J Immunol 127, 2614-2615

SUTTON L, MASON D Y, REDMAN C W G (1983). HLA-DR positive cells in the human placenta. Immunology 49, 103-112

SUTTON L, GADD M, MASON D Y, REDMAN C W G (1986). Cells bearing class II MHC antigens in the human placenta and amniochorion. Immunology 58, 23-29

SYNDER F (1985). Chemical and biochemical aspects of platelet activating factor: a novel class of acetylated ether-linked choline-phospho-lipids. Med Research Rev 5, 107-140

TAKELIOGLU-UYSAL M, EDWARDS R G, KISNISCI H A (1975). Ultrastructural relationships between decidua, trophoblast and lymphocytes at the beginning of human pregnancy. J Reprod Fert 42, 431-438

TAWFIC O W, HUNT J S, WOOD G W (1986). A major soluble suppressor factor produced by uterine cells during murine pregnancy is prostaglandin E2 (PGE2). J Reprod Immunol (abstract), 126

TEISNER B, DAVEY M W, GRUDZINSKAS J G (1983). Interaction between heparin and plasma proteins analysed by crossed immunoelectrophoresis and affinity chromatography. Clin Chim Acta 127, 413-417

TERASAKI P I, MICKEY M R, YAMAZAKI J N, VREDEVOE D (1970). Maternal-fetal incompatibility. I.Incidence of HL-A antibodies and possible association with congenital anomalies. Transplantation 9, 538-543

TERQUI M, PALMER E (1979). Oestrogen pattern during early pregnancy in the mare. J Reprod Fert, Suppl.27, 441-446

THATCHER W W, KNICKERBOCKER J J, BARTOL F F, BAZER F W, ROBETS R M, DROST M (1985). Maternal recognition of pregnancy in relation to the survival of transferred embryos: endocrine aspects. Therionology 23, 129-144

TOJO S, ASHITAKI Y, MARUO T, MATSURA S, NISHIMURA R (1982). "Pregnancy Proteins, Biology, Chemistry and Clinical Application". (Grudzinskas J G, Teisner B, Seppala M Eds). Academic Press, Australia, pp.25-38

TOMASI T B Jr (1978). Suppressive factors in amniotic fluid and newborn serum: Is alpha- fetoprotein involved? Cell Immunol 37, 459-466

TSUDO M, UCHIYAMA T, UCHINO H (1984). Expression of Tac antigen on activated normal human B-cells. J Ex Med 160, 612-617

USZYNSKI M (1979). Anticoagulant activity of peptides from the human placenta. Thromb Res 16, 689-694

VAN VLASSELAER P, VANDEPUTTE M (1985). Local immune response suppression by progesterone. "Early Pregnancy Factors". Ellendorff and Koch (Eds) (Perinatology Press), pp.89-92

VAN VLASSELAER P, GOEBELS J, VANDEPUTTE M (1986). Inhibition of lymphocyte aggregation by progesterone. J Reprod Immunol 9, 111-121

VERNON M W, STRAUSS S, SIMONELLI M, ZAVY M T, SHARP D C (1979). Specific PGF_2 binding by the corpora luteum of the pregnant and non-pregnant mare. J Reprod Fert, Suppl.27, 421-429

VERNON M W, ZAVY M T, ASQUITH R L, SHARP D C (1981). Prostaglandin $F_{2\alpha}$ in the equine endometrium: steroid modulation and production capacities during oestrous cycle and early pregnancy. Biol Reprod 25, 581-591

VOISIN G A, CHAOUAT G (1974). Demonstration, nature and properties of maternal antibodies fixed on placenta and directed against paternal antigens. J Reprod Fert, Suppl.21, 81-103

WATHES D C, SWANN R W (1982)
Is oxytocin an ovarian hormone? Nature 297, 225-227

WEEKE B (1973)
Crossed immunoelectrophoresis. Scand J Immunol, Suppl.1, pp.47-56

WEISSMANN A M, HARFORD J B, SVETLIK P B, LEONARD W L, DEPPER J M, WALDMANN T A, GREENE W C, KLAUSNER R D (1986). Only high affinity receptors for IL-2 mediate internalization of the ligand. Proc Natl Acad Sci USA 83, 1463-1466

WHITE J G, MATLACK K L, MUNDSCHENK D, RAO G H R (1975). Platelet studies in normal and a bleeder horse. Procs First Int Symp Equine Hematol, 209-221

WHYTE A, ALLEN W R (1985). Equine endometrium at preimplantation stages of prenancy has specific glycosylated regions. Placenta 6, 537-542

WOODS G L, BAKER C B, BALDWIN J L, BALL B A, BILINSKI J, COOPER W L, LEY W B, MANK E C, ERB H N (1987). Early pregnancy loss in brood mares. J Reprod Fert, Suppl.35, 455-459

YACHIN S, LESTER E P (1979). Human alpha-fetoprotein and prostaglandins suppress human lymphocyte transformation by different mechanisms. "Carcino-Embryonic Proteins". Ed Lehmann F G, Vol.II, Elsevier-North Holland, Biomedical Press, pp.339-344

ZAVY M T, BAZER F W SHARP D C (1978). Non-surgical technique for collection of uterine fluid from the mare. J Anim Sci 47, 672-676

ZAVY M T, BAZER F W, SHARP D C, WILCOX C J (1979a). Uterine luminal proteins in the cycling mare. Biol Reprod 20, 689-698

ZAVY M T, MAYER R, VERNON M W, BAZER F W, SHARP D C (1979b). An investigation of the uterine luminal environment of non-pregnant and pregnant pony mares. J Reprod Fert, Suppl.27, 403-411

ZAVY M T, SHARP D C, BAZER F W, FAZLEABAS A, SESSIONS F, ROBERTS R M (1982). Identification of stage-specific and hormonally induced polypeptides in the uterine protein secretions of the mare during the oestrous cycle and pregnancy. J Reprod Fert 64, 199-207

ZAVY M T, VERNON M W, SHRAP D C, BAZER F W (1984). Endocrine aspects of early pregnancy in pony mares: a comparison of uterine luminal and peripheral plasma levels of steroids during the oestrous cycle and early pregnancy. J Endocrin 115, 214-219

ZHIHAI Q, ZHEN-QUN Z (1987). Detection of early pregnancy factor in human sera. Am J Reprod Immunol 13, 15-18

ZUCKERMANN F A, HEAD J R (1986). Expression of MHC antigens on murine trophoblast and their modulation by interferon. J Immunol 3, 846-853.

APPENDIX I: MATERIALS

ADP reagent:	Sigma Chemical Co. Ltd
Agarose:	Sigma Chemical Co. Ltd
Barbitone [Diethylbarbituric Acid]:	BDH Chemicals Ltd
Biuret reagent:	BDH Chemicals Ltd
Bovine Serum Albumin, Fr.V.:	Sigma Chemical Co Ltd
Bromophenol Blue:	Fisons Pharmaceuticals
Collagen reagent:	Sigma Chemical Co. Ltd
Cocktail 'O' liquid scintillant:	BDH Chemicals Ltd
Collagenase:	Boehringer-mannheim
Controlled process serum replacement [CPSR-2]:	Sigma Chemical Co. Ltd
Coomassie Brilliant Blue:	Sigma Chemical Co. Ltd
Dulbecco's modification of Eagles medium containing 20 mM/1 HEPES [DMEM]:	Flow Laboratories, Northumbria Biologicals Ltd
Foetal Calf Serum:	Flow Laboratories
Folin and Ciocalteau:	BDH Chemicals Ltd
Freunds complete/incomplete adjuvent:	Sigma Chemical Co. Ltd
GFC Filter paper:	Whatman Ltd
Heparin Ultrogel:	LKB Instruments Ltd
Heparin Sepharose:	Pharmacia Ltd
Incubator [CO_2 humidified]:	W.C. Heraeus Gmbh
Lymphopaque separation medium [relative density 1.084]:	Nycomed UK Ltd
Microtitre plates [96 well]:	Beckton Dickinson UK Ltd
Optiphase Hisafe liquid scintillant:	LKB Instruments Ltd
Penicillin/streptomycin solution:	Flow Laboratories, Northumbria Biologicals Ltd

Phenyl Methyl Sulphonyl Fluoride (PMSF):	Sigma Chemical Co. Ltd
Platelet aggregometer (Model 1002):	ADG Instruments Ltd
Pregnant Mare Serum Gonadotrophin:	Sigma Chemical Co. Ltd, Intervet
PZ-Pro-Leu-Gly-Pro-Arg:	Boehringer-mannheim
Scintillation Counter (LKB Rackbeta 1212):	LKB Instruments Ltd
Semi-automated cell harvester:	Skatron AS, Norway
Sephacryl S-300:	Pharmacia Ltd
Sodium azide:	Koch-Light Ltd
Sodium borohydride:	BDH Chemicals Ltd
Sodium-m-periodate:	Fisons Pharmaceuticals
Tritiated thymidine (^{3}H-Tdr):	Amersham International
Trypsin EDTA:	Flow Laboratories
Vacutainers (143 USP units Li Heparin; 0.105 M sodium citrate):	Beckton Dickinson UK Ltd

All other chemicals were obtained from either BDH Chemicals Ltd or Sigma Chemical Co. Ltd.

APPENDIX II : BUFFERS AND REAGENTS

Agarose gel layer	1% agarose in Tris/Barbitone buffer - gel formed by steaming at 90°C.
BS7 Reagent	1 vol. Biuret reagent in 7 vol. 2.5% w/v sodium carbonate.
Collagenase substrate solution	10 mg PZ-Pro-Leu-Gly-Pro-Arg dissolved in 0.2 ml methanol and make up to 10 ml with tris buffer.
Phosphate buffer (PB)	0.15 M $Na_2HPO_4.12H_2O$ (82%) 0.15 M $NaH_2PO_4.2H_2O$ (18%)
Phosphate buffer saline (PBS)	PB with 0.9% NaCl.
Sodium acetate buffer (0.1M; pH 4.8)	Acetic acid (36%) Sodium acetate (64%)
Tris/Barbitone buffer (0.1 M; pH 8.6)	22.4 g Diethylbarbituric acid 44.3 g Tris 0.533 g Ca - Lactate 0.650 g Sodium azide (NaN_3). Add distilled water to 1 litre to give a concentrated buffer. Dilute 1:4 before use.
Tris (hydroxymethyl) - aminomethane	1.22 g Tris - make up to 100 ml and adjust to pH 7.1 with 1 M HCl.

APPENDIX III: ELISA ASSAY FOR eCG: BUFFERS AND REAGENTS

Plate coating buffer (pH 6.0)	20 mM	Citrate
Wash buffer and glaze (pH 7.0)	2.5 g	lactose
	2.5	BSA
	0.5 g	Sodium azide
	500 ml	Deionized water
Conjugate buffer (pH 8.5)	14.9 g	Trimethanolamine
	52.8 g	Ammonium sulphate
	5.0 g	BSA.
	1.0 g	Magnesium chloride
	1.0	Sodium azide
	1.0 ml	Triton X-705
	1.0 l	Deionized water
Standard buffer (Ph 7.0)	250 ml	PBS
	2.5 g	BSA
	0.25 g	Sodium azide
Sample dilution buffer (pH 7.0)	250 ml	PBS
	2.5 g	BSA
	0.25 g	Sodium azide
	250 ul	Triton X-705
Assay wash buffer (pH 8.0) [20 x concentrate]	158.56	Ammonium sulphate
	20.00	Trizma base
	21.28 g	Trizma HCl
	12.00 ml	Magnesium chloride
	12.00 ml	0.1 M Zinc chloride
	1.00 g	Sodium azide
	1.00 ml	Triton X-705
	1.00 g	SB-14 (Calbiochem)
	975 ml	Deionized water